Oxidative Stress in Applied Basic Research and Clinical Practice

Anna Dietrich-Muszalska
Ved Chauhan
Sylvain Grignon *Editors*

Studies on Psychiatric Disorders

Humana Press

Oxidative Stress in Applied Basic Research and Clinical Practice

Editor-in-Chief
Donald Armstrong

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Note from the Editor-in-Chief

All books in this series illustrate point-of-care testing and critically evaluate the potential of antioxidant supplementation in various medical disorders associated with oxidative stress. Future volumes will be updated as warranted by emerging new technology, or from studies reporting clinical trials.

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The book is dedicated to the memory of my mother Halina Dietrich-Miłobędzka

Preface

Since the discovery of free radicals by M. Gomberg at the University of Michigan, USA (1900), and the suggestion of the role of toxic radicals in the etiology of schizophrenia in the mid-1950s (Hoffer et al.), there has been great progress in the study of oxidative stress in neuropsychiatric disorders. A large body of evidence suggests that free radicals are involved in cell membrane pathology, and may play a role in schizophrenia.

Free radicals play an integral role in cellular signalling; however being highly unstable with unpaired electrons, they have differential oxidative strength and potential to damage cellular proteins, lipids, carbohydrates, and nucleic acids. Under physiological conditions, multiple defence mechanisms exist to protect against these free radicals. Oxidative stress occurs when redox homeostasis is tipped towards an overbalance of free radicals, due to either their overproduction or deficiencies in antioxidant defence. Oxidative stress-induced impairment of neuronal processes has been reported to be involved in neurodegeneration and also in the pathophysiology of neuropsychiatric diseases.

Studies on Psychiatric Disorders shows the current state of the knowledge concerning the role of oxidative stress, mainly in psychiatric disorders. All the chapters in this book discuss the role of oxidative stress in neuropsychiatric diseases.

In the Introduction, the book puts special emphasis on the specific biomarkers of oxidative, nitrosative/nitrative and chlorinative stress (chapter "Oxidative, Nitrosative and Chlorinative Stress: Biomarkers").

Involvement of oxidative stress in the pathophysiology of various psychiatric disorders such as schizophrenia, bipolar disorder, autism spectrum disorders, obsessive-compulsive disorder, attention deficit and hyperactivity disorder, and neurodegenerative disorders is described in this book (Part of Clinical Aspects, chapters "Oxidative Stress in Schizophrenia", "Oxidative Stress in Bipolar Disorder", "Contribution of Oxidative Stress to the Pathophysiology of Autism Spectrum Disorders: Impact of Genetic and Environmental Factors", "Oxidative Stress and Anxiety Disorder", "Relationship Between Oxidative Stress and OCD", "A Relationship Between Oxidative Status and Attention Deficit Hyperactivity Disorder",

"The Role of Oxidative Stress in Neurodegenerative Diseases", and chapter "Antioxidant Interventions in Neuropsychiatric Disorders").

Chapter "Oxidative Stress in Schizophrenia" presents biomarkers of oxidative damage, the role of oxidative stress in numerous abnormalities in biochemical pathways including apoptosis in pathophysiology of schizophrenia, mitochondrial dysfunction and the consequent oxidative damage to biomolecules in bipolar disorders (chapter "Oxidative Stress in Bipolar Disorder"). Chapter "Contribution of Oxidative Stress to the Pathophysiology of Autism Spectrum Disorders: Impact of Genetic and Environmental Factors" reviews the role of oxidative damage coupled with reduced antioxidant defence, genetic susceptibility, oxidative/nitrosative stress, and impact of environmental agents in autism. A link between oxidative stress and pathological anxiety is addressed in chapter "Oxidative Stress and Anxiety Disorder". The manifestations of oxidative stress have been found in obsessive-compulsive disorder patients, and are described in chapter "Relationship Between Oxidative Stress and OCD". Recent findings show that the pathophysiology of attention deficit and hyperactivity disorder may be associated with oxidative stress (chapter "A Relationship Between Oxidative Status and Attention Deficit Hyperactivity Disorder"). It has been proposed that oxidative stress related damage of several macromolecules play a key role in pathogenesis of neurodegenerative diseases. In chapter "The Role of Oxidative Stress in Neurodegenerative Diseases", there is a review of current knowledge regarding the role of reactive oxygen species and oxidative stress in neurodegeneration occurring in neurological diseases such as Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis and multiple sclerosis.

In Part II, various pathophysiological aspects of oxidative stress in neuropsychiatric disorders are presented: (chapters "Magnetic Resonance Spectroscopy Studies in Bipolar Disorder Patients: Focus on the Potential Role of Oxidative Stress", "The Impact of Oxidative Stress on Dopaminergic Neurotransmission", "The Reciprocal Effects of Oxidative Stress and Glutamate Neurotransmission", "Mitochondrial Dysfunction in Psychiatric disorders", "The Kynurenine Pathway at the Interface Between Neuroinflammation, Oxidative Stress and Neurochemical Disturbances: Emphasis in Schizophrenia", "Dysregulation of Glutathione Synthesis in Psychiatric Disorders", "The Role of Nitric Oxide and Nitrosative Stress in Schizophrenia", "Blood Platelet as a Peripheral Cell in Oxidative Stress in Psychiatric Disorders", "Mitochondrial Dysfunction in Autism", "Ultrasound and Autism: How Disrupted Redox Homeostasis and Transient Membrane Porosity Confer Risk", "Animal Model of Autistic Regression: Link to Toxicant-Induced Oxidative Stress", "Genetic Polymorphism Related to Oxidative Stress in Autism", "Telomere Length in Major Psychiatric Disorders. Is there any Relationship Between Telomere Length and Oxidative Stress?", "The Impact of Oxidative Stress on GAD67 Levels and Parvalbumin-positive Neurons", "The Possible Role of Iron in Neurodegeneration", and "Oxidative Stress and Polyunsaturated Lipid Peroxidation Products in the CNS: Focus on Retinal Bisretinoids and DHA-derived Carboxyethylpyrroles as Potential Inducers of Vision-threatening Pathology"). Chapter "Magnetic Resonance Spectroscopy Studies in Bipolar Disorder Patients: Focus on the Potential Role of Oxidative Stress", describes the overview of magnetic resonance spectroscopy

(MRS) studies exploring the glutamate neurotransmission and specific neural markers in bipolar disorder debates on the findings in relation to oxidative stress and mitochondrial dysfunction. Chapter "The Impact of Oxidative Stress on Dopaminergic Neurotransmission" focuses on how redox status impacts the key components of dopamine neurotransmission, and evaluates the relevance of these modifications with respect to the recently revisited dopamine hypothesis of schizophrenia. How glutamate modulates the redox state by effectors such as dynamics, stimulation of mitochondrial NADPH oxidase, nitric oxide and redox modulating glutamate-cystine exchanger, inter alia is shown in chapter "The Reciprocal Effects of Oxidative Stress and Glutamate Neurotransmission". The evidence for mitochondrial dysfunction which plays a role in schizophrenia, major depression, bipolar disorder, personality/mood disorders, Alzheimer disease and autism spectrum disorders is discussed in chapter "Mitochondrial Dysfunction in Psychiatric Disorders". The kynurenine pathway at the interface between neuroinflammation, oxidative stress and neurochemical disturbances is emphasized in schizophrenia and the problems pertaining to these issues is discussed in chapter "The Kynurenine Pathway at the Interface Between Neuroinflammation, Oxidative Stress and Neurochemical Disturbances: Emphasis in Schizophrenia". The purpose of chapter "Dysregulation of Glutathione Synthesis in Psychiatric Disorders" is to review the available literature referring to glutathione synthesis and its multiple functions in the central nervous system. The role of nitric oxide and nitrosative stress in schizophrenia is described in chapter "The Role of Nitric Oxide and Nitrosative Stress in Schizophrenia". The use of platelets, the blood cells with a relatively short half-life time, as a peripheral model to study the mechanisms of cell signalling pathways or the extent of oxidative damage and changes in the central nervous system is described in chapter "Blood Platelet as a Peripheral Cell in Oxidative Stress in Psychiatric Disorders". The evidence for mitochondrial dysfunction in autism, including decreased activities and protein expression levels of mitochondrial electron transport chain complexes, mitochondrial DNA or nuclear DNA mutations, oxidative stress, and calcium-signalling abnormalities in autism is described in chapter "Mitochondrial Dysfunction in Autism" and a possible connection between ultrasound and autism is discussed in chapter "Ultrasound and Autism: How Disrupted Redox Homeostasis and Transient Membrane Porosity Confer Risk". In chapter "Animal Model of Autistic Regression: Link to Toxicant-Induced Oxidative Stress", an animal model of autistic regression with link to toxicant-induced oxidative stress is presented. Chapter "Genetic Polymorphism Related to Oxidative Stress in Autism" concerns an understanding of the genetic etiology of autism in the context of oxidative stress and genetic susceptibility. This review describes polymorphisms of various genes potentially associated with oxidative stress and the susceptibility to autism and Rett's disorder. Is there any relationship between telomere length and oxidative stress? The question is undertaken in chapter "Telomere Length in Major Psychiatric Disorders. Is there any Relationship Between Telomere Length and Oxidative Stress?", where the telomere length in major psychiatric disorders is described. As the next interesting issue, the impact of oxidative stress on GAD67 levels and parvalbumin-positive neurons is the topic of chapter "The Impact of Oxidative Stress on GAD67

Levels and Parvalbumin-positive Neurons". Iron may play a deleterious role in neurodegeneration by triggering oxidative stress via Fenton reaction. Therefore in chapter "The Possible Role of Iron in Neurodegeneration", the results of long-term studies aimed to assess the concentrations of total and labile iron and ferritin in Parkinson disease, Alzheimer disease and progressive supranuclear palsy are presented. Chapter "Oxidative Stress and Polyunsaturated Lipid Peroxidation Products in the CNS: Focus on Retinal Bisretinoids and DHA-derived Carboxyethylpyrroles as Potential Inducers of Vision-threatening Pathology" focuses on retinal bisretinoids and DHA-derived carboxyethylpyrroles as potential inducers of vision-threatening pathology seen from the aspect of oxidative stress and polyunsaturated lipid peroxidation products in the CNS.

A special role of various antioxidants in reduction of oxidative stress in psychiatric disorders is presented in "Therapeutic aspects" (chapters "Antioxidant Interventions in Neuropsychiatric Disorders", "Antioxidant Plant Polyphenols and Cognitive Disorders", "Hyperbaric Oxygen Treatment in Autism Spectrum Disorders", "Effects of Lithium on Oxidative Stress", and "Cryostimulation as Adjunct Treatment in Psychiatric Disorders"), where the different aspects associated with oxidative stress and treatment have been described. Recent evidence suggests that novel therapeutic strategies such as supplementation with antioxidants, ω3 fatty acids or combination of both might improve the neuroplasticity and can be effective for long-term treatment management of neuropsychiatric disorders. In chapter "Antioxidant Interventions in Neuropsychiatric Disorders", an overview of the recent findings on the potential treatment strategies using antioxidants in schizophrenia and bipolar disorder and major depression is presented. The detailed review of research concerning the use of plant polyphenols antioxidants in cognitive disorders in older individuals can be found in chapter "Antioxidant Plant Polyphenols and Cognitive Disorders", suggesting the preventive and therapeutic potential for neurodegenerative diseases of natural plant polyphenols with antioxidant activity. In chapter "Hyperbaric Oxygen Treatment in Autism Spectrum Disorders", a hyperbaric oxygen treatment in autism spectrum disorders is described.

The effects of lithium on oxidative stress (chapter "Effects of Lithium on Oxidative Stress") and antipsychotics on nitrative stress in schizophrenia (subchapter.15.5) are described. Finally, chapter "Cryostimulation as Adjunct Treatment in Psychiatric Disorders" presents the physiology and neuroprotection of hypothermia and the effects of cryostimulation on oxidative stress in psychiatric disorders, especially in depression.

Fifty-one authors, all leading experts from around the world, have contributed their expertise to *Studies on Psychiatric Disorders*.

We believe this volume will prove to be attractive not only to scientists but also to practitioners and students.

Lodz, Poland — Anna Dietrich-Muszalska
Staten Island, NY, USA — Ved Chauhan
Sherbrooke, QC, Canada — Sylvain Grignon

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Anna Dietrich-Muszalska

Contents

Oxidative, Nitrosative, and Chlorinative Stress: Biomarkers 1
Grzegorz Bartosz and Izabela Sadowska-Bartosz

Clinical Aspects

Oxidative Stress in Schizophrenia 43
Anna Dietrich-Muszalska

Oxidative Stress in Bipolar Disorder 73
Gustavo Scola and Ana C. Andreazza

Contribution of Oxidative Stress to the Pathophysiology of Autism Spectrum Disorders: Impact of Genetic and Environmental Factors 89
Ved Chauhan and Abha Chauhan

Oxidative Stress and Anxiety Disorder 121
Marina Čeprnja and Vladimira Vuletić

Relationship Between Oxidative Stress and Obsessive–compulsive Disorder 131
Jolanta Rabe-Jabłońska† and Anna Dietrich-Muszalska

A Relationship Between Oxidative Status and Attention Deficit Hyperactivity Disorder 143
Salik Selek and M. Fatih Ceylan

The Role of Oxidative Stress in Neurodegenerative Diseases 151
Dominika Książek-Winiarek and Andrzej Głąbiński

Pathophysiological Aspects

Magnetic Resonance Spectroscopy Studies in Bipolar Disorder Patients: Focus on the Potential Role of Oxidative Stress 171
Nicola Dusi, Filippo Cecchetto, and Paolo Brambilla

The Impact of Oxidative Stress on Dopaminergic Neurotransmission 197
Jessica Deslauriers, Philippe Sarret, and Sylvain Grignon

The Reciprocal Effects of Oxidative Stress and Glutamate Neurotransmission 211
Sylvain Grignon and Jessica Deslauriers

Mitochondrial Dysfunction in Psychiatric Disorders 231
Daniel A. Rossignol and Richard E. Frye

The Kynurenine Pathway at the Interface Between Neuroinflammation, Oxidative Stress, and Neurochemical Disturbances: Emphasis in Schizophrenia 245
Benjamín Pineda, Victoria Campos-Peña, Rafael Lugo-Huitrón†, Camilo Ríos, and Verónica Pérez-de la Cruz

Dysregulation of Glutathione Synthesis in Psychiatric Disorders 269
Elżbieta Lorenc-Koci

The Role of Nitric Oxide and Nitrosative Stress in Schizophrenia 299
Anna Dietrich-Muszalska, Grzegorz Bartosz, and Izabela Sadowska-Bartosz

Blood Platelet as a Peripheral Cell in Oxidative Stress in Psychiatric Disorders 327
Barbara Wachowicz

Mitochondrial Dysfunction in Autism 355
Abha Chauhan, Feng Gu, and Ved Chauhan

Ultrasound and Autism: How Disrupted Redox Homeostasis and Transient Membrane Porosity Confer Risk 373
Emily L. Williams and Manuel F. Casanova

Animal Model of Autistic Regression: Link to Toxicant-Induced Oxidative Stress 393
Melody Furnari, Constance L.L. Saw, Ah-Ng T. Kong, and George C. Wagner

Genetic Polymorphism Related to Oxidative Stress in Autism 417
Hee Jeong Yoo

Telomere Length in Major Psychiatric Disorders: Is There Any Relationship Between Telomere Length and Oxidative Stress? 435
Dariusz Nowak

The Impact of Oxidative Stress on GAD67 Levels and Parvalbumin-Positive Neurons 451
Jessica Deslauriers and Sylvain Grignon

The Possible Role of Iron in Neurodegeneration 469
Jolanta Galazka-Friedman and Andrzej Friedman

Oxidative Stress and Polyunsaturated Lipid Peroxidation Products in the CNS: Focus on Retinal Bisretinoids and DHA-Derived Carboxyethylpyrroles as Potential Inducers of Vision-Threatening Pathology 479
Jerzy Z. Nowak

Therapeutic Aspects

Antioxidant Interventions in Neuropsychiatric Disorders 505
Anilkumar Pillai and Jeffrey K. Yao

Antioxidant Plant Polyphenols and Cognitive Disorders 521
Dariusz Nowak

Hyperbaric Oxygen Treatment in Autism Spectrum Disorders 553
Daniel A. Rossignol

Effects of Lithium on Oxidative Stress 567
Jolanta Rabe-Jabłońska†

Cryostimulation as Adjunct Treatment in Psychiatric Disorders 575
Elżbieta Miller

Index 593

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Oxidative, Nitrosative, and Chlorinative Stress: Biomarkers

Grzegorz Bartosz and Izabela Sadowska-Bartosz

Abbreviations

AGEs	Advanced glycation end products
AOPP	Advanced oxidation protein products
DNP	Dinitrophenyl
DNPH	Dinitrophenylhydrazine
ELISA	Enzyme-linked immunosorbent assay
ESR	Electron spin resonance
GSH	Glutathione
GSSG	Oxidized glutathione
HPLC	High-performance liquid chromatography
MDA	Malondialdehyde
MS	Mass spectrometry
NOS	Nitric oxide synthase
Nox	NADPH oxidase(s)
OS	Oxidative stress
RCS	Reactive chlorine species

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RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid

1 Introduction

Oxygen is indispensable for our life. However, it exerts also toxic effects; the main reason of its toxicity is due to formation of oxygen free radicals and other reactive oxygen species (ROS) via partial reduction or excitation of the oxygen molecule (Fig. 1). The collective term "reactive oxygen species" (ROS) includes both free radicals (molecules having an odd electron) and species that are not free radicals (like hydrogen peroxide, singlet oxygen, ozone, hypochlorite, and peroxynitrite). ROS can react with and damage all classes of biomolecules: lipids, proteins, nucleic acids, and carbohydrates. In order to prevent such damage, cells and extracellular fluids contain antioxidants and antioxidant enzymes, which keep a low level of ROS. Formation of ROS is not only an inevitable evil of aerobic metabolism; they play an important role in the defense against invading pathogens and in intra- and intercellular signaling as autocrine and paracrine factors (Bartosz 2009; Labunskyy and Gladyshev 2013). In healthy organism there is a balance between the production of ROS on one hand and activities of antioxidant enzymes and antioxidants at the other hand. A shift of this

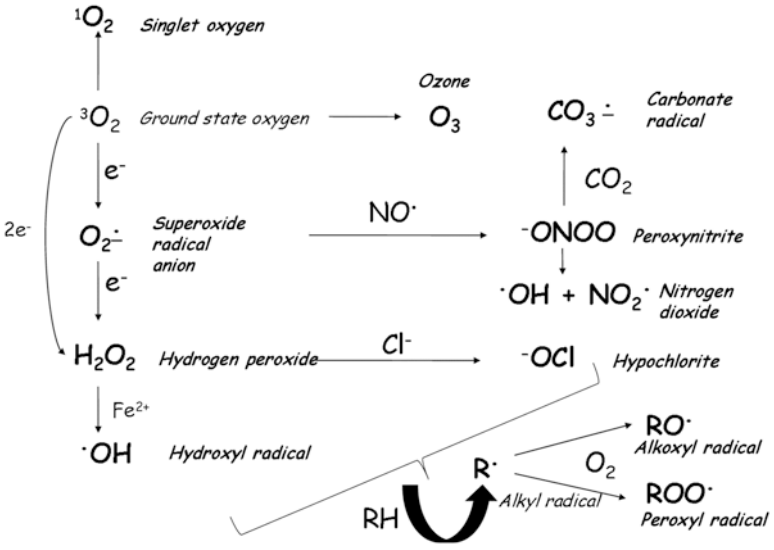


Fig. 1 Main reactive oxygen species occurring in biological systems. After (Bartosz 2009), modified

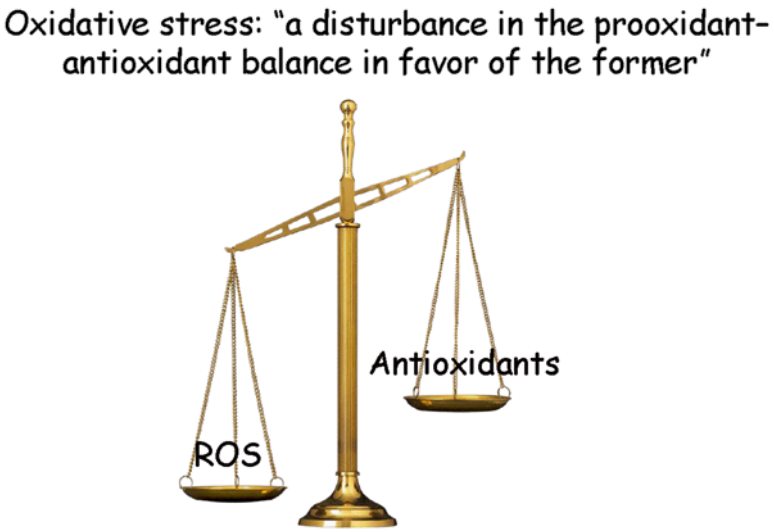


Fig. 2 Definition of oxidative stress, according to Sies (1985)

organism in the prooxidant direction (increased ROS production or diminished antioxidant defense) is referred to as ***oxidative stress*** (OS; Fig. 2) (Sies 1985). OS accompanies numerous diseases, participates in the development of many diseases, and is believed to contribute to aging of cells and organisms.

2 Sources of Reactive Oxygen Species

In the respiratory chain in the mitochondria, an oxygen molecule undergoes four-electron reduction to form two water molecules. However, a fraction of oxygen undergoes one-electron reduction forming ***superoxide radical anion*** $O_2^{\bullet}$. Mitochondria are the main cellular source of superoxide. The magnitude of the fraction of oxygen subject to one-electron reduction in the intact mitochondria has been the question of debate; initial estimates of 1–5 % seem too high and more recent ones correspond to some 0.1 % at the most. Still, taking into account the amount of oxygen used up by respiring cells, these amounts are significant (Dröse and Brandt 2012). Apart from mitochondria, superoxide is produced by microsomal electron transport chains and by some enzymes. Smooth endoplasmic reticulum contains enzymes detoxifying xenobiotics, including drugs, among them cytochrome P-450 isozymes, especially the ethanol-inducible CYP2E1. These enzymes are able to reduce molecular oxygen and produce $O_2^{\bullet}$ and H_2O_2. Nuclear membranes contain cytochrome oxidases and electron transport systems of unknown functions which may also release ROS. Several enzymes can produce superoxide as a by-product.

The most important is xanthine oxidase formed from xanthine dehydrogenase after tissue exposure to hypoxia. This list of enzymes includes aldehyde oxidase, dihydroorotate dehydrogenase, tryptophan dioxygenase and 5-lipoxygenase. Nitric oxide synthases can also produce superoxide in the "uncoupled" state which occurs upon deficiency of cofactor or substrate (tetrahydrobiopterin or L-arginine) (Roe and Ren 2012). A special family of enzymes, NADPH oxidases (Nox), produce superoxide as the main product. Nox2, present in polymorphonuclear leukocytes and other phagocytes, generates large amounts of superoxide for killing invading microorganisms. Other enzymes of this family (Nox1, Nox3-5, Duox1, Duox2) produce much lower amounts of superoxide for signaling purposes (Lambeth et al. 2008; Lambeth 2002, 2007).

Superoxide is also formed by autoxidation of reduced forms of various compounds including thiols, flavins, pteridines, metal ions, adrenaline, diphenols, NAD(P)H and metalloproteins (e.g., hemoglobin, myoglobin and ferredoxin), and some xenobiotics (Riccioni et al. 2011).

Superoxide ion is not very reactive. It can oxidize many compounds but may also reduce such species like Fe^{3+} or tetrazolium dyes. A fraction of the superoxide radical anion is protonated forming the ***hydroperoxyl radical*** $HO_2^{\bullet}$ (Reaction 1) which is a better oxidant than superoxide and, lacking electric charge, can penetrate biological membranes more easily.

$$O_2^{\bullet-} + H+ \rightarrow HO_2^{\bullet} \quad \leftarrow \tag{1}$$

The pK_a value of this equilibrium is 4.88 which means that at pH 4.88 half of the superoxide radical anions is protonated; this proportion decreases with increasing pH.

Superoxide anion can dismutate spontaneously (Reaction 2):

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{2}$$

This reaction is accelerated considerably by superoxide dismutase. A product of this reaction is hydrogen peroxide H_2O_2. Hydrogen peroxide is also formed directly by some oxidases, e.g., D-amino acid oxidase, urate oxidase, L-α-hydroxyacid oxidase, fatty acyl-CoA oxidase, glycolate oxidase. Many of these enzymes are present in peroxisomes. In contrast to superoxide anion which is a free radical (has one odd electron), the molecule of ***hydrogen peroxide*** has an even number of electrons and thus is not a free radical; however, it is more reactive than molecular oxygen and is a ROS. In contrast to the electrically charged superoxide radical anion, this neutral molecule penetrates easily cellular membranes and other barriers and may reach sites where it can be made more reactive. Hydrogen peroxide is a good oxidant; in a cell, one of main targets for hydrogen peroxide are reactive thiol groups of proteins. This reaction is important, especially in the context of signal transduction. Hydrogen peroxide can also react with reduced forms of metals (mainly Fe^{2+}); this reaction (***Fenton reaction***; Reaction 3) produces ***hydroxyl radical***, the most reactive oxidant occurring in living systems:

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + {}^{-}OH + Fe^{3+} \quad (3)$$

If ferric ions produced in this reaction are reduced back to the ferrous form, minute amounts of iron can catalyze the formation of much higher amounts of the hydroxyl radical. Superoxide radical anion is able to reduce Fe^{3+} (Reaction 4) so the simultaneous presence of both ROS in the presence of even trace amounts of ferric/ferrous ions leads to the formation of $^{\bullet}OH$:

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2 \quad (4)$$

Summing up reactions (3) and (4) gives the reaction referred to as ***Haber–Weiss reaction*** (Eq. 5), believed earlier to be responsible for the generation of hydroxyl radical in vivo. In fact, it is too slow to have any real significance, unless catalyzed by metal ions.

$$H_2O_2 + O_2^{\bullet-} \rightarrow {}^{\bullet}OH + {}^{-}OH + O_2 \quad (5)$$

Hydroxyl radical may be also formed by decomposition of peroxynitrite and in the reaction between hypochlorite and hydrogen peroxide (Reaction 6):

$$^{-}OCl + H_2O_2 \rightarrow {}^{\bullet}OCl + {}^{\bullet}OH + {}^{-}OH \quad (6)$$

Another source of hydroxyl radical is ionizing radiation:

$$H_2O(\text{ionizing radiation}) \rightarrow H_2O^* \rightarrow H^{\bullet} + {}^{\bullet}OH \quad (7)$$

where H_2O^* represents an excited water molecule (possessing excess energy deposited by ionizing radiation). Hydroxyl radical may be also produced by UV-induced hemolytic decomposition of hydrogen peroxide

$$H_2O_2(UV) \rightarrow 2\,{}^{\bullet}OH \quad (8)$$

and, albeit with much lower yield, during sonication of water and water-containing substances (Choe and Min 2006).

The velocity of a chemical reaction v between two compounds A and B depends on the concentrations of both compounds ([A], [B]) and the rate constant of the reaction k which is a measure of reactivity of both compounds (Eq. 9).

$$v = k[A][B] \quad (9)$$

Comparison of chosen rate constants demonstrates that hydroxyl radical is much more reactive than other ROS, although the extent of molecular damage depends also on the concentrations of individual species (Table 1). The hydroxyl radical has a very high electrochemical potential (ca +2.3V) which indicates that it is able to

Table 1 Examples of rate constants for reactions of superoxide radical anion, hydrogen peroxide, and hydroxyl radical. After (Bartosz 2003a; Regino and Richardson 2007)

Compound	k [M^{-1} s^{-1}]
Superoxide	
Glutathione	6.7×10^5
Cytochrome c	2.6×10^5
Methemoglobin	6×10^3
Hydrogen peroxide	
Glutathione	18
Papain	62
Metmyoglobin	140
Hydroxyl radical	
Glutathione	8×10^9
Papain	4.7×10^{10}
Hemoglobin	3.6×10^{10}

oxidize almost all molecules occurring in living systems (Halliwell and Gutteridge 1999; Bartosz 2003a).

An important free radical playing a fundamental signaling role is ***nitric oxide*** ($NO^{\bullet}$), being responsible, first of all, for smooth muscle relaxation and lowering blood pressure but also for retrograde signaling in the nervous system and having bactericidal action at high (micromolar) concentrations. Due to its unusual properties, in 1992 $NO^{\bullet}$ was acclaimed as the "molecule of the year 1992" by the *Science* Magazine (Koshland 1992). Nitric oxide is formed mainly by nitric oxide synthases (NOS) (EC 1.14.13.39) from arginine. NOS1 (nNOS) and NOS3 (eNOS) produce low amounts of $NO^{\bullet}$ upon activation by increased cytoplasmic Ca^{2+} concentration. NOS2 (iNOS) is constitutively active and produces large amount of $NO^{\bullet}$ for defensive purposes (Alderton et al. 2001). Small amounts of this compound can be produced by reduction of nitrite; interestingly, dietary nitrite may thus contribute to the beneficial effect of blood pressure lowering associated with consumption of vegetables (Bondonno et al. 2012).

Reaction of superoxide radical anion with nitric oxide produces ***peroxynitrite*** $ONOO^-$, anion of peroxynitrous acid HONOO:

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^- \tag{10}$$

Peroxynitrite is a strong oxidant capable of performing one- or two-electron oxidations of various compounds including antioxidants, lipids, and constituents of proteins and nucleic acids and is also a nitrating and nitrosylating agent. Peroxynitrous acid, when formed by protonation of peroxynitrite, decomposes or isomerizes rapidly (half-life time of about 1 s at neutral pH). Isomerization to nitric acid leads to loss of reactivity while decomposition (believed to represent ca 30 % of the reactivity of peroxynitrous acid) produces hydroxyl radical and nitrogen dioxide:

$$HOONO \rightarrow {}^{\bullet}OH + NO_2^{\bullet} \tag{11}$$

Reactions of peroxynitrite lead to lipid peroxidation, nitrosylation of –SH groups (formation of nitrosothiols –SNO), and nitration (introduction of the nitro group $–NO_2$),

especially of tyrosine and tryptophan residues in proteins and guanine in nucleic acids and also of other compounds, (e.g., γ-tocopherol). The nitration reaction is favored by the presence of carbon dioxide which reacts with peroxynitrite to form 1-carboxylato-2-nitrosodioxidane $ONOOCO_2^-$. The latter compound undergoes dissociation into carbonate radical anion $CO_3^{\bullet-}$ and nitrogen dioxide $NO_2^{\bullet}$:

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow CO_3^{\bullet-} + NO_2^{\bullet} \quad (12)$$

(Calcerrada et al. 2011; Bartosz 1996).

The main amino acid subject to nitration is tyrosine. 3-Nitrotyrosine formed in this reaction absorbs radiation in the wavelength range where tyrosine and tryptophan emit fluorescence (300–450 nm), and it is essentially nonfluorescent. Tyrosine nitration has been shown to alter protein functioning, with a change in catalytic activity, cell signaling, and cytoskeletal organization; proteins containing 3-nitrotyrosine cannot be phosphorylated by tyrosine kinases (Schopfer et al. 2003). Subsequently, signal transduction mediated by tyrosine kinases – such as trophic factors, nerve growth factors, and brain-derived neurotrophic factors – is disturbed and cell apoptosis may occur. Manganese-dependent superoxide dismutase (MnSOD) is nitrated by peroxynitrite in tyrosine-34 by a Mn-catalyzed process, which leads to enzyme inactivation (Yamakura and Kawasaki 2010).

Nitric oxide, other oxides of nitrogen, and peroxynitrite may be classified as ***reactive nitrogen species*** (RNS). Overproduction of reactive nitrogen species is referred to as nitrosative stress (Ridnour et al. 2004). This term does not seem to be the best as RNS induce both nitrosation and nitration, and in some cases (e.g., nucleic acids) nitration is the dominant effect. Some authors prefer to use the term ***nitrative stress*** (Roberts et al. 2009). "Nitrosative/nitrative stress" would be perhaps optimal but clumsy, so the term "nitrosative stress" will be used throughout this chapter.

Enzymatically catalyzed oxidation of chloride ions by hydrogen peroxide leads to the formation of ***hypochlorous acid*** (HOCl)***/hypochlorite*** ($^-$OCl):

$$H_2O_2 + Cl^- \rightarrow HOCl + HO^- \quad (13)$$

The main enzymes catalyzing this reaction are myeloperoxidase and lactoperoxidase. Myeloperoxidase can also oxidize the halides and the pseudohalide thiocyanate (SCN^-) to their corresponding hypohalous acids. However, owing to its high concentration in biological fluids (100–140 mM Cl^-, 20–100 μM Br^-, < 1 μM I^-, 20–120 μM SCN^-), Cl^- is the major substrate for MPO (Yap et al. 2007). As the pK_a value of hypochlorous acid is about 7.5, comparable amounts of hypochlorous acid and its anion are present at near-neutral pH. Hypochlorite is a strong bactericidal, oxidizing, and chlorinating agent and is produced for defensive purposes mainly by granulocytes which are rich in myeloperoxidase. Isolated neutrophils kill most ingested microorganisms rapidly by a myeloperoxidase-dependent mechanism that is almost certainly due to HOCl. However, individuals with myeloperoxidase deficiency rarely have problems with infection. A possible explanation is that HOCl provides a frontline response that kills most of the microorganisms, with survivors killed by non-oxidative processes (Winterbourn 2002; Winterbourn and Kettle 2013).

Reactions of hypochlorite with lipids induce peroxidation; its reactions with thiol groups of proteins lead to the formation of disulfides. Disulfides can also be oxidized by HClO to sulfinic acids (Prütz 1998). Reactions with amino groups of proteins, amino acids, sugars, and nucleosides form ***chloramines,*** among them *N*-chloro- or *N,N*-dichloroamino acids/amino acid residues.

$$R_1NH_2 + HOCl \rightarrow R_1NHCl + H_2O \tag{14}$$

$$R_1NHCl + HOCl \rightarrow R_1NCl_2 + H_2O \tag{15}$$

Chloramines are unstable and decompose spontaneously to aldehydes, with the release of ammonia and carbon dioxide. Their typical half-lives are about 10 min at 37 °C they decompose releasing chlorine (Bernofsky 1991) (Pattison and Davies 2006). Hypochlorite and chloramines are main ***reactive chlorine species*** (RCS) occurring in the body. Overproduction of RCS is referred to as ***chlorinative stress*** (Yap et al. 2007).

HOCl can react with nitrite (which is a breakdown product of NO• metabolism) to generate a less cytotoxic product nitryl chloride, NO_2Cl, possessing oxidizing, chlorinating, and nitrating ability. Nitrite levels in healthy subjects have been reported as around 1 μM in cerebrospinal fluid, and increased levels have been reported in diseased brains. Therefore, HOCl as a major oxidant formed by phagocytes in the presence of myeloperoxidase contributes to both oxidative and nitrative processes by the production of secondary species, •OH and NO_2Cl (Fig. 3). In view

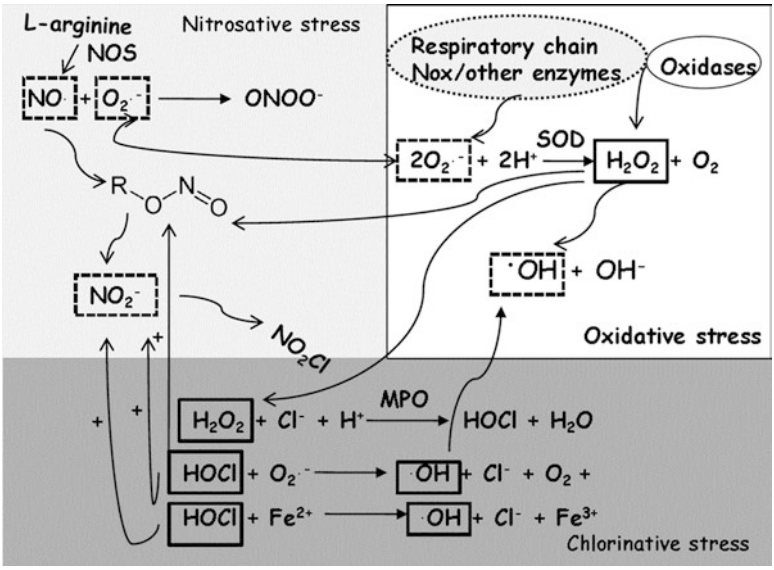


Fig. 3 Relation between oxidative stress, nitrosative stress, and chlorinative stress. MPO, myeloperoxidase. After (Yap et al. 2007), modified

of the above evidences, the highly reactive HOCl that is involved in both oxidative and nitrative processes is likely to be the primary oxidant responsible for the bystander effect of damage to central nervous system during neurodegenerative disorders (Whiteman et al. 2002; Yap et al. 2007).

Singlet oxygen is an excited form of the oxygen molecule formed by the excitation of ground-state (triplet) oxygen molecule with UV radiation or light in the presence of photosensitizers (Pattison et al. 2012; Kruft and Greer 2011).

Ozone is another reactive oxygen species, a minor constituent of the stratosphere but very important due to absorption UV radiation, formed also in the atmosphere mainly by photochemical reactions and electric discharge (Srebot et al. 2009).

3 The Antioxidant System

In an organism, ROS are under control of antioxidant proteins and low-molecular-weight antioxidants, keeping their local concentrations at optimally low levels. In chemical terms, antioxidant is "any substance that when present at low concentrations, compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell et al. 1995). This definition, however, is not sufficient in the biological context where antioxidants are perceived as substances preventing or counteracting the effects of uncontrolled oxidation at the cellular or organismal level. Such a broader definition of antioxidants includes several classes of compounds: (i) antioxidant enzymes which decompose ROS, (ii) nonenzymatic antioxidant proteins and other macromolecules, (iii) low-molecular-weight cofactors of antioxidant enzymes, (iv) low-molecular-mass ROS scavengers, (v) metal chelators, (vi) inhibitors of prooxidant enzymes, and (vii) inducers of biosynthesis of antioxidant proteins. This dynamic, spatially differentiated and overlapping network of antioxidants enables efficient control of ROS reactions allowing for the performance of their signaling functions and attenuating oxidative stress.

3.1 Antioxidant Enzymes

Our body is endowed with enzymes capable of reacting with the less reactive partially reduced oxygen species: superoxide and hydrogen peroxide. There is no protein able to react enzymatically with hydroxyl radical which is obviously impossible due to the high and nonspecific reactivity of this radical. Also, there are no enzymes reacting catalytically with hypochlorite and peroxynitrite. Some heme proteins are able to decompose peroxynitrite in a catalytic reaction, but this reaction is accompanied by other ones leading to protein inactivation. The enzymatic system controlling the cellular level of ROS consists mainly of the "enzymatic triad" of superoxide dismutases, catalases, and glutathione peroxidases and of other enzymes in mammalian cells; among them, thioredoxin peroxidases (peroxiredoxins) are also of considerable importance.

Superoxide dismutases (SODs) (EC 1.15.1.1) accelerate the reaction of dismutation of superoxide radical anion (Reaction 2). This reaction is relatively rapid even in the absence of SOD. Its rate constant is pH dependent, but at optimal pH (about 5) it reaches a value of 10^5 M^{-1} s^{-1}. The rate constant for the reaction of SOD with superoxide exceeds 10^9 M^{-1} s^{-1}, i.e., the enzyme accelerates the reaction by at least four orders of magnitude.

Three types of SOD are present in our body: a cytosolic enzyme containing copper and zinc (CuZnSOD, SOD1), a dimeric protein of molecular weight of about 32,000 composed of two identical subunits; a mitochondrial enzyme containing manganese at the active site (MnSOD, SOD2) being a homotetramer of subunits of molecular weight of about 23,000; and extracellular enzyme (EC-SOD, SOD3), also containing Cu and Zn but being a homotetramer, of molecular weight of about 135,000. CuZnSOD is an unusually stable enzyme, resistant to high temperatures, denaturing agents, and proteolysis, stable in a broad pH range exceeding 5–10 (Zelko et al. 2002).

SODs are ubiquitous in aerobic cells. The reasons for this phenomenon are not obvious because superoxide is not especially reactive and undergoes spontaneous dismutation anyhow. However, the lack of SOD leads to elevation of the steady-state level of superoxide which may lead to augmented damage to various biomolecules. It has been speculated that the efficient decomposition of superoxide may prevent the iron-catalyzed Fenton reaction; but, superoxide is not the only compound able to reduce Fe^{3+}. Perhaps the rapid decomposition of superoxide is important for the prevention of peroxynitrite formation. Indeed, the reaction between superoxide and nitric oxide (Reaction 10) is very rapid, its rate constant being even higher than that of the reaction of SOD with superoxide. Normally, nitric oxide is present at nanomolar levels while the intracellular concentration of SOD is micromolar (SOD is an enzyme present at concentrations higher than those of its substrate) and outcompetes nitric oxide in the reaction with superoxide. Excessive production of nitric oxide may reverse this situation, however.

Mutations of CuZnSOD result in a familial form of amyotrophic lateral sclerosis (Redler and Dokholyan 2012). Mice with MnSOD knockout die several days before or after birth. Deficiency of EC-SOD, leading to an increased level of superoxide, is associated with elevated blood pressure due to the higher conversion of nitric oxide to peroxynitrite (Fattman et al. 2003).

Catalase (EC 1.11.1.6) is a homotetrameric hemoprotein which catalyzes the reaction of dismutation of hydrogen peroxide (Nicholls 2012):

$$2H_2O_2 \rightarrow 2H_2O + O_2 \tag{16}$$

Glutathione peroxidases (EC 1.11.1.9) containing selenocysteine at the active site reduce hydrogen peroxide at the expense of oxidation of glutathione (GSH) to glutathione disulfide (GSSG):

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG \tag{17}$$

They may also reduce organic hydroperoxides LOOH to corresponding alcohols

$$\mathrm{LOOH} + 2\mathrm{GSH} \rightarrow \mathrm{LOH} + \mathrm{GSSG} + \mathrm{H_2O} \tag{18}$$

thus preventing re-initiation of lipid peroxidation chain reaction. There are eight human isoenzymes of glutathione peroxidases showing different tissue localization. The induction of oxidative stress in selenium deficiency is due, to a large extent, to the diminution in activities of glutathione peroxidases (Brigelius-Flohe and Flohe 2003; Brigelius-Flohe 1999).

GSSG produced by glutathione peroxidase is reduced back by ***glutathione reductase*** (EC 1.6.4.2) which oxidizes NADPH (Perham et al. 1991):

$$\mathrm{GSSG} + \mathrm{NADPH} + \mathrm{H^+} \rightarrow 2\mathrm{GSH} + \mathrm{NADP^+} \tag{19}$$

Excess GSSG may be actively transported out of cells by some of transporters of the ABCC (MRP) family.

Glutathione S-transferases (EC 2.5.1.18) catalyze the conjugation of electrophile xenobiotics and also endogenous compounds, including products of lipid peroxidation, to glutathione, forming less toxic glutathione *S*-conjugates, actively exported by ***ABCC transporters*** (Raza 2011).

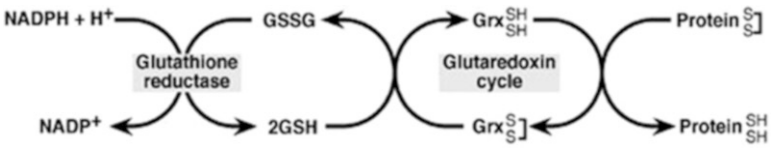
Hydrogen peroxide is also removed by thioredoxin peroxidases (***peroxiredoxins***, EC 1.11.1.15), which catalyze the reaction of oxidation of thiol groups of small proteins, ***thioredoxins***.

$$\begin{array}{l} \text{Thioredoxin} + \mathrm{H_2O_2} \rightarrow \text{Thioredoxin} + 2\ \mathrm{H_2O} \\ \quad\ |\quad\ | \qquad\qquad\qquad\ \ |\quad\ | \\ \quad \mathrm{HS} \quad \mathrm{SH} \qquad\qquad\quad \mathrm{S{-}{-}{-}S} \end{array} \tag{20}$$

Oxidized thioredoxins are reduced by ***thioredoxin reductases*** (EC 1.8.1.9) at the expense of NADPH.

$$\begin{array}{l} \text{Thioredoxin} + \mathrm{NADPH} + \mathrm{H^+} \rightarrow \ \text{Thioredoxin} + \mathrm{NADP^+} \\ \quad\ |\quad\ | \qquad\qquad\qquad\qquad\ \ |\quad\ | \\ \quad \mathrm{S{-}{-}{-}S} \qquad\qquad\qquad\quad \mathrm{HS} \quad \mathrm{SH} \end{array} \tag{21}$$

The thioredoxin system may also reduce oxidized protein thiol groups, although the main role in this case is played by the *glutaredoxin system* in which oxidized ***glutaredoxins*** are reduced by glutathione and oxidized glutathione by glutathione reductase:



(22)

NAD(P)H: quinone oxidoreductase (***DT-diaphorase***; EC 1.6.99.2) is the enzyme catalyzing two-electron reduction of quinones to semiquinones, thus omitting the step of semiquinone free-radical intermediates which are prone to autoxidation

generating superoxide and other ROS. ***Epoxide hydrolases*** (EC 3.3.2.3) convert reactive epoxides into *trans*-dihydrodiols. Both these enzymes perform therefore an antioxidant function (Arand et al. 2005).

Thiol-disulfide oxidoreductase (TD+OR) (EC 1.8.4.2) enzymes, particularly thioltransferase and thioredoxin (TRx), provide the primary cellular mechanism for repair of oxidized protein sulfhydryls by catalyzing the reduction of disulfide bonds or sulfenic acids. This action of TDOR enzymes is termed "dethiolase activity" because it results in the removal of a thiol compound (usually GSH) from a protein mixed disulfide. The thioltransferase catalytic cycle is coupled to glutathione and glutathione reductase, and the thioredoxin redox cycle is coupled to a specific thioredoxin reductase.

Methionine sulfoxide reductases (EC 1.8.4.11) repair methionine residues. ***Sulfiredoxins*** (EC 1.8.98.2) reduce "overoxidized" peroxiredoxins in which thiol groups are oxidized to sulfinic derivatives (believed until recently to be an irreversible step of thiol oxidation). ***DNA repair enzymes*** are very important in the defense against the effects of ROS-induced damage. Proteins (except for repair of oxidized thiol and methionyl groups) are generally not subject to repair, but oxidized proteins are generally better substrates for ***proteolytic*** *enzymes*, including proteasomes, which also play a role in removal of the effects of oxidative stress (Valko et al. 2007; Bartosz 2003a).

3.2 Nonenzymatic Antioxidant Proteins and Other Macromolecules

Proteins are predominant components of cells and extracellular medium in terms of mass fraction and therefore are the main primary target of ROS, especially due to the reactivity of cysteine, methionine, tryptophan, and tyrosine residues. Some proteins seem to play an important antioxidant function, protecting other biomolecules. Such a role has been ascribed to *albumin* in blood plasma. *Nuclear proteins*, especially histones, may act as antioxidants with respect to DNA (Prajapati et al. 2011).

Melanins, dark polymer pigments derived mainly from tyrosine, protect against UV but are also ROS scavengers (Page et al. 2011). Polysaccharides may have also an antioxidant role, e.g., polysaccharides of cell surface with respect to exogenous ROS.

3.3 Nonenzymatic ROS Scavengers

Low-molecular-weight ROS scavengers include glutathione, ascorbic acid (vitamin C), NAD(P)H and uric acid (hydrophilic antioxidants), tocopherols and tocotrienols (vitamin E), carotenoids, bilirubin, dihydrolipoic acid, and ubiquinol (hydrophobic antioxidants). Low-molecular-weight ROS scavengers may intercept reactive oxygen species before they react with critical biomolecules (preventive action), inhibit chain

of free-radical reactions (interventive action), and repair biologically important molecules transformed into free-radical forms (repair action) (Kirsch and De Groot 2001).

3.3.1 Hydrophilic ROS Scavengers

The tripeptide ***glutathione*** (γ-glutamylcysteinylglycine) is the main hydrophilic intracellular antioxidant, present at millimolar concentrations, being a substrate for glutathione peroxidases and glutathione *S*-transferases and a cofactor in the glutaredoxin system, but acting also in a nonenzymatic manner.

Ascorbic acid is present in blood plasma at a concentration of about 50 μM and in some cells at higher concentrations (up to millimolar). Upon hydrogen donation to an oxidant, ascorbic acid forms a relatively stable semi-dehydroascorbic radical which may be further oxidized to dehydroascorbic acid.

Uric acid is the predominant antioxidant in the blood plasma of humans and other primates which lost the ability to metabolize it further; it is present in the plasma at concentrations of several hundred μM.

Carnitine, acting as transporter of fatty acids from the cytosol into the mitochondria and peroxisomes for β-oxidation, often used as a nutritional supplement, is also an efficient ROS scavenger.

Plants contain a variety (over 8,000) of phenolic compounds of antioxidant properties which, however, have limited bioavailability. ***Flavonoids***, the most important polyphenol groups, include flavones, flavonols, flavanones, flavanonols, and anthocyanins. The most ubiquitous flavonoid is quercetin. Soybeans contains high isoflavone levels and are the major dietary source of these compounds (e.g., genistein, daidzein, and glycitein) in humans. Flavonoids inhibit free-radical reactions and complex metal ions (Kris-Etherton et al. 2002; see also chapter "Antioxidant Interventions in Neuropsychiatric Disorders").

Flavonoids are usually found as glycosides; these derivatives are more hydrophilic than aglycones. However, glycosides are not detected in human blood and urine. Hydrolysis of glycosides is performed by microorganisms in the colon, and its efficiency may limit the availability of these compounds. In any case, their antioxidant activity may be important for the digestion process to ameliorate the oxidative stress to the stomach and intestine (Halliwell 2007).

3.3.2 Hydrophobic ROS Scavengers

Vitamin E is the collective name for a set of 8 related α-, β-, γ-, and δ-tocopherols and the corresponding four tocotrienols, Of these, α-tocopherol has been the most studied as it has the highest bioavailability. It has been claimed that α-tocopherol is the most important lipid-soluble antioxidant and that it protects cell membranes from oxidation by reacting with lipid radicals produced in the course of peroxidation thus acting as a chain-breaking antioxidant (Yoshida et al. 2007).

Ubiquinone, a component of the respiratory chain, is blamed for being a major source of superoxide formation in the mitochondria, due to reaction of its ubisemiquinone free-radical form with oxygen. However, its reduced form (***ubiquinol***) is thought to act as a lipid-soluble antioxidant (Chong-Han 2010). A similar role is ascribed to ***lipoic acid*** (Gorąca et al. 2011).

Bilirubin, the end product of heme degradation, was demonstrated to be an efficient antioxidant (although its excess is neurotoxic, especially to newborns). It has been postulated that bilirubin, oxidized by ROS to biliverdin and reduced back at the expense of NADPH by ***biliverdin reductase*** (EC 1.3.1.24), may be an important antioxidant to protect cell membranes in an analogous way as glutathione protects the aqueous compartments of the cell (Sedlak et al. 2009).

Another class of lipid-soluble antioxidants are ***carotenoids***. From over 600 naturally occurring carotenoids, more than 400 are regularly consumed in the diet. There are two classes of carotenoids: carotenes (tetraterpenoid hydrocarbons without oxygen atoms) and xanthophylls (which contain oxygen in the form of hydroxyl, methoxyl, carboxyl, keto, or epoxy groups). ***Lycopene*** and ***β-carotenes*** are typical carotenes while ***lutein*** present in green leaves and ***zeaxanthin*** present in corn are typical xanthophylls. Double bonds in carotenoids are conjugated, and usually the *all-trans* forms are found in plant tissues. Carotenoids are the most efficient singlet oxygen quenchers in biological systems, their efficiency increasing with the increasing number of double bonds. Carotenoids with 7 or less soluble bonds are not effective quenchers. One molecule of β-carotene can quench 250–1,000 molecules of singlet oxygen at a rate of $1.3*10^{10}$ M^{-1} s^{-1}. Singlet oxygen quenching by carotenoids is of physical nature and involves no chemical reaction, generating no oxidizing product (Min and Boff 2002; Riccioni et al. 2012). ***Melatonin*** is a good antioxidant, but its concentrations, even in persons taking it as a supplement, seem too low to make melatonin a significant antioxidant via its direct action. However, induction of antioxidant proteins by this hormone may be of importance (Galano et al. 2011).

3.4 Metal Ion Chelators

As metal ions are important for aggravation of oxidative damage, due to autoxidation and, first of all, participation in the Fenton reaction and decomposition of lipid hydroperoxides, metal-sequestering proteins involved in transport and storage of transition metals (e.g., iron-storage proteins ***ferritin*** and ***lactoferrin***, ***haptoglobin*** responsible for hemoglobin sequestration and ***hemopexin*** responsible for heme sequestration in blood plasma, copper-storage protein ***ceruloplasmin***, ***transferrin*** responsible for iron transport, ***metallothioneins*** binding exogenous metals) are important elements of antioxidant defense. Ceruloplasmin, apart from binding copper, protects also against the prooxidative action of Fe^{2+}, acting as a ferroxidase, i.e., oxidizing Fe^{2+} to Fe^{3+}.

The antioxidant action of the dipeptide ***carnosine*** (β-alanyl-L-histidine) present at high millimolar concentrations in the brain and muscle seems to be due mainly to its metal-chelating activity. Carnitine and many flavonoids are also good metal scavengers (Begum et al. 2005; Jomova and Valko 2011).

4 The Signaling Role of ROS

The signaling role of nitric oxide has been widely recognized. Also ROS, especially hydrogen peroxide, has been demonstrated to participate in signal transmission within and between cells. It has been shown that many cells increase the production of superoxide (and, eventually, hydrogen peroxide) upon stimulation with cytokines, growth factors, and hormones, e.g., interleukin 1β, interleukin 3, interleukin 6, tumor necrosis factor α, angiotensin II, fibroblast growth factor, granulocyte–macrophage colony-stimulating factor, platelet-derived growth factor, nerve growth factor, and transforming growth factor β1, acting as secondary messengers of these factors (Valko et al. 2007; Thannickal and Fanburg 2000). ROS were shown to mediate also the control of such cellular events as cell adhesion and spreading as well as wound healing, affect the activities of transporters and proteinases, and activate heat shock proteins (Bartosz 2009). Induction of ROS production upon action of noxious stimuli such as lipopolysaccharide of gram-negative bacteria, hyperoxia, hyperthermia, UV, and shear stress involves activation of Nox and serves apparently signaling purposes (Park et al. 2006; Parinandi et al. 2003; Sorescu et al. 2004).

Activities of numerous transcription factors are affected by ROS, among them NF-κB, AP-1, Nrf2, HIF-1α, Hic-5, and p53. Nrf2 is the most important regulator of cell defense against chemical and oxidative stress which coordinatively controls expression of cytoprotective genes via the antioxidant-responsive element (ARE), also defined as electrophile-responsive element. The list of genes controlled by Nrf2 includes those coding for detoxification phase II enzymes, glutathione *S*-transferase, and heme oxygenase-1. Under basal conditions, the transcription factor Nrf2 is anchored by the Keap1 protein within the cytoplasm and targeted for ubiquitination and proteasomal degradation. Activation of Nrf2 may occur via two main mechanisms: modification of Cys residues within Keap1 and phosphorylation of Nrf2. Human Keap1 protein contains 27 cysteine residues, 9 of them highly reactive. Alkylation or oxidation of Cys residues of Keap1 leads to Nrf2 activation. ROS oxidize Cys 273 and Cys 288 on Keap1 which leads to dissociation of Nrf2 from the complex, translocation of Nrf2 to the nucleus, and activation of target genes (Copple et al. 2008).

Hydrogen peroxide, the main ROS participating in signal transmission, oxidizes cysteines to disulfides or to sulfenic acids –SOH. Sulfenic acids may be converted to disulfides upon reaction with another thiol. The disulfide bonds can be reduced by glutaredoxins, thioredoxins, or glutathione (GSH) so this modification is reversible. *S*-Glutathionylation (formation of a mixed protein–glutathione disulfide) is

thought to prevent further, irreversible oxidation of cysteine sulfur. It is often viewed as a general reversible protein modification, comparable to protein phosphorylation (Townsend 2007). Glutathionylation and deglutathionylation are catalyzed by glutaredoxins. Further oxidation of a thiol group leads to a sulfinic acid $–SO_2H$ or sulfonic acid $–SO_3H$. Both these reactions were considered to be irreversible; however, ***sulfiredoxins***, enzymes capable of ATP-dependent reduction of sulfinic acid residues, have been identified (Lei et al. 2008). In addition to sulfiredoxins, ***sestrins*** are also able to reduce sulfinic acids (Budanov et al. 2004).

Thiol groups are reactive mainly in their deprotonated form, i.e., as thiolates $–S^-$. The pK_a of most cysteine thiols in proteins and of GSH is about 8.5 so these groups are mainly protonated. Therefore Cys residues of low pK_a, being ionized at physiological pH, may be selectively oxidized, even in the presence of excess of other Cys residues (Kim et al. 2000). Among proteins most susceptible to thiol oxidation are protein phosphatases, especially protein tyrosine phosphatases (PTPs); G proteins; some ion channels; and some transcription factors. PTPs have a catalytically important low pK_a (4.7–5.4) Cys residue which at neutral pH resides as a thiolate anion. This low-pK_a Cys is crucial for the enzyme activity and, at the same time, makes this amino acid susceptible for oxidation. The list of phosphatases consistently reported to be regulated by reversible thiol oxidation includes PTP1B, a phosphatase dephosphorylating, i.e., insulin receptor and mediating insulin resistance, protein phosphatase 2B (calcineurin) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a dual function phosphatase acting on proteins or inositol-3-phosphate (IP_3). Since the level of phosphorylation of substrates is a resultant of the rates of phosphorylation and dephosphorylation, oxidative inactivation of PTPs increases the level of tyrosine phosphorylation of protein substrates. Inactivation of PTEN amplifies the effects of IP_3 signaling (Janssen-Heininger et al. 2008).

If ROS participate in cellular signaling, their presence and transient increases of their local concentrations in the cells are not only unavoidable but also necessary. However, the signaling role of ROS is prone to impairment. Many vascular proinflammatory/proatherogenic states (hypertension, diabetes, hypercholesterolemia) are associated with elevated expression of Nox2 and possibly Nox1 in the vessel wall. Increased generation of ROS by Noxs is involved in the hypertension via augmented scavenging of nitric oxide. Amyloid-β peptide was demonstrated to activate ASK-1 in neurons and Nox2 of microglia, increasing ROS generation. Both effects lead to apoptotic neuronal death. Nox2 was also found to be activated in the spinal cord of patients with amyotrophic lateral sclerosis (Lambeth 2007). Nox, especially Nox2, may be involved in myocardial infarction as Nox2-deficient mice are protected from the deleterious consequences of infarction (Lambeth et al. 2008). Activation of Nox1 and Nox2 was found to play a role in endothelial dysfunction in diabetes (Wendt et al. 2005). Nox2, apart from other sources of ROS, seems to contribute significantly to injuries due to ischemic stroke. Ethanol-induced hepatitis seems to be mediated by Nox activation since expression of Nox4, Duox1, and Duox2 is strongly augmented and Nox inhibitors ameliorate alcoholic liver injury (Lambeth et al. 2008).

Thus, it seems that quite often oxidative stress is in fact due to malfunction of the signaling role of ROS. A redefinition of oxidative stress has been even proposed: oxidative stress is a "disruption of redox signaling and control" (Jones 2006). In any case, however, oxidative stress is characterized by intensification of reactions of uncontrolled oxidations.

5 Markers of Oxidative, Nitrosative, and Chlorinative Stress

The assessment of oxidative, nitrosative, and chlorinative stress in the cause of diseases is often of considerable importance. Though these stress markers rarely have a diagnostic value, they may be useful indicators of the stage/severity of the disease and point to the need of antioxidant supplementation. Oxidative (nitrosative, chlorinative) stress is usually a local event, focused in the affected organ which, however, may not be easily (or at all) accessible for biochemical analysis, as it is with the brain. Therefore, oxidative (nitrosative, chlorinative) stress is most often assessed in patients at the whole-body level, basing on the available material (blood, urine, exhaled breath condensate). Cerebrospinal fluid is a better material to study oxidative stress in the central nervous system, but its acquisition is troublesome. The rationale for the indirect approach is that products of oxidative damage are released into the circulation and excreted (if not subject to metabolism). Sometimes, oxidative (nitrosative, chlorinative) stress is not limited to the affected organ but similar changes occur also in cells of other organs due to common mechanisms of regulation. It is not always the case and results of analysis may be "diluted" with respect to those which could be done in the affected organ. Development of imaging techniques gives a promise to overcome this problem; however, routine analytical methods of today must rely mainly on the assessment of oxidative stress at the whole-body level.

Apparently, the simplest assay of oxidative stress would consist in an estimation of the level of ROS or RNS in the affected organ. This is impossible due to the low levels of ROS/RNS which are below the detection limits of techniques such as electron spin resonance (ESR) or nuclear magnetic resonance (NMR). The only exception concerns nitric oxide production measuring the level of nitrite as a metabolite of nitric oxide. However, this biomarker is far from being specific as nitrite may come from other sources (Viinikka 1996; Tsikas 2007). Estimates of the steady-state levels of superoxide and hydrogen peroxide in erythrocytes brought values of an order of $(2–5)\times10^{-13}$ M and 5×10^{-11} M, respectively (Johnson et al. 2005). Even if these are higher for other cells and elevated in pathology, they are still too low to allow for a direct detection. A plethora of spin traps enabling more sensitive and specific detection of free radicals and fluorescent probes for detection of ROS and RNS in vitro is available (Bartosz 2006; Wardman 2007). In principle, they could be used for ex vivo analyses, but possibilities of artifacts are enormous. Attempts have been made, mainly on animal models, to estimate ROS and RNS production in vivo using exogenous spectroscopic probes, but they pose serious problems due to transport,

metabolism, and toxicity. Therefore the optimal strategy for detection of oxidative stress is nowadays to look for products of reaction of ROS and RNS with endogenous substrates.

Most studies of reactions of ROS, RNS, and RCS with body components have concerned lipids, proteins, and DNA, and markers of oxidative (nitrosative, chlorinative) stress which are products of modification of these molecules. ROS, RNS, and RCS may also deplete cellular and extracellular antioxidants. Decreased levels of antioxidants may be also markers of oxidative stress, although homeostatic compensatory mechanisms must be taken into account; depletion of endogenous antioxidants (e.g., glutathione) may be followed by upregulation of their biosynthesis leading to restoration of the normal level or even an overshoot.

There are several criteria for a good biomarker including markers of oxidative stress in diseases. A valid biomarker should be:

- A major product of oxidative modification that may be implicated directly in the development of disease
- A stable product, not susceptible to artifactual induction or loss during storage
- Representative of the balance between oxidative damage generation and clearance (i.e., the steady state, but also possibly applicable to the measurement of cumulative oxidative damage)
- Determined by an assay that is specific, sensitive, reproducible, and robust
- Free of confounding factors from dietary intake
- Measurable within the limits of detection of a reliable analytical procedure

In practice, all these criteria are rarely met. In particular, the stability of a material stored for determination of oxidative/nitrosative stress biomarkers is limited; some products may be lost, and some formed during storage (Griffiths et al. 2002). The material should be therefore stored at a deep freezer (−80°C), optimally under argon or nitrogen for a limited time, and should not be thawed and refrozen.

5.1 Markers of Lipid Oxidative Lipid Modifications

5.1.1 Introduction

Free radical-induced lipid peroxidation is a chain reaction consisting of three distinctive steps: initiation, propagation, and termination. In the initiation step, the hydrogen atom is removed from a molecule LH and a free-radical form of lipid (alkyl radical) L• is formed:

$$\mathrm{LH} \rightarrow \mathrm{L}^{\bullet} + \mathrm{H}^{\bullet} \tag{23}$$

The hydrogen bound to carbon separating carbon involved in the C=C bond is the easiest to be detached; therefore, polyunsaturated fatty acids or phospholipids containing such residues are most prone to peroxidation. Linoleate was found to be 40 times more prone to peroxidation than oleate and linolenate 2.4 times more prone

than linoleate. Heat, UV and visible light, and metal catalysts can accelerate this step. It is usually initiated by ROS such as hydroxyl, peroxyl or hydroperoxyl radicals (but not superoxide or hydrogen peroxide), or hemoproteins activated to the ferryl state.

Initiation step leads to a shift of double bonds in residues of polyunsaturated fatty acids producing conjugated dienes. In the propagation step, alkyl radical reacts with oxygen to form peroxyl radical which, in turn, can abstract hydrogen atom from another lipid molecule:

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \tag{24}$$

The reaction proceeds rapidly, as characterized by rate constant $k = 3*10^8\ M^{-1}\ s^{-1}$.

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \tag{25}$$

This reaction is relatively slow (rate constant of $10\ M^{-1}\ s^{-1}$).

Reactions between two radicals terminate the reaction:

$$LOO^{\bullet} + L^{\bullet} \rightarrow LOOL \tag{26}$$

$$L^{\bullet} + L^{\bullet} \rightarrow LL \tag{27}$$

Rearrangement of peroxyl radicals and hydroperoxides may lead to the formation of more stable peroxidation products such as isoprostanes which have prostaglandin-like structures but are not synthesized by cyclooxygenase. F_2-isoprostanes, formed from arachidonic acid residues present in phospholipids, are most abundant in blood plasma and urine. Analogs of F_2-isoprostanes may be formed by peroxidation of other polyunsaturated fatty acid residues. Peroxidation of eicosapentaenoic acid (EPA) leads to the production of F_3-isoprostanes while peroxidation of docosahexaenoic acid (DHA) yields F_4-isoprostanes, also termed neuroprostanes due to the high levels of their precursor in the brain. Alternatively, intermediates and products of lipid peroxidation are subject to further reactions producing secondary lipid oxidation products. Homolytic β-scission of the C–C bond of alkoxyl radicals produces further degradation products (aldehydes, ketones, acids, alcohols, and short-chain hydrocarbons). One of the main aldehyde products of lipid peroxidation is 4-hydroxy-2-nonenal (4-HNE), formed by degradation of polyunsaturated fatty acids such as arachidonic and linoleic acids. Less polyunsaturated fatty acids, containing three double bonds, produce 4-hydroxy-2-hexanal. Aldehyde products of lipid peroxidation include malondialdehyde (MDA), glyoxal, and acrolein.

Cholesterol is also oxidized by ROS, although its oxidation rate is relatively slow compared to phospholipids containing polyunsaturated fatty acid residues. Hydroxyl radical abstracts hydrogen from cholesterol, mainly the C-7 hydrogen, producing free radicals. Main cholesterol oxidation products found in food are 7-ketocholesterol, β-epoxycholesterol, and α-epoxycholesterol. Cholesterol oxidation products have been shown to be more injurious to arterial cells than pure cholesterol and more

likely to induce atherosclerosis and coronary heart disease. They also affect cell membrane functions, especially permeability (Hur et al. 2007).

The biological activities of MDA and other aldehydes include cross-linking of proteins and DNA, which alters the function/activity of these molecules. MDA can react with amino and thiol groups. Aldehydes are more diffusible than free radicals and can transmit oxidative damage to distant sites. Aldehydes are quickly removed from cells since several enzymes control their metabolism (Muzio et al. 2012).

Nitration of unsaturated fatty acids/fatty acid residues in phospholipids yields nitro derivatives. These molecules are generated in vivo as an adaptive response to oxidative inflammatory conditions and manifest predominantly anti-inflammatory signaling reactions. The actions of nitrated fatty acids are diverse, with these species serving as a potential chemical reserve of $NO^{\bullet}$, reacting with cellular nucleophiles to modify proteins and other molecules (Baker et al. 2009).

The action of hypochlorite on lipids induces lipid peroxidation but induces also specific modifications. Addition of HOCl to a double bond –C=C– produces relatively stable chlorohydrins –COH–CCl– which may be markers of the action of hypochlorite on phospholipids and cholesterol (Winterbourn and Kettle 2000; Carr et al. 1996).

5.1.2 Malondialdehyde

Determination of the level of MDA with thiobarbituric acid (TBA) in blood plasma or urine is one of the mostly frequently used methods in free-radical research. In the TBA test, MDA reacts with TBA under acidic conditions and elevated temperature, forming an adduct that absorbs at 532 nm. An improved method uses the fluorescence of TBA–MDA adduct, which has an excitation wavelength of 531 nm and emits at 553 nm. There are several problems with this assay. Various laboratories use different protocols for the TBA test, which leads to different color intensity; therefore, results cannot be compared. Some lipid peroxidation products may decompose during the reaction; some protocols recommend addition of antioxidants (especially butylated hydroxytoluene, BHT) to the reaction mixture to prevent this effect. The reaction is fairly nonspecific; many substances such as biliverdin, acetaldehyde, sucrose, and reducing sugars, including deoxyribose, deoxyglucose, methionine, glutamic acid, and many others, react with TBA forming chromogens identical to a product of the reaction between MDA and TBA (Gutteridge 1981). The determination of MDA with the TBA test overestimates the free-radical damage. Determination of the TBA–MDA adduct by HPLC provides more reliable results (Griffiths et al. 2002).

5.1.3 Protein Adducts of Aldehyde Products of Lipid Peroxidation

Aldehyde products of lipid peroxidation including MDA, 4-HNE, and acrolein react with proteins. The level of aldehyde-modified proteins may be a measure of the level of these aldehydes over a period of time. They can be estimated either with specific antibodies or by mass spectrometry (MS) (Sayre et al. 2006; Borovic et al. 2006).

5.1.4 Conjugated Dienes

Conjugated dienes are primary products of the breakdown of fatty acids. They normally do not occur in living cells and are considered to be specific products of free radical-induced lipid peroxidation.

Conjugated dienes can be measured spectrophotometrically at 230–235 nm. A disadvantage of the diene assay is that many other biological substances, even the polyunsaturated fatty acids, absorb in the same UV region. Application of second derivative spectroscopy (Corongiu and Banni 1994) and of HPLC and GC–MS techniques increase the sensitivity and specificity of this assay (Iversen et al. 1985).

5.1.5 F_2-Isoprostanes and Neuroprostanes

It has been claimed that F_2-isoprostanes are the best marker of lipid peroxidation in vivo (Praticò et al. 2001). There are several methods of determination of F_2-isoprostanes including gas chromatography–negative ion chemical ionization–mass spectrometry (GC–NICI–MS), liquid chromatography–mass spectrometry (LC–MS), and immunochemistry (ELISA) (Milatovic and Aschner 2009; Breusing et al. 2010). Similar methods are used to estimate F_4-neuroprostanes and neurofurans, other peroxidation products containing a substituted tetrahydrofuran ring, which provide a unique insight into oxidative lipid damage occurring within neurons (Milatovic and Aschner 2009; Arneson and Roberts 2007).

5.1.6 Nitro-Fatty Acids

Qualitative and quantitative analysis of nitro-fatty acids and their esters is done mainly by LC–MS/MS and GC–MS/MS (Baker et al. 2009; Tsikas et al. 2011).

5.1.7 Chlorohydrins

Chlorohydrins are analyzed by HPLC or gas chromatography with flame ionization detection (FID) and mass spectrometry (MS) (Albert et al. 2009).

5.2 Markers of Protein Modification

5.2.1 Introduction

Proteins are a major target for oxidants as a result of their abundance in biological systems and their high rate constants for reaction. Kinetic data for a number of radicals and non-radical oxidants are consistent with proteins consuming the majority

of these species generated within cells. Oxidation can occur at both the protein backbone and on the amino acid side chains (Davies 2005).

Initial attack of reactive free radicals such as hydroxyl radical on protein leads initially to the formation of free radicals which then react with oxygen to produce peroxyl radicals (Protein-OO•) and eventually protein peroxides (Protein-OOH) (Eqs. 28, 29, and 30):

$$\text{Protein-H} + {}^{\bullet}\text{OH} \rightarrow \text{Protein}^{\bullet} + \text{H}_2\text{O} \tag{28}$$

$$\text{Protein}^{\bullet} + \text{O}_2 \rightarrow \text{Protein-OO}^{\bullet} \tag{29}$$

$$\text{Protein-OO}^{\bullet} + \text{RH} \rightarrow \text{Protein-OOH} + \text{R}^{\bullet} \tag{30}$$

Protein peroxides are relatively stable products in the absence of other reactants (half-life times of hours) but in the cells react further forming final oxidation products (Gebicki and Gebicki 1993). A list of most common side chain protein modifications is given in Table 2. Oxidative modifications of proteins increase protein surface hydrophobicity which enables their recognition and preferential digestion by proteasomes (Pacifici et al. 1993). However, heavily oxidized proteins, especially bound to lipid oxidation products to form lipofuscin, are less prone to proteolytic degradation and may inhibit proteasome activity (Grimm et al. 2012).

5.2.2 Carbonylated Proteins

Protein carbonyls may be generated by the oxidation of several amino acid side chains (e.g., in Lys, Arg, Pro, and Thr) (Dalle-Donne et al. 2003a). They may be also formed through oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains, leading to the formation of a peptide in which the N-terminal amino acid is blocked by an α-ketoacyl derivative. In addition, carbonyl groups may be introduced into proteins by secondary reaction of the nucleophilic side chains of Cys, His, and Lys residues, with aldehydes (4-HNE, MDA, and acrolein) produced during lipid peroxidation or with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a consequence of the reaction of reducing sugars, or their oxidation products with lysine residues of proteins (glycation and glycoxidation reactions), with the eventual formation of the advanced glycation/lipoxidation end products (AGEs/ALEs), that is, glycoxidation products, such as carboxymethyllysine and pentosidine, and lipoxidation products, such as malondialdehyde-lysine and 4-hydroxynonenal-protein adducts (Dalle-Donne et al. 2003b).

Protein carbonyls are widely used as chemically stable biomarkers of oxidative stress. They circulate for longer periods in the blood compared to other oxidized products, and the assay sample can be kept at −80 °C for at least 10 years (Stadtman and Levine 2003).

Table 2 Main oxidative modifications of protein side chains. After (Dalle-Donne et al. 2003a), modified

Amino acid	Modification products
Cysteine	Oxidation of a sulfhydryl group (Cys-SH) to form sulfenic (Cys-SOH), sulfinic (Cys-SO_2H), or sulfonic (Cys-SO_3H) groups
	Formation of a disulfide bond (Cys-S-S-Cys) between two nearby Cys residues within a protein molecule (intramolecular cross-linking) or between two protein molecules (intermolecular cross-linking)
	Formation of a mixed disulfide (Cys-S-S-glutathione) between a sulfhydryl group and glutathione (S-glutathionylation)
Histidine	2-Oxohistidine
Lysine	α-Aminoadipic semialdehyde (a carbonyl derivative)
Arginine	Glutamic semialdehyde (a carbonyl derivative)
Proline	2-Pyrrolidone (a carbonyl derivative)
Threonine	2-Amino-3-ketobutyric acid (a carbonyl derivative)
Lysine, cysteine, histidine	Formation of carbonyl derivatives by secondary reaction with reactive carbonyl compounds derived from oxidation of carbohydrates (glycoxidation products), lipids (malondialdehyde, 4-hydroxynonenal, acrolein), and advanced glycation and lipoxidation end products
Methionine	Methionine sulfoxide
Phenylalanine	*o*-Tyrosine, *m*-tyrosine
Tryptophan	*N*-Formylkynurenine, kynurenine, 5-hydroxytryptophan, 7-hydroxytryptophan, nitrotryptophan
Tyrosine	3,4-Dihydroxyphenylalanine (DOPA), 3-chlorotyrosine, 3-nitrotyrosine, dityrosine (Tyr–Tyr cross-links)

Many assays are available for detection of protein carbonyls. Highly sensitive assays for detection of protein carbonyls involve derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product. The measurement of protein carbonyls following their covalent reaction with DNPH was pioneered by Stadtman et al. (Levine et al. 1990) and has become the most widely utilized measure of protein oxidation in several human diseases. Stable DNP adduct can be detected by various means. The DNP group itself absorbs ultraviolet light (at about 370 nm) so that the total carbonyl content of a protein or mixture of proteins can be quantified by a spectrophotometric assay, which can be coupled to protein fractionation by HPLC to give greater sensitivity and specificity than measuring total carbonyls in a protein mixture (Dalle-Donne et al. 2003b).

Carbonylated proteins can also be analyzed be ELISA (Yan and Forster 2011). Application of this method to tissue homogenates containing nucleic acids can cause artifacts as DNA reacts strongly with DNPH. Treatment of the extracts with DNase + RNase or with streptomycin sulfate to precipitate nucleic acids dramatically reduce the apparent carbonyl content (Luo and Wehr 2009).

In order to identify carbonylated proteins, proteins derivatized with DNPH are subject to gel electrophoresis, Western blotting, and reaction with anti-DNP

antibodies (OxyBlot) (Tezel et al. 2005). Recently, methods based on fluorescent detection of carbonylated proteins have been proposed. Protein carbonyl may react with fluorescein-5-thiosemicarbazide fluorescence hydroxylamine, Alexa 488 fluorescence hydroxylamine, or fluorescent BODIPY, Cy3, and Cy5 hydrazides (Tamarit et al. 2012). N-aminoperylene-3, 4, 9, 10-tetracarboxylic-bismides (APTB) is unique in that the probe itself is not fluorescent but becomes fluorescent upon reaction with carbonyl groups (Yan and Forster 2011; Tamarit et al. 2012; Pazos et al. 2011). Western blot and immunodetection are no longer needed, shortening the procedure and increasing accuracy.

Protein carbonyls can be also analyzed by labeling with tritiated borohydride either in solution or before gel electrophoresis. The radioactive hydrogen can then be detected by standard methods. This method is highly sensitive and specific when applied to samples of purified proteins, but high backgrounds and poor specificity (as tritiated borohydride reacts also with Schiff bases) can complicate its application to unfractionated tissue supernatants (Dalle-Donne et al. 2003b).

5.2.3 Advanced Oxidation Protein Products (AOPP)

In 1996, a novel oxidative stress biomarker, referred to as advanced oxidation protein products (AOPP), was detected in the plasma of chronic uremic patients. AOPP were defined a novel marker of oxidative damage and are considered as a reliable marker to estimate the degree of oxidant-mediated protein damage. Two fractions of AOPP are present in the plasma, corresponding to a molecular mass of 60 (low-molecular-weight AOPP) and 600 kDa (high-molecular-weight AOPP). Formation of AOPPs could be induced in control plasma by chlorinating oxidants such as chloramines or hypochlorous acid but AOPP can be also formed by other oxidants (Alderman et al. 2002; Witko-Sarsat et al. 1996; Witko-Sarsat et al. 1999). The main component of AOPP is albumin; albumin aggregates are mainly linked by dityrosines formed between different protein molecules (Ramasamy et al. 2005).

AOPP are assayed by a simple spectrophotometric assay (Witko-Sarsat et al. 1996), which contributes to the increasing popularity of this biomarker of protein oxidation.

5.2.4 Oxidation of Thiol Groups

Cysteine ($HSCH_2–CHNH_2–COOH$) and cystine ($HOOCCHNH_2–CH_2–S–S–CH_2–CHNH_2–COOH$) have the property of being interconvertible, cysteine being reversibly oxidized to cystine. Both S–S bonds and SH groups are important determinants of conformational and, therefore, functional properties of protein molecules. There are only two covalent linkages between amino acids in nonmodified proteins: the peptide bond and the disulfide bond of cystine. The peptide bond determines the primary sequence of amino acids into chains. The S–S bond serves as the only covalent cross-link between two separate polypeptide chains (interchain disulfide bond) or

between loops of a single chain (intrachain disulfide bond). When these disulfide bridges are cleaved, proteins unfold and their biological activity is altered. The major role of SH and S–S groups lies, then, in determining and stabilizing the three-dimensional structure of proteins thereby preserving their functional properties. Oxidative stress leads to oxidation of protein thiols and formation of new disulfide bonds or sulfenic acids of cysteine residues.

Global oxidation of thiol groups is assessed usually spectrophotometrically with the Ellman reagent (Ellman 1959) or fluorimetrically (Hu 1994; Wright and Viola 1998). However, while this method is appropriate for blood plasma proteins where the contribution of nonprotein thiols is minimal, it poses difficulties for cell and tissue extracts where protein thiols are accompanied by low-molecular-weight thiols (mainly glutathione). Proteomic techniques based, e.g., on the "fluorescence switch" allow not only for estimation of protein thiol oxidation but also for the identification of proteins which undergo oxidation (Baty et al. 2005; Izquierdo-Álvarez et al. 2012).

5.2.5 Other Modifications of Protein Side Chains

Modifications of protein side chains (Table 2) are usually estimated using MS techniques, especially the analysis of peptide fragments by LC–MS/MS (Szuchman-Sapir et al. 2008; Schoneich and Sharov 2006). Proteomic techniques similar to those used to identify proteins subject to cysteine oxidation allow for identification of proteins undergoing other reversible modifications (*S*-glutathionylation, *S*-nitrosylation).

Loss of tryptophan and formation of dityrosines and N-formylkynurenine are sometimes assessed fluorimetrically (Rice-Evans et al. 1991; Robaszkiewicz et al. 2008). This is a simple assay but subject to artifacts and therefore cannot be recommended without reservation.

Blood plasma α1-antitrypsin is inactivated by oxidants due to oxidation of either methionine 581 or methionine 351 or methionine 358. Therefore, measurement of the activity of this protease inhibitor in blood plasma is another biomarker of oxidative stress mediated by methionine oxidation (Taggart et al. 2000).

5.2.6 Protein Fragmentation and Cross-linking

Protein radicals can also undergo peptide bond cleavage. Peptide bond cleavage can occur also by hydroxyl radical-initiated attack of the glutamic acid and proline residues of proteins (Stadtman 2004).

Protein oxidation can also lead to the generation of various types of intra- and intermolecular cross-links. Intermolecular cross-links produce protein aggregates. Oxidation of protein cysteine groups results in disulfide –S–S– cross-linked proteins while the oxidation of tyrosine residues to –Tyr–Tyr– cross-linked derivatives. Direct interaction of two carbon-centered radicals on different protein molecules forms –C–C– protein cross-links (Stadtman and Levine 2000). Interaction of carbonyl groups obtained in direct oxidation of amino acid side chains with lysine amino

groups forms Schiff-based cross-linked products; the interaction of glycation/glycoxidation-derived protein carbonyls with either a lysine or an arginine residue on a different protein molecule results in Schiff-based cross-linked products (Thorpe and Baynes 2003). The Michael addition reaction of either cysteine thiol groups, lysine amino groups, or histidine imidazole groups of proteins with the double bonds of aldehydes obtained by lipid peroxidation (e.g., 4-HNE, MDA) produces Schiff-based cross-linked derivatives (Grune and Davies 2003).

Electrophoresis under reducing and nonreducing conditions coupled with Western blotting and detection of individual proteins with specific antibodies allows to detect protein cross-linking and differentiate between cross-links due to disulfide formation (which are cleaved by dithiothreitol or β-mercaptoethanol) and other types of cross-links.

5.2.7 Protein Nitration

Specific markers of nitrosative/nitrative stress are protein nitration and nitrosylation. While nitrosylation is a reversible modification of thiol groups and nitrosylated proteins may be a reservoir of nitric oxide (Ishibashi et al. 2003), protein nitration is a stable and apparently irreversible protein modification. Tyrosine residues are the main substrate for nitration; also, tryptophan and histidine residues may be nitrated albeit with much lower yield (Nuriel et al. 2011) so nitrotyrosine is the marker of choice. Initially it was believed that the formation of nitrotyrosine is a footprint of peroxynitrite formation; it became obvious then that there are other mechanism of nitration; however, in any case the formation of nitrotyrosine is a marker of nitrosative stress (Castro et al. 2011).

The 3-nitrotyrosine in biological samples has been quantified by a variety of methods. Antibody-based methods (ELISA, Western blotting) are considered to be semiquantitative because there is no strict assay validation (but most commonly used). HPLC with electrochemical detection (ECD), mass spectrometry-based assays (GC–MS) and gas chromatography–tandem mass spectrometry (GC–MS/MS), and liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) are proposed to have adequate sensitivity for quantification of 3-nitrotyrosine (Dalle-Donne et al. 2006).

5.2.8 Protein Chlorination

Although chlorination of tyrosine residues by hypochlorite is less favored than chlorination of amines, these reactions have the advantage of producing more stable end products (3-chlorotyrosine and 3,5-dichlorotyrosine), which may serve as biomarkers of HOCl formation. Their presence may be assessed and semiquantitatively estimated by immunochemical methods (ELISA, Western blot, flow cytometry with the use of anti-chlorotyrosine antibodies (Chapman et al. 2000; Robaszkiewicz et al. 2011)) or by soft ionization MS (Pitt and Spickett 2008) or LC–MS/MS (Delporte et al. 2012).

5.2.9 Protein Glycoxidation

Nonenzymatic protein glycosylation (glycation) is a complex process of covalent damage of proteins usually accompanied by oxidative steps. It involves oxidative reactions so is often called "glycoxidation." The process is initiated by the nonenzymatic reaction between amino groups of proteins and carbonyl groups of reducing sugars (e.g., glucose), their reactive metabolites (e.g., α-oxoaldehydes), or other carbohydrate relatives (e.g., ascorbic acid) and leads to the generation of advanced glycation end products (AGEs) via early (Schiff bases) and intermediate glycation products (Amadori or Heyns products). AGEs are complex and heterogeneous molecules that cause significant changes in the physicochemical properties of proteins including increase in molecular weight due to aggregation and cross-links formation and induction of yellow-brown pigmentation and fluorescence generation (Vlassara and Palace 2003; Schalkwijk et al. 2004).

Protein glycation leads also to secondary production of ROS. AGEs interact with receptors for AGEs (RAGEs), immunoglobulin-like cell surface receptors. Stimulation of these receptors evokes several cellular phenomena, among them is stimulation of Nox leading to production of superoxide and other ROS (Fig. 4).

Due to the complexity of nonenzymatic glycation, there are various methods of its monitoring including quantification of arising metabolic intermediates and AGEs mostly by chromatographic (HPLC or GC), spectrofluorimetric, or

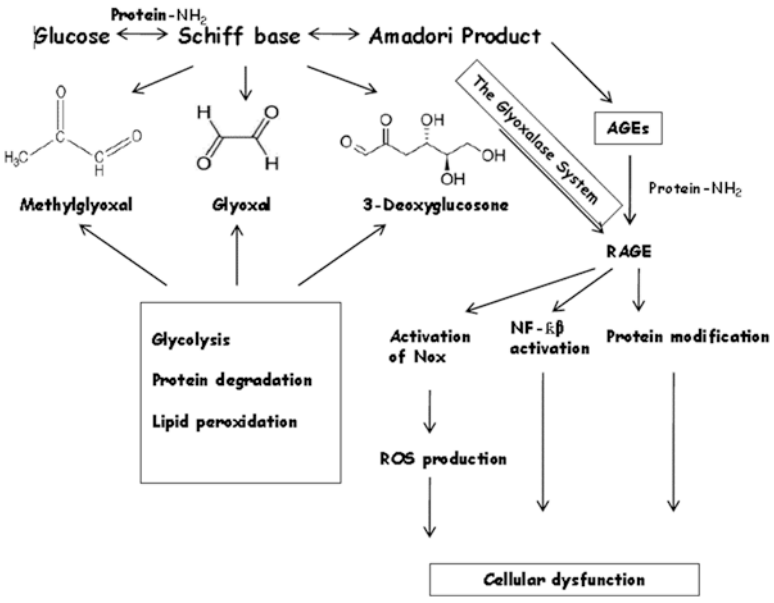


Fig. 4 Protein glycation leads to secondary ROS formation. After (Viinikka 1996; Jack and Wright 2012), modified

immunochemical methods. Pentosidine and *N*-ε-carboxymethyllysine (CML) are commonly used as biomarkers of glycoxidation as well as oxidative stress, respectively (Moreira et al. 2005; Kalousová et al. 2005; Ansari and Moinuddin 2011; Nagai et al. 2008).

5.3 Markers of Oxidative/Nitrative/Chlorinative Damage to Nucleic Acids

5.3.1 Introduction

Free-radical attack upon DNA generates a range of DNA lesions, including strand breaks and base modifications. Hydroxyl radical attack on DNA leads to a large number of pyrimidine- and purine-derived base changes. Some of these modified DNA bases have considerable potential to damage the integrity of the genome. 8-Oxo-2-deoxyguanosine (8-oxo-dGuo) is one of the most critical lesions. The presence of 8-oxo-dGuo residues in DNA can lead to GC to TA transversion, unless repaired prior to DNA replication. The presence of 8-oxo-dGuo may, therefore, lead to mutagenesis and carcinogenesis (Griffiths et al. 2002).

While oxidative damage to DNA is of main importance, RNA oxidation occurs at a broader scale due to the amount of RNA present in cells. The oxidation of free nucleotides occurs also but seemingly does not contribute significantly to the pool of oxidized metabolites measured. Oxidized nucleosides and bases are excised from DNA by repair enzymes and are measured in urine or plasma. The urinary measurement of oxidized nucleic acid metabolites provides a noninvasive measurement of oxidative stress to DNA and RNA. There is a concern, however, that dead cells contribute to the oxidatively modified DNA metabolites in urine (Lindahl 2001). If oxidative damage to cellular DNA is measured, lymphocytes are often used as surrogate cells.

5.3.2 Oxidatively Modified Bases and Nucleosides

8-Oxo-7,8-dihydroguanine (8-oxoGua) is abundant in a lesion excised by oxoguanine DNA glycosylase 1 (OGG1) (EC 3.2.2.23) (Loft et al. 2012). Other modified bases, e.g., 5-hydroxyuracil, are also analyzed (Chen et al. 2005). The analysis of oxidized nucleic acid metabolites can be performed by a variety of methodologies: high-performance liquid chromatography (HPLC) with electrochemical detection (EC) or mass spectrometry (MS) detection, gas chromatography–mass spectrometry (GC–MS), and HPLC tandem capillary electrophoresis and ELISA. The available ELISA methods are not specific and are not recommended (Weimann et al. 2012; Valavanidis et al. 2009).

5.3.3 Nitrative Modifications of Nucleic Acids

RNS both nitrate nucleic bases and oxidize nucleic acids. While it is impossible to distinguish oxidative damage by RNS and ROS, 8-nitroguanine is a marker of nitrative stress (Ohshima et al. 2006). However, estimation of 8-nitroguanine may underestimate nitrative stress since 8-nitroguanine decomposes spontaneously with a half-life of hours (Griffiths et al. 2002).

5.3.4 Chlorinative Modifications of Nucleic Acids

Treatment of DNA with HOCl and histidine chloramine results in the formation of 5-chloro-2′-deoxycytidine (5CldC), 8-chloro-2′-deoxyadenosine (8CldA), and 8-chloro-2′-deoxyguanosine (8CldG). Among the nucleosides, 8CldG is the favored product in each case. Cellular RNA is also a target for hypochlorite and chloramines, and there is evidence for the formation of 5-chloro-cytidine (5ClC) (Stanley et al. 2010).

5.3.5 The Comet Assay

The comet assay is a sensitive and convenient method for detecting DNA strand breaks. It can also be used to measure oxidized bases, with the addition of a step in which DNA is incubated with bacterial repair endonucleases, which recognize and remove damaged bases and make nicks at the resulting abasic (AP) sites in the DNA. Endonuclease III detects oxidized pyrimidines; formamidopyrimidine DNA glycosylase (FPG) recognizes altered purines including 8-oxoguanine (Ravanat et al. 2012; Griffiths et al. 2002).

5.3.6 End Points of Oxidative DNA Damage

Oxidative DNA damage leads ultimately to such sequelae as mutations, chromosome aberrations, micronuclei formation, and sister chromatid exchange. While they are not necessarily specific for oxidative damage, oxidative stress increases the frequency of these phenomena and in many situations they may serve as legitimate markers of oxidative stress (Griffiths et al. 2002; Cassini et al. 2011).

5.4 Effects of Oxidative/Nitrosative/Chlorinative Stress on the Antioxidant System

5.4.1 Introduction

The biological role of antioxidants is to prevent/repair oxidative damage to vital biomolecules. Therefore, they are expected to be consumed first under conditions of oxidative stress unless compensatory biosynthesis occurs. Activities of antioxidant

enzymes are also often measured in oxidative stress. While usually there are no reasons for these enzymes to be more sensitive to ROS/RNS/RCS than many others, assessment of their activities is useful since their decreased activities contribute to and, simultaneously, demonstrate the existence of oxidative stress. Often, these enzyme activities are determined in erythrocytes, which is not a bad choice. These cells are easily available in large amounts and lack protein synthesis; it is an advantage in this case since damaged proteins cannot be replaced by newly synthesized molecules. Therefore, the activities of antioxidant enzymes in erythrocytes reflect the effects of oxidative stress over some time period, taking into account the life span of an erythrocyte (up to 120 days).

5.4.2 Antioxidants

The levels of antioxidants present in extracellular fluids such as vitamin C, urate, or vitamin E are usually assessed in blood plasma or, less frequently, cerebrospinal fluid. The method of choice is HPLC with UV detection (Kanďár et al. 2011), although there are simple colorimetric or fluorimetric methods for estimation of these metabolites (Vislisel et al. 2007; Van der Jagt et al. 1986; Díaz et al. 2006). The estimation of urate level is usually a component of routine laboratory analysis.

Ascorbic acid is oxidized to a relatively stable ascorbyl free radical. Elevated concentration of this radical in biological fluids such as blood plasma, measured by ESR spectroscopy, is also employed as a biomarker of oxidative stress (Pietri et al. 1994; Spasojević 2011).

Extracellular concentrations of glutathione are much lower than intracellular ones; therefore, glutathione concentration in blood is usually determined in erythrocytes. If the essay is done in whole blood, it reflects mainly the intracellular glutathione. In such a case the hematocrit value should be also reported, as decrease in hematocrit would lead to decreased levels of whole blood glutathione. Since glutathione is the main erythrocyte thiol, its level is often estimated by the thiol essay with the Ellman reagent (Ellman 1959). However, such an assay must be performed on the protein-free fraction of blood (erythrocytes); otherwise, protein thiols will also react and hemoglobin may introduce artifacts because it absorbs at 412 nm, the analytical wavelength of the Ellman assay.

Oxidative stress induces oxidation of glutathione (GSH) to glutathione disulfide (GSSG), which increases the GSSG/GSH concentration ratio. Whenever possible, the estimation of both the reduced and oxidized forms of glutathione is recommended as it allows for the calculation of the GSSG/GSH ratio or redox potential of the glutathione couple (Schafer and Buettner 2001), other parameters for the assessment of oxidative stress. This can be done by HPLC, but spectrophotometric and fluorimetric assays are also available (Senft et al. 2000; Akerboom and Sies 1981).

5.4.3 Total Antioxidant Capacity

A convenient alternative to determining the levels of individual antioxidants is the assay of total antioxidant capacity (TAC) of body fluids, measuring the sum of activities of all antioxidants present in a sample. There are various methods to estimate TAC, the most popular at present being the $ABTS^{\bullet}$ decolorization assay (Re et al. 1999), the ferric ions reduction assay (Benzie and Strain 1996), copper reduction assay (Ribeiro et al. 2011), and oxygen radical absorbance capacity kinetic assay based on protection of beta-phycoerythrin or fluorescein against oxidation induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) (Cao et al. 1995; Ou et al. 2001; Bartosz 2003b).

However, there are considerable reservations against the use of TAC as a biomarker of oxidative stress. The variety of methods of TAC assay makes comparisons difficult. In the blood plasma, the main contributor to TAC is uric acid due to its high concentration. Changes in TAC induced by various factors can be therefore masked by changes/lack of changes in the concentration of uric acid. Uric acid dominates also, even to a higher extent, the TAC of urine (Bartosz 2003b, 2010).

5.4.4 Antioxidant Enzymes

Most often, activities of main antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione *S*-transferase, are estimated in erythrocytes by spectrophotometric or fluorimetric methods. There are several spectrophotometric methods for the estimation of superoxide dismutase activity; those based on inhibition of pyrogallol autoxidation (Marklund and Marklund 1974) and oxidation of a water-soluble tetrazolium salt WST-1 (Peskin and Winterbourn 2000) belong to the most convenient ones. Glutathione peroxidase is usually assayed in a coupled assay with glutathione reductase; in this assay oxidation of NADPH by glutathione reductase is monitored at 340 nm (Flohé and Günzler 1984). The assay of glutathione reductase is based directly on this principle (Mannervik and Carlberg 1985). Glutathione *S*-transferase is usually assayed on the basis of conjugation of 1-chloro-2,4-dinitrobenzene with glutathione, although a fluorimetric assay based on monochlorobimane is also available (Hulbert and Yakubu 1983).

Sometimes superoxide dismutase and catalase are determined in blood plasma instead of erythrocytes. Interpretation of such data is not easy as the enzymes may be released to plasma due to hemolysis of erythrocytes or necrosis of other cells.

6 Concluding Remarks

Most of the biomarkers used are common for oxidative/nitrosative/chlorinative stress as nitrosative and chlorinative stress also induces oxidative stress. There are, however, biomarkers specific for nitrosative and chlorinative stress. Multiple biomarkers are used, although the popularity of biomarkers often does not correlate with their specificity and reliability. Simultaneous use of several biomarkers in a study is highly recommended.

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Clinical Aspects

Oxidative Stress in Schizophrenia

Anna Dietrich-Muszalska

Abbreviations

($O_2.^-$)	Superoxide anion
31P MRS	31-Phosphorus magnetic resonance spectroscopy
4-HNE	4-Hydroxynonenal
8-OH-Gua	8-Hydroxyguanosine
AA	Arachidonic acid
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
cAMP	3′-5′-Cyclic adenosine monophosphate (or cyclic AMP)
CAT	Catalase
CNS	Central nervous system
COX-2	Cyclooxygenase 2
CSF	Cerebrospinal fluid
CSH	Cysteine CGSH – cysteinylglycine
Cu	Copper
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
ECT	Electroconvulsive therapy
ELISA	Enzyme-linked immunosorbent assay
EPUFAs	Essential polyunsaturated fatty acids
Fe	Iron
GABA	Gamma-aminobutyric acid

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GCL	Glutamate cysteine ligase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSH/GSSG	Reduced glutathione/glutathione disulfide
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HCSH	Homocysteine
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-6	Interleukin-6
KA	Kynurenic acid
MAO	Monoamine oxidase
MDA	Malondialdehyde
Mn	Manganese
MRI	Magnetic resonance imaging
N_2O	Nitrous oxide
NAC	N-Acetylcysteine
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
PE	Phosphatidylethanolamine
PGE2	Prostaglandin E2
$PGF_2\alpha$	Prostaglandin F2 alpha
PI	Phosphatidylinositol
PLA2	Phospholipase A2
PS	Phosphatidylserine
PUFAs	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAS	Total antioxidant status
TBARS	Thiobarbituric acid reactive substances
TNFα	Tumor necrosis factor alpha
Trx	Thioredoxin
TRYCAT	Tryptophan catabolite

1 Introduction

Schizophrenia is one of the most severe and chronic forms of mental disorders with to date unknown pathophysiology and aetiology, and it affects roughly 1 % of the world's population (Saha et al. 2007). At present, it is fully recognized as a multidimensional illness, with a profound impact on behavior, perception, thinking, emotions, neurocognition, and social function (O'Leary et al. 2000). A complex interaction between genetic and environmental factors appears to be critical to the pathogenesis of schizophrenia (van Os and Murray 2008), and data from many studies progressively contribute to a characterization of its mechanisms. The disorder is characterized by chronic, often recurrent course, and serious deterioration in cognitive and psychosocial functioning occurs in most patients, as early as during the first 5 years (Tamminga and Holcomb 2005).

The etiopathogenesis of this illness due to heterogeneity of patient population, different symptoms, difficulties in diagnosis particularly in the early stage of the disease, long-term treatment, and various side effects is difficult to study. Neurodevelopmental (Murray and Lewis 1987; Lewis and Levitt 2002; Weinberger 1986, 1987), neurodegenerative (Lieberman 1999; Rund 2009), immunological (Kinney et al. 2010; Kliushnik et al. 2009; Strous et al. 2009; O'Donnell 2012), inflammatory (Covelli et al. 2003; Hanson and Gottesman 2005), infectious (Babulas et al. 2006; Brown et al. 2009; Yolken and Torrey 1995; Yolken et al. 2000), and metabolic (De Hert et al. 2009; Fan et al. 2010) hypotheses and various pathophysiological mechanisms have been proposed to explain the etiopathogenesis of schizophrenia. Molecular mechanisms, leading to oxidative stress, involved in the pathophysiology of schizophrenia are intricate and not yet fully explained. This diversity reflects the considerable neurobiological heterogeneity and complexity of clinical syndromes of schizophrenia. The implication of oxidative stress in inflammatory process, neurodegeneration, and neurodevelopment is emerging as an important mechanism underlying numerous pathological processes. This disorder is increasingly regarded as the result of pathological alterations in architecture and function of the brain circuits supporting perceptual, cognitive, and emotional processes (Insel 2009; Lewis and Sweet 2009; Palop et al. 2006) leading to disturbances of neural coordination within these networks. The current psychiatric classification systems, the International Statistical Classification of Diseases and Related Health Problems, 10th Revision (ICD-10; WHO 2008), and the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV; APA 2000), are based solely on the diagnosis of schizophrenia dependent on its clinical symptomatology since there is a lack of specific biological markers. The identification of biological, clinical, or biochemical markers for schizophrenia could facilitate the understanding of its etiopathogenesis as well as its diagnosis. It is important to determine clear biological markers, which could accurately help to predict clinical outcome of schizophrenia and identify at-risk individuals in preclinical stages of the disorder (Schwarz and Bahn 2008). Thus, the search for effective biological markers for schizophrenia seems to be a main challenge for neuroscientific and psychiatric

researches (Lakhan et al. 2010). Recently, markers of oxidative stress have been taken into account. Biochemical alterations in the brain, especially the dopamine system with free radical production and oxidative damage to the brain structure, might be partly responsible for the pathogenesis of this heterogeneous disease. Oxidative stress is involved in different intricate mechanisms of the disease and might contribute to the explanation of various pathological processes integrating some etiopathogenetic hypotheses about schizophrenia.

2 ROS Generation in Schizophrenic Patients

Imbalance between the pro- and anti-oxidative processes is a physiological phenomenon used in many important functions of the body, and this imbalance with a predominance of oxidation, defined as oxidative stress, may be involved in the pathogenesis of many diseases including schizophrenia. Oxidative stress causes changes in the function of several cellular components of the central nervous system (CNS) and is involved in inflammatory and neurodegenerative processes (Ng et al. 2008; Pedrini et al. 2012; Lin and Beal 2006; Halliwel 2006). The brain with its high metabolic rate is particularly vulnerable to oxidative damage (Halliwell 1992). The impairment of redox mechanisms in the brain manifested as an imbalance in the generation and scavenging of reactive oxygen species (ROS) and nitrogen species (RNS) and altered regulation of these fundamental redox mechanisms leading to oxidative stress are involved in the brain pathology in schizophrenia and can contribute to the pathogenesis of the disorder. Moreover, cognitive dysfunction in schizophrenia is accompanied by the changed redox mechanisms (Bitanihirwe and Woo 2011; Zhang et al. 2012). The brain, rich in essential polyunsaturated fatty acids (EPUFAs), is particularly susceptible to damage caused by free radicals, has a high demand for oxygen (20 % of the total oxygen consumed by the body), and produces large amounts of ROS (Halliwell and Gutteridge 1996). ROS are produced in mitochondria as the leak of electrons from the electron transport chain; oxidative phosphorylation in the mitochondrial electron transport chain is highly effective. Incomplete reduction in oxygen during the respiration produces superoxide anion ($O_2.^-$) that is enzymatically dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). Hydroxyl radicals, in turn, are formed via Fenton reaction (Halliwell 1992). Superoxide anion ($O_2.^-$) of these three generated ROS is one of the most reactive and controlled via multiple enzyme systems: SOD, CAT, GSH transferase, and thioredoxin. NADPH oxidase (NOX2) and xanthine oxidase are also involved in the production of free radicals (Halliwell 1992; Halliwel et al. 2006).

In mitochondria (outer membranes), enzymatic oxidation of biogenic amines induced by monoamine oxidase (MAO) generates H_2O_2; dopamine as a substrate for MAO is also a source of ROS. Dopamine is a modulator of oxidative status; it can undergo autoxidation to generate ROS (Dalla Libera et al. 1996; Masserano et al. 2000; Grima et al. 2003; Bošković et al. 2011). During the mitochondrial respiration, the increased Ca ion influx also activates neuronal NO synthase (nNOS) to

generate NO, which has been reported to regulate mitochondrial function. NMDA is also engaged in ROS production (O_2^-). The activation of NMDA receptors in neurons and subsequent Ca ion influx can induce NO generation by nNOS (Nakamura and Lipton 2011). NO, in turn, inhibits the activity of NMDA receptor via S-nitrosylation (Lipton et al. 1993). The malfunction of NMDA receptors may have effect on the redox status in patients with schizophrenia. Damage to mitochondria in brain cells of schizophrenic individuals is connected with the impairment of oxidative phosphorylation and increased ROS generation (Ben-Shachar et al. 1999; Ben-Shachar 2002; Prabakaran et al. 2004; Clay et al. 2011; Martins-de-Souza et al. 2011). Approximately up to 50 % of ROS in the CNS derive from mitochondria. Especially neurons are sensitive to mitochondrial defects because they require high levels of energy (ATP) for maintenance of synapses, survival, and their specialized functions (Nakanishi and Wu 2009).

The impaired redox regulation caused by genetic and environmental factors, with the alterations of antioxidant defense system and oxidative stress in the brains of schizophrenic patients, is associated with various pathophysiological processes including mitochondrial dysfunction, inflammation, epigenetic changes, impairment of cell signaling, hypoactive NMDA receptors, or impairment of GABA interneurons (Benes and Berretta 2001; Reynolds et al. 2004; Picchioni and Murray 2007; Ben Sachar et al. 2002; Nakazawa et al. 2012). Schizophrenia is characterized by mitochondrial dysfunction (Ben-Shachar and Karry 2008) with oxidative damage to the mitochondrial membrane (Yao et al. 2001) and the mitochondrial electron transport chain dysfunction (Ben-Shachar 2002; Prabakaran et al. 2004). Dopamine may also impair mitochondrial membrane potential (Elkashef et al. 2002). The differences between schizophrenic and normal brains seem to be related partly to mitochondrial dysfunction and oxidative changes (Dror et al. 2002). In mitochondria, the toxic hydrogen peroxide is generated from the enzymatically dismutated O_2^- by SOD, and glutathione is oxidized to glutathione disulfide (GSSG) (Dror et al. 2002). Mitochondria do not synthetize GSH and the low GSH level contributes to mitochondrial function impairment and oxidative damage (Griffith and Meister 1985). Recently, Seybolt (2010) has suggested that adjunctive use of alpha lipoic acid (its reduced form dihydrolipoic acid) and niacinamide as components of coenzymes NAD and NADP in the treatment of schizophrenia due to their properties to reduce ROS/RNS and improve the GSH/GSSG ratio could help alleviate mitochondrial dysfunction, reduce oxidative stress, and improve psychiatric symptoms in schizophrenia. S-nitrosylation and subsequent further oxidation of critical cysteine residues can also lead to mitochondrial dysfunction (Nakamura and Lipton 2011). Reduced level of GSH was observed in plasma of patients with schizophrenia (Dietrich-Muszalska et al. 2009c). GSH is the brain's dominant antioxidant implicated in the pathophysiology of schizophrenia. An increased risk of schizophrenia is linked with polymorphisms of genes associated with GSH synthesis (Saadat et al. 2007; Tosic et al. 2006). In some brain structures, the presence of significant quantities of transition metal ions – iron (Fe), copper (Cu), and manganese (Mn) – can also contribute to the formation of ROS (Halliwell 1992). Free radical-induced membrane lipid/phospholipid peroxidation may cause damage to the cell membrane, due to changes in its

biophysical properties, such as fluidity, and inactivation of receptors/enzymes associated with the membrane (Perluigi et al. 2012). These processes, in turn, may lead to disturbances in the physiological function of cells, especially neurons. Neurons are particularly sensitive to oxidative stress, and therefore, the efficient antioxidant defense system plays an important role in their normal functioning. The low antioxidant defense system observed in schizophrenia seems to be responsible in part for oxidative stress in this disorder (Yao et al. 1998a; Yao and Keshavan 2011a).

3 Markers of Oxidative and Nitrative Stress in Schizophrenia: Antioxidants

Multidimensional data support the fact that redox status in schizophrenia can be determined by the level of specific biomarkers of oxidative stress and the level of antioxidants (Lakhan and Kramer 2009). Data for the brain redox status are limited and contradictory. The majority of evidence for oxidative stress in schizophrenia is mostly received peripherally by the assessment of markers in plasma/serum or blood cells. Direct evidence of oxidative stress, especially when tardive dyskinesia occurred, has been obtained in animal models (Harrison 1999). The functional and biochemical consequences of oxidative stress were studied using animal models and postmortem brain analysis, but most measurements of oxidative stress in schizophrenia are in peripheral cells and fluids. They reflect redox processes occurring in the brain. Common markers used to assess the extent of oxidative/nitrative stress in schizophrenic patients are the products of oxidized and changed biomolecules – lipids, proteins, and nucleic acids – and also the activities of antioxidant defense system. The increased level of lipid peroxidation products measured commonly as TBARS or MDA, 4-hydroxynonenal, and isoprostanes (see Table 1), altered proteins and amino acids (estimated as the level of generated carbonyl groups or protein peroxides, 3-nitrotyrosine) (Dietrich-Muszalska and Olas 2009a, b), and the presence of DNA damage products (8-hydroxyguanosine, telomere shortening) (Miller et al. 2011; Jorgensen et al. 2013; Malaspina et al. 2001, 2014) as well as reduced antioxidant defense systems (Yao and Reddy 2011) are observed as the multiple pathophysiological consequences of increased toxicity of oxidative stress. Carbonyl groups formed by the oxidation of proteins may be readily estimated by an immunoassay technique (ELISA), but they seem not to be specific markers, contrary to 3-nitrotyrosine which is a specific and sensitive marker of protein alteration caused by nitrative stress.

The antioxidant defense system consists of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), thioredoxin (Trx), and numerous nonenzymatic antioxidants). Changes in the activity of antioxidant enzymes and in total antioxidant status are presented in Tables 2 and 3. The individual antioxidants may act cooperatively and protect the biomolecules against oxidative damage.

Table 1 Increased levels of lipid peroxidation products (e.g., MDA, TBARS, 4-HNE isoprostanes) in patients with schizophrenia

References	Increased levels of lipid peroxidation products (e.g., MDA, TBARS) in:	Antipsychotic drugs (FGA/SGA)
McCreadie et al. (1995)	Plasma, serum	Antipsychotics
Brown et al. (1998)		Antipsychotics
Mahadik et al. (1998)		Antipsychotics
Khan et al. (2002)		FGA/SGA
Kuloglu et al. (2002)		Drug-naïve
Akyol et al. (2002)		FGA/SGA
Ranjekar et al. (2003)		FGA/SGA
Arvindakshan et al. (2003a)		FGA/SGA
Dietrich-Muszalska et al. (2005)		SGA
Zhang et al. (2006)		FGA/SGA
Gama et al. (2006)		FGA/SGA (haloperidol, clozapine)
Kunz et al. (2008)		FGA/SGA
Dakhale et al. (2008)		–
Huang et al. (2010)		FGA/SGA
Gama et al. (2008)		Clozapine
Dietrich-Muszalska and Olas (2009b)		SGA
Dietrich-Muszalska and Kontek (2010)		SGA (olanzapine, risperidone)
Mahadik et al. (1998)	Red blood cells	Antipsychotics
Altuntas et al. (2000)		FGA
Herken et al. (2001b)		FGA
Arvindakshan et al. (2003b)		Never-medicated
Evans et al. (2003)		SGA
Dietrich-Muszalska et al. (2005)	Blood platelets	SGA
Das et al. (1998)	Fibroblasts	FGA
Srivastava et al. (2001)	PMN	–
Dietrich-Muszalska et al. (2009)	↑ levels of isoprostane (PGF2α) in urine	SGA
Wang et al. (2009)	Postmortem increased (by 47 %) 4-HNE levels in the anterior cingulate cortex	Antipsychotics
	No changes of level of lipid peroxidation products	
Skinner et al. (2005)	CSF	Drug-free
SSRG (2000)	Plasma	Drug-free

MDA malondialdehyde, *TBARS* thiobarbituric acid reactive substances, *4-HNE* 4-hydroxynonenal, *PMN* polymorphonuclear leukocytes, *CSF* cerebrospinal fluid, *FGA* first-generation antipsychotics, *SGA* second-generation antipsychotics

Table 2 Antioxidant enzymes in plasma in patients with schizophrenia

References	Changes of antioxidant enzymes	FGA/SGA or drug-naïve
Decreased SOD level or activity and changes of GPx or CAT		
Raffa et al. (2009)	↓SOD levels, GPx – unchanged, ↓CAT	Antipsychotic-free, first episode
Mukerjee et al. (1996)	↓SOD, GPx, and CAT – no changes	First episode
Akyol et al. (2002)	↓SOD, ↑GPx, CAT – not examined	FGA and SGA
Evans et al. (2003)	↓SOD, GPx – no changes, ↑CAT	Drug-naïve
Ranjekar et al. (2003)	↓SOD, ↓GPx, ↓CAT	SGA
Zhang et al. (2003a, b, c)	↓SOD, GPx, and CAT – no changes	SGA
Dietrich-Muszalska et al. (2005)	↓SOD activity in blood platelets (reduction about 85 %)	SGA
Dietrich-Muszalska and Kwiatkowska (2014)	↓GPx activity in blood platelets (suppressed GPx activity about 67 %)	SGA
Zhang et al. (2006)	↓SOD, ↓GPx, ↓CAT	FGA and SGA
Dadheech et al. (2008)	↓SOD, ↓GPx, and CAT – no changes	–
Ben Othmen et al. (2008)	↓SOD, ↓GPx, ↓CAT	FGA
Raffa et al. (2009)	↓SOD, ↓GPx, ↓CAT	Drug-naïve
Zhang et al. (2010)	↓SOD, GPx, and CAT – no changes	[a]
Raffa et al. (2012a)	↓SOD, ↓GPx, and CAT – no changes	–
Raffa et al. (2012b)	↓SOD, ↓GPx, ↓CAT	Mostly by FGA
Increased SOD level or activity and changes of GPx or CAT		
Reddy et al. (1991)	↑SOD, GPx – not examined, ↓CAT	Chronic schizophrenics FGA
Yao et al. (1998a)	↑SOD	Chronic schizophrenics
Yao et al. (1998c)	↑SOD, ↑GPx, and CAT – no changes	Drug-free treatment with haloperidol
	Both SOD and GPx activities were higher in the drug-free condition compared to the treatment	
Abdalla et al. (1986)	↑SOD	FGA
Altuntas et al. (2000)	↑SOD	FGA and drug-naïve
Kuloglu et al. (2002)	↑SOD	Drug-naïve
Zhang et al. (2003)	↑SOD	Chronic schizophrenics
Gama et al. (2006)	↑SOD	Drug-naïve, first-episode, and chronic schizophrenics on antipsychotics[b]
Wu et al. (2012)	↑SOD	Drug-naïve, first-episode, and chronic schizophrenics on antipsychotics[b]

(continued)

Table 2 (continued)

References	Changes of antioxidant enzymes	FGA/SGA or drug-naïve
No changes[1] of SOD level or activity and changes of GPx or CAT		
Yao et al. (1998c)	SOD and CAT – no changes	–
Srivastava et al. (2001)	SOD – no changes	–
Herken et al. (2001b)	SOD – no changes, ↑GPx, ↑CAT	FGA
Evans et al. (2003)	SOD and GPx – no changes, ↑CAT	SGA
Raffa et al. (2011)	SOD – no changes, ↑GPX, ↓CAT	Drug-naïve
Micó et al. (2011)	SOD – no changes, ↑GPx, CAT – no changes	Antipsychotic-free

[1] significant, *CAT* catalase, *GPx* glutathione peroxidase, *SOD* superoxide dismutase, *FGA* first-generation antipsychotics, *SGA* second-generation antipsychotics, ↑ increased protein level of the enzyme or its activity, ↓ decreased protein level of the enzyme or its activity
[a]Meta-analysis
[b]Haloperidol or clozapine

Table 3 Total antioxidant status or total antioxidant capacity and antioxidant levels in plasma in patients with schizophrenia

References	Changes of TAS or TAC and antioxidant levels in plasma
Suboticanec et al. (1990)	↓ ascorbic acid levels
McCreadie et al. (1995)	↓ alpha-tocopherol levels
Yao et al. (1998a, b)	↓ TAS, ↓ uric acid, albumin, and bilirubin
Yao et al. (2000)	↓ uric acid, albumin, and bilirubin
Reddy et al. (2003)	↓ uric acid, albumin, and bilirubin (independent of smoking status)
Virit et al. (2009)	↓ TAS
Zhang et al. (2009a)	↑ thioredoxin in serum in acute phase of schizophrenia (thioredoxin positively correlated with positive symptoms of schizophrenia)
Li et al. (2011)	↓TAS (never-medicated first-episode patients)
Zhang et al. (2013)	Thioredoxin normalized in chronic schizophrenics (on long-term treatment with antipsychotics)
Owe-Larsson et al. (2011)	↑ thioredoxin concentration in plasma (patients with first episode of psychosis (acute phase))
	↑ thioredoxin concentration in plasma (on long-term treatment with antipsychotic)

TAC total antioxidant capacity, *TAS* total antioxidant status, ↑ increase of level, ↓ decrease of level, *CSF* cerebrospinal fluid

To evaluate the antioxidant defense system in plasma reflecting the total activity of all antioxidants present in plasma, the total antioxidant status (TAS) should be measured together with the estimation of individual antioxidants. TAS depends mainly on a high concentration of albumin in plasma and the presence of ascorbic and uric acids, bilirubin, tocopherol, melatonin, and various exogenous antioxidants derived from diet, especially numerous polyphenols (see Chapter "Antioxidant Plant Polyphenols and Cognitive Disorders").

The level of uric acid, a product of purine metabolism present in plasma, is relatively high and reaches approximately over half of the free radical scavenging activity in human blood (Korte et al. 1998; Murr et al. 2002; Chittiprol et al. 2010). Properties of uric acid include quenching of superoxide and singlet oxygen and protecting against oxidation of ascorbic acid through the chelation of iron. The ability of ascorbic acid to repair oxidized uric acid leads to synergy for maintaining antioxidant capacity by these two antioxidants. Due to its properties, uric acid is an important CNS antioxidant, since neurons contain high concentrations of ascorbic acid (Suboticanec et al. 1990; Rose and Bote 1993). Moreover, a lower serum level of this antioxidant may indicate that there is a significant loss of protection against NO and toxic peroxynitrite derived from NO because uric acid is a scavenger of peroxynitrite (Szabó et al. 2007). The level of uric acid in the CSF is about ten times lower than in serum (Bowman et al. 2010). This suggests that this purine metabolite is generated peripherally, and its migration to the CNS is limited by the blood-brain barrier (BBB). In schizophrenic patients, the lower level of uric acid has been reported (Yao et al. 1998b; Reddy et al. 2003).

Oxidative DNA damage may cause single- and double-stranded DNA breaks and modification of DNA components such as bases and sugar. The estimation of guanosine derivative is the most common method used (Cooke et al. 2003). Oxidative/nitrative changes induced by oxidative stress in schizophrenic patients are not restricted only to the brain, but may be observed in blood and peripheral cells: plasma, erythrocytes, and blood platelets. These elements are used to estimate the level of oxidative damage markers in schizophrenia. These markers may give clues about the relation of oxidative damage to the onset and progression of schizophrenia. Data from animal models and from postmortem studies in humans show an increase in the level of lipids, proteins, and DNA oxidation products implicated in oxidative stress in the brains of schizophrenic individuals. However, the results of the estimation of oxidative markers are often contradictory (see Tables 1 and 2). The discrepancies may depend on several reasons, such as differences in techniques of estimation, tested biological material (plasma, cell), the use of a single marker that may not accurately reflect the totality of oxidative damage, the choice of markers that can lack specificity and precision to detect oxidative stress in vivo, or measurement methods that may not be able to detect low levels of oxidative damage. Moreover, sample preparation methods may introduce artifactual oxidation during storage of samples. Improper matching of cases to the controls may also lead to the discrepant results, when lifestyle, diet, and ethnic origin are not taken into account. It is extremely important to study and compare the samples from schizophrenic patients in different stages of disease progression (naïve, first, chronic patients) and also after prescribed treatment, especially after different antipsychotics (both first and second generation). Altered gene expression may be attributed to the pathogenesis of schizophrenia (Konat 2003; Tsankova et al. 2007; Jiang et al. 2008). Genetic analysis has shown that the antioxidant pathway genes especially for GSH are involved in schizophrenia. Oxidative damage results in gene silencing expression of an affected genomic region. The mechanisms responsible for gene silencing are likely epigenetic and may be mediated through the alteration

of DNA methylation (Lu et al. 2004). High level of homocysteine in patients with schizophrenia seems to be associated with DNA hypomethylation and changes in gene expression (James et al. 2002). Polymorphism in the glutathione S-transferase gene has been reported to be responsible partly for vulnerability to develop psychosis (methamphetamine abuse) leading to schizophrenia (Hashimoto et al. 2005). Polymorphism in the glutamate cysteine ligase gene has been also presented (Gysin et al. 2007). Moreover, in patients with schizophrenia, the capacity for the synthesis of GSH is lower than in controls and it may reflect a dysregulation of redox with an increased susceptibility to oxidative stress that may be of a genetic origin (Do et al. 2010; Gysin et al. 2007, 2009, 2011).

4 Lipid Peroxidation in Schizophrenia

Lipid peroxidation through free radical oxidation of unsaturated fatty acids (arachidonic acid, docosahexaenoic acid) is presumed to be involved in the oxidative injury in schizophrenia. Several methods have been developed for the measurement of the products of free radical-induced lipid peroxidation, such as lipid peroxides, 4-hydroxynonenal, malondialdehyde (MDA), and thiobarbituric acid reactive substances (TBARS), to assess oxidative injury in vivo in schizophrenia. The common colorimetric method used for the estimation of lipid peroxidation products is based on the reaction of unsaturated fatty acid products, mainly aldehydes with thiobarbituric acid, and the expression of the results as TBARS or MDA. The level of lipid peroxidation in peripheral cells and body fluid of schizophrenic patients is presented in Table 1. The assessment of MDA and TBARS, in spite of the lack of specificity leading to the overestimation of MDA, is still the most widely used marker of oxidative stress in clinical studies. Most of the published data concerning the oxidative stress and oxidative damage to lipids in schizophrenia are based on this assessment. There are other markers of free radical-induced lipid peroxidation: 4-hydroxynonenal, ethane (Puri et al. 2008; Ross et al. 2011), and isoprostanes (Dietrich-Muszalska and Olas 2009b). Grignon and Chianetta (2007) performed a meta-analysis of studies on MDA levels in schizophrenic patients published up to July 2006 and showed very large heterogeneity of the results. The analysis confirmed the value of these markers in the assessment of oxidative stress in schizophrenia.

The contribution of oxidative injury to the pathophysiology of schizophrenia has been indicated by the increase in lipid peroxidation products in the plasma and CSF of patients, and the altered levels of both enzymatic and nonenzymatic antioxidants in chronic, naïve, and first-episode patients (Mahadik and Scheffer 1996; Khan et al 2002; Dietrich-Muszalska et al. 2005; Zhang et al. 2010; Tsai et al 2013; see Tables 1 and 2). Recently, the increased level of breath ethane in patients with schizophrenia has been reported. The preliminary evidence suggests that oxidative stress can be assessed noninvasively by estimating breath ethane levels – the omega-3 PUFA oxidation product (Ross et al. 2011) and breath hydrocarbon analysis may represent a simple, noninvasive means to monitor the metabolic processes occurring in the course and treatment of schizophrenia.

According to Kartalci et al. (2011), the effects of electroconvulsive treatment (ECT) used in schizophrenic patients indicate that ECT, contrary to the effects of many antipsychotic drugs, may be a safe treatment alternative in terms of oxidative stress, inducing significant clinical improvement in severity of the disease associated with a significant decrease in the MDA serum level.

4.1 Isoprostanes

Isoprostanes (a group of prostaglandin isomers) belong to a family of prostaglandin derivatives synthesized from arachidonic acid in vivo through the nonenzymatic free radical-catalyzed mechanism. They circulate in peripheral blood and are excreted into the urine. Isoprostanes are generated in situ directly as an esterified form in membrane phospholipids and released as a free form, mainly by phospholipases. Phospholipase A_2 has a dominant role in the release of arachidonic acid from phospholipids, removing the acyl chain of arachidonic acid from β-position of phospholipids. This nonenzymatic peroxidation of arachidonic acid associated with the creation of prostaglandin isomers (including the rearrangement) is important for the escalation of oxidative processes and damage to cellular biomolecules (Morrow and Roberts 1997; Chen et al. 1999; Brame et al. 1999). Morrow's discovery of F_2-isoprostanes (a group of isomers of $PGF_2\alpha$) in 1990 and the development of sensitive detection methods (immunoenzymatic assay) made their measurement a recommended, reliable method for assessing the status of oxidative stress in vivo (Morrow et al. 1990; Montuschi et al. 2004; Cracowski et al. 2002). F_2-isoprostanes are important and recommended biomarkers of oxidative stress, and the method of measuring them is specific, highly sensitive, and noninvasive. The formation of isoprostanes indicates that the free radical oxidation of arachidonic acid occurs in patients with schizophrenia. The measurement of F_2-isoprostanes is more sensitive than TBARS/MDA, lipid hydroperoxide, and conjugated diene level estimation, which is in vivo hampered by various methodological limitations (Morrow 2006; Morrow and Roberts 1997; Roberts et al. 1996; Roberts and Morrow 2000). Therefore, the method of F_2-isoprostane evaluation can be used to monitor oxidative stress in patients with schizophrenia, according to clinical status of the disease (type of schizophrenia, predominance of positive or negative symptoms, first episode or recurrence, an acute episode or remission).

Isoprostanes (prostaglandin isomer $PGF_2\alpha$ such as 8-iso-$PGF_2\alpha$) are the primary end-products of lipid peroxidation in vivo. The measurement of isoprostanes can be useful for monitoring the effectiveness of schizophrenia treatment. The study (Dietrich-Muszalska and Olas 2009b) on the assessment of the increased F_2-isoprostane concentration (8-iso-$PGF_2\alpha$) in patients with schizophrenia was the first one showing that the oxidation of arachidonic acid occurs through the nonenzymatic pathway (not associated with cyclooxygenase), as a result of free radical attack on membrane structures (Dietrich-Muszalska and Olas 2009b).

5 Cell Membrane Phospholipids and Polyunsaturated Fatty Acids (PUFAs) in Schizophrenia

Abnormal antioxidant enzyme activities and total antioxidant status, as well as greater abundance of oxidation products with the reduced level of PUFA, have been reported in schizophrenia (Yao et al. 2002b, 2003c; Ross et al. 2011). Metabolism of phospholipids is the most extensively studied in schizophrenia (Gattaz et al. 1995; Keshavan et al. 1993; Glen et al. 1994, 1996; Mahadik et al. 1994; Yao et al. 2002; Ripova et al. 1997, 1999; Strunecká and Rípová 1999; Rybakowski and Lehmann 1997). Phospholipids in the brain are extremely rich in polyunsaturated fatty acids (PUFAs), which in the neuronal membrane constitute as high as 65 % compared with other cells (15–35 %) (Horrocks et al. 1992). Membrane phospholipids such as phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) are highly rich in arachidonic (AA) and docosahexaenoic (DHA) acids, which are predominantly released by phospholipase A2 (PLA2) after the stimulation of cell receptors (Skosnik and Yao 2003).

Polyunsaturated fatty acids (AA and DHA acids) as components of membrane phospholipids play an important role in cell membrane dynamics (Rana and Hokin 1990; Du Bois et al. 2005); their defects caused partly by oxidative stress have been observed in schizophrenic patients during the medication and in the course of the illness, suggesting PUFA dysregulation in schizophrenia (Skosnik and Yao 2003). The correlation between peripheral erythrocyte polyunsaturated fatty acids and cerebral phospholipid metabolism estimated by 31-phosphorus magnetic resonance spectroscopy (31P MRS) has also been documented for first-episode neuroleptic-naïve schizophrenic patients (Pettegrew et al. 1991; Yao et al. 2002; Fukuzako et al. 1999) and was selective for bilateral prefrontal cortex regions (Do et al. 2000). The postmortem cortical tissue study also implicates abnormal fatty acid composition in the frontal cortex in schizophrenic patients (McNamara et al. 2007; Yao and Keshavan 2011a). The altered composition of membrane phospholipids may be one of the most significant factors in the etiopathogenesis of schizophrenia, since it leads to disturbances in signal transduction dependent on receptors of numerous neurotransmitters (e.g., dopamine, serotonin, glutamate, acetylcholine, gamma-aminobutyric acid, and noradrenaline) and nerve growth factor (NGF) (Mahadik et al. 2001). Arachidonic acid, docosahexaenoic acid, and their metabolic products such as diacylglycerol (DAG), phosphatidylinositol, and prostaglandins act as second messengers and physiological mediators. The abnormalities in signal transduction caused by free radicals may be associated with altered membrane fluidity depending on phospholipid components or with changes in second messengers (arachidonic acid, docosahexaenoic acid, diacylglycerol, phosphatidylinositol, prostaglandins, cytokines, endocannabinoids), derived from membrane phospholipids and generated by neurotransmitters and growth factors (Du Bois et al. 2005; Yao and van Kammen 2003c).

It seems that the reduced level of second messengers derived from membrane arachidonic acid caused by oxidative stress may be a key factor in modified signal

transduction and neuronal deficits in schizophrenia (Horrobin 1998; Skosnik and Yao 2003). The impairment of membrane phospholipids induced by free radicals and their dysfunction leading to disturbance of signal transduction can be also linked with function of neurotransmitters in schizophrenia, especially glutamatergic and serotoninergic systems (Du Bois et al. 2005; Skosnik and Yao 2003). Biochemical studies of blood platelets obtained from patients with schizophrenia have shown abnormalities in cellular phosphatidylinositol system, which is a second messenger for serotonin (5HT2) receptors (DeClerk 1990; Strunecká and Rípová 1999; Yao et al. 2004). The dysregulation of glutamatergic mechanisms, particularly hyperactive in exacerbation of psychosis and the related glutamate excitotoxicity, may be associated with oxidative stress and changes in the composition of membrane phospholipids (Tai et al. 1998). Membrane arachidonic acid is a precursor of second messengers in signal transduction of several neurotransmitters (dopamine, serotonin, acetylcholine, norepinephrine) (Axelrod 1990), and its oxidation may cause changes in signal transduction. Moreover, membrane phospholipid composition altered by oxidative stress may affect the activities of ion channels and enzymes, such as Na^+/K^+-ATPase and adenylate cyclase, leading to the production of cyclic AMP (Bourre et al. 1992). The omega-6/omega-3 PUFA ratio in membrane phospholipids has an effect not only on fluidity of membrane but also can affect the ligand-receptor interaction, possibly by increasing the availability of surface protein receptors and/or by increasing the concentration of receptors in the membrane (Farkas et al. 2002).

6 Nitrative Stress in Schizophrenia in Chapter 15

6.1 Glutathione

Glutathione (GSH), a tripeptide composed of glutamate, glycine, and cysteine, is involved in a number of diverse functions that include disulfide bond formation, detoxification, and antioxidant defense (Dringen 2000). The antioxidant function of GSH is due to the redox-active thiol group that becomes oxidized when GSH reduces target molecules. GSH may be oxidized to form glutathione disulfide (GSSG). The GSH/GSSG ratio is a marker of the redox status, which is low in schizophrenia (Dietrich-Muszalska et al. 2009c). GSH deficiency seems to be a major cause of oxidative stress in this disease. An impairment of the synthesis of glutathione seems to be one of the central causes of increased oxidative stress in schizophrenia (Do et al. 2009). GSH as a major nonprotein antioxidant in the brain plays a very important role in protecting the neurons against damage caused by ROS (which in the brain are produced additionally as a result of dopamine metabolism). The decrease in low-molecular-weight thiols in schizophrenic patients shows a significant reduction in the antioxidant defense system (Dietrich-Muszalska et al. 2009c), where GSH and thiols are important. The deficit of GSH can lead to the

Table 4 Glutathione (GSH) in brain and cerebrospinal fluid of patients with schizophrenia: postmortem and spectroscopy studies

References	Brain and CSF	Antipsychotic drugs or drug-naïve
	Postmortem studies	
Yao et al. (2006)	40 % depletion of GSH in the caudate nucleus	Treated earlier with antipsychotic drugs
Gawryluk et al. (2011a)	Reduced levels of GSH in prefrontal cortex	Treated earlier with antipsychotic drugs
Do et al. (2000)	Reduced levels of GSH by 52 % in prefrontal cortex and by 27 % in cerebrospinal fluid (MRS studies)	Drug-naïve
	Spectroscopy studies	
Terpstra et al. (2005)	No changes in GSH levels in:	(c) Treatment with antipsychotics
Matsuzawa et al. (2008)	a. Anterior cingulate cortex	
Wood et al. (2009) (c)	b. Posterior medial frontal cortex	
	c. Medial temporal lobe	

CSF cerebrospinal fluid, *MRS* magnetic resonance spectroscopy, *GSH* glutathione

peroxidation of membrane lipids and microdamage in dopaminergic terminals, causing the loss of synaptic connectivity (Grima et al 2003). The reduced GSH levels in cerebrospinal fluid and prefrontal cortex in patients with schizophrenia have been described (Do et al 2000; Woo et al. 2008), and the deficit of GSH and its metabolite, γ-glutamylglutamine, in the cerebrospinal fluid of patients with schizophrenia, who were drug-naïve or drug-free at that time, was also found (see Table 4). In magnetic resonance spectroscopy studies in vivo, the significant decrease in GSH levels in the prefrontal cortex in patients with schizophrenia was proved (Do et al. 2000; Gawryluk et al. 2011a, b).

Glutathione may exert its antioxidant effects through several mechanisms. GSH as redox state-regulating antioxidant is involved in the detoxification of drugs and storage of cysteine and may affect gene expression and development of neurons (Dringen 2000). Moreover, glutathione also enhances the action of glutamate at the *N*-methyl-D-aspartate receptor (NMDA) (Köhr et al. 1994; Papadia et al. 2008); thus, the reduction in GSH concentration could also contribute to the hypofunction of NMDA receptor in the brain (Steullet et al. 2006). Metabolism of dopamine also plays a pathological role in schizophrenia. It was shown that dopamine in cultured cortical neurons decreased the GSH level by 40 % due to conjugation (Grima et al. 2003). Postmortem studies have revealed reduction in GSH level (about 40 %) in the caudate nucleus of schizophrenic patients (Yao et al. 2006) and in the prefrontal cortex (Gawryluk et al. 2011). The detoxication of ROS and harmful xenobiotics by GSH occurs either by nucleophilic scavenging or as a result of the reaction catalyzed by glutathione peroxidase (GPx). GSH acts as a cofactor for antioxidant enzymes such as glutathione peroxidase and glutathione transferase (Lu 2009); regenerates other crucial antioxidants, vitamins C and E; and may directly eliminate ROS

Table 5 Decreased glutathione level (GSH) or thiol group concentration in blood cells and plasma

References	Blood cells (erythrocytes, platelets) and plasma	Antipsychotic drugs or drug-free
Altuntas et al. (2000)	↓GSH level in erythrocytes	Antipsychotic-free and chronic treatment of FGA or SGA
Dietrich-Muszalska and Olas (2009b)	↓GSH in plasma	
Dietrich-Muszalska and Olas (2009a)	↓ concentration of thiol groups in platelet proteins	
Zhang et al. (2007)	↓GSH level in plasma	
Raffa et al. (2009, 2011)	↓GSH level in erythrocytes	
Raffa et al. (2009)	↓GSH level in plasma	
Micó et al. (2011)	↓GSH level in erythrocytes	
Raffa et al. (2012a)	↓GSH level	

GSH glutathione, ↓ decrease of level

(Do et al. 2009). In our work (Dietrich-Muszalska et al. 2009c), the significant reduction in the amount of low-molecular-weight thiols such as glutathione and its precursors, cysteine (CSH), and cysteinylglycine (CGSH) was described, while a significant increase in the amount of homocysteine (HCSH) in plasma of schizophrenic patients occurred. Oxidative stress in schizophrenia seems to be partly associated with the impairment of the synthesis of GSH, since a number of polymorphisms in the gene coding the key enzyme for GSH synthesis (glutamate cysteine ligase) have been demonstrated (Tosic et al. 2006; Saadat et al. 2007). The decreased level of GSH in blood cells (erythrocytes, platelets) from schizophrenic patients is presented in Table 5. Recently, Steullet et al. (2010) have demonstrated in the mouse model that redox dysregulation affected the ventral but not the dorsal hippocampus. GSH deficit caused impairment of parvalbumin neurons with concomitant reduction in gamma oscillations in the hippocampus (Steulett 2010). A novel treatment target in schizophrenia and other psychiatric disorders seems to be glutathione that possesses a dominance as the important cellular antioxidant. Recently, the results of several studies indicate the efficacy of N-acetylcysteine (NAC), a glutathione precursor, which is a useful agent in the treatment of various psychiatric disorders including schizophrenia (Matsuzawa and Hashimoto 2011; Berk et al. 2008a,b). The mechanism of NAC action is not clearly understood. NAC, which is capable of restoring thiol stores by shifting the redox balance in favor of GSH, may act as precursor of GSH and may be involved in the modulation of glutamatergic, neurotrophic, and inflammatory pathways.

7 Oxidative Stress and Pathological Mechanisms in Schizophrenia

Schizophrenia is a progressive disorder, as suggested by the clinical evidence, and is generally considered as a neurodevelopmental disorder. Many factors play an important role in its pathophysiological mechanisms. Despite the enormous

advances that have been achieved, the pathogenesis of schizophrenia is still not clear. The brain pathology in schizophrenia is a neurodevelopmental, genetic, and environmental origin.

Biochemical alternations, especially in dopamine system in the brain with free radical production and ROS/RNS generation, leading to oxidative/nitrative damage to numerous biomolecules, might be partly responsible for the pathogenesis of this heterogeneous disorder. Oxidative stress with lower antioxidant defense and oxidative damage, reported by numerous studies, supports pathophysiological progression of schizophrenia and is consistent with the neurodegeneration hypothesis (Lieberman 1999). Inflammation has been postulated to be a factor in the pathophysiology of this disorder leading to the overproduction of prostaglandins from arachidonic acid, especially PGE2, and production of proinflammatory cytokines (IL-6) (Pedrini et al. 2012). The activity of cyclooxygenase 2 (COX-2) responsible for the synthesis of prostaglandins is also elevated.

Infection-induced developmental neuroinflammation may be pathologically relevant beyond the neonatal periods and may contribute to disease progression associated with the gradual development of the disease. Elevated risk of schizophrenia following prenatal exposure to infection is connected with cytokine-associated inflammatory events (prenatal cytokine hypothesis). The existence of the chronic inflammatory syndrome in schizophrenia has been described (Körschenhausen et al. 1996; Müller and Schwarz 2010). There is also increasing evidence that chronic inflammation, mediated by cytokines, contributes to the pathophysiology of this disorder (Potvin et al. 2008; Kim et al. 2000, 2009). Cytokines are an essential element of cell-to-cell communication in the immune system but also in the interaction between the immune system and the brain. Cytokines affect the differentiation and survival of neurons (Marx et al. 2001). Disturbances of brain development caused by prenatal maternal infections seem to be the result of cytokine-related inflammatory events (Meyer et al. 2008). The origins of increased cytokine levels in schizophrenia are not known yet. Schizophrenic patients had higher level of cytokines IL-2 and IL-6 than healthy controls (Lin et al. 1998; van Kammen et al. 1999; Behrens et al. 2008; Meyer et al. 2011). The elevated superoxide anion level in schizophrenia (with lower SOD activity) acts as a second messenger to activate NF-kB which initiates the transcription of many genes involved in the synthesis of cytokines such as IL-6, IL-1, and TNFα (Zhang et al. 2002).

Developmental neuroinflammation may affect processes that are pivotal for normal brain maturation, including myelination, synaptic pruning, and neuronal connectivity, all of which occur to a great extent during the postnatal brain maturation (Kasper and Papadimitriou 2009). Microglia that are resident macrophage of the brain are involved directly in the neuronal degeneration by producing various proinflammatory cytokines and free radicals (Yao et al. 2003; Bernstein et al. 2009). The neuropathology of schizophrenia is closely associated with microglial activation (Stefano et al. 2004). Changes in immune-inflammatory pathways with the activation of microglia increase proinflammatory cytokine generation and oxidative stress, autoimmune responses and activation of the tryptophan metabolite (TRYCAT) pathway and consequent modulation of NMDA receptors, and glutamate production

(Yao et al. 2010b). These factors may account for the higher neurodevelopmental pathology in schizophrenia.

Various lines of evidence suggest immune dysfunction in schizophrenia (Yolken and Torrey 1995) and the association of schizophrenia with autoimmune disorders and increased levels of cytokines IL-1, IL-6, and IFNγ (Zhang et al. 2009b). Neopterin that is the catabolic product of GTP serves as a marker for immune system activation and might elucidate the interaction between immune pathogenesis and oxidative stress in schizophrenia (Yao et al. 2010a). It is produced in monocytes/macrophages upon stimulation with cytokine interferon γ. High level of neopterin is associated with increased production of ROS. Conflicting findings were presented by Chittiprol et al. (2010). They have reported that antipsychotic-naïve patients with schizophrenia had significantly higher levels of neopterin nitrates and lower levels of antioxidants; after treatment with antipsychotics, there were a significant decrease in the neopterin levels and an increase in antioxidants. These studies support the view that oxidative stress in schizophrenia might be linked with immune pathogenesis (Zhang et al. 2009b).

Tryptophan/kynurenine metabolism with kynurenic acid (KA) and neurotoxic TRYCAT production is regulated by cytokines (see Chapter "The Kynurenine Pathway at the Interface Between Neuroinflammation, Oxidative Stress and Neurochemical Disturbances: Emphasis in Schizophrenia"). Increased KA and TRYCAT pathway has the effect on NMDA receptor dysfunction and neuroprogression (Anderson and Maes 2013; Najjar et al. 2013). Maternal infection and subsequent immune-inflammatory responses are also associated with oxidative stress and lower level of an important antioxidant – glutathione. This process contributes to alterations in neurodegeneration and myelination. Oxidative stress and TRYCAT pathway could modulate the CNS glial-neuronal interactions that determine synaptic plasticity as well as myelin generation and maintenance (Anderson and Maes 2013). Schizophrenia is a brain disease with extensive abnormalities found in the cognitive function and brain structures. The use of magnetic resonance imaging (MRI) allowed to measure the amount of gray and white matter. In schizophrenic patients (first episode), the loss of gray matter is accelerated, particularly in the frontotemporal cortical regions and sulcal and ventricular expansion (Kasper and Papadimitriou 2009). However, there is considerable inhomogeneity of brain abnormalities in this disorder, and the alterations in mean volume of neuron and glial cell densities in different brain regions and changes in neurotransmitter/receptor systems, growth factors, hormones, regulatory proteins, and brain energy metabolism with dysfunction of mitochondria and ROS production have been reported (Rezin et al. 2009). There is some evidence that oxidative stress supports pathophysiological progression in schizophrenia. It is consistent with neurodegeneration hypothesis.

Based on the role of oxidative stress and lipid peroxidation, Horrobin in 1998 presented two hypotheses that:

1. Alteration in membrane phospholipid composition and the lipid metabolism results in neuronal dysfunction leading to disruption of the membrane and cell damage.

2. Deficiency in antioxidant defense in the cortex of maturational development and stressful physiological activity lead to increased concentrations of ROS, which cause cellular injury dysfunction and potentially cell death.

These hypotheses highly theoretical are supported by described oxidative stress in schizophrenia and integrate basic neurobiological mechanisms with the clinical dimensions of the illness. Clinical deterioration is manifested by the development and increasing severity and persistence of psychotic and negative symptoms and cognitive impairment.

Although a number of hypotheses have been proposed in an attempt to explain the pathophysiology of schizophrenia, no single theory seems to account for all aspects of the disease.

Each hypothesis explains only some of the phenomena associated with schizophrenia; however, many variables described in these hypotheses interact to produce a disorder characterized by heterogeneous symptomatology and its progression. Converging lines of evidence including reduced neuropil suggest that disrupted cortical synaptic circuitry is a central deficit in schizophrenia (Lewis and Lieberman 2000) and apoptotic mechanisms may also be involved in this process and the pathophysiology of schizophrenia (Jarskog et al. 2005). Cortical pyramidal neurons from individuals with schizophrenia exhibit smaller somal volume, decreased spine density, decreased dendritic length, and decreased terminals compared to healthy control. These postmortem findings contribute to the hypothesis that schizophrenia stems from altered synaptic circuitry. The cortical neuropathology of schizophrenia includes neuronal atrophy, decreased neuropil, and alterations in neuronal density with suggestion of altered synaptic circuitry (Kasper and Papadimitriou 2009). Moreover, neuroimaging studies also indicate that a progressive loss of cortical gray matter occurs in the early course of schizophrenia (Lewis and Sweet 2009; Lewis 2012). The underlying mechanisms of the defects and synaptic dysfunction suggest that the dysregulation of neuronal apoptosis may contribute to the pathophysiology of the disorder. Usually, the activation of complex apoptotic pathway may lead to rapid neuronal death. The dysregulation of neuronal apoptotic cascade of pro- and antiapoptotic proteins could lead to a limited form of apoptotic pathway in terminal neuritis and individual synapses to cause synaptic elimination without the cell death and with synaptic deficit and cortical dysfunction in schizophrenia. Apoptotic mechanism seems to be responsible for progressive gray matter volume loss (first onset of psychosis) when antioxidant activity is low (Glantz et al. 2006, 2010).

Apoptotic mechanisms that can influence synaptic connectivity, and neuronal complexity seem to support the apoptotic hypothesis of schizophrenia connected also with oxidative stress. Apoptotic hypothesis proposing that limited apoptotic activity with apoptotic dysregulation can contribute to a gradual reduction in neuronal viability and to synaptic deficits without causing neuronal death might be taken into account. Oxidative stress could contribute to complex apoptotic mechanisms.

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Oxidative Stress in Bipolar Disorder

Gustavo Scola and Ana C. Andreazza

Abbreviations

5-HT	Serotonin
5meOHC	5-hydroxymethylcisteine
8-OHdG	8-hydroxy-2-deoxyguanosine
BD	Bipolar disorder
BER	Base excision repair
DNA	Glycosylase 1
ETC	Electron transport chain
GWAS	Genome-wide association studies
LPA	Lysophosphatic acid
MD	Mitochondrial dysfunction
MDD	Major depressive disorder
NER	Nucleotide excision repair
Ogg1	Oxyguanosine
PAF	Platelet-activating factor
PKA	Protein kinase A
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCZ	Schizophrenia

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1 Introduction

Bipolar disorder (BD) is characterized by mood fluctuations between episodes of mania and depression. It is also characterized by a larger loss of disability-adjusted life-years than all forms of cancer or major neurologic conditions (Merikangas et al. 2011), which elevates the health-care costs four times higher than that of the general population (Altamura et al. 2011). Therefore, BD is becoming a foremost health concern. The complex pathophysiology of BD has brought interest in several areas of research to investigate the causes and consequences of this mood fluctuation to the brain.

Several hypotheses have been postulated during this journey to identify the etiology and pathophysiology of BD, which includes inflammatory responses (Goldstein et al. 2009), genetic modifications (Schulze 2010), alteration in calcium signaling (Kato 2008a), and decrease of density and size of neurons and glia (for review see Gigante et al. 2010). Mitochondrial dysfunction, mitochondrial DNA abnormalities, protein expression of mitochondrial electron transport chain, reduced pH, and decreased levels of high-energy phosphates in the brain, as well as increased oxidative stress status, have been a common feature identified in several recent investigations carried out on patients with BD (Andreazza et al. 2008; Clay et al. 2010). Therefore, mitochondrial dysfunction and the consequent oxidative damage to biomolecules could be associated with the verified neuronal or glial impairment in BD (Beal 2002; Clay et al. 2010; Gigante et al. 2010). In the following section of this chapter, the most relevant results to date for BD and how calcium plays a role in this scenario will be discussed.

2 Evidence of Oxidative Stress in Bipolar Disorders

The central nervous system presents high amounts of oxidizable substrates, high oxygen tension, and relatively low antioxidant capacity making it extremely vulnerable to oxidative damage (Halliwell and Whiteman 2004; Sies 1991). When the cytoplasmic enzymatic and nonenzymatic antioxidant and mitochondria systems are overwhelmed by high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), the DNA, lipids, and proteins can be damaged (Lenaz 2001).

Mitochondria are intracellular organelles that play a crucial role in ATP production carried out by the electron transport chain (ETC) complexes I, II, III, IV, and V in the inner membrane through a process known as oxidative phosphorylation (Chinopoulos and Adam-Vizi 2010). Mitochondria are not only essential for energy control but also for maintaining calcium homeostasis (Kato 2008b), regulating apoptosis, and generating reactive oxygen species (ROS) (Jeong and Seol 2008).

ATP production occurs through the flow of electrons along ETC complexes transferring protons across the inner membrane, producing a large mitochondrial membrane potential. The energy lost by the reentering protons in the mitochondrial matrix, through the ATP synthase protein, is used to form ATP (Green and Kroemer 2004; Lenaz 2001; Reeve et al. 2008). Single electrons escape during the ETC transfer, resulting in a single electron reduction of molecular oxygen forming superoxide anion (O_2^-), especially in complex I (NADH:ubiquinone oxidoreductase) (Green and Kroemer 2004). Superoxide dismutase (SOD) converts the mitochondrial O_2^- into hydrogen peroxide (H_2O_2), which in the presence of ferrous iron (Fe^{+2}) results in the production of hydroxyl radicals ($OH^\bullet$) via Fenton reaction ($H_2O_2 + Fe^{+2} \rightarrow Fe^{+3} + OH^- + OH^\bullet$). Another relevant event is the reaction of $O_2^{\bullet -}$ with nitric oxide ($NO^\bullet$), reactive nitrogen species produced by microglia and astrocytes, to form peroxynitrite ($ONOO^-$) (Naoi et al. 2005).

2.1 Protein Oxidation

Proteins can have their structure and functionality modified by oxidative damage (Beal 2002; Lee et al. 2009). In BD, many proteins are targets for oxidative damage, which may include synaptic function key proteins, such as synaptophysin (Mallozzi et al. 2009). The mitochondrial ETC proteins are more vulnerable to nitrosative damage, suggesting a functional relationship between mitochondrial dysfunction and nitrosative damage (Murray et al. 2003).

In the hippocampus of patients with BD, the neuronal nitric oxide synthase I, the enzyme that generates $NO^\bullet$, was found to be upregulated, in addition to increased serum levels of $NO^\bullet$ in subjects with the same disorder (Selek et al. 2008). Protein oxidative damage can be induced by reaction with hydroxyl free radical ($OH^\bullet$), which is catalyzed by Fe^{+2} and Cu^{+2}, introducing carbonyl groups (Beal 2002). Protein nitration occurs by reaction of $ONOO^-$ with sulfhydryl and hydroxyl residues (Naoi et al. 2005). Such modifications might inactivate the membrane signaling pathways and key enzymes (Naoi et al. 2005). The tyrosine residues nitration produces 3-nitrotyrosine in proteins that serves as a marker of $ONOO^-$ oxidative damage induced in vivo (Naoi et al. 2005). This oxidative damage affects protein functionality, altering, for example, enzyme activities (Beal 2002), and susceptibility to proteolytic degradation (Naoi et al. 2005).

Results from our group reported increased levels of 3-nitrotyrosine in postmortem prefrontal cortex (Andreazza et al. 2010) and also found increased serum levels of 3-nitrotyrosine in patients with BD in both early (0–3 years) and late (10–20 years) stages of the illness (Andreazza et al. 2009). In addition, other evidences give support to the vulnerability of mitochondrial protein to nitrosative damage. A functional relationship between complex I activity and nitration was shown in mitochondrial membranes from bovine heart, where $ONOO^-$ targeted mainly

complex I subunits, resulting in significant inhibition of complex I activity (Murray et al. 2003). This is further supported by the report by Naoi et al. (2005) of increased 3-nitrotyrosine levels in the mitochondrial complex I subunits, but not other mitochondrial proteins, and of SH-SY5Y cells incubated with $ONOO^-$. Other studies demonstrated increase of the oxidative stress markers such as protein carbonylation, lipid peroxidation, and 3-nitrotyrosine levels in the brain and peripheral blood cells of BD subjects (Machado-Vieira et al. 2007; Wang et al. 2009; Andreazza et al. 2009, 2010).

2.2 DNA Oxidation

DNA is also vulnerable to oxidative damage; hydroxyl radicals react with DNA causing single- or double-strand breaks (Halliwell and Gutteridge 2007) or promote oxidation to C-8 position of deoxyguanosine on DNA, forming 8-hydroxy-2-deoxyguanosine (8-OHdG). DNA oxidation can also be induced by $ONOO^-$, which forms strand breaks and base oxidation products and cause deamination of G and A leading to formation 8-nitro-deoxyguanosine (Burcham and Harkin 1999). DNA lesions are rapidly detected by the DNA damage response system (Barzilai and Yamamoto 2004). This response culminates in activation of cell-cycle checkpoints and the appropriate DNA repair pathways (Iliakis et al. 2003). Oxidative DNA damage is mostly repaired by base excision repair (BER) and nucleotide excision repair (NER) enzymes (Halliwell and Gutteridge 2007). Most of the damage is removed before the cell reach replication preventing damage transmission to new cells (Evans et al. 2000). If this system is overwhelmed by free radicals, mutations to adenine or cytosine (A:T to G:C or G:C to T:A transversion mutations) will occur and consequently activate the apoptosis machinery (Halliwell and Gutteridge 2007).

Recently, oxidative stress and deficiency of oxyguanosine DNA glycosylase 1 (Ogg1), an enzyme responsible to repair the damage resulting from 8-OHdG, are considered to be a crucial factor in the process of aging and aging-related diseases, such as Alzheimer (Mao et al. 2007). Mitochondrial oxidative phosphorylation subunits are assembled from proteins encoded in both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Complex I is assembled from 45 subunits, 7 of those are encoded by mtDNA and the rest by nDNA; complex II is formed from 4 nDNA polypeptides; complex III has 11 subunits and cytochrome C is encoded by mtDNA; and complex IV also has only one subunit from the 13 encoded by mtDNA (Wallace 2010). Therefore, to maintain an efficient energetic metabolism, the cells have to protect both mtDNA and nDNA from damage. Damage to mtDNA and nDNA can lead to various levels of mitochondrial dysfunction (loss of control, but still functional) and disorder (loss of functionality) (Campbell and Mahad 2012).

mtDNA mutation rate is usually higher than nDNA, which can be due to the lack of protection from histones and the proximity to mitochondrial ROS production (Wallace 2010). It is reasonable to expect that mtDNA repair system would be higher

than nDNA. On the other hand, the cells do not invest just enough energy to save the mtDNA till cell reproduction or death (Wallace 2007). Thus, it has been observed that mtDNA has higher levels of oxidation than nDNA (Bohr and Dianov 1999). The importance of mtDNA to keep a healthy CNS is highlighted by the number of mtDNA disorders, where complex II (nDNA) is spared. The section "Mitochondrial Dysfunction" of this chapter will describe the findings of mitochondrial DNA mutation in BD. Andreazza et al. (2008) have demonstrated an increased DNA fragmentation in lymphocytes from patients with BD during different episodes of the diseases. Additionally, Andreazza et al. (2008) reported a positive correlation between Young Mania Rating Scale (YMRS) and the intensity of DNA damage, highlighting the importance of illness severity for these findings. The technique used in this study was COMET assay, which is an easy method to detect DNA double- and single-strand breaks or damage. Interestingly, BD is associated with other known DNA damage diseases as cardiovascular, diabetes, and obesity. Che et al. (2010) found elevated oxidative damage to nucleic acids (8-hydroxy-2'-deoxyguanosine) in CA1, CA3, and dentate gyrus regions of the hippocampus among patients with BD, schizophrenia (SCZ), and major depressive disorder (MDD). Supporting the involvement of DNA damage in BD, Buttner et al. (2007) demonstrated increased DNA fragmentation in non-GABAergic neurons in postmortem anterior cingulate cortex from patients with BD. Finally the authors suggested that the increased DNA damage may be attributed to high oxidative stress associated with BD.

Telomere shortening has been a well-thought-out sign of growing oxidative stress and a marker of antioxidant defense capacity (Saretzki and Von Zglinicki 2002). Passos et al. (2007) utilized the replicative senescence model as a reliable cellular model of aging. He verified that mitochondrial generation of ROS is crucial for determining telomere shortening. To further support the accumulative effect of oxidative damage occurring in BD, Simon et al. (2006) found that the amount of telomere shortening in patients with chronic mood disorders corresponds to 10 years of accelerated aging. As Higuchi (2004) stated that oxidative DNA damage is an intermediate step to cellular apoptosis and knowing that telomeres are located in the end of mammalian chromosomes, it is believed that the link between mitochondrial ETC dysfunction, oxidative stress, DNA damage, and cell death is a promising field in the investigation of the pathophysiology of BD.

3 Sources of Oxidative Stress Damage in Bipolar Disorder

3.1 Mitochondrial Dysfunction

Mitochondrial dysfunction (MD) is a dysregulation of the ETC complex, which can be caused by genetic alteration; different toxins capable of inhibiting mitochondrial ETC complex, such as 6-hydroxydopamine, a sub-product of dopamine oxidation; or simply impaired activity (Halliwell and Whiteman 2004). BD may be associated with the susceptibility of oxidative stress; a downregulation of several complex I

subunits occurs in BD. Human complex I is composed of 45–46 different subunits and divided into three functional modules (dehydrogenase, hydrogenase, and transporter). Finally, the transporter module is responsible for translocation of protons across the membrane (Brandt et al. 2003). Interestingly, Iwamoto et al. (2004) and Sun et al. (2006) reported that NDUFV2, NDUFS1, NDUFS7, and NDUFS8 present decreased expression in BD in the dehydrogenase module. These results suggest that patients with BD may have reduced ability to oxidize NADH and to transfer electrons to ubiquinone. This means that O_2^- is produced because electrons may persist for sufficient time to react with molecular oxygen (Boveris and Chance 1973; Turrens and Boveris 1980; Andreazza et al. 2010). Together, the organization of complex I, downregulation of complex I subunits, and diminished antioxidant levels support the susceptibility of proteins from mitochondrial oxidative damage in BD. These oxidations on protein residues can alter protein function or lead to deleterious intermolecular aggregates (Beal 2002). Decreased expression of genes involved in proteasome degradation process was found in the prefrontal cortex of subjects with BD, suggesting a faster accumulation of carbonylated proteins (Konradi et al. 2004). The etiology and/or progression of several chronic central nervous system disorders, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis, is associated with the accumulation of carbonylated proteins (Castegna et al. 2002a, b).

Many lines of studies suggest that MD plays a role in BD pathophysiology. In 1995, McMahon et al. (1995) speculated that mtDNA and imprinted DNA can be inherited from the mother and increase 1.3- to 2.5-fold the risk of illness for the offspring of affected mothers. Additionally, a deletion on mtDNA of 4,977 bp, known as the "common deletion," was reported to be associated with BD and SCZ. In the cortex of BD probands and suicide victims, significant increases in the prevalence of the common deletion were found (Kato et al. 1997). Kakiuchi et al. (2005) reported controversial results failing to find the association of common deletion levels with BD and schizophrenia individuals. Another genetic alteration is the polymorphism in the mt-ND1 gene (366T>C), supporting the involvement of mtDNA mutations in ETC functionality, associated with BD. The mt-ND1 alteration causes a decreased mitochondrial membrane potential and complex I activity (Munakata et al. 2004). A decreased mitochondrial matrix pH has been linked to 10398A>G polymorphism in BD and, also, to higher baseline and poststimulation mitochondrial Ca^{2+} levels (Kato and Kato 2000; Kato et al. 2003; Kazuno et al. 2006, 2008). Interestingly, mtDNA copy number can be modulated by the energy requirement for the cell. For example, Liu et al. (2003) reported that human leukocytes increase the mtDNA copy number in response to oxidative stress. Wallace (2007) reported that mutation in mtDNA accumulated with the aging process and might be a response to increment of oxidative damage during this process.

For instance, in BD, many mRNAs coding for electron ETC complexes I–V subunits, especially complex I, presented decreased expression (Clay et al. 2010). Postmortem hippocampus (Konradi et al. 2004) and frontal cortex (Iwamoto et al. 2005; Sun et al. 2006) revealed decreased expression of several mRNAs coding for ETC complexes I–V subunits by DNA microarray analyses. Iwamoto et al. (2005)

reported that mRNA levels in the prefrontal cortex of subjects with BD such as complex I subunit NDUFS1, complex III subunit UQCRC2, and complex IV subunit COX15 were decreased. Konradi et al. (2004) also identified nuclear mRNA coding for mitochondrial proteins and genes regulating oxidative phosphorylation and the adenosine triphosphate-dependent process of proteasome degradation. Sun et al. (2006) reported a downregulation of 8 mitochondrial ETC-related genes, using high-density cDNA spot microarrays, consisting of NDUFS7 and NDUFS8 (complex I), UQCRC2 (complex III), COX5A and COX6C (complex IV), and ATP5C1 and ATP5J (complex V) and confirmed that mRNA levels of NDUFS7 were decreased using real-time quantitative PCR. Moreover, evidence from several genotyping studies suggest that polymorphisms of complex I subunit NDUFV2 may be associated with BD, thus supporting the involvement of mitochondrial complex I dysfunction in BD (Xu et al. 2008; Washizuka et al. 2009). Moreover, other recent studies in subjects with BD have demonstrated alterations in a diverse set of oxidative stress parameters, such as alterations in antioxidant enzymes (Kuloglu et al. 2002; Savas et al. 2006; Andreazza et al. 2008), increased lipid peroxidation (Kuloglu et al. 2002; Savas et al. 2006; Machado-Vieira et al. 2007; Andreazza et al. 2008), increased DNA fragmentation (Andreazza et al. 2008; Buttner et al. 2007), and increased levels of nitric oxide (Savas et al. 2002; Selek et al. 2008) on peripheral blood cells. Oxidative damage modifies the structure and function of proteins, suggesting that such alterations might be connected with disease outcome (Beal 2002; Lee et al. 2009).

3.2 Calcium Metabolism

Several critical cellular responses are controlled by Ca^{2+}, which is a key element in signal transduction (Murray et al. 2003). Thus, Ca^{2+} ion levels are transported across the plasma membrane and the membranes of intracellular organelles through a number of tightly controlled channels, pumps, and exchangers. Calcium is mainly buffered by two organelles: endoplasmic reticulum and mitochondria (Adam-Vizi and Starkov 2010). Growing attention has been put toward increasing the understanding of the mechanisms involved in Ca^{2+} ion uptake by the mitochondria. Mitochondrial machinery is activated by the accumulation of Ca^{2+} leading to increased ATP synthesis and ATP levels in the cytosol (Rizzuto et al. 1999). The accumulation of calcium accelerates H^+ extrusion and activates oxidative phosphorylation (Hansford 1985; Santo-Domingo and Demaurex 2010), which can in turn increase ROS production (Adam-Vizi and Starkov 2010).

The ROS production induced by calcium can be triggered by different pathways including *(1)* activation of mitochondrial electron transport chain and thus increase the probability of leaking O_2^- in mitochondrial complex I and complex III; *(2)* stimulation of nitric oxide synthase (NOS) that increases the production of $NO^{\cdot}$ by catalyzing the oxidation of a guanidine nitrogen of L-arginine (L-Arg) producing L-arginine as an intermediate and $NO^{\cdot}$ as a final product; and *(3)* stimulation of

calcium-dependent endonucleases, which can induce DNA fragmentation, an important event in apoptosis. On the other hand, H_2O_2 can increase intracellular Ca^{2+} levels through the opening of TRPM2 cation channels resulting in cellular loss if not interrupted. The prolonged increase of Ca^{2+} can also induce mitochondrial permeability transition pore, leading to mitochondrial swelling and cytochrome C release resulting in cell death by apoptosis (Giorgi et al. 2012; Miller and Zhang 2011).

In BD, elevated intracellular Ca^{2+} and abnormal Ca^{2+} signaling have been recognized as markers (Kato 2008). The first report of elevated calcium concentration in BD was found in platelets (Dubovsky et al. 1994). Also, cells of patients afflicted with both depression and mania had increased free intracellular calcium ion concentrations (Dubovsky et al. 1994). Euthymic lithium-treated patients presented increased total serum and ionized calcium when compared to healthy controls (El Khoury et al. 2002). Besides, elevated basal calcium concentrations have been detected in transformed B lymphoblasts in BD-I compared with those with BD-II, major depression, or healthy controls (Emamghoreishi et al. 2000). In all mood states of BD, calcium homeostasis appears altered. Further, thrombin, serotonin (5-HT), and platelet activating factor (PAF) are agonist-induced calcium influx and are enhanced in cells derived from patients with BD regardless of the agonist used (Kato 2008). In peripheral blood cells of patients with BD, thapsigargin-induced cytosolic Ca^{2+} response was found to be increased (Hough et al. 1999; Kato et al. 2003; Perova et al. 2008). Perova and colleagues (2010) stimulated B lymphoblast cell lines from patients with BD-I with lysophosphatic acid (LPA), showing increased Ca^{2+} mobilization.

Another piece of evidence that supports the calcium involvement in BD come from genome-wide association studies (GWAS) and linkage studies. GWAS of large groups of patients and controls are a very promising strategy to identify relevant genetic biomarkers. GWAS meta-analyses showed that CACNA1C and ANK3 (ankyrin 3) are the major candidate risk loci in BD (Sklar et al. 2008; Ferreira et al. 2008; Kempton et al. 2009; Schulze et al. 2009; Bigos et al. 2010). CACNA1C gene encodes for the alpha-1 subunit of an L-type voltage-dependent calcium channel named Cav1.2. This gene, nearly 300 kb, including 44 invariants and 6 alternative exons with a coding region of over 8 kb is located on chromosome 12q13.3. Some studies showed in patients with BD intriguing associations between CACNA1C SNP Rs1006737 (A/A genotype) and higher gray matter volume (Kempton et al. 2009), increased gray matter density in the right amygdala and hippocampus with some equivocal results (Bigos et al. 2010), and increased limbic activity during an emotional or reward task in fMRI (Jogia et al. 2011).

Intracellular calcium levels are tightly regulated via L-type (cav1.2 and Cav1.3) calcium channels and consist of 24 transmembrane segments, which are activated by membrane depolarization and mediate cellular Ca^{2+} influx (Catterall 2000). Cav1.2 is a complex protein containing four subunits in the cardiac form (an α1 subunit of 190–250 kDa, a transmembrane disulfide-linked complex of α2 and δ subunits, a β intracellular subunit, and a γ transmembrane subunit) and three subunits in the neuronal form (α1, α2δ, β). The expression of voltage-dependent Ca^{2+} channels is regulated

through the phosphorylation pathway by a second messenger-activated protein (Catterall 2000). Ser1928 in the C-terminal domain is a target for phosphorylation through protein kinase A (PKA) (De Jongh et al. 1996) and plays a pivotal role in the functionality of Cav1.2, at least, in the cardiac isoform, as α1C subunit was found to bind to calmodulin, modulating Ca^{2+}-dependent inactivation and facilitation of the channel (Kameda et al. 2006). Supporting the integration between mitochondria and calcium channels, Koh et al. (2003), using myocytes from cerebral arteries, demonstrated that mitochondria sense IP3R-mediated sarcoplasmic reticulum Ca^{2+} release to control NF-κB-dependent Cav1.2 channel expression.

4 Concluding Remarks: Perspectives of Oxidative Stress

As described above, the regulation of energy metabolism through decreased mitochondrial electron transport chain functionality and consequent increased oxidative stress damage to lipids, proteins, and DNA, as well as calcium metabolism, may be central to the pathophysiology of BD (Fig. 1). Oxidative stress can cause structural modifications to DNA purine and pyrimidine bases or induce posttranslational

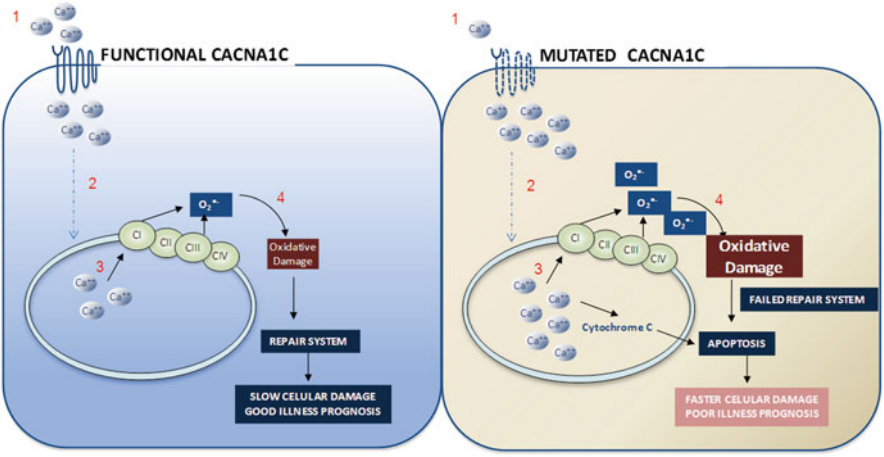

Fig. 1 Oxidative stress, mitochondrial dysfunction, and calcium voltage-dependent channels: an integrative model for bipolar disorder. *1*. Calcium influx is controlled by CACNA1C. When it is mutated, CACNA1C loses its ability to control Ca^{2+} influx. *2*. Protein interactors of CACNA1C will guide calcium signaling. *3*. Mitochondria can sense increased levels of Ca^{2+} to buffer it. Ca^{2+} is essential for normal mitochondrial electron transport chain (mETC) functionality. Under normal physiology, the leakage of superoxide ($O_2^{\bullet -}$) is controlled by the antioxidant repair system and the oxidative damage to biomolecules is under control. Excessive intra-mitochondrial Ca^{2+} concentration over-activates mETC, producing an excessive $O_2^{\bullet -}$. *4*. Combining excessive free radical production with failed antioxidant repair system, the oxidative damage to biomolecules will happen and it will affect protein functionality, leading to apoptosis if not stopped by antioxidant enzymes or repair system

modifications in proteins. Interestingly, epigenetic changes also play an important role in the etiology of BD. Epigenetics is characterized as a process that modifies gene expression through alteration in DNA methylation and chromatin structure without changing the genomic DNA sequence (Tseng et al. 2008). Using an epigenome-wide approach to verify DNA methylation of specific genes, Cui et al. (2007) found epigenetic differences at genes involved in neuronal development and loci implicated in oxidative stress and mitochondrial dysfunction. Recently, Nohesara et al. (2011) found that similar to their previous findings in the prefrontal cortex, MB-COMT promoter was hypomethylated (~50 %) in DNA derived from the saliva in SCZ and BD, compared to controls.

Emerging evidence suggests an interaction between oxidative stress and DNA methylation (Yucel et al. 2008). Cytosine (i.e. 5-hydroxymethylcytosine) or guanine oxidation (i.e. 8-hydroxy-2'-deoxyguanosine) promotes DNA demethylation through decreasing the affinity of the methyl group binding to DNA CpG islands, thus inducing changes in the expression of several genes (Fig. 2). Therefore, future studies evaluating the connection between oxidative damage to DNA and DNA methylation are essential to explain whether the oxidative stress can play a role in the well know decreased gene expression in BD (Wang et al. 2009).

Our understanding of molecular defects leading to BD is limited, which significantly prevents the development of new treatments for this illness. The evolution of

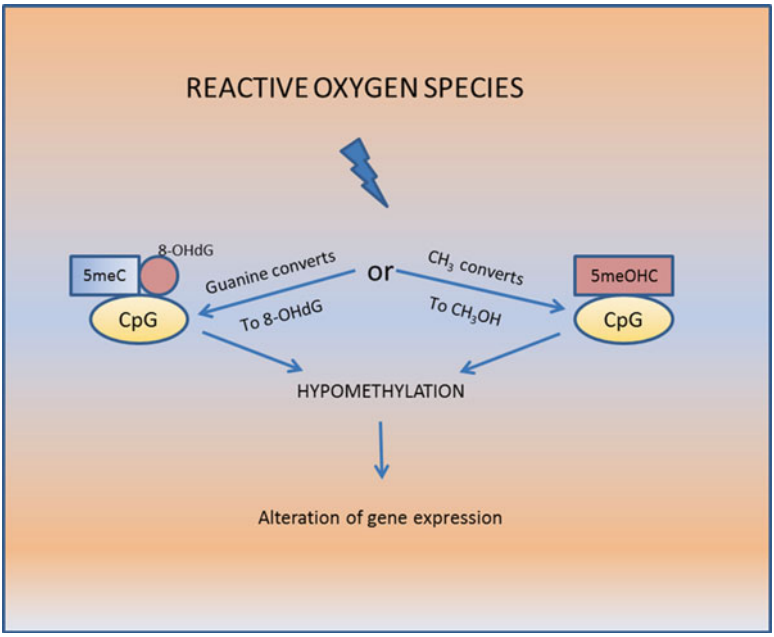

Fig. 2 Oxidative stress and DNA methylation/demethylation pathways. Reactive oxygen species (ROS); 5-meC, 5-methylcytosine; 5-meOHC, 5-hydroxymethylcytosine; 8-OHdG, 8-hydroxy-deoxyguanosine. Reactive oxygen species (ROS) induce redox alterations to guanine (8-OHdG) or cytosine (5-meC/5-meOHC), leading to DNA aberrations and alterations to gene expression

studies exploring the relationship among oxidative stress, DNA methylation, and gene expression will ultimately open avenues to the development of new strategies that may prevent oxidative stress damage to biomolecules, thus translating the knowledge from the bench to the clinic.

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Contribution of Oxidative Stress to the Pathophysiology of Autism Spectrum Disorders: Impact of Genetic and Environmental Factors

Ved Chauhan and Abha Chauhan

Abbreviations

ADHD	Attention deficit hyperactivity disorder
ASDs	Autism spectrum disorders
BPA	Bisphenol A
CNS	Central nervous system
COX	Cyclooxygenase
DNA	Deoxyribonucleic acid
EDCs	Endocrine-disrupting chemicals
ER	Estrogen receptors
ETC	Electron transport chain
GABA	Gamma aminobutyric acid
Glo 1	Glyoxalase 1
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidized glutathione
GSTM1	Glutathione S-transferase M1
H_2O_2	Hydrogen peroxide
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAOA	Monoamine oxidase A

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MD	Mitochondrial dysfunction
MDA	Malonyldialdehyde
mGluR5	Metabotropic glutamate receptor 5
MMP	Mitochondrial membrane potential
MTHFR	Methylene tetrahydrofolate reductase
NO	Nitric oxide
$ONOO^-$	Peroxynitrite anions
PBDEs	Polybrominated diphenyl ethers
PCBs	Polychlorinated biphenyls
PDD-NOS	Pervasive developmental disorder-not otherwise specified
PDDs	Pervasive developmental disorders
PDE	Phosphodiesterase
PE	Phosphatidylethanolamine
RBC	Red blood cell
RFC	Reduced folate carrier
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAH	*S*-adenosinehomocysteine
SAM	S-adenosylmethionine
SNPs	Single nucleotide polymorphisms
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TNF	Tumor necrosis factor
VPA	Valproic acid
XO	Xanthine oxidase

1 Autism Spectrum Disorders (ASDs)

ASDs are neurodevelopmental disorders characterized by impairments in social interactions and in verbal and nonverbal communication skills and by restricted, repetitive, and stereotyped patterns of behavior (Lord et al. 2000). ASDs include autistic disorder (also called "classical" autism), Asperger syndrome, and pervasive developmental disorder-not otherwise specified (PDD-NOS). Pervasive developmental disorders (PDDs) is a broader category, which includes the ASDs above, plus childhood disintegrative disorder and Rett syndrome. According to a recent report from the Centers for Disease Control and Prevention, the prevalence of ASDs in the USA is 1 in 68 children (Wingate et al. 2014). The symptoms of ASDs are typically present before the age of 3 years and are often accompanied by abnormalities in cognitive functioning, learning, attention, and sensory processing. Some children first show signs of normal social and language development, but these developmental skills are lost at 15–24 months and they develop autistic behavior, a condition known as regressive autism (Ozonoff et al. 2005). The reported incidence of regressive autism varies from 15 to 62 % of cases in different studies (Goldberg

et al. 2003; Hansen et al. 2008; Lord et al. 2004; Stefanatos 2008). In a few cases, regression may significantly affect language, with a lesser impact in other domains such as social interaction or imaginative play (Goldberg et al. 2003; Stefanatos et al. 1995). On the other hand, some children may regress particularly in social functions and not in language (Luyster et al. 2005).

While the cause of autism remains elusive, autism is considered a multifactorial disorder that is influenced by multiple genes and environmental factors. Some studies have suggested prenatal and perinatal onset for developmental abnormalities in autism (Kolevzon et al. 2007; Kinney et al. 2008; Miller et al. 2005). Although autism is behaviorally defined, many biochemical and immunological abnormalities have been reported in autism (Chauhan and Chauhan 2006; Chauhan et al. 2009a, 2011b, 2012, 2012a, b; Pardo-Villamizar and Zimmerman 2009; Onore et al. 2012; Rossignol and Frye 2012a, b). Extensive evidence from our and other groups suggests that oxidative stress may serve as a common link between susceptibility genes and environmental risk factors, resulting in the clinical development of autism (Chauhan and Chauhan 2006; Chauhan et al. 2009a; Deth et al. 2008; Herbert 2010).

2 Oxidative Stress

Under normal conditions, a dynamic equilibrium exists between the production of free radicals and the antioxidant capacity of the cell. Free radicals include reactive oxygen species (ROS) (such as superoxide and hydroxyl) and reactive nitrogen species (RNS) (such as peroxynitrite and nitrite) (Fig. 1). Oxidative stress occurs when ROS levels exceed the antioxidant capacity of the cell. Elevated ROS levels can be due to increased ROS generation or decreased antioxidant capacity or both.

3 Oxidative Stress in Autism: Increased Oxidative Damage Coupled with Reduced Antioxidant Defense

Numerous studies have provided evidence for elevated oxidative damage and reduced antioxidant defense in autism. Any condition that generates imbalance of free radicals will lead to oxidative stress. We hypothesized that increased vulnerability to oxidative stress by endogenous or environmental prooxidants in conjunction with genetic susceptibility factors may contribute to the development and clinical manifestations of autism (Chauhan and Chauhan 2006). In fact, the markers of lipid peroxidation, protein oxidation, and/or DNA oxidation have been reported to be increased in blood and urine and in postmortem brain samples from subjects with autism. We reported increased levels of malonyldialdehyde (MDA), a marker of lipid peroxidation in the plasma of children with autism compared to their typically developing siblings (Chauhan et al. 2004a), as well as increased lipid peroxidation (Chauhan et al. 2011b; Muthaiyah et al. 2009), DNA oxidation (Chauhan

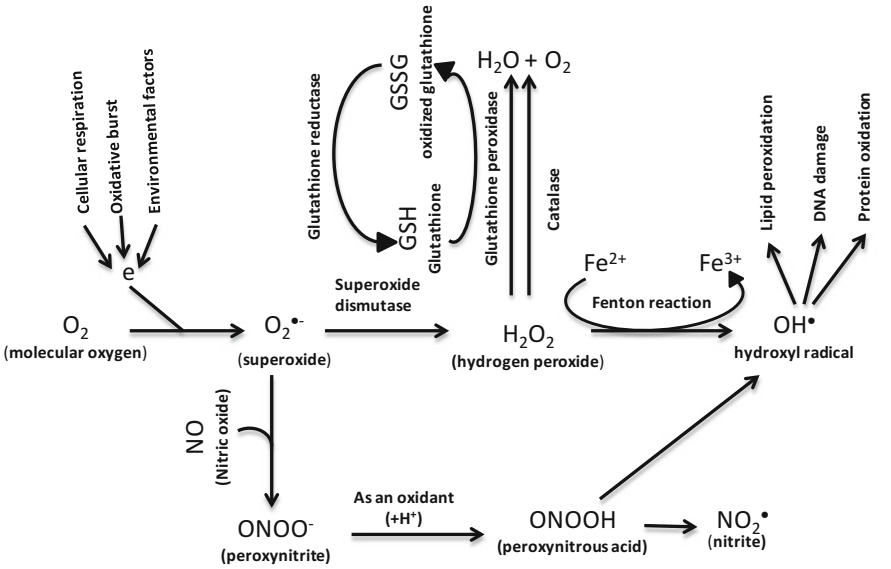

Fig. 1. The generation of free radicals (ROS and RNS) and antioxidant defense system. Molecular oxygen is reduced by an electron, which is provided by cellular respiration, oxidative burst, or environmental factors, and as a result, superoxide radicals are generated. Superoxide is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 either is neutralized to H_2O and O_2 by glutathione peroxidase (GPx) and catalase, or it can produce hydroxyl radical ($OH^{\bullet}$) by Fenton reaction. During conversion of H_2O_2 into H_2O, reduced glutathione (GSH) is oxidized by GPx. Oxidized glutathione (GSSG) is then converted back to its reduced form by glutathione reductase. RNS are produced when nitric oxide (NO) reacts with superoxide to generate peroxynitrite ($ONOO^-$), a free radical. $ONOO^-$ is an oxidant and gives rise to peroxynitrous acid (ONOOH), which generates nitrite and hydroxyl free radicals. Hydroxyl radicals are the most potent free radicals that oxidize lipids, proteins, and DNA, leading to cellular damage

et al. 2011a), and protein oxidation (Chauhan et al. 2010) in the cerebellum and frontal and temporal regions of the brain in autism. Other studies also indicated increased levels of lipid peroxidation and protein oxidation markers in autism, thus confirming increased oxidative stress in autism. Zoroglu et al. (2004) reported increased thiobarbituric acid (TBA)-reactive substances in the erythrocytes of autism subjects as compared to normal controls. Ming et al. (2005) reported increased excretion of 8-isoprostane-F2α in the urine of children with autism. Isoprostanes are produced from the free radical oxidation of arachidonic acid through nonenzymatic oxidation of cell membrane lipids. Evans et al. (2008) reported increased levels of lipid-derived oxidative protein modification, i.e., carboxyethyl pyrrole and iso 4-levuglandin E2-protein adducts, in the brain, primarily in the white matter of autistic subjects. Sajdel-Sulkowska et al. (2009) reported increased levels of 3-nitrotyrosine (a specific marker for oxidative damage of protein) in the cerebellum of autistic subjects. The density of lipofuscin, a matrix of oxidized lipid and cross-linked protein, was also observed to be greater in the

cortical brain areas involved in social behavior and communication in autism (López-Hurtado and Prieto 2008).

As represented in Fig. 1, many enzymes participate in the elimination of free radicals. Alterations in the enzymes that play a vital role in the antioxidant defense mechanism against damage by ROS have also been reported in autism. For instance, compared to control subjects, individuals with autism showed decreased activity of glutathione peroxidase (GPx) in erythrocytes and plasma (Yorbik et al. 2002; Pasca et al. 2006) and decreased activities of catalase (Zoroglu et al. 2004) and superoxide dismutase (SOD) (Yorbik et al. 2002) in erythrocytes. We also reported increased oxidative damage and free radical generation, coupled with reduced activities of antioxidant enzymes, in lymphoblastoid cells from autistic subjects compared with age-matched control subjects (Essa et al. 2009).

In a study of Egyptian children, oxidative stress was found in 88.64 % of autistic children, as revealed by elevated plasma F2-isoprostane and/or reduced GPx levels (Mostafa et al. 2010). In Saudi children with autism, decreased glutathione (GSH) levels and SOD activity in red blood cells (RBCs) were observed (Al Gadani et al. 2009). Meguid et al. (2011) recently reported lower levels of SOD and GPx and increased lipid peroxidation in blood samples from autistic children as compared with control subjects.

Glutathione is the most important endogenous antioxidant for detoxification and elimination of environmental toxins and free radicals. Several clinical studies have reported lower levels of reduced glutathione (GSH), higher levels of oxidized glutathione (GSSG), and a lower redox ratio of GSH/GSSG in the plasma of individuals with autism (Adams et al. 2009, 2011; Al Gadani et al. 2009; Bertoglio et al. 2010; Geier et al. 2009; James et al. 2004, 2006). Recently, we have reported reduced levels of GSH, increased levels of GSSG, and a decrease in the ratio of GSH/GSSG in the cerebellum and temporal cortex of autistic subjects compared with age-matched control subjects (Chauhan et al. 2012a, b). In another study, James et al. (2009) reported a decreased ratio of GSH/GSSG in the lymphoblastoid cells from autistic subjects.

Ceruloplasmin (a copper-transporting protein) and transferrin (an iron-transporting protein) are major antioxidant proteins that are synthesized in several tissues, including the brain (Loeffler et al. 1995; Arnaud et al. 1988). Ceruloplasmin inhibits the peroxidation of membrane lipids catalyzed by metal ions, such as iron and copper (Gutteridge 1983). It also acts as ferroxidase and SOD, and it protects polyunsaturated fatty acids in RBC membrane from active oxygen radicals (Arnaud et al. 1988). Transferrin acts as an antioxidant by reducing the concentration of free ferrous ion (Loeffler et al. 1995). Ferrous ion contributes to oxidative stress by catalyzing the conversion of H_2O_2 to highly toxic hydroxyl radicals by the Fenton reaction (McCord and Day 1978). In addition, the Fe^{3+}-protoporphyrin (heme) group is also present in the four protein subunits of catalase enzyme (Chance and Schonbaum 1962). We have reported reduced levels of ceruloplasmin and transferrin in the serum of children with autism as compared to their developmentally normal siblings (Chauhan et al. 2004a). Interestingly, the levels of ceruloplasmin and transferrin were reduced more effectively in children with regressive autism who had lost

previously acquired language skills (Chauhan et al. 2004a). Other preliminary studies have also suggested altered serum Cu/Zn ratios in autism (McGinnis 2004).

Aberrant metabolism of the methionine cycle has also been suggested in autism. Methionine is a main amino acid in the metabolism of glutathione. Hyperhomocysteinemia can cause oxidative stress via a number of mechanisms such as auto-oxidation of homocysteine to form ROS (Heinecke et al. 1987), increased lipid peroxidation (Jones et al. 1994), and reduced production of GPx (Upchurch et al. 1997). Pasca et al. (2006) reported higher levels of total homocysteine in the plasma of children with autism compared with control subjects. In the autistic group, a strong negative correlation was observed between homocysteine levels and GPx activity, suggesting an association between high levels of homocysteine and oxidative stress in autism. Within the methionine cycle, there are redox-sensitive enzymes, i.e., methionine synthase, betaine homocysteine methyltransferase, and methionine adenosyltransferase, which are downregulated by oxidative stress. Clinical studies have reported lower concentrations of methionine, homocysteine, cystathionine, and cysteine as well as a decreased ratio of S-adenosylmethionine (SAM)/*S*-adenosinehomocysteine (SAH), an indicator of methylation capacity in the plasma of children with autism (Adams et al. 2011; Geier et al. 2009; James et al. 2004, 2006). An increased vulnerability to oxidative stress and a decreased capacity for methylation (significantly lower ratio of SAM to SAH) is, therefore, suggested in autism. According to the "redox/methylation hypothesis of autism" proposed by Deth et al. (2008), oxidative stress initiated by environmental factors in genetically vulnerable individuals leads to impaired methylation and neurological deficits secondary to reductions in the capacity for synchronizing neural networks. Interestingly, the parents of autistic children were found to share similar metabolic deficits in methylation capacity and GSH-dependent antioxidant/detoxification capacity, as observed in autistic children (James et al. 2008).

Xanthine oxidase (XO) is an endogenous prooxidant that produces superoxide radicals during conversion of xanthine to uric acid (Kellogg and Fridovich 1975). Increased XO activity has been reported in the erythrocytes of autistic subjects (Zoroglu et al. 2004).

Nitric oxide (NO) is another free radical that can react with superoxide anion and generate cytotoxic peroxynitrite anions ($ONOO^-$) (Fig. 1). NO is known to affect the development and function of the central nervous system (CNS). Its role has been implicated in neurotransmitter release (Lonart et al. 1992), neurite growth (Hindley et al. 1997), synaptogenesis (Truman et al. 1996), memory and learning (Holscher and Rose 1992), and macrophage-mediated cytotoxicity (Hibbs Jr. et al. 1988). The expression of inducible nitric oxide synthase (iNOS) and the production of NO are also known to affect inflammatory processes (Wong and Billiar 1995). The induction of iNOS is mediated by the cytokines, namely, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β (Nussler et al. 1992). Sogut et al. (2003) reported increased NO levels in the RBCs of autistic subjects and suggested that NOS may be activated in autism. Elevated plasma levels of nitrite and nitrate in autism were also reported by Zoroglu et al. (2003) and Sweeten et al. (2004). A positive correlation was observed between nitrates and IFN-γ levels in the autistic

subjects, suggesting an association of elevated plasma NO with IFN-γ activity in autism (Sweeten et al. 2004).

It was reported that cholinergic receptors known to be sensitive to NO• toxicity were decreased in the cortex of subjects with autism (Perry et al. 2001). Additionally, treatment with cholinergic agonists improved behavioral abnormalities in autism (Hardan and Handen 2002). In other studies, gamma aminobutyric acid (GABA) receptors that are sensitive to oxidative stress were reduced in the hippocampus of individuals with autism (Blatt et al. 2001), and an association between a GABA(A) receptor beta 3 (GABRβ3) polymorphism with autism was suggested by Buxbaum et al. (2002). Fatemi et al. (2011) reported that upregulation of metabotropic glutamate receptor 5 (mGluR5) is associated with the underexpression of both fragile X mental retardation protein (FMRP) and GABRβ3 in autism. It has been suggested that a dysfunction of GABAergic signaling in early development may lead to a severe synaptic excitatory/inhibitory imbalance in neuronal circuit, which may be a contributing factor in the behavioral deficits observed in ASDs (Pizzarelli and Cherubini 2011).

4 Genetic Susceptibility to Oxidative/Nitrosative Stress in Autism

The cause of autism is not known, but genetic and environmental factors have been suggested to contribute to the etiology of autism. Gene mutations or deletions, copy number variations, and other genetic abnormalities are all persuasively linked to autism (Sutcliffe 2008). It has been suggested that a genetically susceptible population may be vulnerable to oxidative/nitrosative stress in autism. In addition, mutations in genes involved in oxidative/nitrosative stress may also facilitate oxidative stress in autism. Kim et al. (2009b) genotyped nine single nucleotide polymorphisms (SNPs) in the NOS-I gene and nine SNPs in the NOS-IIA gene, and conducted a transmission disequilibrium test (TDT) and haplotype analysis in 151 Korean ASD trios. They reported significant evidence for an association between NOS-IIA and ASD in the Korean population.

Glyoxalase 1 (Glo 1) plays a critical role in the detoxification of dicarboxylic compounds, thereby reducing the formation of advanced glycation end products. Glo 1 uses GSH as a cofactor to detoxify cytotoxic 2-oxoaldehydes, such as methylglyoxal, which are produced by lipid peroxidation, glycation, and degradation of glycolytic intermediates (Thornalley 2003). While two studies reported an SNP in Glo 1 in autism (Junaid et al. 2004; Sacco et al. 2007), other studies did not find an association between Glo 1 gene and autism (Rehnstrom et al. 2008; Wu et al. 2008).

Monoamine oxidase A (MAOA) catalyzes the oxidation of endogenous amine-containing neurotransmitters such as serotonin and norepinephrine (Fitzpatrick 2010). The role of MAOA in autism is of particular interest because this enzyme affects the levels of serotonin, which are known to be abnormal in some individuals with autism (Cohen 2010; Hranilovic et al. 2007). A 30-base pair (bp) repeat polymorphism

(uVNTR) within the MAOA promoter region consists of 3, 3.5, 4, or 5 copies. Expression studies have indicated that the number of repeats determines the transcriptional efficiency of the MAOA gene. In comparison to other alleles, the 3-repeat allele is associated with reduced transcription and, therefore, reduced activity of MAOA (Sabol et al. 1998; Denney et al. 1999). An association of the 3-repeat MAOA-uVNTR allele (low activity) with increased severity of autism has been reported (Cohen et al. 2003; Cohen 2010; Cohen et al. 2011). Yoo et al. (2009) also reported preferential transmission of the 3-repeat allele of an MAOA-uVNTR marker in ASDs in a Korean population. Furthermore, Davis et al. (2008) reported an association between MAOA-uVNTR polymorphism and brain growth in autism. A magnetic resonance imaging (MRI) study showed an increase in the volume of white matter in the brain of children with autism who have the low-activity, 3-repeat allele compared to those with a high-activity, 4-repeat allele.

Cyclooxygenase (COX) is an enzyme that is responsible for the formation of important biological mediators called prostanoids, including prostaglandins, prota-cyclin, and thromboxane. During inflammation, COX-2 is rapidly induced by growth factors, cytokines, and inflammatory molecules. Yoo et al. (2008) examined the relationship between ASDs and polymorphism of PTGS2 (the gene encoding COX-2) in 151 Korean family trios including children with autism. They reported a significant association of one intronic SNP (OS2745557) and the GAAA haplotypes with ASDs.

The role of the folate gene polymorphism has also been suggested in autism. Adams et al. (2007) reported that the 19-bp deletion polymorphism of dihydrofolate reductase may act independently or in concert with related folate polymorphisms as a significant risk factor for autism. James et al. (2006) reported differences in allele frequency and/or significant gene-gene interactions for genes encoding the reduced folate carrier (RFC) and methylene tetrahydrofolate reductase (MTHFR). Recently, Schmidt et al. 2012 reported association of maternal periconceptional folic acid intake with reduced ASD risk, which was strongest for mothers and children with MTHFR 677 C>T variant genotypes. Folate deficiency can increase oxidative stress by increasing the levels of homocysteine, and it can also contribute to increase in cytosolic calcium and to subsequent mitochondrial and DNA damage.

5 Role of Mitochondria in Free Radical Generation and Mitochondrial Dysfunction in Autism

Free radicals are generated endogenously during oxidative metabolism and energy production by mitochondria in the cell (Cadenas and Davies 2000; Lenaz 2001). The mitochondrial respiratory chain, also known as the electron transport chain (ETC), consisting of five enzymes, i.e., complexes I–V, is responsible for the generation of energy in the form of ATP (Bertram et al. 2006; Szewczyk and Wojtczak 2002). ETC complexes I–IV generate a proton gradient, i.e., mitochondrial membrane potential (MMP), which is needed by complex V (ATP synthase) for ATP

production. The mitochondria are also one of the main sources of free radicals, i.e., ROS and RNS, and they induce oxidative stress and trigger apoptosis (Brookes et al. 2002; Cadenas and Davies 2000; Lenaz 2001; Polster and Fiskum 2004). As shown in Fig. 1, molecular oxygen during cellular respiration is reduced to superoxide radical. Mitochondrial ETC complexes I and III are the main sites of production of superoxide radical by mitochondria (Barja 1999; Muller et al. 2004). While oxidative phosphorylation in the mitochondria generates superoxide anions, enzymatic oxidation of biogenic amines by MAO in the outer mitochondrial membrane produces H_2O_2.

Mitochondria play an important role in regulating developmental processes, including neurite outgrowth, axonal plasticity, and synaptic plasticity (Mattson and Liu 2002). The brain has a high demand for energy, and neurons contain a large number of mitochondria, especially in synapses. Therefore, neurons' function and plasticity rely on mitochondria, which are localized in synapses. Alterations of the number, morphology, or function of synaptic mitochondria can be detrimental to synaptic transmission (Polster and Fiskum 2004).

In a recent meta-analysis of data in the literature, Rossignol and Fyre (2012a) reported the strongest evidence for immune dysregulation/inflammation and oxidative stress, followed by toxicant exposures and mitochondrial dysfunction (MD) in autism. Several studies with blood, muscle biopsy, and postmortem brain tissue samples have suggested mitochondrial dysfunctions in a subset of individuals with autism (Chauhan and Chauhan 2012; Chauhan et al. 2011b, 2012b; Gargus and Imtiaz 2008; Haas 2010; Palmieri and Persico 2010; Rossignol and Frye 2012b). The review of previous reports and meta-analysis conducted by Rossignol and Frye (2012b) suggested deficiencies of complexes I, III, V, IV, and II in 53 %, 30 %, 23 %, 20 %, and 9 % of children with ASD and concomitant MD, respectively. Multiple complex deficiencies were reported in 36 % of the children with ASD/MD. In the lymphoblastoid cells from autistic subjects, we reported reduced MMP and increased free radical generation (Chauhan et al. 2009b). Recently, we also reported brain region-specific changes in the levels of mitochondrial ETC complexes, in subjects with autism. In autistic children (4–10 years of age), we observed significantly lower levels of complexes III and V in the cerebellum of complex I in the frontal cortex and of complexes II, III, and V in the temporal cortex as compared to age-matched control subjects (Chauhan et al. 2011b). These studies suggest that MD in autism may occur due to ETC abnormalities, which in turn may induce oxidative stress (Chauhan et al. 2012b).

6 Environmental Risk Factors in Autism

Genetic abnormalities alone cannot account for the majority of autism cases. Therefore, environmental factors either alone or collectively with susceptibility genes have been suggested to be involved in the etiology of ASDs (Chauhan and Chauhan 2006; Chauhan et al. 2009a; Daniels 2006; Deth et al. 2008). Both prenatal and/or postnatal exposures to certain environmental factors, such as metals, viruses, maternal

drugs, bisphenol A (BPA), organophosphate insecticides, polychlorinated biphenyls (PCBs), phthalates, etc., have been linked to the developmental problems related to autism. In a recent review, Herbert (2010) also stressed the need to explore the link between genetics, environmental factors, and oxidative stress in autism. Many of the hypotheses regarding the pathogenesis of ASDs involve a functional deficit caused by alterations in specific brain structures occurring in utero during defined temporal windows of vulnerability (Polleux and Lauder 2004). Some of the environmental factors that may contribute to the etiology of autism are discussed below.

6.1 Metals

Toxic metals have long been suspected to be involved in autism. We reported previously that levels of transferrin (an iron-transporting protein) and ceruloplasmin (a copper-transporting protein) are decreased in the serum of children with autism. Interestingly, there was a relationship between reduced levels of these proteins and regression in autism (Chauhan et al. 2004a). In another study, we reported reduced levels of phosphatidylethanolamine (PE), a membrane phospholipid in the erythrocyte membrane of children with autism (Chauhan et al. 2004b; Chauhan and Chauhan 2009). Among the various metal cations (copper, iron, calcium, cadmium, and zinc) studied, only copper was found to oxidize and decrease the levels of membrane PE. The action of copper on PE oxidation was time- and concentration-dependent (Chauhan et al. 2008). Other investigators have also reported abnormalities of copper metabolism in children with autism. Lakshmi and Geetha (2011) studied 45 autistic children with different grades of severity, i.e., low-, medium-, and high-functioning autism, and reported a correlation between copper burden and severity of disease. They also observed significantly higher levels of lead and mercury in hair and nail samples of children with autism as compared to normal control subjects. In another study, of 230 children with autistic disorder, PDD-NOS, and Asperger syndrome, Faber et al. (2009) reported increased levels of copper, decreased levels of zinc, and reduced zinc/copper ratio in the plasma of children with autism. However, Jackson and Garrod (1978) did not observe alterations in plasma levels of zinc and copper in the children with autism compared to control subjects. Similarly, in a cross-sectional case–control study and a meta-analysis, including the present and previous similar studies, De Palma et al. (2011) excluded any association of autism with concentrations of mercury, cadmium, selenium, lithium, and copper in the hair. The discrepancy in results between different studies can be attributed to the different ages of subjects studied and/or the severity of ASDs. Among all metals, the role of exposure to mercury from consumption of contaminated seafood during pregnancy, dental amalgams, and the thimerosal (mercury-based preservative) used in childhood vaccines (until recently) and flu vaccines remains a most controversial issue in autism. In particular, the measles–mumps–rubella (MMR) vaccination in children as a risk factor for the development of autism has been a subject of great debate. However, large-scale studies have not found any credible evidence for a link between vaccines and autism (Heron and Golding 2004;

Hertz-Picciotto et al. 2010; Honda et al. 2005; Kaye et al. 2001; Madsen et al. 2002; Taylor et al. 1999; Thompson et al. 2007).

6.2 Maternal Infections

There is a large body of epidemiological data suggesting an association between maternal infections (both bacterial and viral) during pregnancy and increased incidence of neuropsychiatric disorders such as autism and schizophrenia (Arndt et al. 2005; Cannon and Clarke 2005; Patterson 2011). There is also considerable epidemiological evidence for the possibility that specific gestational periods may correspond to the time window with differing vulnerability to infection-mediated disturbances in fetal brain development (Meyer et al. 2007). In autism, maternal infections in the first few weeks of gestation may lead to abnormalities in fetal brain development and a higher risk of autism in the offspring (Arndt et al. 2005; Libbey et al. 2005; Miller et al. 2005). Autism in children with congenital rubella syndrome due to maternal rubella infection during pregnancy has also been attributed to disturbance in early fetal brain development (Chess 1971; Chess and Fernandez 1980; Ueda et al. 1979).

The studies above are also supported by experiments conducted in animal models. Behavioral, cognitive, and psychopharmacological abnormalities have been detected in mice and rats following prenatal exposure to the bacterial endotoxin lipopolysaccharide (LPS) (Fortier et al. 2004; Golan et al. 2005), human influenza virus (Shi et al. 2003), and the viral mimic polyriboinosinic–polyribocytidilic acid (Meyer et al. 2006; Shi et al. 2003). Studies on prenatal exposure to rubella and other viral agents have also alluded to a possible environmental etiology of autism (Assumpcao and Kuczynski 2002; Hwang and Chen 2010; Libbey et al. 2005). In contrast, no significant correlation between prenatal viral exposure and occurrence of autism was found in other studies (Anlar et al. 1994; Chen et al. 2004; Deykin and MacMahon 1979). Nevertheless, several researchers believe that environmental insults such as maternal infections may exacerbate genetic vulnerabilities in some individuals, or they may cause alterations in genes and/or protein expression, precipitating the abnormal phenotypes observed in autistic individuals (Chauhan and Chauhan 2006; Chauhan et al. 2009a; Fatemi et al. 2008, 2009; Herbert 2010).

6.3 Maternal Drugs

6.3.1 Thalidomide

This drug was originally introduced as a sedative that was typically used to cure morning sickness in pregnant women. Later, this drug was withdrawn due to its teratogenicity and neuropathic effects. There is now growing clinical interest in thalidomide for its role as an immunomodulatory agent. In a study of 100 subjects

of embryopathy in the Swedish thalidomide registry (Miller 1991; Stromland et al. 1994), five of these individuals had autism, and they all were from a group of 15 subjects with evidence of exposure during the 20th–24th day of gestation, which implicates a 33 % rate of autism in this subpopulation. This particular period (20th–24th day of development) falls during the closure of the neural tube and also coincides with the production of the first neurons that form the motor nuclei of the cranial nerves. Injury to the motor nuclei or their projections has been reported in autistic subjects (Rodier et al. 1997). Research on thalidomide suggests that autism may be caused by a very early injury to the developing brain. It also suggests that an animal model of autism may be developed on the basis of disrupting CNS development during neural tube closure. After thalidomide treatment of rats at embryonic day 9 (E9), a dramatic shift was observed in the distribution of serotonergic neurons in the dorsal raphe nucleus on postnatal day 50. This alteration is suggested to reflect abnormalities of serotonergic neuronal differentiation and migration in autism (Miyazaki et al. 2005). In another study, Narita et al. (2002) reported an increase in levels of hippocampal serotonin, frontal cortex dopamine, and hyperserotonemia in rats exposed to thalidomide at E9, suggesting that thalidomide-induced alteration of monoamine metabolism may be associated with the pathogenesis of autism. Another mechanism proposed to explain the teratogenic effects of thalidomide is oxidative stress (Ito et al. 2011; Knobloch et al. 2011), which is one of the core characteristics of autism.

6.3.2 Valproic Acid (VPA)

VPA is an anticonvulsant and mood-stabilizing drug that is used primarily for the treatment of epilepsy and bipolar disorder and less commonly for major depression. Epidemiological studies suggest that VPA exposure during the first trimester of pregnancy may result in higher incidence of autism in the offspring. In a study on long-term prenatal exposure to several antiepileptic drugs in Aberdeen (UK), Rasalam et al. (2005) reported that VPA was most commonly associated with ASDs. In another study, Bromley et al. (2008) reported that in utero exposure to VPA resulted in a sevenfold greater incidence of ASDs in the children.

The studies above have also been reproduced in the animal models, showing that prenatal exposure to VPA can result in autistic-like behavior (Markram et al. 2008; Schneider et al. 2008), cerebral pathology (Rodier et al. 1996), and altered levels of monoamines (Narita et al. 2002). In mice, both prenatal and postnatal treatment of VPA resulted in behavioral alterations (Wagner et al. 2006; Yochum et al. 2008). Prenatal injection of VPA induced a delayed motor maturation and impairment of learning and memory (Wagner et al. 2006). Postnatal injection of VPA resulted in impaired social behavior as well as increased apoptosis in the cerebellum and hippocampus (Yochum et al. 2008). Recently, Mehta et al. (2011) reported that prenatal exposure to VPA resulted in increased repetitive and anxiety-like behaviors in mice. In rats, prenatal VPA exposure resulted in dysmorphology similar to that observed

in the brain of children with autism (Lukose et al. 2011). In another study, prenatal exposure to VPA in rats induced demethylation in the promoter regions of wnt1 and wnt2 (proteins involved in embryogenesis) in prefrontal cortex and hippocampus of offspring (Wang et al. 2010).

Several studies suggest that prenatal exposure to VPA in animals can cause biochemical abnormalities similar to those observed in human subjects with autism. Altered expression of phosphodiesterase (PDE) 4A and 4B has been reported in the brain of subjects with autism (Braun et al. 2007). Similarly, decreased expression of PDE subtypes was also observed in VPA-treated rats (Fatemi et al. 2010). Consistent with findings of hyperserotonemia in many subjects with autism (Hranilovic et al. 2007), rats exposed to VPA prenatally also showed serotonergic impairment (Dufour-Rainfray et al. 2010; Miyazaki et al. 2005). Furthermore, prenatal exposure to VPA led to reduced expression of synaptic adhesion molecules neuroligin 3 in mice, which has also been implicated in genetic studies of autism (Kolozsi et al. 2009).

One of the core biochemical features of autism is the presence of oxidative stress (Chauhan et al. 2004a; Chauhan and Chauhan 2006; Chauhan et al. 2009a; Chauhan and Chauhan 2012; Chauhan et al. 2012a). Exposure of humans, animals, and cell cultures to VPA has also been reported to induce oxidative stress. Increased oxidative stress was reported in children who were receiving VPA (Michoulas et al. 2006). In the embryonic cultures, VPA exposure increased ROS formation and induced apoptosis in postimplantation embryos (Tung and Winn 2011). VPA treatment also induced oxidative stress and inflammation in patients with epilepsy (Ounjaijean et al. 2011). Kiang et al. (2011) reported glutathione depletion after increase in oxidative stress in hepatocytes treated with VPA. Fu et al. (2010) reported that VPA induced oxidative stress and autophagy in glioma cells and that oxidative stress occurred upstream of autophagy. Glutathione S-transferase M1 (GSTM1) is a gene that codes for an enzyme involved in the management of toxicant-induced oxidative stress and is associated with increased risk of autism. When GSTM1 knockout mice and wild-type control mice were exposed to VPA, GSTM1 knockout mice showed increased behavioral abnormalities as compared to wild-type animals (Yochum et al. 2010).

6.4 Endocrine-Disrupting Chemicals (EDCs)

EDCs are the chemicals that interfere with the endocrine system (or hormone system). Humans are regularly exposed to chemicals with estrogenic effects because EDCs are found in low doses in various commonly used products. The chemicals detected in humans include BPA, pesticides such as endosulfan, PCBs, polybrominated diphenyl ethers (PBDEs), and phthalates. While most studies suggest that exposure to these chemicals poses a health risk to humans (Colborn 2004; Frye et al. 2012; Sharpe and Irvine 2004; Solomon and Schettler 2000), one study does not support such risk (Safe 2000). Nevertheless, there is a general consensus that these chemicals have the potential to cause neurodevelopmental abnormalities such as autism.

6.4.1 EDCs and Neurodevelopmental Abnormalities

Many of these EDCs are organic in nature and can mix easily with lipids. The lipid solubility of EDCs results in accumulation of these chemicals in fatty tissues such as the brain. Furthermore, these chemicals can readily transfer across the placenta prenatally and are also present in breast milk. A recent review by Frye et al. (2012) describes the effects of EDCs on behavior and the potential mechanism of their action. Many behaviors and the neuroendocrine pathways that regulate them are sexually dimorphic, i.e., different in males and females. Exposure to EDCs can alter sexually dimorphic behaviors and affect neurodevelopmental processes, leading to increased developmental, cognitive, and/or emotional disabilities (Frye et al. 2012; Schettler 2001). Hence, development of psychological disorders that are prevalent in a specific gender may be associated with the disruption of developmental trajectory and/or maturation of sexually dimorphic brain (Bale et al. 2010). Exposure to EDCs that disrupt hormone function during critical periods of life, such as intrauterine, perinatal, or juvenile periods, may influence susceptibility to sex- and/or hormonally differentiated aspects of behavior (Frye et al. 2012; Richter et al. 2007; Swan et al. 2010).

Exposure to EDCs in early life can lead to long-term changes in social and sensory function, which are commonly observed in developmental disorders. Sensory impairment is higher in children with neurodevelopmental disorders than in the general population (Carvill 2001). In individuals with ASDs, sensory abnormalities are highly prevalent (30–100 %) (Reynolds and Lane 2008). In addition to sensory abnormalities, children with developmental disabilities often manifest social problems, such as aggression (Tyrer et al. 2006).

6.4.2 Bisphenol A (BPA)

BPA (4,4'-dihydroxy-2,2-diphenylpropane) is used in the production of polycarbonated plastics (used in some food and drink containers) and epoxy resins (used in most food and beverage metal cans) (Brede et al. 2003; Carwile et al. 2011; Kang et al. 2003). It is also found in plastics used for children's toys, CDs, DVDs, dental sealants, and household electronics (Joskow et al. 2006; Suzuki et al. 2000). Global production of BPA was estimated to be more than 2.2 million tons in 2009. Several reports indicate that frequent hydrolysis of ester bonds in plastic and resins during normal use of food and drink containers and baby bottles, which is further accelerated with time, elevated temperature, and pH extremes, leads to leaching out of BPA from tin cans and plastic containers into food and beverages (Brede et al. 2003; Carwile et al. 2011; Kang et al. 2003). The primary exposure of BPA in humans occurs orally, due to leaching of BPA from incomplete polymerization of epoxy resins or degradation of the weak ester bonds that link BPA monomers.

The exposure data from several countries including the USA suggest that the human body is continually exposed to BPA (Vom Saal and Hughes 2005). The amount of BPA exposure in humans varies depending upon the consumption of food

items in plastics and metal cans. In humans, BPA has been found in biological fluids, including blood, urine, placental tissue, follicular fluid, umbilical cord blood, fetal serum, and amniotic fluid, suggesting that BPA can pass through the placenta (Ikezuki et al. 2002; Kang et al. 2006). The concentration of BPA was fivefold higher in amniotic fluid at 15–18-week gestation compared with other fluids in humans (Ikezuki et al. 2002). According to the National Toxicology Program Expert Panel Report, infants (0–12 months old fed with liquid formula) and children (1.5–6 years old) are among the most exposed and can consume up to 13 and 14.7 μg BPA/kg body weight/day, respectively (Alderson 2008). On the other hand, estimated BPA intake was much lower in breast-fed infants (0.2–1 μg/kg body weight/day) and in adults (0.008–1.50 μg/kg body weight/day).

The reports by government-sponsored panels have raised concerns for the effects of BPA on the brain, behavior, and prostate gland in fetuses, infants, and children at current environmentally relevant doses of BPA (National Toxicology Program 2007). In a randomized crossover trial of canned food consumption and urinary excretion of BPA, BPA was detected in 77 % of people who ate canned soup (Carwile et al. 2011). Several studies have reported behavior abnormalities and cognitive impairment in animals exposed to BPA and suggested that BPA exposure in humans may increase the risk for autism, schizophrenia, and attention deficit hyperactivity disorder (ADHD) (Brown 2009; Masuo et al. 2004; Wetherill et al. 2007).

BPA is a known endocrine disruptor, which binds to both estrogen receptors ERα and ERβ, and it causes disruption of cellular function during neurodevelopment (Wetherill et al. 2007). In addition, BPA exposure has been shown to enhance oxidative stress, a condition known to be involved in the etiology of autism (Chauhan and Chauhan 2006; Chauhan et al. 2009a, 2012a, b). The lipid peroxidation was increased in the brain, kidney, and testis in mice exposed to BPA during fetal life and infancy (Kabuto et al. 2004). In another study, mice injected with BPA showed increased GSSG and a decreased GSH/GSSG ratio in the brain (Kabuto et al. 2003). Other studies have shown that BPA induces oxidative stress in women (Yang et al. 2009), in zebrafish embryo (Wu et al. 2011), and in rats (Korkmaz et al. 2010; Minamiyama et al. 2010).

6.4.3 Polychlorinated Biphenyls (PCBs)

All PCBs are chlorinated biphenyl molecules. The exposure to PCBs in humans is from residual PCBs in the diet, air, water, and soil, especially by PCBs used as dispersants in pesticides and as coolants or heat transfer agents in electrical transformers (Ritchie et al. 2003; Slim et al. 2000). PCBs have also been used in microscope immersion oil and in carbonless copy paper.

PCBs are persistent pollutants with immunological and neurological effects (Crinnion 2011; Kimura-Kuroda et al. 2007). It has also been reported that PCBs increase the steady-state levels of ROS (Hennig et al. 2002), oxidative stress, and cytotoxicity that can be mitigated by antioxidants (Zhu et al. 2009). The neurological and immunological abnormalities as well as oxidative stress have been observed

in individuals with autism (Chauhan and Chauhan 2006, 2012; Chauhan et al. 2009a, 2012b; Pardo-Villamizar and Zimmerman 2009; Onore et al. 2012). In vitro studies showed that PCBs are potent inducers of apoptosis for monocytes (Shin et al. 2000) and thymocytes (Tan et al. 2003). Dietary PCB supplements in the form of contaminated whale blubber resulted in diminished mitogen response, decreased phagocytosis, and diminished numbers of CD8+ cells, indicating PCB-induced immunosuppression (Fournier et al. 2000). Animals exposed to dioxin-like PCBs also developed thymic atrophy and immunosuppression (Davis and Safe 1990). PCBs also reduced available SOD and oxidative stress and diminished the number of neutrophils and reduced cellular immunity (Narayanan et al. 1998). Both prenatal and postnatal exposures to PCBs showed a reduced number of circulating polynuclear neutrophils (Leijs et al. 2009) and increased the incidence of middle-ear disease (Chao et al. 1997), suggesting that exposure to PCBs has a lasting effect on cell-mediated immunity.

Neurological consequences of PCB exposure are more pronounced when exposure occurs in utero. The children with prenatal exposure to PCBs exhibited intellectual disabilities, impaired mental and motor neurological development, cognitive defects, and poorer gross motor function (Jacobson et al. 1985; Jacobson and Jacobson 1997; Walkowiak et al. 2001). Intelligence quotient (IQ) levels were lower in children exposed to PCBs than in children without such exposure (Chen et al. 1992, 1994; Lai et al. 2002). In mice, neonatal PCB exposure also resulted in long-term neurological problems. In utero PCB exposure adversely affected learning and memory function when exposed mice reached adulthood (Eriksson and Fredriksson 1998). In addition, postnatal exposures to PCBs can also cause neurological problems (Plusquellec et al. 2010).

6.4.4 Polybrominated Diphenyl Ethers (PBDEs)

PBDEs are widely used as flame-retardant chemicals in furniture foam, carpet pads, and the plastics surrounding electronics such as computers, cell phones, and televisions. In humans, high levels of PBDEs have been detected in breast milk, placenta, adipose tissues, and blood, including fetal blood (Frederiksen et al. 2009; Gomara et al. 2007; Mazdai et al. 2003). The levels of PBDEs in the environment and in humans are approximately tenfold higher in North America than in Europe and Asia (Frederiksen et al. 2009). Humans are exposed to PBDEs via inhalation of household dust and intake of vegetables and animal products (Frederiksen et al. 2009). Children are exposed to larger amounts of PBDEs than are adults, because of child-specific hand-to-mouth behavior and frequent ground contact (resulting in the ingestion of house dust), and their serum levels of PDBEs have been reported to be higher than adults' (Fischer et al. 2006; Toms et al. 2009). An additional source of exposure for infants is breast milk, (Frederiksen et al. 2009). Prenatal exposure to PBDEs can also occur through placental transfer (Gomara et al. 2007; Mazdai et al. 2003).

Exposure to PBDEs has been associated with neurotoxicity, especially in young children (Eriksson et al. 2001). Recent studies showed adverse effects on cognitive and neurodevelopmental parameters in humans exposed to PBDEs. In a study of Dutch children, motor, cognitive, and behavioral performance correlated with maternal serum levels of PBDEs measured in the 35th week of pregnancy (Roze et al. 2009). In another study of U.S. children (0–6 years of age), the scores of yearly tests of mental and physical development were lower among those children exposed prenatally to higher concentrations of PBDEs (Herbstman et al. 2010).

Several studies suggest that exposure to PBDEs also results in increased oxidative stress. Zhong et al. (2011) reported that PBDE metabolites, especially 6-OH-BDE85, caused cytotoxicity in human L02 cells, which was related to the degree of oxidative stress. In rodents, exposure to PBDEs also resulted in increased oxidative stress and decreased nerve conduction (Vagula et al. 2011). A study with primary neuronal cultures showed that PBDEs increased the rate of apoptosis, expression of P38 MAPK, calcium ion concentration, ROS and NO levels, and lipid peroxidation (Chen et al. 2010). Several lines of evidence have suggested abnormalities in signal transduction in autism (Chauhan and Chauhan 2009). Taken together, all of these studies implicate the deleterious effects of early childhood exposure to PBDEs and also suggest that exposure to PBDEs may contribute to elevated oxidative stress and biochemical and behavioral changes, similar to those observed in children with autism.

6.5 Phthalates

Phthalates—diesters of 1,2-benzenedicarboxylic acid (phthalic acid)—are a group of synthetic chemicals with a wide spectrum of industrial and commercial uses, e.g., as primary plasticizers for polyvinyl chloride and solvents in personal care products (such as shampoos, cosmetics, and fragrances) (Wormuth et al. 2006). Phthalate plasticizers are slowly emitted into the surrounding environment (Wormuth et al. 2006), thus constituting an indoor pollutant (Bornehag et al. 2005). Phthalates can be ingested through food or inhaled through contaminated air or dust. Dermal contact with products that contain phthalates and polymer coating in some medications are also potential sources of its exposure (Hernandez-Diaz et al. 2009).

After entering the body, phthalates undergo rapid metabolism to monoesters and can also be further oxidized to oxidative metabolites (Engel et al. 2010). The metabolites of phthalates have been detected in all biological fluids, including amniotic fluid, breast milk, semen, blood, and urine (Frederiksen et al. 2007). The maternal transmission of phthalates to offspring has been demonstrated because these compounds have been found in the amniotic fluid and fetal circulation in human (Huang et al. 2009; Wittassek et al. 2009). It has been estimated that infants may experience higher exposures to phthalates in relation to their body weight (Wormuth et al. 2006).

The exposure to phthalates (prenatal, postnatal, infancy, or childhood) has raised concerns because these chemicals have been associated with developmental and reproductive toxic effects (Borch et al. 2006; Gray et al. 2000; Engel et al. 2009, 2010). Although no study has been conducted to evaluate phthalates as a risk factor for ASDs, recent studies have reported an association of phthalate exposure with neurodevelopment. Prenatal exposure to phthalates has been associated with poor birth outcomes (Wolff et al. 2008), neurological problems in the neonate (Engel et al. 2009), behavioral abnormalities (Engel et al. 2010), reduced masculine play in boys (Swan et al. 2010), and childhood social impairment (Miodovnik et al. 2011). Phthalates also caused hyperactivity and impulsivity in rats, which resembled the clinical features observed in ADHD (Ishido et al. 2004; Masuo et al. 2004). A cross-sectional survey also reported associations between phthalate metabolites and intelligence scores (Cho et al. 2010) as well as ADHD symptoms in school-aged children (Kim et al. 2009a). Phthalates have also been suggested to interfere with the thyroid hormone system (Ghisari and Bonefeld-Jorgensen 2009; Huang et al. 2009), a system vital to normal brain development in the fetus and infant. All of these findings suggest that exposure to phthalates may cause disturbances in the normal developmental trajectory of the fetal and infant brain.

6.6 Pesticides

A large number of agricultural pesticides are known to have neurological effects (Weiss et al. 2004), which raises the possibility that gestational exposure to these compounds may be involved in the etiology of ASDs and related neurodevelopmental disorders. Although most of these compounds are used in a specific area, they are prone to drift, and detectable levels are often observed in air samples for extended periods at locations beyond the site of application (Lee et al. 2002). Elevated levels of agriculture pesticides in household dust, and their metabolites in urine, have been associated with residential proximity to treated fields (Loewenherz et al. 1997; Lu et al. 2000; Simcox et al. 1995). Accumulating evidence suggests an association of residential proximity or parental occupational exposure to pesticides with pediatric diseases, most notably for neurodevelopmental disorders (Eskenazi et al. 2007; Grandjean et al. 2006) and cancer (George and Shukla 2011).

Many environmental toxins are transferred through placenta, and the blood–brain barrier remains relatively permeable to many of these compounds until the first year of life (Andersen et al. 2000). It has been reported that prenatal exposure to organophosphate pesticides is negatively associated with cognitive development, particularly perceptual reasoning, with evidence of effects beginning at 12 months and continuing through early childhood. Paraoxonase 1 may be an important susceptibility factor for these deleterious effects (Engel et al. 2011). A study of 465 children with ASDs born in California during 1996–1998 showed a link between proximity to organochlorine pesticide applications and incidence of ASDs (Roberts et al. 2007).

Several studies suggest that pesticides are also involved in inducing elevated oxidative stress. Subchronic exposure to malathion (an organophosphate) resulted in increased levels of hepatic lipid peroxidation, protein carbonyl groups (protein oxidation marker), and 8-deoxyguanosine (DNA oxidation marker) (Mostafalou et al. 2012). Insecticides such as endosulfan are also known to cause oxidative stress (Saxena et al. 2011; Velki et al. 2011; Zervos et al. 2011).

7 Conclusions

The incidence of autism has risen dramatically in the last 20 years. While the cause of autism remains unknown, autism is considered a multifactorial disorder that is influenced by genetic and environmental factors and increased vulnerability to oxidative stress. Although genetic factors may play a role in the etiology of ASDs, not all autism cases have pathogenic mutations or copy number variants. As discussed in this review, prenatal or postnatal exposure to environmental factors such as metals, maternal drugs, infections, and endocrine disruptors, alone or in combination, is most likely to cause ASDs, at least in a subset of vulnerable individuals. Several independent studies have provided evidence of increased oxidative damage and deficient antioxidant defense mechanism in the children with autism. Emerging evidence from our and other groups has also shown mitochondrial dysfunction in autism. It is suggested that environmental factors may act as triggers for interactions of genetically susceptible alleles in autism, whereas mitochondrial dysfunction and oxidative stress may serve as common links between susceptibility genes and environmental factors, leading to behavioral abnormalities and clinical development of autism (Fig. 2).

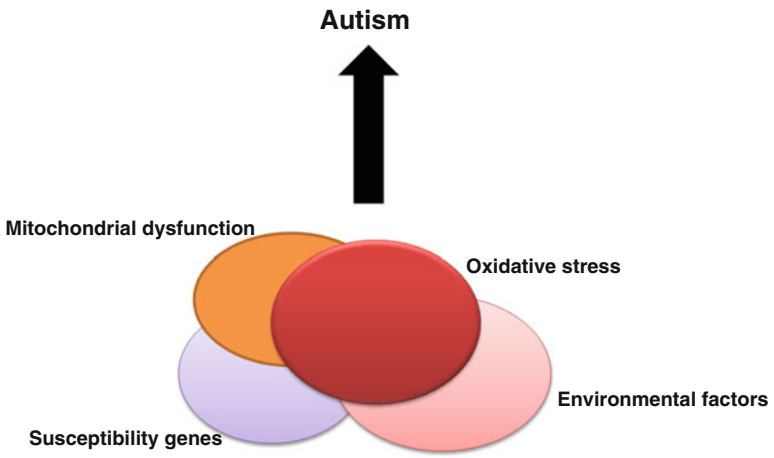

Fig. 2. Gene-environment interactions in autism. Environmental factors may interact with genetically susceptible alleles in autism, whereas oxidative stress and mitochondrial dysfunction may provide a common link between susceptibility genes and environmental factors, resulting in clinical development and behavioral symptoms of autism

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Oxidative Stress and Anxiety Disorder

Marina Čeprnja and Vladimira Vuletić

Abbreviations

BSO	Buthionine-S,R-sulfoximine
CAT	Catalase
FR	Free radicals
GABAergic	Gamma-aminobutyric acid–ergic
GSH	Glutathione
GSH-Px	Glutathione peroxidase
OCD	Obsessive-compulsive disorder
PTSD	Post-traumatic stress disorder
ROS	Reactive oxygen species
SOD	Superoxide dismutase

1 Anxiety Disorders

Anxiety disorders describe a group of related mental illnesses. It is a normal emotional response to a threat or potential threat. When this emotion is inappropriate and persistent, it is classified as pathological. In response to threatening situations,

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the feeling of the emotion that constitutes the subjective feature of anxiety is accompanied by emotional stress, which involves behavioral, expressive, and physiological features, such as an avoidance of the source of the danger, assuming defensive postures, and an increase in blood pressure. Anxiety is implicated in a number of psychiatric disorders, such as depression, panic attacks, phobias, generalized anxiety disorder, obsessive-compulsive disorder (OCD), and post-traumatic stress disorder (PTSD) (Hovatta et al. 2005; Bouayed et al. 2009; http://www.heretohelp.bc.ca/publications/factsheets/anxiety).

2 Oxidative Stress

Free radicals (FR) are species normally produced during cellular metabolism in aerobic cells, changing their physicochemical characteristics (Marzatico and Cafè 1993). It has been found that oxidative stress and the activities of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and glutathione (GSH) are disturbed due to the growing oxidative stress (Dadheech et al. 2008). Overbalance of ROS induces oxidative damage on cellular components, so oxidative stress has been implicated in a number of diseases (Bouayed et al. 2009). FRs have relatively short half-lives, and thus the determination of their levels is difficult. Therefore, they can be evaluated indirectly by measurement of some antioxidant enzyme levels such as SOD, catalase (CAT), or GSH-Px; by products of lipid, proteins, or DNA peroxidation; or by some transition metal levels such as copper, zinc, and iron (Atmaca et al. 2008). In the presence of oxidative stress, the lipid-rich constitution of brain favors lipid peroxidation that results in decrease in membrane fluidity and damage in membrane proteins inactivating receptors, enzymes, and ion channels (Valko et al. 2007; Delattre et al. 2005; Halliwell 2006). Oxidative stress can alter neurotransmission, neuronal function, and overall brain activity (Lebel and Bondy 1991; Cardozo et al. 1999).

3 Oxidative Stress and Anxiety Disorder

Oxidative stress is related to probably more than 100 human diseases including male infertility, autoimmune diseases, atherosclerosis, cardiovascular troubles, diabetes, and cancer. Recent research has observed a close relationship between oxidative stress and anxiety in human patients suffering from anxiety disorder, as well as in animals displaying high trait anxiety (Bouayed 2011). The brain is highly vulnerable to oxidative stress due to its high O_2 consumption, its modest antioxidant defenses, and its lipid-rich constitution. Human brain utilizes 20 % of oxygen consumed by the body even though this organ constitutes only about 2 % of the body weight. The brain tissues contain large amounts of polyunsaturated fatty acids and

catecholamines, which are thought to be target molecules for FR-induced peroxidation and neural cell damage (Ng et.al 2008). Recent researches observed a close relationship between oxidative stress and anxiety in human patients suffering from anxiety disorder. Brain oxidative stress disturbances might be a plausible pathogenesis and risk factor for several specific diseases of the nervous system including behavioral troubles and disorders (Bouayed et al. 2009; Ng et.al 2008).

High oxygen-derived species production (through respiration and metabolism) produces toxicity in brain, owing to its intrinsic oxidative vulnerability (Bouayed et al. 2009; Ng et.al 2008).

Most studies have shown that anxiety is controlled by the nervous system and that gamma-aminobutyric acid–ergic (GABAergic) and serotoninergic systems play important roles in the regulation of anxiety (Weinberger 2001; Gingrich 2005). Recent findings emphasized that patients with anxiety disorders (panic disorder), compared to healthy controls, have higher activity levels of the antioxidant enzymes SOD and GSH-Px as well as higher lipid peroxidation activity, which established a link between oxidative stress and certain anxiety disorders. It showed that oxidative metabolism can affect the regulation of anxiety. This hypothesis has gained interest due to the intrinsic oxidative vulnerability of the brain. When the production of ROS prevails over the brain defense systems, the lipid-rich constitution of the brain may favor lipid peroxidation, constituting a FR chain reaction that may result in decrease in membrane fluidity and damage in membrane proteins inactivating receptors, enzymes, and ion channels and even disrupting membrane integrity resulting eventually in cell death. Therefore, brain oxidative damage might be also a plausible pathogenic factor for certain multifactorial neurological diseases including neuropsychiatric troubles, highlighting that oxidative stress disturbances could be implicated in the pathophysiology of conditions that are more specific for the nervous system impairment. A statistically significant positive correlation between aggressive behavior in the resident/intruder test and cell oxidative status in adult male mice has been reported (Rammal et al. 2010). Study results confirm that there is a relationship between the level of intracellular ROS in peripheral blood cells and anxiety-related behavior in mice.

These results show that anxiety in mice is accompanied by markedly elevated levels of ROS in neuronal and glial cells within the cerebellum and hippocampus. That imbalance in the redox system of anxious mice plays a role in neuroinflammation and neurodegeneration. While some data demonstrate that there is a link between oxidative stress and high-anxiety-related behavior, a cause-effect relationship has yet to be completely established. Some of these studies suggest that oxidative stress causes anxiety-related behaviors but do not explain the underlying mechanisms. The potential causal role of oxidative stress on anxiety may generate interest in antioxidants (Bouayed et al. 2009).

Several studies have demonstrated that peripheral oxidative status markers in human erythrocytes and plasma significantly correlated with human age, and as a result of that they have been proposed as biomarkers of the aging process, which is characterized by an increase of oxidative stress with age (Pandey and Rizvi 2010).

Other recent studies have focused on the link between redox status and normal anxiety and also on a possible causal relationship between cellular oxidative stress and emotional stress using rodents as animal model. Mice and rats are often used as translational models for studying anxiety in humans due to the similarity in the extremely complex mechanisms involved in anxiety in these species. The principal brain areas (e.g., the amygdala) implicated in the processing of fear and anxiety, the comparable brain circuits involved with anxiety, and the similar neurochemical substrates (GABA, serotonin) among others make rodents a good model to study anxiety in humans (Bouayed 2011). A close correlation between brain expression of genes of the antioxidative defense system (glutathione reductase 1 and glyoxalase 1) and anxiety-related phenotypes is demonstrated. It is found that the activity of the antioxidative enzymes of glutathione reductase 1 and glyoxalase 1 is highest in the most anxious strain and lowest in the least anxious strains. Abnormalities in the regulatory systems of anxiety in rodents (GABAergic and serotoninergic systems) can result in anxious behavior (Weinberger 2001). A link between oxidative stress and emotional stress is not surprising, since it is well accepted that oxidative damage in the brain causes an impairment of the nervous system. The contribution of oxidative pathophysiology in anxiety disorders is supported by animal models and also by some human biochemical data; however, it is still an area of interest that is being investigated (Ng et.al 2008; Cardozo et al. 1999). The mechanism by which these antioxidant defensive enzymes regulate anxiety is of great interest. Hovatta et al. (2005) used glyoxalase 1, which is an enzyme of the glyoxalase system, as a marker of oxidative stress. Enzymatic activity of glyoxalase 1 aims to protect against carbonyl stress (resulting from excessive accumulation of reactive dicarbonyl compounds) (Hovatta et al. 2005). Carbonyl stress leads to protein and nucleotide damages by dicarbonyl glycation, which is associated with several pathologies including diabetes (Thornalley 1996, 2006). GSH, which is a major antioxidant in the brain, constitutes a determinant cofactor for the enzymatic reaction that is catalyzed by glyoxalase 1. A close relation between oxidative stress and carbonyl stress was established. Other findings from another laboratory (Krömer et al. 2005) have complicated the understanding of the role of glyoxalase 1 in trait anxiety because they are discordant with those of Hovatta et al. Contradictorily, they have proposed that the level of expression of glyoxalase 1 could be used as a physiological marker of trait anxiety level, with high protein expression indicating low trait anxiety level and low expression for high trait anxiety (Bouayed 2011). It is found that high anxiety level was associated with a significant generation of ROS in the peripheral blood lymphocytes, granulocytes, and monocytes in mice compared to low anxiety level (Rammal et al. 2008a). These results confirm that there is a relationship between the level of intracellular ROS in peripheral blood cells and anxiety-related behavior in mice. These results warrant further studies of oxidative status of the brain in mice with distinct levels of anxiety. Using the same behavioral approach to distinguish between anxious and non-anxious mice, it is found that anxiety levels are associated with oxidative status in both neuronal and glial cells in the cerebellum and hippocampus, in neurons of the cerebral cortex, and in peripheral leucocytes (monocytes, granulocytes, and lymphocytes) (Rammal et al. 2008b). These results clearly indicated

the presence of oxidative stress in the central and peripheral systems of anxious mice. Oxidative stress in the brain and blood immune cells could predispose anxious mice to neuroinflammation and neurodegeneration as well as recurrent infections. In keeping with the animal experiments, the link between oxidative stress and human trait anxiety was also determined. Yasunari et al. (2006) found a significant relationship between trait anxiety and ROS formation in monocytes of hypertensive individuals (Yasunari et al. 2006).To study the causal relation between oxidative stress and anxiety, Masood et al. (2008) provoked oxidative stress by depleting GSH in mice using buthionine-S,R-sulfoximine (BSO), and afterwards they assessed the impact of BSO treatment on the level of anxiety. The NADPH oxidase was suggested to be the principal oxidative pathway responsible for the anxiogenic behavior following BSO treatment (Masood et al. 2008). Depletion of GSH was also reported to cause cognitive impairment in rodents (Weinberger 2001). Despite the fact that GSH is considered as a major antioxidant in aerobic cells functioning as an important cellular redox buffer, GSH depletion can cause other cellular stresses, including nitrosative and carbonyl stresses, as GSH is also an important determinant of the nitrogen and dicarbonyl metabolism. Excessive production of ROS induces oxidative damage of cellular structures; production of reactive nitrogen species triggers nitrosylation reactions, which can alter the structure of proteins to inhibit their normal function; excessive accumulation of reactive dicarbonyl compounds leads to damage of protein and nucleotides by dicarbonyl glycation. GSH may also have an additional double role in the central nervous system by acting as a neurotransmitter and neuromodulator, for example, by regulating the release of other neurotransmitters such as dopamine and GABA, which is an important regulator of anxiety (Bouayed 2011). Other studies have mentioned that oxidative stress state could cause anxiogenic behavior. Desrumaux et al. (2005) showed that vitamin E deficiency in the mouse brain significantly causes brain oxidative stress, resulting in anxiogenic behavior without abnormalities in the locomotor performance of the mice (Desrumaux et al. 2005).

Human studies show that social phobia (SP) is associated with elevated antioxidant enzymes and malondialdehyde levels as a lipid peroxidation product (Atmaca et al. 2008). Atmaca et al. (2008) have shown in their study that patients with SP have significantly higher antioxidant enzyme (SOD, CAT, and GSH-Px) activity and malondialdehyde levels compared with those in controls. Pharmacologic studies suggest that the metabolism of serotonin, noradrenaline, and dopamine might be affected in patients with SP (Atmaca et al. 2008). Also, there are studies that suggest an oxidative imbalance in panic disorder. For instance, Ersoy et al. (2008) aimed to investigate the role of oxidative and antioxidative parameters in etiopathogenesis and prognosis of panic disorder (PD), using methods for measurement of total oxidant and antioxidant statuses. Both total antioxidant status and oxidative stress index and ceruloplasmin levels of PD patients were significantly higher in PD patients. Total oxidant status and oxidative stress index decreased after treatment. So, this study suggests an oxidative imbalance in PD and that treatment can reverse overall oxidative imbalance (Ersoy et al. 2008).

4 Oxidative Stress and PTSD

PTSD is an anxiety disorder that can develop after experiencing or witnessing a life-threatening event, such as accident, disaster, war trauma, violence, or abuse (family, sexual, physical, and/or psychological), or any situation that seriously threatens the integrity of a person (Dietrich-Muszalska and Olas 2007; Dietrich-Muszalska et al. 2009). The disorder, however, does not develop in every person exposed to traumatic experience. Stress results from an interaction between the mind and the body. The brain is the organ that determines reaction to stress as it decides on what is stressful and controls biological and physiological responses to stress. These responses vary from one person to another due to differences in their biological, genetic, environmental, and psychological characteristics, as well as their personal history. Stress causes neuroanatomical and neurochemical changes in the brain. Early traumatic experience may affect the brain structures and functions so as to make a person vulnerable to negative stressful events and more prone to later development of PTSD or other anxiety-related disorders. A particular genetic profile also plays a role in vulnerability or resilience to stress. PTSD has two main groups of symptoms. The first group consists of core PTSD symptoms, which include persistent re-experiencing of traumatic event (flashbacks, nightmares), persistent avoidance of stimuli, hyperarousal, and withdrawal. The second group comprises secondary symptoms, which include impaired functioning, poor coping skills, and psychiatric comorbidity, which is present in around 80 % of the cases (Kozaric-Kovačić 2008).

Epidemiological and clinical studies have shown that PTSD commonly occurs with other psychiatric disorders. The most prevalent diagnoses were alcohol abuse, major depressive disorder, anxiety disorders, panic disorders and phobias, psychosomatic disorders, psychotic disorders, drug abuse, and dementia. Psychotic symptoms have been reported for 30–40 % of patients with combat-related PTSD.

Because the diagnostic procedures of PTSD rely mostly on the subjective reporting of symptoms, clinical history, interview, and mental condition, PTSD is most difficult to malinger. Clinicians need to have additional tools not only to enhance their ability to identify the patient at risk for PTSD but also to dynamically monitor the clinical condition of individual PTSD patient during treatment.

Complexity of the molecular mechanisms of the disease and the undefined validation process makes this progress of the search for PTSD biomarkers slow and often frustrating, but advances in molecular biology, material science, and coating technology create a most supportive environment for the investigation of the molecular mechanisms of PTSD and development of a biomarker test for PTSD. Biomarkers may indicate PTSD or PTSD characteristics, including the level or type response resulting from exposure to a traumatic stress, genetic susceptibility, genetic responses to traumatic stress exposure, markers of subclinical or clinical state, or indicators of response to therapy (Zhang et al. 2009).

PTSD is a chronic disease and disabling anxiety disorder that occurs after a traumatic event. The diagnosis for PTSD is established on the basis of a patient's clinical history, mental status examination, duration of symptoms, and clinician-administered symptom checklists or patient self-reports. There are still no well-established laboratory

biomarker tests for PTSD. A simple blood test that could detect PTSD in its earliest and potentially most treatable stages would be beneficial for physicians and patients. In new researches, many potential biomarkers have been identified in the animal model or in patients with PTSD (Ceprnja et al. 2011).

Literature data suggest that patients with anxiety disorders have an increased level of markers of oxidative stress (Zhang et al. 2009). There are studies that intended to examine, analogously, oxidative stress in PTSD patients. These studies showed that markers of oxidative stress in PTSD patients were not significantly elevated in comparison with control group. These data support the thesis that PTSD, as an acquired disorder, has a different etiopathogenesis than other anxiety disorders that were not preceded by a traumatic event. It is necessary to expand research to a larger scale of patients, following different treatment outcomes, in order to be able to define the clinical value of oxidative stress markers, especially protein carbonyl, as a possible PTSD diagnostic marker. A recent study showed statistically significant difference in the protein carbonyl concentration between the PTSD group and the control group. Concentrations were significantly lower in the PTSD group than in the control group, which was not in accordance with the expected values. The clinical significance of these results was examined using ROC analysis, but the ROC curves did not separate the groups in a satisfactory manner so we concluded that this link should be tested on a larger sample of patients, including data from different psychopharmacotherapy; it would be useful to determine the effect of the treatment type on oxidative stress markers in the future studies and verify the importance of these data (Ceprnja et al. 2011).

5 Methodological Considerations

It is well known that oxidant and antioxidant imbalance occurs in smokers and alcohol consumptions, so the difference between the anxiety of patients group and control group for smoking status should be tested (Reale et al. 2011). For the same reason, patients and controls with a diagnosis of alcoholism should not be included in studies. Considering that first-generation antipsychotics treatment may affect lipid metabolism and exhibit pro-oxidant effect, while second-generation antipsychotics do not show such an effect and may have antioxidant effects, it would be useful to determine whether the type of therapy affects the results. It would be also useful to determine the effect of the treatment type on oxidative stress markers in the future studies (Ceprnja et al. 2011).

6 Conclusion

The imbalance between FR production and antioxidant capacity leads to the oxidative damage of proteins, lipids, and DNA and plays an important role in the pathogenesis of many diseases including psychiatric diseases (Ceprnja et al. 2011).

Studies demonstrate that there is a link between oxidative stress and high-anxiety-related behavior, but relationship between cause and effect has yet to be completely established. Literature data suggest that patients with anxiety disorders have increased level of markers of oxidative stress and that the regulation of FR metabolism and antioxidant capacity were altered as compared to healthy population (Carney and Carney 1994; Gao et al. 2010). The available data are consistent in causal relationship established between anxiogenic effect and oxidative stress. It remains to be determined whether PTSD has a different etiopathogenesis than other anxiety disorders that were not preceded by a traumatic event, given that markers of oxidative stress of PTSD patients did not appear to be significantly elevated in comparison with control group (Ceprnja et al. 2011).

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Relationship Between Oxidative Stress and Obsessive–compulsive Disorder

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Abbreviations

5HT2A, 5HT1Dbeta	Serotonin receptor genes
BDNF	Brain-derived neurotrophic factor
BDV	Borna disease virus
CAT	Catalase
DRD2, DRD3, DRDD4	Dopamine receptor genes
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders IV edition
GSH-Px	Glutathione peroxidase
hSERT	Human serotonin transporter gene
IL-1β	Interleukin-1 β
IL-6	Interleukin-6
MDA	Malondialdehyde
MnSOD	Manganese superoxide dismutase
NAC	N-acetylcysteine
OCD	Obsessive–compulsive disorder
OSI	Oxidative stress index

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RP-HPLC	Reversed-phase high-performance liquid chromatography
Se	Selenium
SOD	Superoxide dismutase
SRIs	Serotonin reuptake inhibitors
TAS	Antioxidant status
TBARS	Thiobarbituric acid reactive substances
TNF-α	Tumor necrosis factor α
TOS	Total oxidant status
UCP-2	Uncouple-2 antioxidant genes
UV–VIS spectroscopy	Ultraviolet–visible spectroscopy
YBOCS	Yale–Brown Obsessive–Compulsive Scale

1 Introduction

1.1 Etiology of OCD

Obsessive–compulsive disorder (OCD) is a common psychiatric disorder defined primarily by the presence of obsessive thoughts (obsessions) and repetitive compulsive actions (compulsions). Lifetime and 12-month prevalence estimates for DSM-IV OCD are 2.3 % and 1.2 %, respectively (Ruscio et al. 2010). Various psychological, social, and biological (both genetic and biochemical) factors are thought to be involved in the etiology of OCD (Denys et al. 2004; Hall et al. 2003; Insel and Winslow 1992).

Previous pathogenetic concepts of OCD focused mainly on the serotonergic and dopaminergic systems. Studies have revealed that OCD patients have an insufficient level of serotonin, one of the brain's neurotransmitters (Shohag et al. 2012; Westenberg et al. 2007). The hypothesis that an abnormality in serotonergic neurotransmission underlies OCD arose from the observation that clomipramine, which inhibits both serotonin and norepinephrine reuptake, relieved symptoms, whereas noradrenergic reuptake inhibitors did not. This result is supported by the efficacy of serotonin reuptake inhibitors (SRIs) in the treatment of OCD (Bloch et al. 2010). In fact, standard first-line therapies for OCD generally still include a combination of SRIs and psychotherapy. Up to 20 % of OCD patients are treatment resistant (Bloch et al. 2010).

Although there is some evidence to suggest that glutamatergic abnormalities are present in OCD patients, further characterization is required to determine whether this is a primary, causal effect or a by-product of hypermetabolism and altered neurotransmission in other pathways (Chakrabarty et al. 2005; Janaky et al. 2007). It has been demonstrated that the glutamatergic system may exert an immunomodulatory effect which corresponds to the immunological profile of OCD (Chakrabarty et al. 2005).

Glutamate acts as an immunomodulator and, more particularly, as an important regulator of T-cell function (Moran et al. 2005; Murphy et al. 1989, 2006). Extracellular glutamate promotes enhanced production of cytokines. Genetic altera-

tions of the glutamatergic system may facilitate BDV-induced immunopathological reactions in OCD patients (Pacheco et al. 2007).

In recent years, a neurobiological model of OCD has been described which postulates that its pathogenesis is based around the glutamatergic system or, more specifically, the roles played by relevant neuronal circuits and the cytotoxic effect of glutamate (Ting and Feng 2008). Molecular genetic studies suggest an association between the glutamate transporter gene and predisposition to the illness (Shugart et al. 2009). Treatment possibilities involving the use of drugs influencing the glutamatergic system such as memantine, D-cycloserine, N-acetylcysteine, riluzole, topiramate, and lamotrigine have been presented (Grant et al. 2007; Pasquini and Biondi 2006; Pittenger et al. 2006; van Ameringen et al. 2006; Wilhelm et al. 2008).

Insel's hypothesis regarding neuroanatomical changes in OCD was partly confirmed by the results of imaging studies, which identified an abnormal pattern of activity in a certain area of the brain in OCD patients (Insel and Winslow 1992; Insel 1992). It has been proposed that unusually increased activity in the head of the caudate nucleus inhibits globus pallidus fibers which ordinarily dampen thalamic activity. The resulting increase in thalamic activity produces increased activity in the orbitofrontal cortex, which, via the cingulate gyrus, completes the circuit to the caudate and produces increased activity in the head of the caudate (Husted et al. 2006; Tekin and Cummings 2002). Magnetic resonance imaging and positron emission tomography have revealed increased blood flow and metabolic activity in the orbitofrontal cortex, limbic structures, caudate, and thalamus, with a trend toward right-sided predominance of the OCD patients (Tekin and Cummings 2002).

A recent review shows several notable discrepancies between findings from cognitive studies, neuroimaging studies, and the present theoretical model proposed to underlie OCD. Currently available evidence suggests that the orbitofronto-striatal model may not be sufficient to explain the neurological basis of OCD (Menziesa et al. 2008; Rotge et al. 2010).

There is strong evidence which indicates OCD might be inherited. Studies conducted on twins have shown a significantly higher concordance rate in monozygotic twins (80–87 %) than in dizygotic twins (47–50 %). Approximately 35 % of first-degree relatives of individuals with childhood-onset OCD are affected by the disorder. OCD patients have a 25 % chance of having a first-degree relative who also has the disorder (Inouye 1965; Grados et al. 2003; van Grootheest et al. 2005).

Recent studies revealed some genes that can be associated with OCD: a glutamate transporter gene called SLC1A1, which encodes EAAC1, which in turn regulates the flow of glutamate in and out of the brain cells; human serotonin transporter gene (hSERT) which controls how the body uses serotonin; and the myelin oligodendrocyte glycoprotein gene (MOG) involved in the autoimmune system. OCD is also associated with a mutation on several dopamine receptor genes (DRD2, DRD3, DRDD4), serotonin 2A receptor 5HT2A, 5HT1Dbeta receptor gene and brain-derived neurotrophic factor (BDNF) gene – a protein active in the hippocampus, cortex, basal forebrain, and peripheral nervous system (Arnold et al. 2004, 2006; Frisch et al. 2000; Hall et al. 2003; Han et al. 1997; Janaky et al. 2007; Prasad et al. 2005; Rotge et al. 2010; Stewart et al. 2007; Shugart et al. 2009; Zai et al. 2004).

2 Oxidative Stress in OCD: Review of Studies

There is mounting evidence indicating that reactive free radical species are involved in the initiation and development of OCD. A study by Kuloglu et al. was one of the first studies whose aim it was to examine the importance of free radicals in the pathogenesis of OCD (Kuloglu et al. 2002). This study evaluated the activity of four antioxidant enzymes in patients with OCD: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), as well as malondialdehyde (MDA), a product of lipid peroxidation. The patients were divided into two subgroups: those suffering from OCD and MDD (major depressive disorder) and those with only OCD, diagnosed according to DSM-IV. The MDA and antioxidant enzyme levels in both patients and controls were determined. SOD activity levels were significantly higher in the OCD + MDD group compared with the controls and the OCD group. GSH-Px activity levels were significantly higher in both groups compared with controls. Similarly, there was a significant difference in GSH-Px activity levels between the OCD + MDD and the OCD groups. CAT activity levels were slightly higher in the OCD + MDD group compared with the OCD and control groups. In addition, MDA levels in both groups were significantly higher than in controls. The difference in MDA levels between both groups was statistically significant. The results of this study suggest that the presence of OCD is associated with free radicals.

The role of free radicals in OCD was investigated also by Ersan et al. (2006). The aim of their study was to assess whether antioxidant vitamin (E, C, and A) activity and MDA levels were associated with OCD. The study cohort comprised 48 OCD patients, diagnosed according to DSM-IV, and 48 healthy volunteers acting as controls. Serum levels of vitamins E and A were determined using RP-HPLC, whereas vitamin C was estimated by phenylhydrazine spectrophotometry. The MDA level was measured using UV–VIS spectroscopy. Significant differences in serum vitamin E and C levels were found between the OCD group and controls ($p < 0.05$), and MDA levels were found to be significantly higher in the OCD subjects ($p < 0.05$). These results demonstrate an overall imbalance in antioxidant vitamin level in OCD patients, which may have a potential role in the etiopathogenesis of the disorder.

In 2008 Selek et al. evaluated total oxidant–antioxidant status in OCD patients (Selek et al. 2008). Their study was cross-sectional, and its aim was to assess whether antioxidant–oxidant status is associated with OCD and can be used as a biological marker. Thirty-seven OCD patients diagnosed according to DSM-IV and 40 healthy controls were included in the study. Venous blood samples were collected once. Nine of the patients had psychiatric comorbidities: 5 patients had other anxiety disorders; 2, depression; 1, schizoaffective disorder; and 1, more than one psychiatric disorder. Comorbid patients were included in the study when the other psychiatric conditions were in remission. The total oxidant status (TOS), antioxidant status (TAS), and oxidative stress index (OSI) of the blood plasma were measured using a novel automated colorimetric method. The result of this study was very interesting because no significant difference was found between patients with only OCD and all patients in all measures. TAS levels were higher

than controls in both the "only OCD" group and all patients ($p<0.001$, $p<0.001$, respectively). The TOS and OSI values of both patient groups were significantly lower than controls (TOS $p<0.001$; OSI $p<0.001$) and TOS ($p<0.001$; OSI $p<0.001$). In OCD group, illness duration was correlated with TOS and OSI (rho (0) = 0.44, $p=0.023$, $n=26$ and rho (0) = 0.44, $p=0.026$, $n=26$, respectively) but not with TAS volume.

In a longitudinal case control study, Chakraborty et al. evaluated the oxidative stress in 30 newly diagnosed OCD patients and in 30 control patients (Chakraborty et al 2009). Serum TBARS and plasma ascorbate were assessed to evaluate oxidative stress. The Yale–Brown Obsessive–Compulsive Scale (YBOCS) was used for assessment of OCD severity. Oxidative stress and OCD severity were assessed before and after treatment with fluoxetine at an average dosage of 40 mg/day. An improvement in YBOCS by about 43 % after 12 weeks of treatment was associated with significantly decreased TBARS and increased plasma ascorbate values ($p<0.05$). OCD subjects had higher serum TBARS and lower plasma ascorbate levels than the controls. The results suggest that oxidative stress plays a significant role in OCD and show that successful treatment with fluoxetine not only improves the clinical state but also reduces oxidative stress.

Ozdemir et al. investigated whether serum selenium (Se), antioxidant enzyme (GSH-Px, SOD, and CAT) activity, and plasma MDA levels, a product of lipid peroxidation, were associated with OCD (Ozdemir et al. 2009). Twenty-eight OCD patients who had been drug free at least for a month and a control group of 28 healthy subjects, matched with respect to age and sex, were included in this study. In both groups, the levels of erythrocyte MDA, GSH-Px, SOD, Se, and CAT were measured. The levels of MDA and SOD were significantly higher ($p<0.01$, $p<0.05$, respectively) in OCD subjects than controls. The activities of CAT, GSH-Px, and serum Se levels were significantly lower ($p<0.0001$, $p<0.001$, and $p<0.001$, respectively) in OCD patients than controls. There was a positive correlation in OCD patients between plasma GSH-Px activity and Se concentration ($r=52$, $p=0.001$). In OCD patients, CAT and SOD activities significantly and negatively correlated with MDA levels ($r=-0.45$, $p=0.017$ for CAT and $r=-0.54$, $p=0.020$ for SOD). The results of this study indicate a significant relationship between OCD and oxidative stress.

The relationship between possible stress-related biochemical markers and oxidative–antioxidative status in OCD were studied by Behl et al. (2010). This study determined the oxidative–antioxidative status of 20 OCD patients and 20 healthy subjects aged between 20 and 40 years, the aim being to assess whether oxidative–antioxidative status may be used as a biological marker of OCD. A significant difference in SOD levels was observed between the OCD group and controls ($p<0.05$). MDA levels were also significantly higher in OCD subjects ($p<0.05$). The results reaffirmed those of earlier studies. An overall oxidative imbalance was found in OCD, especially regarding antioxidants and specifically SOD, which has a protective role in oxidative stress. This phenomenon may suggest that oxidative stress could have a significant role in OCD etiology. Hence, therapy specifically targeting MDA production will have a beneficial effect in overcoming

oxidative stress in OCD. Authors suggest that the overall oxidative imbalance shifted toward the antioxidant side, which may be due to either a rebound phenomenon or chronicity of the condition.

A study by Shohag et al. examined serum antioxidant vitamins and MDA levels in OCD patients (Shohag et al. 2012). The aim of this study was to assess whether antioxidant vitamin activity (vitamins E, C, and A) and MDA levels were associated with OCD. Forty-eight OCD patients diagnosed according to DSM-IV and 48 healthy volunteers (controls) were included in this study. The serum levels of vitamins E and A were determined using RP-HPLC, whereas vitamin C was estimated by phenylhydrazine spectrophotometry. The MDA level was measured using UV–VIS spectroscopy. Significant differences in serum vitamin E and C levels were observed between the OCD and controls ($p<0.05$). MDA levels were found to be significantly higher in OCD subjects ($p<0.05$). These results confirm an overall imbalance in antioxidant vitamin level in OCD patients, which may have a potential role in the etiopathogenesis of the disease.

In order to understand the association of mitochondrial disorders with oxidative stress in OCD, Orhan et al. examined genetic variants of manganese superoxide dismutase (MnSOD), uncouple-2 antioxidant genes, MDA, and glutathione, all markers of oxidative stress (Orhan et al. 2012). A group of 104 OCD patients and a group of 110 healthy controls were included in the study. For MnSOD, the CT (Ala/Val) genotype ($p<0.01$) was significantly less common in OCD patients than in controls. In contrast, the CC (Ala/Ala) genotype was significantly more frequent in patients than controls ($p<0.05$). For uncouple-2 I/D, the frequencies of the ID genotype ($p<0.01$) and I allele ($p<0.05$) were lower in OCD patients than in controls. The DD genotype was more prevalent in OCD patients than controls ($p<0.01$). While whole blood glutathione was significantly diminished ($p<0.0001$), serum MDA was significantly elevated in OCD patients compared with controls ($p<0.0001$). MDA levels were significantly elevated in subjects with the DD genotype of UCP-2 I/D ($p<0.05$) and CC genotype of MnSOD ($p<0.05$) as compared with II or ID and TT or CT genotype, respectively. MDA levels in OCD patients with CC ($p<0.05$) or CT ($p<0.05$) genotype were significantly higher than OCD subjects with the TT genotype. The CC genotype of MnSOD or DD genotype of UCP-2 might result in mitochondrial disorders by increasing oxidative stress in OCD.

2.1 Limitations of Studies

Studies regarding oxidative stress in OCD patients have some limitations. They include relatively small groups of OCD patients with symptoms of varying severity and duration of disorder, who are usually in course of treatment with various antidepressants, especially SRIs (in various doses), which probably have antioxidant properties.

3 Antioxidant Properties Antidepressants Used in OCD Patients Treatment

In preclinical studies of animals, antidepressants of different classes have been shown to replenish, to varying degrees, the glutathione depletion seen in the inescapable shock behavioral paradigm of depression (Pal and Dandiya 1994). Venlafaxine was associated with the correction of several oxidative markers in the rat cortex (Herken et al. 2007). A proteomic study using rats has found multiple protein modulations in the hippocampus after venlafaxine or fluoxetine administration. Antioxidant and antiapoptotic proteins were among those identified (Khawaja et al. 2004). In another animal study, escitalopram was shown to improve depression-related GSH-Px, glutathione and vitamin C depletion, and increased lipid peroxidation (Eren et al. 2007).

Studies have not been unanimous in associating normalization of oxidative parameters in patients with antidepressant treatment. However, a small group of clinical studies has provided evidence for the antioxidant effects of these drugs by demonstrating reversals of antioxidant and oxidative disturbances after antidepressant treatments (Bilici et al. 2001; Herken et al. 2007; Khanzode et al. 2003). Consequently, oxidative parameters have been nominated by some authors to be candidate markers of antidepressant efficacy and may be relevant in OCD treatment (Bilici et al. 2001).

One comparatively larger study found that 6 weeks of antidepressant treatment did not affect oxidative–antioxidative systems, regardless of the response or remission status of the patients (Sarandol et al. 2007).

4 Oxidative Stress and Autoimmune System in OCD

Recent research has implicated the role played by the immune mechanism in the pathophysiology of OCD. Alterations in pro- and anti-inflammatory cytokines, including interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α, have been reported in OCD (Denys et al. 2004; Konuk et al. 2007; Murphy et al. 2001, 2006; Oka et al. 1993). Despite increasing evidence concerning the involvement of cytokine release in OCD, the results of the studies are inconsistent. The results of a study by Konuk et al. (2007) show increased production of the proinflammatory cytokines, IL-6, and TNF-α in OCD patients (Konuk et al. 2007).

N-acetylcysteine (NAC) has been shown to have anti-inflammatory properties that are linked to oxidative pathways, which may provide another potential mechanism of action for NAC in psychiatry (Dean et al. 2011). Increased TNF-α and IL-1β levels were reduced following NAC treatment in rat models of both traumatic brain injury and focal cerebral ischemia (Khan et al. 2004). NAC has also been shown to improve outcomes in lipopolysaccharide models of inflammation. Pretreatment with NAC was found to prevent oxidative stress and loss of long-term

potentiation following exposure to prenatal inflammation (Paintlia et al. 2008). Furthermore, lipopolysaccharide treatment results in inhibited oligodendroglial cell development and myelination that is attenuated by NAC administration in rat mixed glial cultures (Lante et al. 2008).

The reductions in inflammatory cytokines by NAC treatment may be a potential mechanism by which NAC modulates the symptoms of psychiatric disorders, including OCD. This may be directly associated with the inflammatory pathway, or it might work through the oxidative processes associated with inflammation.

NAC is converted to cysteine and modulates CNS glutathione levels. The uptake of cysteine by glia causes glial release of glutamate into the extrasynaptic space where it appears to stimulate inhibitory metabotropic glutamate receptors on glutamatergic nerve terminals and thereby reduces the synaptic release of glutamate. NAC may enhance clearance of glutamate by glial cells at the synapse. Elevated level of glutamate depletes glutathione within glial cells, impair cysteine transport, and thereby increase the vulnerability of glia to oxidative stress. Preclinical studies demonstrate that NAC protects glial cells against glutamate toxicity, repletes levels of glutathione, and attenuates toxic levels of glutamate (Noble and Mayer–Proschel 1996; Oka et al. 1993).

At present, there is only 1 case report regarding the use of NAC in an OCD patient, which showed significant benefits in a patient who was refractory to treatment (Lafleur et al. 2006). The patient experienced partial improvement after treatment with fluvoxamine and continued fluvoxamine during a 13-week trial of 3 g of NAC (including dose titration to 3 g). During the course of the trial, the participant improved on both the YBOCS and Hamilton Rating Scale for Depression scores. Continued treatment with fluvoxamine and NAC led to improvement in frequency and severity of compulsions and obsessions.

5 Summary

A growing body of evidence suggests that oxidative stress plays an important role in the etiology of OCD. There have been reports of oxidative stress in OCD patients, including increased lipid peroxidation; decreased levels of vitamin E, CAT, GPx, and Se; increased SOD; and changes in overall oxidative status. Some of these alterations have been linked to the severity of OCD symptoms. However, these studies have some important limitations. The range of designs, comorbid influences in some cases, and the patients' drugs might make it difficult to determine a clear outcome. The cause of the oxidant–antioxidant imbalance disorder is not known. Oxidative processes can be associated with inflammation and dysfunction in the glutamatergic system in OCD. Overall oxidative imbalance can shift toward the antioxidant side, which may be due to either a rebound phenomenon or chronicity of the condition. Antidepressants used in the treatment of OCD may well have antioxidant properties. NACs, which have anti-inflammatory properties that are linked to oxidative pathways, may improve the clinical state of OCD patients who were refractory to treatment.

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A Relationship Between Oxidative Status and Attention Deficit Hyperactivity Disorder

Salik Selek and M. Fatih Ceylan

Abbreviations

ADHD	Attention deficit hyperactivity disorder
CAT	Catalase
CSF	Cerebrospinal fluid
DA	Dopamine
GSH-Px	Glutathione peroxidase
MDA	Malondialdehyde
MPH	Methylphenidate
NAc	Nucleus accumbens
NO	Nitric oxide
OSI	Oxidative stress index
PUFA	Polyunsaturated omega-3 fatty acids
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAS	Total antioxidant status
TOS	Total oxidative status

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1 Introduction

Attention deficit hyperactivity disorder (ADHD) is one of the most prevalent childhood psychiatric disorders in the world. ADHD is frequently a chronic disorder, which leads to a negative impact on functioning in adulthood as well. Follow-up studies into adulthood show that only 10 % individuals diagnosed with ADHD are functionally remitted (less than five symptoms) (Biederman et al. 2000). Therefore, the burden of the disease continues throughout the life span.

The encephalitis pandemics after World War I revealed many ADHD-like cases whose symptoms match with the characteristics of currently defined ADHD (Goldman et al. 1998). Despite the relationship between organic diseases, no "obvious" organic causes were found in 1940s; thus, Strauss and Lehtinen named the disease as "minimal brain dysfunction" (Strauss and Lehtinen 1947). This definition did not set the disease apart from brain either. Several neurochemical and genetic mechanisms are believed to be involved in ADHD (Tripp and Wickens 2009). Neurotransmitter dysfunctions, neural network dysfunctions, environmental factors such as brain injury and perinatal events, hormonal imbalances, and neurological and genetic factors have been discussed in the etiology of ADHD although the basis of the disease remains unclear (Bulut et al. 2007; Faraone 2004).

While aerobic life depends on oxygen, sometimes it may be lethal for living beings which is known as "oxygen paradox" (Davies 1995). That is, during oxidation–reduction reactions for life energy, free radicals called oxidants are produced. These oxygen-based compounds are called reactive oxygen species (ROS) and they generally have very short half-life (Gutteridge and Halliwell 2006). Despite their short half-lives, they interact with basic components of living cells and damage the cellular function or architecture. Oxidants are overcome or removed by antioxidant defense mechanisms. There are mainly three subtypes of antioxidants: primary (preventing ROS production), secondary (removal of oxidants or inhibiting the radicals' reactions), and tertiary (repairing the cellular injury) antioxidants (Valko et al. 2007). An imbalance of oxidative metabolism is called oxidative stress (Valko et al. 2006). Thus, oxidative stress may be overproduction of oxidants or failure of antioxidant defense mechanisms. There's a dynamic equilibrium in a healthy living being. Herman Denham's free radical theory of aging was one of the milestones of oxidative research in humans, inspiring numerous efforts to identify the biochemical reactions in diseases and clinical outcome in 1950s (Horrum et al. 1987). Up till now, oxidative stress has been associated with numerous diseases: atherosclerosis (Kunt et al. 2006), infectious disorders like hepatitis (Horoz et al. 2006), and neurodegenerative disorders such as Huntington's disease (Akyol et al. 2004). Studies on brain disorders are not new. For example, researchers have evaluated the reduction–oxidation (redox) changes of CSF in brain disorders before (Brodskaia et al. 1975). The association between oxidative stress and psychiatric disorders such as schizophrenia, bipolar disorder, depression, and obssesive compulsive disorders has been reported (Dietrich-Muszalska et al. 2009; Andreazza et al. 2008; Herken et al. 2007; Selek et al. 2008a; Selek et al. 2008b). Most of the studies evaluated specific aspect

of oxidative stress such as several oxidants and antioxidants (Andreazza et al. 2008; Herken et al. 2007; Ross et al. 2003), and few have focused on whole oxidative–antioxidative status (Ersoy et al. 2008; Selek et al. 2008b; Yanik et al. 2004). However, studies of ADHD are relatively few in both aspects. A PubMed search with "oxidative stress + ADHD" terms yields more than 30 results, whereas "oxidative stress + schizophrenia" yields more than 300, and systematic meta-analysis of findings is still missing in ADHD. Therefore, this chapter aims to summarize the findings of oxidative stress in ADHD and discuss its possible neurobiological role.

2 Oxidative Stress in ADHD

Ross and his colleagues found higher rates of oxidative breakdown of n-3 polyunsaturated fatty acids in ADHD via exhalant ethane levels measurement which is a noninvasive method, but levels of butane, a marker of protein oxidation, remain unchanged (Ross et al. 2003). However, the sample size was relatively low with 10 patients and 12 healthy controls, and the measurement method was an indirect approach. Anecdotal reports of use of endogenous or exogenous supplements which have antioxidant properties in ADHD have also been reported at the beginning of the millennium (Rohdewald 2002; Sawada and Shimohama 2000).

Specific molecules of oxidants and antioxidants were studied in ADHD. Malondialdehyde (MDA) is the breakdown product of the major chain reactions leading to oxidation of polyunsaturated fatty acids (PUFAs) and thus serves as a reliable marker of oxidative stress. That is, higher MDA levels indicate oxidative stress. Bulut et al. (2007) found higher MDA levels in adult patients, and MDA levels were moderately correlated with the number of hyperactivity criteria met ($n=20$, $p=0.01$, Ro = 0.56) and with the total score for hyperactivity/impulsivity ($n=20$, $p=0.02$, Ro = 0.51). On the other hand, a study in children found no difference of MDA levels when compared to healthy controls (Oztop et al. 2012). In this study, patient group consisted of 27 boys and 3 girls, whereas healthy control group consisted of 18 boys and 12 girls. Another study reported diminished values of plasma MDA accompanied by increased concentrations of gamma-tocopherol in patients. The authors concluded that significant changes occur in the lipid and lipoprotein profiles, as well as in the oxidant–antioxidant status of ADHD patients; however, the fatty acid distribution does not reflect n-3 fatty acid deficiency (Spahis et al. 2008). Larger studies are needed in order to verify whether lipid peroxidation is altered in ADHD or not.

Nitric oxide (NO) is an oxidant, but it also has been implicated in a number of physiological functions such as noradrenalin and dopamine release, memory, and learning (Akyol et al. 2004). Superoxide dismutase (SOD) is an antioxidant whose major function may be to prevent $ONOO^-$ formation from NO. We found remarkable high levels of oxidant NO and low SOD activities which may suggest an oxidative imbalance in adult patients (Selek et al. 2008). Higher NO levels but not lower SOD activity findings were replicated also in child patients (Ceylan et al. 2010).

Ceylan et al. (2010) also found altered levels of other antioxidants such as glutathione peroxidase (GSH-Px) and catalase (CAT), but CAT activity is not significantly different from controls.

Studies have also been conducted in saliva and urine as well (Kawatani et al. 2011). Archana et al. (2012) found a significant increase in the salivary thiol proteins and pseudocholinesterase levels in ADHD children. Increased salivary thiol proteins are believed to be a marker of oxidative stress due to increased turnover degradation.

Studying several specific molecules of oxidative stress leads to diverse results in multiple studies. The replicated findings are relatively new. One or two molecules may not show the general oxidative status in the body. Therefore, total evaluation of oxidative metabolism is essential. Various methods are used to measure the total oxidative status. Erel described novel automated methods for measuring both total oxidative (TOS) and antioxidative status (TAS) which have been cited more than 350 times (Erel 2004, 2005). In this method oxidants present in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ion. He also described the oxidative stress index (OSI) that reflects the general oxidative–antioxidative balance (Kosecik et al. 2005) (Fig. 1). OSI is especially useful in order to identify the degree of oxidative stress when antioxidants are also elevated reacting against the oxidant increase. In some cases reactive increase of oxidants also called as rebound phenomenon may not overcome the oxidants that are seen as high OSI (Selek et al. 2008c).

We found that TAS, TOS, and OSI were significantly higher than controls in adult patients. There was not a significant difference between comorbid cases and only adult ADHD patients in terms of measured values (Selek et al. 2012). Although TOS was found to be increased in several other psychiatric disorders, the remarkable increase (4.2 times) in adult ADHD, no difference between comorbid patients and pure ADHDs suggests a more strong association between adult ADHD and oxidative stress than other psychiatric disorders (Andreazza et al. 2008; Ersoy et al. 2008). The altered oxidative status in ADHD both in our study and previous studies is attributed to several neurobiological bases such as dopaminergic pathways'

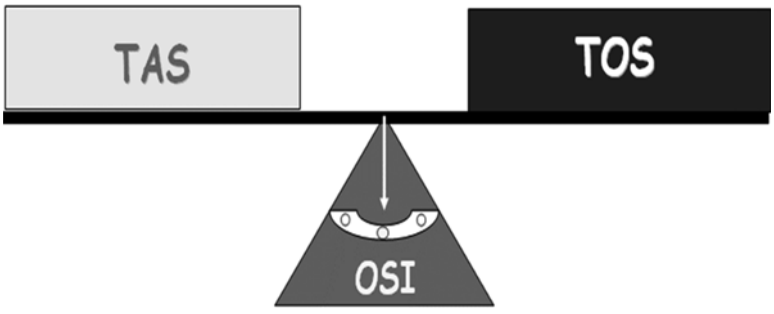

Fig. 1 The relation between oxidative stress markers, total oxidative status (*TOS*), total antioxidative status (*TAS*), and oxidative stress index (*OSI*) (Reprinted with permission from Selek et al. (2012))

susceptibility to auto-oxidation and mitochondrial pathologies (Selek et al. 2012). However, the exact cause remains unclear.

Another area of interest was the treatments' effect on oxidative metabolism in ADHD. Methylphenidate (MPH) is one of the most commonly used medications in ADHD. MPH increases extracellular concentrations of dopamine (DA) within key portions of brain reward circuits, including the nucleus accumbens (NAc) and related regions (Abikoff et al. 2004). Not only clinical trials but also neuroimaging studies have shown the efficacy of MPH in ADHD (Durston 2003). Martins et al. have found that chronic MPH exposure (28 days) induces oxidative stress in healthy young rat brains. In the same study, they have found that oxidative stress was reduced in healthy adult rats both in hippocampus and cerebellum during chronic treatment (Martins et al. 2006). Andreazza et al. found MPH-induced oxidative stress in both adult and young rats (Andreazza et al. 2007). On the other hand, the previously reported observations of oxidative damage in brain tissue of rats exposed to MPH for 28 days were not confirmed in Witt's study (Witt et al. 2010). Were conducted on healthy animals and MPH's effect on ADHD brain from oxidative aspect therefore, the controversial results need to be replicated both in healthy and ADHD rats.

Urinary 8-oxo-7,8-dihydroguanine, a marker or DNA oxidation in the mitochondria and cell cytoplasm, is found to be elevated in some ADHD children (Chovanová et al. 2006). Altered levels of DNA base damage products as well as the expression of the main repair enzyme 8-hydroxyguanine glycosylase 1 have been related with oxidative stress. Amphetamine, methylphenidate, and atomoxetine were found to have protective role in prevention of oxidative stress via decreasing the levels of this enzyme (Schmidt et al. 2010). There are still few studies on other ADHD medications.

Antioxidative supplements gained interest in the treatment of psychiatric disorders as well as in ADHD. Trebatická et al. reported French maritime pine bark extract's (Pycnogenol) efficacy on ADHD that is known with its antioxidant effects. The therapeutic effect was disappeared after cessation of the treatment and relapse of symptoms were observed (Trebatická et al. 2006). However, Pycnogenol failed to show efficacy when compared to standard ADHD medications such as MPH (Tenenbaum et al. 2002). Current data does not support use of Pycnogenol in ADHD (Schoonees et al. 2012). Another supplement which has antioxidative properties is polyunsaturated omega-3 fatty acids (PUFA). Although abnormal blood PUFA levels were reported in a number of studies, weighted comparisons of PUFA status showed no significant differences overall between people with mental health problems and controls (Milte et al. 2009). On the other hand, large randomized placebo-controlled trials of antioxidant use in bipolar disorder, in which oxidative stress data is well accumulated, failed to show any significant difference against placebo (Berk et al. 2012). The researchers claimed that these findings may be confounded by limitations. In general, today the therapeutic use of antioxidants in ADHD remains unclear. Even if there are anecdotal positive reports, the administration dose, duration of use, and other pharmacological issues need to be investigated further.

Most of the researchers have focused on pathophysiology and treatment of oxidative stress. Few studies evaluated the diagnostic performance of those parameters. In fact, establishing a blood test for psychiatric disorder is a challenging area, since there is still no blood or imaging test for psychiatric diagnosis. In our study we also evaluated the diagnostic performance of oxidative parameters. TOS levels above 9.8575 μmol H_2O_2 Eqv./L were highly predictive for the disease state (PPV =86 %) and negative predictive value was also high (100 %) (Selek et al. 2012). Archana et al. also reported higher sensitivity and specificity rates of salivary thiol proteins in identifying the disease state (Archana et al. 2012). But, both of the studies are preliminary and need to be replicated in larger samples.

3 Conclusion

There are several studies that show the oxidative status is altered in ADHD. Medications and antioxidative supplements on oxidative status are controversial. Some of the oxidative parameters are candidates for diagnostic tests. More researches are needed to clarify these issues in ADHD.

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The Role of Oxidative Stress in Neurodegenerative Diseases

Dominika Książek-Winiarek and Andrzej Głąbiński

Abbreviations

8-OHG	8-hydroxyguanine
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ApoE	Apolipoprotein E
APP	β-amyloid precursor protein
Aβ	Amyloid-β
BER	Base excision repair
CD	Caudate nucleus
CHMP2B	Charged multivesicular body protein 2B
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cu/Zn SOD	Copper/zinc superoxide dismutase
DA	Dopamine
FUS/TLS	Fused in sarcoma/translated in liposarcoma
GFAP	Glial fibrillary acidic protein
GS	Glutamine synthetase
GSH	Glutathione
HNE	4-hydroxynonenal
Hsp	Heat shock protein
L-DOPA	L-3,4-dihydroxyphenylalanine
LRRK2	Leucine-rich repeat kinase 2

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MCI	Mild cognitive impairment
mETC	Mitochondrial electron transport chain
MS	Multiple sclerosis
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NF-L	Neurofilament-light
NFTs	Neurofibrillary tangles
NO	Nitric oxide
Nrf2	Nuclear erythroid 2-related factor 2
PD	Parkinson's disease
PINK-1	PTEN-induced putative kinase 1
ROS	Reactive oxygen species
SN	Substantia nigra
SOD2	Superoxide dismutase2
TDP-43	TAR DNA-binding protein
VAPB	Vesicle-associated membrane protein B

1 Oxidative Stress and Neurodegenerative Diseases

Oxidative stress is a result of an imbalance between the production of reactive oxygen species and the ability of the system to neutralize or repair the damage caused by oxygen radicals. ROS constitute a range of oxygen-derived molecules formed by the incomplete reduction of oxygen during oxidative metabolism (Murphy 2006). ROS may have some essential functions in normal, non-pathologic conditions (Le Belle et al. 2011), but they are more attributed to the pathological events that lead to macromolecules' damage and disturbed cell function as well as cell death (Halliwell and Jenner 1998; Jenner 1998). They are implicated in neuroinflammatory, as well as neurodegenerative diseases (Brown and Bal-Price 2003; Zipp and Aktas 2006).

Neurodegenerative diseases are a heterologous group of debilitating diseases, which are so far incurable. Such disorders are characterized by slow and progressive loss of specific subpopulations of neuronal cells and/or loss of their specific functions (Coppedè et al. 2006). The increase in adult life expectancy leads to more frequent incidence of neurodegenerative disorders, like Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis, making them important health and economical problems. The brain is the main place where neurodegenerative immune response occurs. This organ is particularly sensitive to oxidative stress due to some specific features. The brain uses 20 % of the total oxygen to produce energy by oxidative phosphorylation in mitochondria. A major reason for the high oxygen uptake is the enormous amount of ATP needed to maintain neuronal intracellular ion homeostasis towards to all the openings and closings of ion channels (Halliwell 2006). During oxidative phosphorylation, neurons are highly vulnerable to oxidative damage because of their high metabolic activity, low antioxidant capacity, and non-replicative nature. The brain has high levels of transition metals and ascorbate, which can act as potent pro-oxidants. The brain has also

high content of phospholipids containing polyunsaturated fatty acids. Peroxidation of membrane lipids leads to generation of oxidized phospholipids and reactive aldehydes, which may result in increase blood–brain barrier permeability (Miller et al. 2011; Usatyuk and Natarajan 2012). Recent studies also showed that oxidized phospholipids may induce monocyte binding to brain endothelium (Usatyuk and Natarajan 2012). One of the sources of the ROS in the brain may be activated microglia, the resident innate immune cells, releasing hydrogen peroxide or superoxide (Moss and Bates 2001). In microglial cells, ROS can be produced by several sources including intracellular peroxidases, oxidative processes in mitochondria, and NADPH oxidase activity at the cell surface membrane (Block and Hong 2007). The adaptation and survival of cells and organisms requires the ability to sense proteotoxic insults and to coordinate protective cellular pathways and chaperone networks due to protein quality control (Calabrese et al. 2009a). Because the brain is particularly vulnerable to oxidative stress-mediated damage of macromolecules, the protective genes called "vitagenes" play a special role, which control aging, thus linking stress and protein homeostasis with the health antidegenerative mechanisms (Calabrese et al. 2010). The main example of such "vitagenes" are those coding heat shock proteins (Hsp), which can lead to stress tolerance or cytoprotection (Calabrese et al. 2009b). Several reports indicated that in neurodegenerative diseases, the brain regions selectively vulnerable to neurodegeneration demonstrated increased level of oxidative damage and lowered level of antioxidant mechanisms suggesting direct correlation between oxidative stress and neuronal death. The role of oxidative stress in the pathology of neurodegenerative diseases is discussed below.

1.1 Alzheimer's Disease (AD)

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disorder whose clinical manifestation includes cognitive decline and dementia (Harman 2006). The main pathological hallmark in AD is brain atrophy and gradual loss of neurons mainly in hippocampus, frontal cortex, and limbic areas. AD pathology is associated with neuroinflammation, oxidative damage, and astrogliosis with upregulation of the glial marker GFAP (glial fibrillary acidic protein) (Korolainen et al. 2005; Eikelenboom et al. 2000). About 90 % of AD cases are of sporadic nature, whereas between 5 % and 10 % are of familial origin. Differential susceptibility and course of the disease, as well as late age of onset in sporadic AD, indicate multifactorial etiology involving epigenetic and environmental components. AD pathology is characterized by the presence of extracellular senile plaques and intracellular accumulation of hyperphosphorylated tau protein in the form of neurofibrillary tangles (NFTs). The major component of senile plaques are 39- to 42-amino-acid peptides called amyloid-β (Aβ), derived from a large membrane-bound protein – β-amyloid precursor protein (APP) (Masters et al. 1985; Glenner and Wong 1984). Of these peptides, Aβ consisted of 42 amino acids is thought to be the most amyloidogenic. Familial AD involves mutations in genes associated with APP biosynthesis and proteolytic processing (Eckman et al. 1997; Haass et al. 1994).

The genetic studies of AD indicated that early-onset AD is associated with mutations in APP or the presenilins, whereas late-onset AD is associated to an apolipoprotein E (ApoE) polymorphism (Bertram and Tanzi 2004). It was also shown that genetic mutations of the APP or presenilin-1 increase the rate of Aβ formation.

There is established role of amyloid plaques and neurofibrillary tangles in pathology of AD. Tau protein functions as a regulator of microtubule assembly and transport. However in AD, it dissociates from microtubules and in consequence leads to their destabilization and dysfunctional axonal transport. The amyloid cascade hypotheses suggest that accumulation of Aβ is the cause of the disease. More recently, some studies showed that oligomeric Aβ, rather than extracellular fibrillary Aβ, may be the most toxic entity (Haass and Selkoe 2007; LaFerla et al. 2007). Although most of the studies relate to Aβ deposits in the pathogenesis of AD, one study reported poor correlation between Aβ deposits and sporadic AD (Castellani and Smith 2011). Additionally, there is growing body of evidence that alterations in redox balance of AD brains are also of great importance. Oxidative stress may influence Aβ formation in two ways: by increasing APP levels or by affecting Aβ processing through modulation of the activity and levels of the key proteolytic enzymes such as β-secretase and γ-secretase. Aβ itself promotes the formation of ROS and, by inducing more oxidative stress, creates positive feedback on APP levels and on its proteolytic pathway (Pratico 2008). Amyloid-β plaques and NFTs are also considered to be associated with reactive astrocytes and activated microglia (McGeer and McGeer 2001), which can stimulate, in response to Aβ presence, the production of ROS (Weiner and Frenkel 2006).

Mitochondrial functional deficiency is one of the earliest and most prominent features of AD (Nunomura et al. 2001). These organelles play important functions in cell survival or death, especially in long-lived cells such as neurons (Santos et al. 2010). Altered function of mitochondria caused by physiological or environmental stimuli may have severe consequences for neuronal function and survival. Indeed, downregulation of complex I genes has been suggested to be directly responsible for a decrease in energy production in AD brains (Manczak et al. 2004). As a result, ROS production is significantly elevated, which leads to Ca^{2+} buffering impairment and the release of proapoptotic proteins (Reddy and Beal 2005). Moreover, progressive accumulation of Aβ within mitochondria of AD brain cells may lead to abnormal synaptic morphology and deficits in long-term synaptic plasticity (Devi et al. 2006). Aβ and APP can also directly disturb respiration, which in consequence results in elevated ROS production (Yao et al. 2011; Reddy and Beal 2008). With use of in vivo and in vitro approaches, it was demonstrated that Aβ colocalize with complex II of the respiratory chain (Manczak et al. 2006; Tillement et al. 2006; Loo et al. 1993).

Elevated levels of oxidative damage of macromolecules such as DNA, proteins, and lipids and depletion of antioxidants such as glutathione (GSH) have been observed in the AD brains (Mandal et al. 2012). Several studies indicated the presence of increased levels of lipid peroxidation markers, such as malondialdehyde and 4-hydroxynonenal (HNE), in brains of AD and mild cognitive impairment

(MCI–AD precursor disorder) patients and in cerebrospinal fluid (CSF) (Subbarao et al. 1990; Lauderdack et al. 2001; Williams et al. 2006). High levels of free HNE as well as elevated levels of protein-bound HNE were reported (Sayre et al. 1997; Perluigi et al. 2009; Lovell et al. 1997). It was also observed that ROS-oxidized proteins such as carbonyls are elevated in the frontal and parietal lobes and in the hippocampus of AD and MCI subjects (Hensley et al. 1995; Butterfield et al. 2006). Furthermore, it was shown that levels of 3-nitrotyrosine, another marker of protein oxidation, are elevated in brains from MCI subjects compared with controls (Butterfield et al. 2007). Elevated levels of oxidative damage markers observed in brains from MCI subjects indicated that oxidative stress initiated in MCI brain is an early event contributing to the progression of AD.

It was shown that DNA damage accumulates in older neurons and this damage may play a role in late-onset AD. Several studies reported twofold increase in nuclear DNA (nDNA) strand breaks from cortical neurons in AD brains compared to normal control brains (Mullaart et al. 1990) and higher levels of oxidized DNA and RNA bases in nuclear and mitochondrial DNA (mtDNA) in AD brains (Wang et al. 2005). The oxidation of mitochondrial DNA was shown to be approximately 10-fold higher than nuclear DNA bases. High levels of mtDNA oxidation support the notion that mitochondrial abnormalities in the AD brain may contribute to the increased ROS leakage (Beal 1998). Oxidative DNA damage was also found in MCI (Coppede and Migliore 2009). Additionally, it was shown that base excision repair (BER), the main repair system for oxidative DNA damage, is deficient in brains of AD and MCI patients (Weissman et al. 2007). This may explain the higher level of oxidative DNA damage in AD tissues. However, other factors may also be important, such as altered transcription, translation, antioxidant system, or protein processing, leading to aberrant regulatory pathways and additional transcriptional defects (Colangelo et al. 2002; Chow et al. 1998). Oxidative damage also occurs to RNA molecules. In AD hippocampus, frontal and occipital neocortex high levels of 8-hydroxyguanine (8-OHG) were observed in cytoplasmic RNA, and this has inversely correlated with the Aβ load, which suggested that RNA damage is an early event in AD (Nunomura et al. 1999; Shan et al. 2003). Shan and Lin showed 30–70 % oxidation of the mRNAs in the frontal cortex of the AD brain (Shan and Lin 2006). Moreover, an increase of 8-OHG was also reported in the frontal cortex of familial AD cases (Nunomura et al. 2004). In addition to the oxidation of mRNA, an increase in rRNA oxidation has also been observed in the AD brains (Ding et al. 2006).

Decreased total brain mass resulting from loss of brain cells in AD patients has been observed (Katzman and Saitoh 1991). One of the proposed mechanisms that contribute to the neuronal cell loss is excitotoxicity. It was observed that oxidative modification of glutamine synthetase (GS) leads to its altered function in MCI and AD brains, and in consequence to continuous excitation of postsynaptic neurons, Ca^{2+} accumulation, and free radical formation, resulting in impairment of neurotransmission and excitotoxic neuronal cell death (Hensley et al. 1995; Lafon-Cazal et al. 1999).

1.2 Parkinson's Disease (PD)

Parkinson's disease (PD) is a progressive, degenerative disease of the central nervous system (CNS) resulting in impairment of motor skills and speech, as well as other functions (Jellinger 2001). There are two main forms of PD: sporadic and familial. The latter is characterized by alterations in genes such as α-synuclein, leucine-rich repeat kinase 2(LRRK2), parkin, PTEN-induced putative kinase 1 (PINK-1), or DJ-1 (Polymeropoulos et al. 1997; Sun et al. 2006; Bonifati et al. 2008; Weng et al. 2007; Abou-Sleiman et al. 2003). Mutations in PINK1, DJ-1, and parkin cause early-onset autosomal recessive disorder, while mutations in LRRK2 and α-synuclein cause autosomal dominant PD (Savitt et al. 2006). The sporadic form arises rather from environmental factors, like exposure to toxins, head trauma, and infection, or from unknown etiology (Hirsch et al. 2005; Mosley et al. 2006; Bartels and Leenders 2007).

We do not know the exact cause leading to PD; however, it is stated that primary symptoms of this disorder result from insufficient formation and action of dopamine (DA) that leads to decreased stimulation of the motor cortex by the basal ganglia. The loss of dopamine produced mainly by the dopaminergic neurons of substantia nigra (SN) region and striatum of the brain results in tremors, muscle rigidity, cognitive dysfunction, changes in speech patterns, and a dynamic progressive dysfunction that can lead to a loss of physical movement and ultimately death (Goldberg et al. 2005; Baik et al. 1995; Wang et al. 2000; Jenner 2003).

Peterson et al., with use of rodent models of DA neurodegeneration, have shown that the oxidative stress plays a significant role in the etiology of PD (Liu et al. 2003; Qian et al. 2007; Peterson and Flood 2012). Furthermore, studies with animals suggested that the generation of ROS is an upstream event regulating production of other proinflammatory factors (Loeffler et al. 1994; Sanlioglu et al. 2001). Studies conducted on human postmortem brains from PD patients showed lower glutathione and mitochondrial function, increased astrogliosis, protein oxidation and nitration, and lipid peroxidation in SN compared to caudate nucleus (CD) (Mythri et al. 2011). Also increased iron levels were observed in PD brains (Riederer et al. 1989) and elevated superoxide dismutase 2 (SOD2) (Radunovic et al. 1997). These results suggest that brain region-specific imbalance between oxidant and antioxidant markers during aging might determine the vulnerability to degeneration in PD (Venkateshappa et al. 2011).

Significant evidence has now been accumulated that microglial activation leads to dopaminergic neurons loss in PD patients (Block and Hong 2007; Czlonkowska et al. 1996). Activated microglial cells release a wide variety of inflammatory mediators, including ROS and the related nitric oxide (NO) (Baldwin 2001). Recent data indicated that the progression of PD is due to oxidative stress damage that results not only from microglia activation but also from the neurons themselves in large part due to increased dopamine turnover (Maguire-Zeiss et al. 2005). The standard treatment of PD is the administration of L-DOPA (L-3, 4-dihydroxyphenylalanine) – the precursor of dopamine, which relieves many of the PD symptoms for a short

term but has been found to induce an oxidative stress response in DA neurons (Jin et al. 2010). Dopamine can be oxidized and H_2O_2 is generated as a by-product for the catabolism of DA by monoamine oxidase (Chinta and Andersen 2008). In addition, dopamine oxidation can result in the formation of dopamine quinone which can directly modify proteins (Stokes et al. 1999).

Elevated levels of reactive oxygen and nitrogen species are thought to damage lipids, proteins, and DNA as was shown in studies conducted on PD brains samples, where high levels of lipid peroxidation products, oxidized and damaged proteins, as well as oxidized DNA were observed (Jenner 2003; Isobe et al. 2010). Evidences from studies of Jenner et al. (1992) suggest that oxidative damaged proteins form toxic aggregates of α-synuclein – Lewy bodies. Formation of these protein aggregates has been accompanied by selective degeneration of nigral neurons in rodents and primates PD models (Dawson and Dawson 2003; Lin and Beal 2006). Studies conducted on PD patients revealed that the level of 8-OHG is elevated compared to age-matched controls (Alam et al. 1997). It was also shown that activity of complex I, a major component of mitochondrial electron transport chain (mETC), is decline in the SN and frontal cortex in PD patients (Schapira et al. 1990; Parker et al. 2008). Similar deficits in respiratory transport chain complex I have also been observed in peripheral cells, such as myocytes and platelets (Krige et al. 1992). Different cell and animal models indicated that mitochondrial damage or deficiencies may contribute to cell death and neurodegeneration (Ekstrand et al. 2007; Trimmer et al. 2004; Bove et al. 2005).

1.3 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), often referred as motor neuron disease, is one of the most common adult-onset neurodegenerative diseases. It is a relentless progressive neuromuscular failure, caused by degeneration of both upper motor neurons in the motor cortex and lower motor neurons connecting the spinal cord and brain stem to muscle fibers, leading to muscle denervation and atrophy (Leigh 2007). Although there may be affected discrete motor neuron population at the onset of the disease, during progression of ALS both upper and lower motor neurons will be eventually dysfunctional. ALS pathology is characterized by loss of motor neurons, with ubiquitinated inclusions, abnormal mitochondrial function, neurofilament aggregates, and glial activation. The exact etiology of ALS is still unknown. Most of the cases accounts for a sporadic form of this disorder, whereas about 5–10 % are of familial origin. Several gene mutations have been identified and associated with familial form of ALS (Valdmanis and Rouleau 2008), of which the most common are mutations in copper/zinc superoxide dismutase (Cu/Zn SOD), accounting for 10–20 % of autosomal dominant cases (Rosen et al. 1993). Other genes include fused in sarcoma/translated in liposarcoma (FUS/TLS) (Kwiatkowski et al. 2009), TAR DNA-binding protein (TDP-43) (Kabashi et al. 2008), charged multivesicular body protein 2B (CHMP2B) (Parkinson et al. 2006), vesicle-associated

membrane protein B (VAPB) (Nishimura et al. 2004), and angiogenin (Greenway et al. 2006). Much of our understanding of the mechanisms underlying neurodegeneration in ALS arises from cell and animal models of this disease. We can distinguish the mechanisms implicated in ALS development and progression as: oxidative stress, excitotoxicity, mitochondrial dysfunction and protein aggregation. Disruption of neurofilament network, involvement of non-neuronal cells from the vicinity of motor neurons, and defects in RNA processing are also implicated in ALS pathogenesis (Barber and Shaw 2010).

Motor neurons have an unusually high metabolic demand that may contribute to their vulnerability to cumulative oxidative stress. Pathological studies have showed the presence of morphologically altered mitochondria in motor neurons from ALS patients (Siklos et al. 1996; Sasaki and Iwata 1996), suggesting their role in disease pathogenesis. Studies conducted on mutant SOD1 mouse and on cell models also revealed abnormal mitochondria (Kong and Xu 1998; Martin et al. 2007) and decreased electron transport chain activity and mitochondrial membrane potential (Carri et al. 1997; Jung et al. 2002), altered calcium homeostasis (Carri et al. 1997), increased mtDNA damage (Martin et al. 2007), and reduced mitochondrial antioxidant defense mechanisms (Wood-Allum et al. 2006). Furthermore, a higher frequency of mtDNA mutations has been observed as well in the motor cortex and spinal cord of ALS patients compared to controls (Dhaliwal and Grewal 2000; Wiedemann et al. 2002).

Numerous studies have reported an increase in oxidative stress in ALS postmortem tissue compared to control samples. Elevated protein carbonyl levels have been observed in the spinal cord (Shaw et al. 1995b) and motor cortex (Ferrante et al. 1997) from sporadic ALS cases, and increased 3-nitrotyrosine levels, a marker for peroxynitrite-mediated damage, were found within spinal cord motor neurons in both SOD1–familial ALS and sporadic ALS patients (Beal et al. 1997). The presence of lipid oxidation markers in the spinal cord from sporadic ALS patients has also been reported, which were absent in control spinal cords (Shibata et al. 2001). Studies conducted on CSF from ALS patients enable measurement of oxidative stress markers at earlier stages of the disease. These studies have reported elevated levels of 8-OHG (Ihara et al. 2005), 4-hydroxynonenal (Simpson et al. 2004), and ascorbate free radical (Ihara et al. 2005). Additionally oxidation of mRNA has been recently identified in ALS patients and transgenic mice expressing SOD1 mutations contributed to familial ALS (Chang et al. 2008). mutSOD1 was also observed to bind and sequester mitochondrial Bcl-2 and cytosolic Hsps rendering them unavailable for antiapoptotic functions (Pasinelli et al. 2004).

Oxidative stress has been widely implicated in the pathogenesis of the ALS, as it is interconnected with other pathological mechanisms, such as excitotoxicity. It was revealed that exposure to ROS was sufficient to reduce uptake of glutamate through glutamate transporters on both glial and neuronal cells (Trotti et al. 1998; Rao et al. 2003). Glutamate is the major excitatory neurotransmitter in mammalian CNS and is contributed to synaptic transmission of nerves impulses in motor neurons. Imbalance between release and reuptake of glutamate leads to excitotoxicity, which results in increased intracellular free calcium concentrations, neuronal injury, and death (Heath and Shaw 2002). Increased glutamate levels have been observed in a

subset of ALS patients (Shaw et al. 1995a; Spreux-Varoquaux et al. 2002), and also elevated levels of intracellular calcium have been observed in motor nerve terminals in ALS patients (Siklos et al. 1996) and mutant SOD1 mice (Siklos et al. 1998).

Another process altered by oxidative stress is protein aggregation. A wide range of protein aggregates have been described in ALS, the most common of which is the ubiquitinated inclusion (Wood et al. 2003). One of the major protein components of such inclusions is TDP-43 (Arai et al. 2006) and mutant SOD1 (Shibata et al. 1996). The neuronal intermediate filament network, which is responsible for maintaining cell shape and axonal caliber, is also a target for ROS. Abnormal neurofilament accumulation is observed in spinal cord motor neurons in human ALS (Hirano et al. 1984) and in mutant SOD1 mouse (Zhang et al. 1997). ROS produced by SOD1 have been implicated in neurofilament-light (NF-L) alterations, mainly dityrosine cross-link formation and aggregation (Kim et al. 2004).

Evidence of oxidative stress implication in ALS pathology also comes from a microarray study of motor neuronal cells expressing mutant SOD1, which showed downregulation of genes involved in the antioxidant response, including nuclear erythroid 2-related factor 2 (Nrf2) (Kirby et al. 2005). Reduced Nrf2 mRNA and protein expression has also been reported in spinal cord neurons from ALS patients (Sarlette et al. 2008). Nrf2 leads to increase expression of proteins involved in antioxidant defense systems (Nguyen et al. 2009). Downregulation of Nrf2 expression may contribute to decreased ability of cells to remove ROS generated in normal, as well as in the pathological processes, resulting in a gradual increase in oxidative stress over time.

1.4 Multiple Sclerosis (MS)

Multiple sclerosis is a chronic demyelinated inflammatory and neurodegenerative disease of the CNS which is characterized by myelin loss and progressive disability. Patients with MS often displayed changes in sensation, visual problems, muscle weakness, and difficulties with coordination and speech. MS is mediated by a T-cell-dependent process (Witherick et al. 2010). MS pathology is characterized by infiltration of lymphocytes and macrophages leading to myelin damage and axon degeneration, which is the underling clinical course of the disease. The most prevalent form of MS is relapsing–remitting form which is characterized by recurrent and reversible episodes of neurological dysfunction affecting one or several sites. Usually, later in time, this form transforms into a secondary progressive phase with continuous and progressive neurological decline (Tumani et al. 2009).

There is a growing body of evidence indicating that oxidative stress plays a major role in the pathogenesis of MS. Results of several studies showed that relapsing–remitting and progressive clinical phases of MS may be caused by two distinct mechanisms: (a) focal inflammation which contributes to relapse and (b) diffuse axonal degeneration which seems to be the main contributor to disease progression. Oxidative stress is thought to be involved in both processes (Gilgun-Sherki et al.

2004). ROS may contribute to myelin and oligodendroglial degeneration, which are pathological hallmarks of MS (Amorini et al. 2009). They can induce several other changes important for MS development, such as cytoskeletal rearrangements, loss of blood–brain barrier integrity, tight-junction alterations, and the extravasation of leukocytes into the CNS (Witherick et al. 2010; van Horssen et al. 2011). Inflammation and oxidative stress are thought to promote damage in MS. However, there are some results indicating that oxidative damage is not accompanied by inflammatory activity rather it precedes the inflammatory response (Castegna et al. 2011).

Although various mechanisms may lead to demyelination and neurodegeneration in MS, it was recently shown that mitochondrial injury and subsequent energy failure highly contribute to tissue injury (Dutta et al. 2006; Mahad et al. 2008). It was indicated that profound alterations of mitochondrial ETC and deletions in mtDNA are present in neurons, especially in the progressive stage of MS (Campbell et al. 2011). Mitochondrial alterations may result from demyelination, as this process enhances the energy consumption in axons and thereby affects mitochondrial activity (Mahad et al. 2008).

It was shown that elevated levels of oxidative stress markers and/or decreased levels of antioxidant enzymes and small antioxidant molecules are present in the blood, serum, and CSF of patients with MS (Sayre et al. 2008). Also increased lipid peroxidation products have been shown in biological fluids of MS patients (Ferreti et al. 2005). Furthermore Haider et al. observed elevated levels of oxidized lipids and 8-OHG in MS brains compared to control subjects (Haider et al. 2011), whereas Miller et al. observed a significant decrease in total antioxidant capacity (Miller et al. 2010). Antioxidant deficiencies may occur during the clinical course of MS, as a result of chronic inflammation that is accompanied by increase in oxidative stress (Van Meeteren et al. 2005).

2 Conclusions

Oxidative stress resulting from an imbalance between ROS and antioxidants is a factor contributing to development of various degenerative diseases. As the brain is particularly susceptible to harmful effects of ROS, it is suggested that oxidative stress may play an unique role in development and progression of various neurodegenerative disorders, such as AD, PD, ALS, or MS. Oxidative stress is an important factor in such diseases, as the damage of the neurons could be due to either an increase in oxidative process or a decrease in antioxidant defenses or both. Oxidative stress could be generated as a secondary effect of a preexisting diseased condition or could be the central cause of the disease itself. ROS can induce changes in mitochondria morphology and functions, resulting in lower ATP level and increased oxidative stress. They can also alter the structure and function of various macromolecules such as lipids, proteins, or nucleic acids. As it was frequently shown, oxidative stress is not a separate event in the pathogenesis; rather it is interconnected to other pathological mechanisms contributing to disease onset and progression.

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Pathophysiological Aspects

Magnetic Resonance Spectroscopy Studies in Bipolar Disorder Patients: Focus on the Potential Role of Oxidative Stress

Nicola Dusi, Filippo Cecchetto, and Paolo Brambilla

Abbreviations

ACC	Anterior cingulated cortex
ATP	Adenosine triphosphate
BD	Bipolar disorder
CAT	Catalase
Cho	Choline
CK	Creatine kinase
Cr	Creatine
DLPFC	Dorsolateral prefrontal cortex
ETC	Electron transport chain
GABA	Gamma aminobutyric acid

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Gln	Glutamine
Glu	Glutamate
GPC	Glycerophosphocholine
GPx	Glutathione peroxidase
GSH	Glutathione
MRS	Magnetic resonance spectroscopy
NAA	N-acetyl-aspartate
NADPH	Nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
$ONOO^-$	Peroxynitrite
PCh	Phosphocholine
PCr	Phosphocreatine
PME	Phosphomonoester
ROS	Reactive oxygen species
SOD	Superoxide dismutase
WHO	World Health Organization

1 Introduction

Bipolar disorder (BD) is a chronic and recurrent mental illness that determines an increasing cause of disability worldwide, according to the World Health Organization (WHO) (Kessler et al. 2009). As the diagnosis is still based on syndromic and statistical categories, there's a lack of knowledge about its etiology (Judd et al. 2002). Clinical presentation, longitudinal outcome, and treatment response vary among patients due to the heterogeneity of the disease. Therefore, an urgent need of a better understanding of the biology of this illness is required, allowing more detailed and tailored analyses of genetic implications and molecular underpinnings (Fekadu et al. 2009).

Several lines of research report neuroanatomical alterations in BD. These abnormalities pertain brain networks that modulate emotional processing (Townsend and Altshuler 2012), such as amygdala, prefrontal cortex, cingulate, thalamus, and striatum (Lane et al. 1997; Chen et al. 2011). In this regard, there is evidence that such anomalies in BD might be associated with dysfunctions in synaptic efficiency; cellular plasticity, including neuronal and glial cells; resilience; and adaptation to stress factors (Schloesser et al. 2009). Many hypotheses have been proposed to explain the pathophysiology of BD, such as monoamines, gamma aminobutyric acid (GABA), glutamate, and second-messenger signaling pathways. In this perspective, a possible unifying mechanism can be the oxidative stress (Andreazza et al. 2007), an altered cellular "redox" state resulting from an imbalance in prooxidant (such as free radicals or Reactive Oxygen Species, ROS) and antioxidant factors. Though playing a key role in modulating cellular signaling, immunological response, and mitosis, free oxygen radicals are highly reactive and can potentially

damage all cellular components such as proteins, lipids, carbohydrates, and nucleic acids (Filomeni and Ciriolo 2006), ultimately leading to cellular toxicity and death. Free radicals are physiologically produced by cells during oxidative phosphorylation within mitochondria through the enzymatic reactions of the mitochondrial respiratory chain (Mattson et al. 2008) and are usually scavenged by cellular defense and replacement mechanisms. Though mitochondria are a main source of them, free radicals are also produced during cellular reactions that involve other enzymes, such as NADPH oxidases (Muller 2000), which take part in host defense, post-translational processing of proteins, cellular signaling, regulation of gene expression, and cell differentiation (Bedard and Karl-Heinz 2007).

A controlled and limited oxidative damage is part of the normal cellular life and underlies aging processes, but an imbalance in oxidative homeostasis might result in cellular damage such as mitotic arrest, apoptosis, or necrosis (Davies 2000). Oxidative stress in BD might be the result of several interconnected causes, such as an overregulation of glutamate neurotransmission, elevated extracellular levels of glutamate through the so-called glutamate-induced excitotoxicity (Gigante et al. 2012), mitochondrial DNA mutations (Kato 2000), reduced antioxidant defenses (Andreazza et al. 2008), or exogenous factors such as alcohol intake (Kato 2000). Glutamate, one of the major neurotransmitters, is involved in energetic metabolism and it is the precursor of glutamine, gamma aminobutyric acid (GABA), and glutathione (Bak et al. 2006). Excessive glutamate concentration can cause oxidative stress determining an increased intracellular calcium concentration, which in turn activates Ca^{2+}-dependent enzymes such as nitric oxide synthase, thus enhancing the production of free oxygen radicals (Frey et al. 2007). Whenever not efficiently removed, an over-quota of free radicals can primarily damage mitochondrial structures as mitochondrial DNA, possibly inducing mutation in key genes involved in oxidative phosphorylation (Kato 2000; Clay et al. 2011), and mitochondrial membranes (Wallace 2005; Andreazza et al. 2010) directly interfering with electron transport chain (ETC) and mitochondrial adenosine triphosphate (ATP) synthesis (Steckert et al. 2010). Thus, in this perspective, ROS generation is strictly and reciprocally associated to mitochondrial functioning, representing, at the same time, a source and a consequence of mitochondrial dysfunction.

Mitochondria are intracellular organelles, which are involved in synthesis of energetic compounds such as ATP, creatine, and phosphocreatine, during oxidative phosphorylation. They are also involved in other crucial processes such as apoptosis and calcium homeostasis (Horn and Barrientos 2008) and are the main sources of free oxygen radicals through the respiratory chain (Mattson et al. 2008). Over the past few decades, several studies have suggested the hypothesis that mitochondrial dysfunction can be present in BD patients, representing a significant pathophysiological feature of this disorder. This is supported by the evidence of changes in mitochondrial morphology (Cataldo et al. 2010) and brain metabolism in BD (Stork and Renshaw 2005; Manji et al. 2012) and by comorbidity between affective symptoms and mitochondrial disorders (Grover 2006). In particular MRS studies strongly contribute to provide insight on brain metabolic abnormalities in BD. This is a noninvasive technique that allows quantification of neurotransmitters or intracellular

compounds through the detection of magnetic resonance signals produced by atomic nuclei located within molecules in living tissue, particularly including hydrogen (^{1}H) or phosphorus (^{31}P) (Dager et al. 2008). Choline, phosphocholine and phosphomonoester, N-acetyl-aspartate, creatine, pH, and lactate have been extensively investigated in BD patients as they all represent valid markers of cellular metabolic and energetic status, reflecting a possible underlying mitochondrial dysfunction (Stork and Renshaw 2005).

The focus of this chapter is to summarize recent MRS findings on oxidative stress involvement in BD and to comment on their research and therapeutic implications. First, we collected MRS studies on glutamatergic neurotransmission, since glutamate-induced excitotoxicity has been considered a primary source of oxidative stress in BD (Andreazza et al. 2008; Nicholls 2009; Gigante et al. 2012; Zádori et al. 2012; Mehta et al. 2012). Second, since oxidative stress and excitotoxicity have been hypothesized to be associated with mitochondrial dysfunction and cellular metabolic failure (Nicholls 2009; Clay et al. 2011; Mehta et al. 2012), we summarized MRS studies that explored metabolic/energetic cellular markers such as N-acetyl-aspartate, choline, intracellular pH, lactate, and phosphomonoester (Kato 2000; Stork and Renshaw 2005; Jou et al. 2009; Minuzzi et al. 2011).

2 Glutamatergic Neurotoxicity

Glutamate is critically involved in human brain functioning, being the most abundant excitatory neurotransmitter and, as an amino acid, being involved in neuronal metabolism (Yüksel and Öngür 2010) (Table 1). It plays a major role in determining neural activity through excitatory postsynaptic currents (ESPCs) and it participates to processes of brain maturation and neuroplasticity (Gigante et al. 2012). Nonetheless, whenever is overregulated, glutamatergic stimulation can even provoke cellular toxicity and neuronal death through a complex process named glutamate-related excitotoxicity (Mehta et al. 2012).

As recently reported in Yuksel and Ongur's review (2010), glutamate (Glu) and glutamate-related compounds are usually evaluated in two different ways, (1) as a composite spectral peak (named Glx) that includes glutamate, glutamine (Gln), and a small amount of other metabolites such as GABA or (2) separately as single peaks (termed Glu, Gln, GABA). Most of the studies revealed a significant increase in Glx or Glu among BD patients regardless the medication and clinical states (depressed, euthymic, manic, and mixed mood) in several brain areas, such as in frontal and temporal lobes (Castillo et al. 2000), basal ganglia (Dager et al. 2004), cingulate gyrus—especially the anterior portion— (Dager et al. 2004; Frye et al. 2007b; Ongür et al. 2008; Soeiro-de-Souza et al. 2013), occipital cortex (Dager et al. 2004; Bhagwagar et al. 2007; Senaratne et al. 2009), dorsolateral prefrontal cortex (DLPFC) (Michael et al 2003b, 2009; Nery et al. 2010), and parietal-occipital cortex (Ongür et al. 2008). Similarly, Glu peak elevation has been reported in hippocampus and DLPFC (Colla et al. 2009; Lan et al. 2009). Although it's not definitely

Table 1 ^{1}H-MRS studies on cerebral glutamate and glutamate-related compounds in bipolar disorder

Study	Study subjects/ clinical state	N patients (males)/N controls (males)	Mean age of patients/mean age of controls	Medication status	Region(s) of interest (ROI)	Glu studied	Summary of results
Castillo et al. (2000)	BD/ not declared	10(9)/10(8)	8/not declared	Mf	Frontal lobes Temporal lobes	Glx/Cr	↑Glx in frontal and temporal lobes of BD versus HC
Davanzo et al. (2001)	BD I 9, II 2/manic 9, hypomanic 2	11/11	11.4/not declared	M	ACC	Glx/Cr	↔Glx in BD versus HC
Michael et al. (2003a)	BD I 7, II 7 NOS 1/ depressed	15(6)/15(5)	54.1/52.2	Mf	Left amygdala	Glx	↔Glx in BD versus HC
Michael et al. (2003b)	BD/manic	8(6)/8(6)	40.1/40.7	Mf	L-DLPFC	Glx	↑Glx in BD versus HC
Dager et al. (2004)	BD I 11, II 17/ depressed and mixed mood	32 (14)/26 (12)	30.3/31.9	Mf	Whole brain	Glx	↑Glx in BD versus HC
					CG		
					OCC		
					Insula		
					Basal ganglia		
					Thalamus		
DelBello et al. (2006)	BD I/manic or mixed	19(6)/10(6)	14.42/15	M	ACC	Glx	↔Glx in BD versus HC
Bhagwagar et al. (2007)	BD I/euthymic	16(6)/18(9)	37.0/37.6	Mf	OCC	Glx	↑Glx in BD versus HC
Frey et al. (2007)	BD type I (20) and type II (12), manic, depressed, hypomanic, mixed mood	32 (11): 7 manic, 17 depressed, 7 hypomanic, 1 mixed /32 HC (10)	33.8/33.8	M	L-DLPFC	Glu	↔Glu in BD versus HC

(continued)

Table 1 (continued)

Study	Study subjects/ clinical state	N patients (males)/N controls (males)	Mean age of patients/mean age of controls	Medication status	Region(s) of interest (ROI)	Glu studied	Summary of results
Frye et al. (2007a)	BD/manic	16(10)/17(11)	37.5/32.9	M	ACC	Glx, Glx/Cr	↔Glx in BD versus HC
					Basal ganglia		
					OCC		
Frye et al. (2007b)	BD/depressed	23 (17) /12 (7)	35.6/32.8	Mf	ACC/medial prefrontal cortex	Glx, Glx/Cr, Glu, Glu/Cr	↑Glx and ↑Glu in BD versus HC
Ongür et al. (2008)	BD I/manic	15(7)/21(11)	36.3/34.3	M	ACC	Gln/Glu	↑Gln/Glu in BD versus HC
					POC		
Port et al. (2008)	BD I 8, BD II 9, NOS 4/euthymic 6, manic 10, depressed 6	21(8)/21(8)	30.8/31.1	Mf	Caudate and lentiform nuclei thalamus	Glx	↓Glx in frontal WM, lentiform nucleus in BD versus HC
					ACC		
					OCC		
					Frontal and parietal WM		
Colla et al. (2009)	BD I/euthymic	21 (10)/19 (9)	54.2/54.6	M	Hippocampus (bilaterally)	Glu	↑Glu in BD versus HC
Lan et al. (2009)	BD postmortem sample type I 8, type II 1, NOS 1	10(7)/10 (7) normal postmortem controls	39.1/45.6	5 M, 5 Mf	DLPFC	Glu	↑Glu in BD versus controls
Kaufman et al. (2009)	BD/euthymic 10, depressive 2, manic 1	13(8)/11 (7)	40.5/41.2	M	Basal ganglia	Glx	↔Glx in BD versus HC
					Whole brain		

Michael et al. (2009)	BD II/ rapid cycling RC (6) in different mood states, BD non-RC (6) depressed	12 (2)/6 (1)	53.5/51.5	M	DLPFC	Glx	↑Glx in BD RC versus BD non-RC versus HC, ↔Glx in BD non-RC versus HC
Senaratne et al. (2009)	BD I and II/euthymic	12 (3)/12(3)	42.1/37.9	M	OFC hippocampus OCC	Glx	↑Glx only in OCC in BD versus HC
Nery et al. (2010)	BD I and II/euthymic 10, depressive 10, manic 6	26 (5) / 54 (14)	39.8/38.7	M	DLPFC	Glx, Glu	↑Glx in BD versus HC
Singh et al. (2010)	BD I 18, II 2/not declared	20(13)/20(15)	15.89/15.1	M	ACC	Glx, Glx/Cr, Glu, Glu/Cr	↓Glx in BD versus HC
Soeiro-de-Souza et al. (2013)	BD I/euthymic	40 (14)/40(21)	28.9/26.6	M	ACC	Glu/Cr, Glx/Cr	↑Glu/Cr ↑Glx/Cr in BD versus HC

ACC anterior cingulated cortex, *BD* bipolar disorder, *CG* cingulated gyrus, *Cr* creatine, *DLPFC* dorsolateral prefrontal cortex, *Gln* glutamine, *Glu* glutamate, *HC* healthy controls, *L* left, *M* medicated, *Mf* medication free, *NOS* not otherwise specified, *OCC* occipital cortex, *OFC* orbitofrontal cortex, *POC* parieto-occipital cortex, *RC* rapid-cycling, ↑ increase, ↓ decrease, ↔ no statistically significant differences

established what proportion of Glx or Glu signal corresponds to the synaptic glutamate pool, it's highly conceivable that Glx or Glu peak increase could reflect an increased extracellular/synaptic concentration of glutamate (Gigante et al. 2012) suggesting a glutamatergic dysregulation in BD patients.

Therefore, abnormal glutamatergic neurotransmission might play a major role in BD pathophysiology, representing a significant feature of the neurobiological underpinnings of this disorder.

Different clinical states are associated with a partially different anatomical localization of Glx or Glu increase. Studies exclusively focused on manic patients revealed a Glx increase in left DLPFC (Michael et al. 2003b) as well as a glutamine increase (with normal glutamate) in anterior cingulated cortex (ACC) and parieto-occipital cortex (Ongür et al. 2008). Depressed BD patients showed an increase in ACC/medial prefrontal cortex (Dager et al. 2004; Frye et al. 2007a) as well as those in euthymic phase (Soeiro-de-Souza et al. 2013) who additionally showed a Glx increase in occipital cortex (Bhagwagar et al. 2007; Senaratne et al. 2009) and hippocampus (Colla et al. 2009). Taken together these studies suggest that an increased Glx is present across all mood states of BD and probably reflect an underlying neurobiological susceptibility or a trait vulnerability to the disease rather than an epiphenomenon (Bhagwagar et al. 2007).

However, it should be noted that there are also conflicting findings. Indeed, two studies found a Glx reduction in BD patients. Port et al. (2008) reported a lower Glx in right and left frontal white matter and right lentiform nucleus, whereas no difference was found in other cerebral regions of interest such as thalamus, ACC, and occipital cortex. Likewise, Singh et al. (2010) demonstrated a Glx reduction in ACC of younger medicated bipolar patents in ACC. Also, some other few studies reported preserved Glx levels in BD patients in ACC, amygdala, basal ganglia, thalamus, DLPFC, and parietal-occipital cortices (Davanzo et al. 2001; Michael et al. 2003a; DelBello et al. 2006; Frye et al. 2007a, b; Kaufman et al. 2009).

In general, ^{1}H-MRS studies may be influenced by potential confounders such as sample size and medication status (Friedman et al. 2004; Shibuya-Tayoshi et al. 2008). Indeed, most of the negative studies included small samples of medicated patients (less than 20 patients) (Davanzo et al 2001; Frey et al. 2007a, b; Kaufman et al 2009; Singh et al. 2010), whereas 8/14 studies that found increased glutamatergic levels had larger populations (>20), also composed by medication-free patients. Finally, since creatine has usually been used as an internal reference, it cannot be excluded that changes in creatine levels might influence glutamate concentration when reported as a ratio to creatine itself (Brambilla et al. 2005; Frey et al. 2007).

To sum up, there is evidence of elevated glutamatergic neurotransmission in BD in DLPFC, temporal lobes, and cingulate gyrus. This supports the hypothesis that overactivity in glutamatergic system can be a plausible feature of this disorder (Bhagawar et al. 2007; Gigante et al. 2012). In this context, a possible underlying mechanism of glutamate-related reactive oxygen species production and mitochondrial dysfunction has been found in glutamate-induced excitotoxicity (Vincent and

Mulle 2009; Lau and Tymianski 2010; Mehta et al. 2012). Excitotoxicity is a cellular process that involves (i) glutamate-signaling through glutamate receptors NMDA (N-methyl-D-aspartate), (ii) increased intracellular calcium concentration (Ca^{2+}), (iii) generation of ROS and peroxynitrite, and (iv) disruption of mitochondrial bioenergetic reactions (as ATP synthesis), ultimately leading to cell death (Lipton 2008; Dong et al. 2009; Heneka et al. 2009). A supraphysiological activation of NMDA provokes a massive Ca^{2+} ion influx from extracellular space, resulting in a cytosolic overload of calcium and dysregulation of homeostatic control of intracellular Ca^{2+} (Chen et al. 2000). Elevated intracellular Ca^{2+} exerts its toxic effect through impairment of mitochondrial functioning and hyper-activation of Ca^{2+}-dependent enzymes (Mehta et al. 2012). Ca^{2+} overload has indeed deleterious effects on mitochondrial membrane function (Orrenius et al. 2003), increases mitochondrial membrane permeabilization (Clay et al. 2011), depolarizes mitochondrial membrane (Lemus-Molina et al. 2009), and activates ROS and nitric oxide (NO) generation (Kumar et al. 2011; Zádori et al. 2012). This, in turn, leads to further mitochondrial injury (and consequent ATP production impairment) and ultimately contributes to cell apoptotic death (Orrenius et al. 2003). Furthermore, since mitochondria are greatly involved in Ca^{2+} handling and homeostasis (Chen et al. 2000; Parihar and Brewer 2007), a calcium-induced mitochondrial dysfunction can even result in an increased cytoplasmatic concentration of Ca^{2+} itself (Castilho et al. 1999). On the other hand, activity of Ca^{2+}-dependent phospholipase A_2 (Miller et al. 2010) as well as of nitric oxide synthase activity (NOS) (Andreazza et al. 2008; Gautier et al. 2012) can be enhanced by cellular Ca^{2+} overload, resulting in ROS and NO production and contributing to cellular toxicity and death. In conclusion high levels of synaptic glutamate are mainly responsible for excitotoxicity, leading to ROS generation (Parihar and Brewer 2007), mitochondrial dysfunction, ATP synthesis decrease, and, ultimately, cell death (Wang et al. 2009; Mehta et al. 2012).

Conversely, the altered extracellular concentration of glutamate found in BD patients could occur as a consequence of cellular metabolic failure rather than being an expression of an over-regulated glutamatergic neurotransmission (Gigante et al 2012). In this scenario, mitochondrial dysfunction and cellular metabolic failure would play a primary role through impaired ATP production (Stork and Renshaw 2005). Indeed, the block of mitochondrial respiratory chain using cyanide increases extracellular glutamate, suggesting that normal oxidative phosphorylation and ATP synthesis are crucial both for release and reuptake of glutamate (Clausen et al. 2001). Also, decreased ATP production may lead to a failure of $Na^{+/}K^{+}$ pump resulting in a drop of the Na^{+} transmembrane gradient which in turn maintains glutamate within neuronal cell, preventing an increase in extracellular glutamate level (Nicholls 2009). It should also be kept in mind that some studies have revealed that an excessive amount of glutamate is not strictly necessary to generate excitotoxicity. Since $Na^{+/}K^{+}$ pump failure (due to ATP fall) leads to a more depolarized cellular membrane, NMDA voltage-dependent activation become more probable even at a normal extracellular glutamate levels (Mehta et al. 2012; Nicholls 2008, 2009).

3 Energetic Abnormalities

3.1 NAA (N-Acetyl-Aspartate)

NAA is an "amino-acid derivative" originated in neuronal mitochondria through the NAA-synthase-catalyzed acetylation of aspartate (Frye et al. 2007b; Patel and Clark 1979) and represents the most prominent spectroscopic peak of ^{1}H-MRS spectra (Table 2). It is probably involved in myelin formation (Clark 1998) as well as in mitochondrial energetic reactions (Madhavarao et al. 2003) and it has therefore been traditionally considered as a reliable spectroscopic marker of neural viability and mitochondrial function (Minuzzi et al. 2011; van der Knaap et al. 1992).

^{1}H-MRS studies reported conflicting findings about NAA levels in BD patients. NAA reduction has been detected in different cerebral regions such as hippocampus (Bertolino et al. 2003; Deicken et al 2003), basal ganglia (Frye et al. 2007a; Port et al. 2008), occipital cortex (Bhagwagar et al. 2007), frontal lobe (Cecil et al. 2002), orbitofrontal cortex (Cecil et al. 2002), and DLPFC (Sassi et al. 2005; Winsberg et al. 2000; Sassi et al. 2005), including younger medicated bipolar patients (Chang et al. 2003). On the contrary, other studies reported preserved NAA concentrations in BD in DLPFC (Bertolino et al. 2003; Brambilla et al. 2005; Frey et al. 2005, 2007; Ongür et al. 2008), hippocampus (Colla et al. 2009; Senaratne et al. 2009), basal ganglia (Kato et al. 1996; Ohara et al. 1998; Hamakawa et al. 1998; Bertolino et al. 2003; Malhi et al. 2007), frontal and temporal lobes (Castillo et al. 2000; Cecil et al. 2002; Bertolino et al. 2003; Port et al. 2008), thalamus (Port et al. 2008), insula (Dager et al. 2004), and ACC (Davanzo et al. 2001, 2003; Bertolino et al. 2003; Dager et al. 2004; Frye et al. 2007a; Ongür et al. 2008; Patel et al. 2008; Port et al. 2008; Brady et al. 2012). Finally, when compared to healthy controls, increased NAA levels were also shown in basal ganglia (Sharma et al. 1992), putamen (Dager et al. 2004), and thalamus (Deicken et al. 2001; Forester et al 2008), potentially being sustained by lithium treatment (Moore et al. 2000b; Sassi et al. 2002; Sharma et al. 1992; Silverstone et al. 2003; Brambilla et al. 2004; Yildiz-Yesiloglu and Ankerst 2006; Forester et al. 2008). For instance, Brambilla et al. (2004) found an increase in NAA DLPFC levels only in those patients who underwent a lithium treatment compared to the untreated subgroup. Therefore, lithium treatment should be considered as a possible confounder factor in NAA spectroscopic detection in BD due to its potential neurotrophic effects.

Although findings on NAA are controversial, abnormalities in NAA concentration seem to be present in BD patients across different brain areas, even though these findings could be partially confounded by pharmacological treatments. Since NAA level alterations have been considered an epiphenomenon of mitochondrial dysfunction (Stork and Renshaw 2005), it is arguable that mitochondrial alteration can be present in BD patients. As mentioned above, NAA probably plays multifaceted role in neuronal functioning, myelin formation (Clark 1998), protection from osmotic stress (Baslow 2003), synthesis of N-acetyl-aspartyl-glutamate (NAAG) (Blakely et al. 1998), and mitochondrial ATP production via mini-citric acid cycle

Table 2 ^{1}H-MRS and ^{31}P-MRS studies on cerebral levels of NAA, choline-containing compounds, *myo*-inositol, and ^{31}P-containing compounds in bipolar disorder

Study	Study subjects, clinical state	N patients (males)/N controls (males)	Mean age, patients/mean age controls	Medication status	Region(s) of interest (ROI)	Neurometabolites studied (other than glutamate and related compounds)	Summary of results
Kato et al. (1998)	BD type I (4), type II (3), euthymic state	7(3)/60(27)	44.1/39.6	Mf	Whole brain	PME, Pi, PDE, PCr, β-ATP	↓intracellular pH
							↔PME in BD versus HC
Castillo et al. (2000)	BD/not declared	10 (9)/10 (8)	8/not declared	Mf	Frontal lobes	Cho, NAA, phospholipids	↔NAA and Cho in BD versus HC,
					Temporal lobes		↑PL in frontal lobes of BD versus HC
Winsberg et al. (2000)	BD I 10, II 10/ euthymic	20(9)/20(9)	37.9/33.5	Mf	DLPF	NAA, Cr, Cho	↓NAA/Cr in DLPF bilaterally in BD versus HC
Cecil et al. (2002)	BD I/manic 9, mixed mood 8	17 (6)/21(9)	22/21.7	M	Orbitofrontal (GM + WM)	NAA, Cho	↓NAA in GM of BD versus HC
							↓Cho in GM of BD versus HC
							↔mI in BD versus HC
Bertolino et al. (2003)	BD I/all mood states	17(10)/17(10)	40.1/37.6	M	Hippocampus	NAA/Cr, NAA/Cho, Cho/Cr	↓NAA/CRE only in hippocampus of BD versus HC
					Prefrontal regions		
Chang et al. (2003)	BD/most euthymic	15(13)/11(6)	12.6/12.6	M	DLPFC	NAA/Cr	↓NAA/Cr only in the right DLPFC of BD versus HC
						Cho mI	↔mI and Cho in BD versus HC

(continued)

Table 2 (continued)

Study	Study subjects, clinical state	N patients (males)/N controls (males)	Mean age, patients/mean age controls	Medication status	Region(s) of interest (ROI)	Neurometabolites studied (other than glutamate and related compounds)	Summary of results
Davanzo et al. (2003)	BD I/manic and mixed mood	10(8) /13(8)	9.8/11.7	M	ACC	mI, NAA, Cho, Cr	↑mI/Cr and ↑mI in ACC of BD versus HC
					OCC		↑Cho in ACC of HC versus BD
Deicken et al. (2003)	BD I/euthymic	15(15)/20(20)	39.3/36.0	M	Hippocampus (bilaterally)	NAA, Cho, Cr	↓NAA and ↓Cr ↔Cho in BD versus HC
Michael et al. (2003b)	BD/manic	8(6)/8(6)	40.1/40.7	Mf	L-DLPFC	NAA, Cho, Cr	↔NAA
							↔Cho
							↔Cr in BD versus HC
Dager et al. (2004)	BD I 11, II 17/ depressed, mixed mood	32 (14)/26 (12)	30.3/31.9	Mf	Frontal	Lactate, Cre, Cho, NAA, myo-inositol	↑lactate in BD versus HC
					WM		
					ACC		
					Insula		↑NAA in left putamen of BD versus HC
					Caudate		
					Putamen		
					Thalamus		
					Parietal WM		
Brambilla et al. (2005)	BD I 8, II 2/ euthymic 9, depressed 1	10(2)/10(2)	36.6/35.5	M	L-DLPFC	NAA, Cho, CRE	↔NAA
							↔Cho
							↔Cr in BD versus HC
							↑NAA/CRE in Li treated subgroup of BD versus non-treated BD

Sassi et al. (2005)	BD I 10, II 3, NOS 1/euthymic 13, depressed 1	14(6)/18(11)	15.5/17.3	M	L-DLPFC	NAA, Cho, Cr	↓NAA in BD versus HC
							↔Cho
							↔Cr
Bhagwagar et al. (2007)	BD I/euthymic	16(6)/18(9)	37.0/37.6	Mf	OCC	NAA, Cr	↓NAA/Cr in BD versus HC
Frey et al. (2007)	BD type I (20) and type II (12), manic, depressed, hypomanic, mixed mood	32 (11): 7 manic, 17 depressed, 7 hypomanic, 1 mixed/32 HC (10)	33.8/33.8	M	L-DLPFC	NAA, Cho, mI, Cr	↓Cr
							↓Cho
							↔myo-inositol and NAA in BD versus HC
Frye et al. (2007a)	BD/manic	16(10)/17(11)	37.5/32.9	M	ACC	NAA, Cr	↓NAA and NAA/Cr in basal ganglia of BD versus HC
					R-basal ganglia		
					L-OP (WM)		
Patel et al. (2008)	BD/depressed	28(5)/10(4)	15.5/14.6	Mf	ACC	NAA, Cr, Cho, mI	↑NAA
					VLPFC		↑Cho and ↑Cr in L-VLPFC
							↑mI and Cr in R-VLPFC in BD versus HC
Port et al. (2008)	BD I 8, BD II 9, NOS 4/euthymic 6, manic 10, depressed 6	21(8)/21(8)	30.8/31.1	Mf	Caudate and lentiform nuclei Thalamus	NAA, Cr, Cho, mI	↓NAA in caudate nuclei and left lentiform nucleus
					ACC		
					OCC		↓Cho and ↓Cr in right caudate
					Frontal and parietal WM		↑mI in left caudate of BD versus HC

(continued)

Table 2 (continued)

Study	Study subjects, clinical state	N patients (males)/N controls (males)	Mean age, patients/mean age controls	Medication status	Region(s) of interest (ROI)	Neurometabolites studied (other than glutamate and related compounds)	Summary of results
Colla et al. (2009)	BD I/euthymic	21 (10)/19 (9)	54.2/54.6	M	Hippocampus (bilaterally)	NAA, Cho, Cr	↔NAA, Cho, Cr in BD versus HC
Michael et al. (2009)	BD II/rapid cycling RC (6) in different mood states, BD non-RC (6) depressed	12 (2)/6 (1)	53.5/51.5	M	DLPFC	NAA, Cho, Cr	↑NAA, Cho, Cr In BD versus HC
Senaratne et al. (2009)	BD I and II/ euthymic	12 (3)/12(3)	42.1/37.9	M	OFC Hippocampus OCC	NAA, Cho	↑Cho in BD in Hippocampus and OFC of BD versus HC ↔NAA
Brady et al. (2012)	BD I/manic 7, euthymic 7	14 (6)/6 (3)	38.6/35.2	M	ACC POC	Lactate/Cr, NAA/Cr	↓Lactate/Cr in euthymic versus manic and HC

ACC anterior cingulated cortex, *BD* bipolar disorder, *CG* cingulated gyrus, *Cho* choline, *Cr* creatine, *DLPFC* dorsolateral prefrontal cortex, *GM* gray matter, *HC* healthy controls, *L* left, *M* medicated, *Mf* medication-free, *mI* myo-inositol, *NAA* N-acetyl-aspartate, *NOS* not otherwise specified, *OCC* occipital cortex, *OFC* orbitofrontal cortex, *OP* occipital-parietal cortex, *PCr* phosphocreatine, *POC* parieto-occipital cortex, *PME* phosphomonoester, *PL* phospholipids, *VLPFC* ventrolateral prefrontal cortex, *WM* white matter, ↑ increase, ↓ decrease, ↔ no statistically significant differences

(Madhavarao et al. 2003). Furthermore, inhibition of mitochondrial oxidative phosphorylation results in decreased NAA concentration (Bates and Van Woerkom 1996).

3.2 Choline and Choline-Containing Compounds

Spectroscopic choline (Cho) peak represents intracellular choline and choline-containing compounds phosphocholine (PCh) and glycerophosphocholine (GPC). Since choline and choline-related compounds play a crucial role in synthesis and maintenance of cell membranes, Cho resonance signal has been considered as a valid marker of cellular membrane status (Moore and Galloway 2002).

Elevated Cho signal has been reported in several studies on BD patients across a variety of cerebral structures of interest, such as basal ganglia (Kato et al 1996; Hamakawa et al. 1998; Moore et al. 2000a; Yildiz-Yesiloglu and Ankerst 2006), ACC (Davanzo et al. 2003), left ventrolateral prefrontal cortex (Patel et al. 2008), DLPFC (Michael et al. 2009), hippocampus (Senaratne et al. 2009), and orbitofrontal cortex (Senaratne et al. 2009). Elevated Cho signal could reflect (1) an accelerated phospholipid metabolism related to a mitochondrial dysfunction and/or (2) a membrane breakdown accompanied by a release of membrane choline compounds (Farber et al. 2000; Stork and Renshaw 2005; Senaratne et al. 2009). Reduction in Cho levels has also been found in DLPFC (Frey et al 2007), in right caudate (Port et al. 2008), and in orbitofrontal gray matter (Cecil et al. 2002), potentially representing insufficient energetic mitochondrial contribution. However, some other MRS studies did not report any differences in Cho signal in frontal and temporal lobes (Castillo et al. 2000), hippocampus (Bertolino et al. 2003; Deicken et al. 2003; Colla et al. 2009), and DLPFC (Winsberg et al 2000; Chang et al. 2003; Michael et al. 2003b; Brambilla et al. 2005; Sassi et al. 2005) of BD patients in respect to healthy controls.

Although results on Cho are not consistent and lithium treatment might influence Cho levels (Sharma et al. 1992), alterations of Cho concentration in BD patients may represent impairment in phospholipid metabolism indicating an underlying energetic deficit subsequent to mitochondrial dysfunction (Stork and Renshaw 2005).

3.3 Intracellular pH and Lactate

Intracellular pH and lactate are indicators of cellular energetic status and mitochondrial oxidative phosphorylation (Stork and Renshaw 2005). Respectively, intracellular pH could be estimated indirectly from ^{31}P-MRS measurements, whereas lactate levels could be evaluated with ^{1}H-MRS technique.

^{31}P-MRS studies (Kato et al. 1998; Hamakawa and Murashita 2004) found reduced intracellular pH in euthymic BD subjects when compared to normal controls, but not in manic or depressed patients. Lower intracellular pH has also been linked to increase in lactate levels (Kato et al. 1998; Stork and Renshaw 2005). In the study of Dager et al. (2004), an elevated lactate level has been found in gray matter of medication free BD patients, suggesting that an energetic shift from oxidative phosphorylation to glycolysis can be present. Taken together these preliminary findings provides evidence that pH reduction and elevated lactate represent markers of a metabolic/energetic shift from oxidative phosphorylation to glycolysis, possibly related to a cellular energetic impairment as proposed by Dager et al. (2004) and Stork and Renshaw (2005).

3.4 Creatine

In ^{1}H-MRS, creatine resonance signal (Cr) derives from the contribution of creatine itself and phosphocreatine (PCr), whereas in ^{31}P-MRS phosphocreatine can be detected separately as a single peak. Since creatine and phosphocreatine play a significant role in rapid regeneration of ATP consumption by cell, their resonance signals may represent an indicator of cellular energetic status. PCr is synthesized by the creatine kinase (CK) enzyme within mitochondria from Cr and ATP and is then transported to the cytosol where it operates as a phosphate donor to ADP to rapidly restore ATP (Aubert and Costalat 2002; Minuzzi et al. 2011). Creatine and phosphocreatine are therefore indicators of cellular ATP consumption and, indirectly, of brain energy metabolism.

It has been reported that individuals with BD show decreased PCr or Cr signals in frontal lobes (Kato et al. 1994, 1995), particularly in left DLPFC (Frey et al. 2007), hippocampus, and caudate (Deicken et al. 2003; Port et al 2008), although not in all studies (Hamakawa et al. 1999; Cecil et al. 2002; Michael et al. 2003b; Brambilla et al. 2005; Sassi et al. 2005; Colla et al. 2009). Also, two studies reported an increased Cr signal in BD patients (Michael et al. 2009; Patel et al. 2008). Reduced Cr levels could represent both an increase in energy requirement (such as during a period of acute neuronal activity) or, if prolonged, a larger abnormality in cellular metabolism possibly due to mitochondrial dysfunction and impaired ATP production (Stork and Renshaw 2005). On the other hand, increased Cr peak could equally reflect abnormal metabolism, confirming that Cr is not fully appropriate as an internal standard for other ^{1}H-MRS metabolites in BD.

3.5 Phosphomonoester (PME)

PME resonance signal is formed by the contribution of phosphocholine, phosphoethanolamine, phosphoserine, and inositol-1-monophosphate and represents a reliable marker of cellular membrane status (Yildiz et al. 2001). A decreased PME level

in frontal and temporal lobes has been reported in euthymic BD patients when compared both to manic and depressed as well as to normal controls (Deicken et al 1995; Kato et al. 1998; Yildiz et al. 2001; Stork and Renshaw 2005). As the same studies reported higher PME signals in manic and depressed state than in healthy controls, it has been suggested that PME alteration could be state-dependent. Similarly to Cho signal, a probable biological significance of PME peak alterations in BD could be found in alteration of normal membrane metabolism due to mitochondrial dysfunction (Stork and Renshaw 2005).

Taken together, these findings suggest that energetic impairment plays a major role in the pathophysiology of BD. In this regard, the hypothesis of a "mitochondrial dysfunction" has been proposed (Kato 2000; Stork and Renshaw 2005; Jou et al. 2009; Steckert et al. 2010; Clay et al. 2011; Minuzzi et al. 2011; Manji et al. 2012). It is generically defined as an impaired mitochondrial functioning that involves impaired oxidative phosphorylation and ATP synthesis, decreased substrate availability, and altered membrane metabolism. Interestingly, alterations in number and morphology of mitochondria (Uranova et al. 2001; Cataldo et al. 2010) and mutations of nuclear DNA genes involved in mitochondrial respiratory chain have been shown in BD (Xu et al. 2008; Andreazza et al. 2010). Moreover, indirect evidence is offered by the high comorbidity between mitochondrial disease (due to specific mitochondrial DNA mutation) and psychotic/affective symptoms (Grover 2006; Mancuso et al. 2008) (Fig. 1).

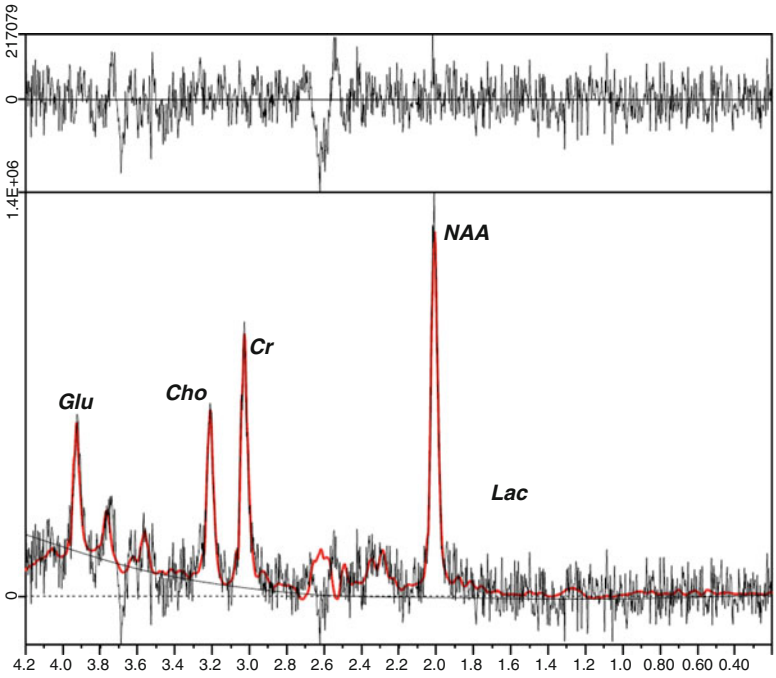

Fig 1 NMR spectrum of most neuro-metabolites presented above

4 Perspectives

The main purpose of this chapter was to investigate spectroscopic evidence of oxidative stress in BD. Changes in glutamatergic and energetic levels have been observed by 1H-MRS studies in BD. They are considered to correlate, respectively, with oxidative stress and mitochondrial abnormalities (Clay et al. 2011). The term "oxidative stress" defines a biochemical scenario of altered redox state resulting from an imbalance in pro-oxidant/antioxidant agents (Sies 1991). Increased pro-oxidants (especially an excess of ROS- and NO-derived reactive compounds, such as peroxynitrite $ONOO^-$) as well as decreased antioxidant cellular defenses (superoxide dismutase SOD, catalase CAT, glutathione peroxidase, GPx, glutathione, GSH) could both lead to oxidative stress (Andreazza et al. 2008). ROS are oxygen-containing compounds mainly derived from mitochondrial respiratory processes, in particular from ETC (Moro et al. 2005). To a certain extent, ROS generation is mostly a physiological consequence of normal oxidative phosphorylation processes (1–5 % of all oxygen burst generates free radical) (Lee and Wei 2005). ROS rapidly and greatly interact with all cellular major macromolecules exerting irreversible and structural damages to membrane lipids (through peroxidation), proteins, carbohydrates, and nucleic acids (Clay et al. 2011; Mehta et al. 2012). Whenever not properly buffered by antioxidant cellular defense, an excess of ROS can provoke a threatening toxic effect potentially undermining cellular survival. As they are mainly produced in mitochondria, ROS firstly and largely affect mitochondria structure and DNA (Wallace 2005; Andreazza et al. 2010), leading to mitochondrial membrane permeabilization (Swamy et al. 2009; Molochnikov et al. 2012) and ultimately resulting in release of mitochondrial pro-apoptotic proteins such as procaspases (Clay et al. 2011).

Though consistently suggesting that oxidative stress and mitochondrial dysfunction play an important role in the pathophysiology of bipolar disorder, some limitation among the studies presented in this review have to be pointed out: (1) the recruitment of relative small and treated samples of patients, often expressing diverse clinical states; (2) the MRS protocols; and (3) the use of Glx/Cr or NAA/Cr ratio instead of absolute values (Frey et al. 2007; Patel et al. 2008). In this perspective it is interesting to note that lithium treatment may reduce brain Glx (Friedman et al. 2004) and, along with lamotrigine, may increase NAA levels in BD patients (Brambilla et al. 2005; Frye et al. 2007b). This suggests that mood stabilizers can normalize glutamatergic neurotransmission and NAA levels (Manji et al. 2000; Lai et al. 2006). In this regard, recent studies have demonstrated that lithium treatment exerts a neuroprotective effect by contrasting the NMDA glutamate-induced activation (Manji et al. 2012). Similarly, studies on rat neuronal cells reported that valproate has a protective effect against glutamate-induced excitotoxicity and oxidative stress (Lai et al. 2006). Likewise, in a double-blind placebo-controlled study, it has been reported that the administration of N-acetylcysteine, an antioxidant amino-acidic compound, ameliorates global functioning and depressive symptoms of BD patients when compared with placebo treatment after a period of 24 weeks (Berk et al. 2008). Further, interesting evidence of possible therapeutic scenarios results

from studies on herbal compounds. A study on rat cerebral cortex cultured neurons revealed that Mangifera indica L. extract (a mixture of antioxidant compounds, such as polyphenols and xanthone mangiferin) contrasts glutamate-related excitotoxicity by scavenging free radicals, preventing lipid peroxidation and reducing Ca2+ mitochondrial influx (Lemus-Molina et al. 2009). Likewise, it has been demonstrated that isoliquiritigenin (a flavonoid extracted from Glycyrrhiza uralensis) in hippocampal neurons prevents ROS production and mitochondrial depolarization and modulates the expression and release of pro-apoptotic proteins (Yang et al. 2012). Therefore, the effect of therapeutic compounds adopted in treating BD, such as mood stabilizers, could be at least partially ascribed to modulation of glutamatergic neurotransmission, glutamate-induced excitotoxicity, and oxidative stress.

At present, less evidence on specific mitochondrially targeted drugs has been accumulated. Some animal studies revealed that specific compounds could exert a mitochondrial-protecting effect by targeting specific mitochondrial receptors and modulating calcium flux, mitochondrial permeability transition pore formation, mitochondrial-induced apoptosis, electron transport, and ROS generation and stimulating cellular antioxidant pathways (see Manji et al. 2012 for a comprehensive review).

5 Conclusions

Although robust evidence on oxidative stress in BD has been accumulating during the last decade, further research is needed to clarify its causative factors and the interplay with mitochondrial dysfunction (Tosic et al. 2006). Future MRS studies should therefore involve large samples of subjects and focus on investigating glutamatergic and energetic levels in drug-free BD patients before and after the administration of mood stabilizers, such as lithium, valproate, or lamotrigine.

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The Impact of Oxidative Stress on Dopaminergic Neurotransmission

Jessica Deslauriers, Philippe Sarret, and Sylvain Grignon

Abbreviations

AP-1	Activator protein 1
ATP	Adenosine triphosphate
AMPT	α-para-methyl-tyrosine
AP	Antipsychotic
CAT	Catalase
COMT	Catechol-*O*-methyl transferase
D1R	D1 receptor
D2R	D2 receptor
DOPA	Dihydrophenylalanine
DA	Dopamine
DRRF	Dopamine receptor regulating factor
DAT	Dopamine transporter
GSHPx	Glutathione peroxidase
GPCR	G protein-coupled receptor

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HO	Heme oxygenase
H_2O_2	Hydrogen peroxide
HIF	Hypoxia inducible factor
HRE	Hypoxia responsive element
MDA	Malondialdehyde
MAO	Monoamine oxidase
NRSE	Neuron restrictive silencer element
NF-kB	Nuclear factor-kappa B
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
Sp1	Specificity protein 1
SOD	Superoxide dismutase
SN	Substantia nigra
TAS	Total antioxidant status
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area
VMAT2	Vesicular monoamine transporter

1 Introduction

Dopamine (DA) is a major neurotransmitter in the central nervous system, with prominent effects on multiple brain functions such as motor control, reward and cognitive processes, to name a few. Dopaminergic neurotransmission has been linked to bona fide neurodegenerative disorders, such as Parkinson's and Huntington's diseases, in line with its prominent oxidative potential and direct neurotoxic effects in some contexts. It also forms the basis of the influential DA hypothesis of schizophrenia which was initially put forward on pharmacological grounds and has subsequently received empirical support from preclinical models as well as genetic and neuroimaging studies. How redox status modulates DA neurotransmission and impacts pathophysiological processes, notably in the context of schizophrenia, is the topic of ongoing investigations and will be reviewed in the present chapter.

2 Dopamine Transmission: Overview and Relevance for the Pathophysiology of Severe Mental Disorders Pathophysiology

DA neurotransmission in the nervous system is organized around four main pathways. The nigrostriatal pathway originates in the *substantia nigra* (SN) and projects to the motor part of the striatum. The mesolimbic and mesocortical pathways originate from the ventral tegmental area (VTA, neuron group 10) and project to parts of the limbic system, including the diencephalic structures (lateral hypothalamus,

lateral mammillary body), basal forebrain (nucleus accumbens, ventral pallidum, olfactory tubercle, amygdala, bed nucleus of the stria terminalis, lateral septum), allocortex (hippocampal complex) and perirhinal, prefrontal and anterior cingulate cortices. The tuberoinfundibular pathway originates in the hypothalamic periventricular zone (periventricular nucleus and arcuate nucleus) and releases DA to the anterior pituitary through portal vessels, where it negatively controls prolactin synthesis and secretion (Torre and Falorni 2007).

DA is synthesized by the dehydration of the amino acid tyrosine to dihydrophenylalanine (DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH) followed by the decarboxylation of DOPA by the aromatic L-amino acid DOPA decarboxylase. TH activity is inhibited by end product (i.e. catecholamine) binding and its binding to synuclein induces protracted activation. Cytoplasmic DA is then transported into secretory vesicles by the vesicular monoamine transporter (VMAT2) for calcium-dependent exocytosis. Released DA can be taken up by dopamine transporters (DATs) or catabolized by either the extracellular catechol-*O*-methyl transferase (COMT) or the mitochondria bound monoamine oxidases (MAO).

DA acts through two families of G protein-coupled receptors (GPCRs). D1-like receptors (D1 and D5) are postsynaptic receptors that activate adenylate cyclase through protein Gs ($\alpha_{s/olf}$), while the pre- and postsynaptic D2-like family (D2-4) inhibits adenylate cyclase through $\alpha_{i/o}$. Of note, a different transduction pathway, independent of G protein signalling, has also been described and involves an arrestin/PI3kinase/Akt/GSK cascade. In the striatum, DA neurons synapse on GABAergic medium spiny neurons, the most abundant (>90 %) population in this structure. There is a significant segregation of D1 and D2, and co-localization occurs in less than 20 % of cases. Similarly, co-expression of D1 and D2 receptors on pyramidal neurons is a relatively scarce (~25 %) occurrence.

Functionally, DA effects are generally described as exerting a modulatory influence on a series of cortex/striatum/thalamus/cortex loops involved in the control of motor, motivational, affective or cognitive processes. At the cellular level, the effect of DA can be described as increasing the signal-to-noise ratio with a suppression of background and weaker inputs and relative enhancement of larger signals (Nicola et al. 2000). DA effects in the prefrontal cortex are generally considered to be mostly D1-dependent. The relationship of prefrontal functional (e.g. cognitive) efficiency to D1R tone is usually described as an inverted U curve (Vijayraghavan et al. 2007).

Apart from schizophrenia, which will be discussed later, DA neurotransmission is involved in many pathological phenomena. The best described aspects pertain to the loss of nigral dopaminergic neurons in Parkinson's disease and have given rise to a large literature on DA-induced oxidative toxicity acting in conjunction with presynaptic partners such as synuclein, Pink-1 and DJ-1. Another neurodegenerative disorder, Huntington's disease, is associated with a massive death of postsynaptic, GABAergic medium spiny neurons, in which D1 and D2 influences have been described (Deyts et al. 2009; Tang et al. 2007). DA also appears to be involved in the pathophysiology of Tourette's syndrome (McNaught and Mink 2011). Disturbed reward processes certainly play a role in drug abuse (Volkow et al. 2011) and eating disorders (Volkow et al. 2008; Bello and Hajnal 2010).

3 Dopamine as a Modulator of Oxidative Status

3.1 Effects on Dopaminergic Neurons

Under normal conditions, DA formed in the cytoplasm is readily taken up and safely stored in secretory vesicles. In the absence of proper sequestering, however, cytoplasmic DA can undergo auto-oxidation to form reactive oxygen species (ROS) and a quinone derivative, notably in the presence of metal ions. Moreover, DA is a substrate for the outer mitochondrial membrane-bound MAO, which stoichiometrically produces hydrogen peroxide (H_2O_2) as well. The quinone derivative can react and form adducts with a variety of cellular proteins (such as α-synuclein, parkin, multiple mitochondrial proteins notably in complexes I and III, DATs) and DNA (Hastings 2009; Belluzzi et al. 2012), as well as polyunsaturated fatty acids (Liu et al. 2008). A further consequence is the induction of phase 4 mitochondrial respiration, with uncoupling from adenosine triphosphate (ATP) production and subsequent increase in ROS production. These considerations on intracellular dopaminergic neuron toxicity apply mostly to Parkinson's disease models and involve the specific disruptive effect of α-synuclein on DA vesicular transport. Similar phenomena, however, have been described as regards the fate of extracellular DA including spontaneous and enzymatic oxidation. Indeed, amperometric detection of H_2O_2 after electrically stimulated DA release shows a biphasic response, with an initial, fast component ascribed to auto-oxidation and a slower phase decreased by MAO inhibition. Of note, both components were almost completely abolished when DA synthesis was inhibited by α-methyl-para-tyrosine (AMPT) (Kulagina and Michael 2003).

3.2 Striatal Redox Status

Possibly due to its high dopamine content, the striatum appears to exhibit intrinsically high levels of oxidative stress markers, when compared to other brain regions. For instance, striatal malondialdehyde (MDA) levels were reported to be >2.50 nmol/mg protein in adult rats, in contrast with contents in the 0.30–0.70 nmol/mg range in other brain regions and plasma (Husain and Somani 1998). Similar results were reported in young adults and aged rats where striatal MDA levels were consistently superior by ~50 % in the striatum compared to other regions. Interestingly, superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT) levels as well as total antioxidant potential, were similar to other regions, suggesting that the striatum does not mount superior antioxidant responses, despite higher lipid peroxidation (and presumably overall excessive oxidative status) (Siqueira et al. 2005).

Formal demonstration that DA dynamics do indeed elicit an increase in striatal *extracellular* oxidation has been obtained through the use of tyrosine conjugated to linoleic acid (L-Tyr), a redox-sensitive compound, which does not readily cross

membranes and is therefore selectively sensitive to extracellular oxidative phenomena. High-dose amphetamine induced an approximate sevenfold increase in extracellular DA concentrations (an increase, it must be said, that substantially exceeds the levels reached in schizophrenia or after autoreceptor D2 blockade by antipsychotic (AP) drugs), which translated into a strong (~threefold) increase in L-Tyr oxidation and a *decrease* of the intracellular oxidative marker 7-keto cholesterol, thought to arise from depleted DA contents (Aluf et al. 2011).

4 Oxidative/Nitrative Stress (OS) and TH Activity

The transcriptional control of TH is a complex process, with significant species differences and cell-type specificities as well. The specific regulation of TH development is under the general control of Nurr1 and Pitx3, which coordinate the ontogenesis of the dopaminergic system (Kadkhodaei et al. 2009), and under the negative control of neuron restrictive silencer element (NRSE). Basal and activity-dependent control of transcription is under the dependence of CRE/CaRE, activator protein 1 (AP-1) and specificity protein 1 (Sp1). Egr1 binding is involved in stress sensitivity, while induction by hypoxia engages AP-1/Sp1 binding, as well as hypoxia inducible factor (HIF) binding to hypoxia responsive element (HRE). Transcriptional control by glucocorticoids and estradiol has also been described (Lenartowski and Goc 2011).

In the presence of peroxynitrite, TH has been shown to undergo tyrosine residues nitration, whose mutation did not prevent inactivation (thereby pointing to a potential modulating, rather than toxic, role). Peroxynitrite was also found to induce oxidation of cysteines (Ara et al. 1998) and formation of glutathione adducts, which were mostly responsible for oxidative/nitrative inactivation (reviewed by Daubner et al 2011).

The transcriptional and post-transcriptional control of TH activity is often studied in the perspective of Parkinson's disease and hypoxia, but has recently shown its relevance in the modelization of schizophrenia as well. Heme oxygenases (HO) catalyze the degradation of heme to carbon monoxide, iron and biliverdin which is subsequently transformed to bilirubin, itself an antioxidant molecule. Two forms are known, the constitutive HO2 and the inducible HO1. HO1 overexpression induces oxidative stress. In a recent work (Song et al. 2012), a transgenic model targeting HO1 to astrocytes (GFAP-HMOX1) has been shown to exhibit a range of behavioural abnormalities relevant to schizophrenia, notably increased locomotor activity and attenuated prepulse inhibition of the acoustic startle, a measure of sensory gating which has been shown to be disturbed in schizophrenia patients. Anatomically and histologically, the mice displayed trophic alterations of the hippocampal region, in the absence of overt neuroinflammation. Moreover, the GFAP-HMOX1 mice were found to have enduring disturbed patterns of TH, up to 48 weeks of age, with a consistent, strong increase at the striatal level, and a relative decrease followed as well by up-regulation in the substantia nigra/VTA.

DAT expression followed a similar pattern (which, in the presence of preserved VMAT-dependent sequestration, could have a protective effect against the DA potentiation of extracellular oxidative stress), and striatal DA levels were indeed increased by two- to threefold. Overall, these results provide evidence that chronic ambient oxidative stress induces TH to levels sufficient to increase the DA releasable pool, in spite of eventual inactivating post-transcriptional influences.

5 Oxidative Stress Effects on Dopamine Transporters

Like most genes involved in dopaminergic neurotransmission, DAT1 is under the influence of a TATA-less, GC-rich promoter and as well under the developmental control of Nurr1 and Pitx3, although the promoter sequence involved in Nurr1 responsivity lacks the cognate NGFI-B responsive element, which suggests an indirect mechanism (Bannon et al. 2001). Sp1 and Sp3 transcription factors seem to exert a critical control on DAT transcription, with a more robust effect of Sp3 and a repressing activity of Sp1 in some contexts (Wang and Bannon 2005). Among the potential transcription modulators, a 40-base variable number tandem repeat (9 or 10) in the 3′ non-coding sequence has attracted a considerable interest and was shown to enhance transcription in vitro (Michelhaugh et al. 2001). In vivo, its impact on striatal DAT expression appears more elusive (Pinsonneault et al. 2011; Costa et al. 2011).

The negative effect of oxidative stress on DAT function has been well documented (Berman et al. 1996; Park et al. 2002) and could be amplified by the loss of α-synuclein inhibition of membrane localization during oxidative stress (Park et al. 2002), which could be protective for intracellular components at the cost of increased extracellular DA levels. Conversely, chronic oxidative stress appears to increase DAT transcription (Song et al. 2012) in certain, but not all (Kuperstein et al. 2008), contexts.

6 Oxidative Stress and D1-Like Receptors

D1 receptors are the most abundant D1-like receptors; they are expressed at high levels in the nigrostriatal, mesolimbic and mesocortical pathways, including the substantia nigra, the ventral tegmental area (VTA), limbic areas and prefrontal cortex.

Human D1 is transcribed from two promoters, separated by a short non-coding exon. The intronic, downstream promoter is significantly more efficient than the upstream promoter and drives more stable transcripts (Lee et al. 1996). Peripheral D1 receptor transcription appears to depend exclusively on the downstream receptor and give rise to shorter transcripts (lacking the first exon), while the use of the upstream receptor appears restricted to the brain. The redox-sensitive transcription factor Sp1 has a clear activating effect, with repression achieved by Sp3 and the zinc finger protein Zic2 (Yang et al. 2000).

Data relative to oxidative stress-induced D1 receptor transcriptional regulation in the brain are relatively scarce. D1 receptor expression was recently shown to be differentially regulated in the cortex and cerebellum in diabetes induced by streptozotocin, a condition where significant oxidative stress has been shown to occur not only in the periphery but also in the brain. In the cortex, there was a strong increase in D1 receptor transcript levels, which contrasted with a significant decrease in the cerebellum. The dependence of this D1 response pattern on oxidative stress was suggested by the normalization achieved with the antioxidant curcumin (Kumar et al. 2010). Conversely, receptor expression levels appeared relatively resistant in another, more specific, system, the methionine sulfoxide reductase knockout mouse. Methionine sulfoxide reductase reverses the oxidation of methionine residues, thereby protecting them from the effects of oxidative stress. In this system, while D2 striatal receptors were significantly affected (see below), no changes occurred in the binding potential, affinity or functional responsiveness of D1 receptors (Oien et al. 2010). Post-transcriptional modifications of D1 receptors have been extensively studied in peripheral systems, notably in the kidney, where oxidative stress uncouples D1 receptors from Gs and contributes to renal hypertension (Fardoun et al. 2006). Similar results were obtained in striatal membranes, on which the neurotoxic lipid peroxidation product 4-hydroxynonenal (in the 5–30 μM range, i.e. close to neurotoxic concentrations) severely decreased D1 binding sites and abolished DA-induced adenylate cyclase activation (Shin et al. 2003).

7 Oxidative Stress and D2-Like Receptors

7.1 Transcriptional Regulation

Like the D1 receptor (D1R), the D2 receptor (D2R) is under the control of a GC-rich, TATA-less promoter. In rodents, D2 receptor expression has been shown to be negatively controlled by the dopamine receptor regulating factor (DRRF or Krüppel-like factor 16 (Lee et al. 2003)), while another member of the same family, KLF11, exerts positive control over D2 receptor transcription (Seo et al. 2012). Besides, the D2R promoter is under positive control from transcription factors Sp1 and AP-1 (Wang et al. 1997), while the kinases MAPK and CAMKII also induce up-regulation (Takeuchi et al. 2002). Inactivation of the p50 subunit of the redox-sensitive transcription factor nuclear factor-kappa B (NF-κB) decreases the expression of the D2 receptor, while a lipid-rich diet (a condition that promotes brain oxidative stress and lipid peroxidation (Stranahan et al. 2011; Park et al. 2010)) increases it (South and Huang 2007).

In humans, the situation is less clear, notably because the effects of KLFs have not been described with similar precision, but it has been argued that the GC-rich sequences in human and mouse promoters are 95 % conserved (Seo et al. 2012). A significant role of the transcription factor NF-κB in the up-regulation of the D2R induced by nerve growth factor on cultured prolactinomas was also demonstrated,

leading to the characterization of two functional binding sites for NF-kB in the D2R promoter (Fiorentini et al. 2002; Bontempi et al. 2007). In cultured human Jurkat and SH-SY5Y cell lines, we have also shown a prominent role of Sp1-dependent transcription on basal and oxidative stress-induced D2R levels, which were significantly affected by mithramycin, a compound that selectively binds GC-rich sequences and is therefore used to assess Sp1-dependent transcription (Patrick Bérubé, Philippe Sarret, Sylvain Grignon, unpublished results). Interestingly, expression levels of Sp1 have been shown to be perturbed in schizophrenia (Ben-Shachar and Karry 2007). Of note, all the transcription factors mentioned above are activated by pro-oxidant conditions (Ryu et al. 2003; Aggeli et al. 2006; Rojo et al. 2004).

The impact of hypoxic/oxidative conditions has been studied in a developmental context. Indeed, perinatal hypoxia was shown to induce early up-regulation of D2R mRNA levels, which could have some relevance for the well-described relationship between obstetrical complications and further vulnerability for schizophrenia (Gross et al. 2005). Also relevant for the epidemiology and treatment of schizophrenia (van der Kemp et al. 2012; Hedelin et al. 2010; Amminger et al. 2010) are the consequences of gestational or perinatal n-3 polyunsaturated fatty acids (PUFAs) deprivation, which induced a very robust increase in D1R and D2R levels, in parallel with a steady decrease of TH and VMAT levels, and relatively preserved DAT levels. While these phenomena are certainly of multifactorial origin, it should be noted that they were associated with indirect signs of inflammatory/oxidative stress, namely, microglial and NF-κB activation (Kuperstein et al. 2008).

To address more directly the role of oxidative status in D2R regulation, we incubated the human SH-SY5Y neuroblastoma cell line with 100 μM H_2O_2 for 24 h and indeed confirmed a transcriptional activation and increased protein expression of D2R (Larouche et al. 2008). In another context, we have demonstrated that the antioxidant lipoic acid was able to normalize D2R levels after haloperidol incubation of the same cell line, in parallel with oxidative status normalization (Deslauriers et al. 2011, 2012). More recently, we have addressed the same question in a double-hit animal model relevant for the pathophysiology of schizophrenia: pregnant C57BL/6 mice were intraperitoneally injected with polyinosinic:polycytidylic acid (poly I:C) or saline at gestational day 12, and their offspring were submitted to two hours daily restraint stress from postnatal days 33 to 35, a condition that increases oxidative stress (Kim et al. 2005). The conjunction of the two conditions induced a significant increase in D2R mRNA cortical and striatal levels, in correlation with increased levels of protein carbonylation, a marker of oxidative stress (Jessica Deslauriers, Annie Larouche, Philippe Sarret, Sylvain Grignon, manuscript submitted for publication and unpublished results).

7.2 Post-Transcriptional Aspects

In contrast to different examples of transcriptional activation of the D2R in oxidative conditions, post-transcriptional effects generally lead to loss of function, with a decrease in binding potential (Alfaro et al. 2004) and reduced G protein activation

(Joseph et al. 1998). Inactivation of methionine sulfoxide reductase allows for a more specific assessment of protein-directed oxidative stress. D2 receptors, which bear more oxidable methionines on their intracellular segments, were the most affected in this model: while there were increased D2R protein levels and binding potential for the antagonist raclopride, the binding of the agonist quinelorane was decreased by 50 % with a defective G protein coupling. Interestingly, the authors also investigated the consequences of protein oxidation on presynaptic D2R function: in MsrA knockout animals, DA release was fully insensitive to the inhibitory effect of quinelorane, attesting to loss of autoreceptor function, and exhibited a paradoxical decrease in the presence of the antagonist sulpiride, which induced a robust and sustained increase in wild-type animals (Oien et al. 2010).

8 Oxidative Stress and Dopaminergic Transmission: Relevance for the Pathophysiology of Schizophrenia

The dopamine hypothesis of schizophrenia is now entering its third version and has been recently the object of influential reviews (Howes and Kapur 2009; Howes et al. 2012). Within the specific scope of the present chapter, it is interesting to critically examine the relevance of oxidative status vis-à-vis the development of these abnormalities.

8.1 Increased Dopamine Release

A robust (effect size 0.79) increase in presynaptic DA function is the main finding of the recent meta-analysis, by Howes et al., on "the nature of dopamine dysfunction in schizophrenia" (Howes et al. 2012). The prominent explanation for increased DA release in schizophrenia can be interpreted in the context of a defective corticostriatal control, most likely glutamatergic in nature, and relies on the classical distinction between tonic and phasic DA release, with a decrease of the former priming DA systems for an increase in the latter (Grace 1991, 2000). However, as exemplified above, chronic ambient oxidative stress by itself could elicit increased presynaptic function comparable to that observed in schizophrenia (Song et al. 2012).

8.2 Increased D2 Binding Potential

The meta-analysis by Howes et al. (2012) also concluded that a small increase (effect size 0.26) in D2R binding potential exists in patients with schizophrenia. The findings, however, were somewhat less robust, notably because drug naïve patients did not exhibit definite increase in this parameter, which suggests that part of the effect could be ascribed to antipsychotic-induced D2R up-regulation. It should be

kept in mind, however, that these *normal or increased D2R levels* exist in the presence of *increased presynaptic function* (i.e. presumably increased synaptic DA concentrations), which in preclinical models predicts a *decrease in D2R expression* (Fauchey et al. 2000; Ghisi et al. 2009). It remains therefore perfectly legitimate to investigate the mechanisms and consequences of increased postsynaptic D2R expression, a parameter which we have shown above to be significantly influenced by oxidative status.

The investigation of the neurochemical characteristics of transgenic DRD2-EGFP mice has provided interesting insights into the consequences of D2R up-regulation: these mice were found to have a ~40 % increase in binding potential and a twofold increase in D2R mRNA levels. They displayed behavioural (locomotor activity) and cellular (outward current) hypersensitivity to the effects of quinelorane and quinpirole, respectively; this suggests that under the conditions prevailing in the striatum, an increase in the level of D2R protein is appropriately transduced and results in a gain of function (Kramer et al. 2011).

8.3 D2 Receptor Up-Regulation and Striatocortical Effects

Moreover, the effects of D2R up-regulation are not restricted to striatal response: in a series of influential papers, Kellendonk et al. have explored the prefrontal cellular and cognitive effects of transgenic D2R overexpression (40 %) restricted to the striatum and confirmed the existence of working memory deficits or loss of cognitive plasticity, which are (1) subserved by the frontal cortex (2) induced by striatal DA dysfunction (3) and neurodevelopmentally relevant since switching the transgene off in adulthood does not reverse them (Kellendonk et al. 2006; Bach et al. 2008). Recently, the same group went on to show that this paradigm also induces deficits in prefrontal GABAergic neurotransmission (Li et al. 2011). Although it must be said that at the moment, demonstration of the neurochemical hallmark of schizophrenia, glutamic acid decarboxylase (GAD67) and parvalbumin (PV) down-regulation, is still lacking, these results provide a fascinating complement to our understanding of schizophrenia pathophysiology.

9 Concluding Remarks

The recent confirmation of robust neuroimaging dopaminergic abnormalities, in conjunction with the demonstration of relevant striato-cortical influences, has rekindled interest in the dopaminergic aspects of schizophrenia pathophysiology. Due to inherent oxidative conditions prevalent in the striatum, the investigation of redox effects on dopaminergic parameters should be of paramount importance; the wealth of data in this domain has been devised to mimic neurodegenerative disorders, chiefly Parkinsons's and Huntington's diseases, and does not extrapolate well to

psychiatric disorders. In parallel with their ongoing demonstration in post-mortem (Gigante et al. 2010; Wang et al. 2009) and clinical (Do et al. 2000) contexts, a careful investigation of oxidative phenomena, clinically relevant in intensity and duration, in well-chosen animal models should continue to provide important contributions to our understanding of the dopaminergic aspects of schizophrenia pathophysiology.

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The Reciprocal Effects of Oxidative Stress and Glutamate Neurotransmission

Sylvain Grignon and Jessica Deslauriers

Abbreviations

4-HNE	4-hydroxynonenal
AMPA	α-amino-5-methyl-3-hydroxy-4-isoxazole propionic acid
ATP	Adenosine triphosphate
cGCL	Catalytic subunit of glutamate cysteine ligase
CNS	Central nervous system
COX2	Cyclooxygenase-2
DNA	Deoxyribonucleic acid
Drp1	Dynamin-related protein 1
DTNB	5, 5′-dithio-bis[2-nitrobenzoic acid]
DTT	Dithiothreitol
EAAC	Excitatory amino acid carriers
Egr-1	Early growth response protein
EPSC	Excitatory post synaptic currents
ERK	Extracellular signal-regulated kinase
H_2O_2	Hydrogen peroxide
iNOS	Nitric oxide synthase, inducible form

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KA	Kainate
MPTP	1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine
NAC	N-acetylcysteine
NFκB	Nuclear factor-kappaB
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
NOX2	NADPH oxidase
NRF1	Nuclear respiratory factor 1
Nrf2	NF-E2-related factor
$O2^{-}$	Superoxide anion
ONOO	Peroxynitrite
OPA1	Optical atrophy protein 1
PKG	Protein kinase G
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SIN-1	3-morpholinosydnonimine
Sp1	Specificity protein 1
t-BHQ	*tert*-Butylhydroquinone
t-bOOH	*tert*-Butylhydroperoxide

1 Introduction

Abnormalities of glutamate neurotransmission are the focus of intense research for the neurobiological approach of neuropsychiatric conditions. A large body of research has demonstrated the implication of glutamate in pathological phenomena such as neurodegeneration, excitotoxicity, and apoptosis as well as its role in neuronal trophicity, synaptic plasticity, and long-term potentiation, to name a few. As specifically regards psychiatric conditions, glutamatergic mechanisms have also received ample attention. The investigation of psychotic states induced by phencyclidine or its analogues unraveled their common property of antagonism at the N-methyl-D-aspartate (NMDA) receptors and provided the initial argument for the glutamatergic hypothesis of schizophrenia, which has since received empirical support from animal, genetic, neuroimaging, and interventional studies. Conversely, the NMDA receptor antagonist ketamine has attracted interest as a therapeutic (as opposed to psychotomimetic) agent in the field of resistant depression (Zarate et al. 2006), and glutamatergic aspects of mood disorders pathophysiology are also intensely studied (Sanacora et al. 2012).

Beside classical aspects of excitotoxicity, calcium mobilization, and programmed cell death, reactive oxygen species (ROS) or reactive nitrogen species (RNS) production upon glutamate receptors stimulation has also attracted early attention (Coyle and Puttfarcken 1993). A distinct line of investigation, the impact of oxidative stress on glutamate neurotransmission, has also produced significant advances.

One subsequent topic, which is also beginning to be addressed experimentally, is the emergence of vicious circles between ambient oxidative stress, gain of glutamatergic function, and subsequent increase in oxidative stress.

2 A Brief Overview of Glutamatergic Neurotransmission

Glutamate is the main excitatory transmitter in the central nervous system (CNS). Glutamate is synthesized in neurons from glutamine under the action of the enzyme phosphate-activated glutaminase (brain/kidney phosphate-activated glutaminase product of the GLS1 gene) and from α-ketoglutarate by mitochondrial aspartate aminotransferase. Within astrocytes, glutamine synthetase converts glutamate to glutamine. The newly formed glutamine is released from astrocytes and taken up by glutamatergic neurons, where new glutamate is synthetized.

2.1 Ionotropic Receptors

Glutamate acts through two families of receptors, namely, ionotropic and metabotropic receptors. Ionotropic receptors have been defined by their preferential ligands. **AMPA** (α-amino-5-methyl-3-hydroxy-4-isoxazole propionic acid) **receptors** are usually heterotetramers (although homotetramers have been documented) of AMPA-R subunits GluR1-4 (or GluA1-4) and some bias towards the inclusion of GluA2 dimers. Functional properties such as calcium permeability, current kinetics, and pharmacology are strongly influenced by subunits composition, alternative splicing, and accessory subunits (Shepherd and Huganir 2007). AMPA receptor kinetics provide the basis for the fast, high-frequency component of excitatory postsynaptic currents (EPSC) in the CNS (Geiger et al. 1997); the control of the trafficking and membrane density of AMPA receptors is also a central mechanism in synaptic plasticity and homeostatic adjustments of EPSC strength (Shepherd and Huganir 2007). The **kainate receptors** have been nominated after their defining preferential agonist, the seaweed toxin kainic acid. They are heterotetramers formed by subunits GluR5-7 (GluK1-3) and KA1-2 (GluK4-5). GluR5-7 subunits undergo substantial editing and alternative splicing. KA1-2 subunits bear high affinity sites for kainite binding, but are unable to form homotetramers in recombinant systems, at odds with the Glu5-7 subunits. They are more sparsely distributed in the CNS than other glutamate receptor types, and their electrophysiological contribution must be "unmasked" from the larger contribution of AMPA currents. Nevertheless, they are involved in many important functions such as synaptogenesis, control of neuronal excitability (including rhythmic activity), neurosecretion (through their presynaptic component), and some forms of synaptic plasticity (Pinheiro and Mulle 2006; Jane et al. 2009). Kainate receptors also impact the properties of critical CNS networks and could play a role in the pathophysiology or treatment of epilepsy (Vincent and Mulle 2009). The **N-Methyl-D-aspartate (NMDA) receptor** has

attracted by far the most attention in the field of excitotoxicity, and in diverse aspects of normal glutamatergic neurotransmission, the most notable being its implication in long-term potentiation and other forms of neuronal plasticity. NMDA receptors are heterotetramers comprising two obligate NR1 subunits, two NR2 subunits (NR2A-D) and an accessory NR3 subunit. At normal membrane polarization, the receptor is blocked by magnesium, and a mild depolarization is necessary to relieve this block, with a half effect at −20 mV. NR1 subunits bear binding sites for the obligate coagonist glycine. Besides, NMDA receptors are endowed with a rich complement of modulatory sites enabling redox, zinc, neurosteroid, and polyamine modulatory effects. The impact of subunit composition (NR2A vs. NR2B) and cellular localization (synaptic vs. extrasynaptic) on the function and neurotoxic effects of NMDA receptors is the focus of intense research (Kohr 2006).

2.2 Metabotropic Receptors

Metabotropic receptors are G protein-coupled receptors. The group I receptors, including mGluR1 and 5, are widespread in neurons (type 1) and/or astrocytes (type 5) and predominantly postsynaptic. They couple to Gαq/11 to induce phosphoinositide breakdown and also signal through β-arrestin and extracellular signal-regulated kinase (ERK) activation (Emery et al. 2010). They enhance NMDA-mediated responses and increase neuronal excitability. Group II receptors (mGluR2-3) are pre- and postsynaptic and typically couple to Gi/o. They decrease neurotransmitter release and neuronal excitability. Group III receptors (mGluR4, 6–8) are located presynaptically (active zone) and also decrease transmitter release through Gi/o coupling.

2.3 Reuptake

Upon release, glutamate can be taken up by two neuronal excitatory amino acid carriers, EAAC1 (or EAAT3), whose quantitative contribution to the overall glutamate uptake appears quantitatively modest (Holmseth et al. 2012), but functionally important in some pathological contexts (Nafia et al. 2008; Ross et al. 2011). The main uptake process, however, is contributed by astrocytes ensheathing the synaptic process, mostly through excitatory amino acid transporter 2 (EAAT2 or GLT1) and EAAT1 (or GLAST) (Kanai and Hediger 2004).

2.4 Neuroenergetics

The disposition and metabolism of glutamate in astrocytes is a complex and compartmented process positioned at the interface of metabolic (e.g., tricarboxylic acid cycle, purine nucleotide cycle, glutathione synthesis), structural (incorporation into

proteins), or neurochemical (glutamine synthesis) functions and has been reviewed in detail (McKenna 2007). Glutamatergic and GABAergic neurons have been estimated to make up to 80–90 % of the CNS neuron complement, with glutamatergic neurons constituting the large majority. The fast turnover, energy-dependent uptake, and glutamine/glutamate recycling have been estimated to make up to 60–80 % of brain energetic consumption (Rothman et al. 2003), with significant glial contribution (including astrocytic involvement in glutamatergic "tripartite synapses"), and involve cooperation between neuronal and glial metabolic processes (notably the tricarboxylic acid cycle) and the glutamate/glutamine cycle (Serres et al. 2008).

3 The Impact of Oxidative Status on Glutamatergic Neurotransmission

3.1 Glutamate Dynamics

3.1.1 Glutamate Release

The release of glutamate due to the depletion of adenosine triphosphate (ATP), resulting from the transmembrane sodium gradient and inversion of membrane glutamate transport systems, is a defining feature of the excitotoxic component of ischemic phenomena and will not be discussed here.

Classical studies explored the effect of oxidative status modification on synaptosomal [^{3}H]aspartate release and showed that 0.01 % hydrogen peroxide (H_2O_2) increased depolarization-induced calcium-dependant release above 200 % of their basal values (Gilman et al. 1994). This effect could not be replicated at substantially lower (100 μM) H_2O_2 concentration, although in this preparation peroxide synergized with a sodium load achieved by veratridine, again in a fashion more relevant to ischemia/reperfusion events (Tretter and Adam-Vizi 2002). However, in addition to ischemia/reperfusion phenomena, the influence of inflammatory and oxidative/nitrative status effects has been studied in recent work. Bal-Price and Brown (2001) used a coculture model of cerebellar granule neurons and activated glia (lipopolysaccharide and interferon-γ stimulation) to demonstrate a massive neuronal death caused by neuronal glutamate release (as evidenced by MK-801 prevention) proceeding from nitric oxide (NO)-induced impairment of mitochondrial respiration (as evidenced by the preventive effect of two distinct inducible nitric oxide synthase (iNOS) inhibitors). These effects were mimicked by the NO donor NOC-18, which induced a doubling of extracellular glutamate concentrations in primary neuronal cultures, and an increase from virtually undetectable levels to ≈9 μM in neuronal/glial cocultures, as well as a decrease in ATP levels more pronounced in pure neuronal culture than in cocultures (Bal-Price and Brown 2001). Mitochondrial involvement in glutamate release induced by oxidative phenomena has been confirmed by the use of sodium cyanide (Dong et al. 2012), which inhibits mitochondrial cytochrome c oxidase (Leavesley et al. 2008). In cortical synaptosomes, cyanide elicited a strong glutamate release, which was completely reversed by the free radical scavengers melatonin and

manganese (III) 5,10,15,20-tetrakis (4-benzoic acid) porphyrin, as well as the H_2O_2 scavenger EUK134. Indeed, this effect was replicated by H_2O_2, albeit at the considerable concentration of 600 μM. The NMDA receptors antagonist AP5 ((2*R*)-amino-5-phosphonovaleric acid; (2*R*)-amino-5-phosphonopentanoate) prevented the effect of H_2O_2 on glutamate release, but was only partially efficient vis-à-vis the effect of cyanide, suggesting a complementary mechanism in this case. Additional results suggested that ATP synthesis inhibition was a likely mechanism to account for this discrepancy: cyanide decreased ATP synthase by more than 50 %, while the ATP synthase inhibitor 3,3′-diindolylmethane increased glutamate release. The authors suggest a role for lipid peroxidation products (also increased by cyanide) as a potential mechanism for the loss of ATP synthase activity, which seems plausible given the particular sensitivity of this enzyme to, for instance, 4-hydroxynonenal (4-HNE) modification (Perluigi et al. 2009).

Another source of reactive oxygen species (ROS) whose activity has recently been linked unequivocally to increased glutamate release is the superoxide-generating enzyme NADPH oxidase (NOX2). Acute ketamine administration in rodents is known to elicit behavioral abnormalities reminiscent of schizophrenia, to increase oxidative stress as well as glutamate release in cortical areas. Some of these effects have been convincingly linked to NOX2 activation and are indeed prevented by its inhibitor apocynin (Behrens et al. 2007). Sorce et al. have compared the effects of ketamine in wild-type and NOX2 knockout mice (NOX2-KO): compared to controls, NOX-KO mice were protected against the behavioral and neurochemical effects of ketamine, notably the hallmark increase in glutamate release (Sorce et al. 2010). Therefore, superoxide production by NOX2 is shown to be a necessary step in the behavioral and neurochemical effects of ketamine.

Overall, it appears that neuronal glutamate release can be increased in an oxidative environment by distinct pathways, namely, disruption of mitochondrial respiration, superoxide production by NOX2, and ATP synthase inhibition, the latter potentially due to lipid peroxidation products.

3.1.2 Glutamate Reuptake

Conversely, there is ample evidence that glutamate uptake processes are the target of redox modulation. Early work using different systems showed that glutamate uptake could be significantly inhibited by H_2O_2 (at concentrations as low as 100 μM) or by enzymatic ROS-producing systems such as glucose oxidase or xanthine oxidase (Piani et al. 1993; Volterra et al. 1994a). This effect could be fully reversed by the reducing agent dithiothreitol (DTT), suggesting that redox-sensitive sulfhydryl groups were involved in the phenomenon (Volterra et al. 1994b). Further mechanistic insights were provided by the incubation of astrocytic cultures with the lipid peroxidation product 4-HNE, which resulted in a dose-dependent inhibition of glutamate uptake. Again, this effect could be reversed by DTT (as well as glutathione). It was associated with the formation of adducts between 4-HNE and the glial glutamate transporter GLT-1, as well as dimerization (up to four times) of the latter.

Evidence of post-translational modifications associated with oxidative stress and uptake impairment has also been obtained for the neuronal transporter EAAC1 (or EAAT3) in the 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine (MPTP) Parkinson's model. In this case, the authors demonstrated an increase in protein tyrosine nitration. Since cysteine is also a permeant of this transporter and a precursor of glutathione synthesis, a parallel decrease in cellular glutathione content was observed, further compromising cellular redox status (Aoyama et al. 2008).

While post-translational oxidative modifications are generally associated with loss of function, a more complex picture emerges from studies of transcriptional control of the same molecules. In the primary astrocytic culture model, it was shown that mRNA and protein levels for all three transporters EAAT1, EAAT2, and EAAT3 were left unchanged by peroxide or tert-butyl hydroperoxide (t-bOOH), although the same conditions led to a decrease in glutamate uptake (Miralles et al. 2001). EAAT1 levels were decreased in a rat model of thiamine deficiency and partially rescued by the antioxidant N-acetyl-cysteine (NAC) (Hazell et al. 2010). They were also downregulated by arsenic exposure, although in this case, direct evidence of oxidative stress was lacking (Castro-Coronel et al. 2011). EAAT2 was also downregulated and rescued by NAC in the thiamine deficiency model (Hazell et al. 2010); exposure of astrocyte cocultures to excitotoxic (S)-5-fluorowillardiine (and oxidative [3-morpholinosydnonimine (SIN-1)]) resulted in a biphasic response in EAAT2 levels, with an increase at 24 h followed by a decrease at 48 h (Wallis et al. 2012). Mechanistically, EAAT2 transcription seems to be under the dependence of the transcription factor nuclear factor-kappaB (NFκB), whose sensitivity to the redox status is well known (Janssen-Heininger et al. 2000). Increases of EAAT2 transcription levels have been documented after ceftriaxone incubation (which induces NFκB activation) as well as in response to tumor necrosis factor alpha (TNFα), although in this case a repressing influence was also suspected (Sitcheran et al. 2005; Lee et al. 2008). The results are less equivocal for EAAT3. Upon exposure to L-sulforaphane and *tert*-butylhydroquinone (t-BHQ), there was a strong upregulation of EAAT3 in C6 glioma culture. This effect could be replicated in vivo and could be ascribed to the activation of the transcription pathway NF-E2-related factor 2 (Nrf2)/antioxidant response element; this response was lost in Nrf2-KO mice and could be mimicked by overexpression of Nrf2 (Escartin et al. 2011).

3.2 Redox Status and NMDA Modulation

Among the many modulating influences that have been described on the NMDA receptor, the redox site has attracted early recognition and interest. In the initial description of the phenomenon, Aizenman et al. (1989) showed that currents elicited in cultured rat cortical neurons by a combination of NMDA (10–100 μM) and glycine (1 μM) were significantly enhanced by pretreatment with the reducing agent DTT, up to 250 % above basal traces. Conversely, the oxidizing agent 5,

5′-dithio-bis[2-nitrobenzoic acid] (DTNB) induced a decrease in the signal (−22 %), which could always be restored by DTT.

Further work addressed the electrophysiological substrate of this response. Using CHO cells culture, it was shown that DTT induced an increase in opening frequencies for all three recombinant subunit combinations tested (NR1/NR2A, NR1/NR2B, or NR1/NR2C) with an increase in open dwell time only for the NR1/NR2A combination (Brimecombe et al. 1997). The redox sensitivity of NMDA receptors could be partially ascribed to two NR1 cysteines (Cys744 and 798) (Sullivan et al. 1994), whose mutation abolished the redox sensitivity of NR1/NR2C combination, and decreased that of NR2A/NR2B pairs. The coexpression of NR2A, however, rescued the redox sensitivity of mutated (C744A, C798A) NR1. This suggests that NR2A subunits also bear redox sites, which could also explain the different kinetic response (increased open dwell time) (Brimecombe et al. 1999).

Apart from the mechanistic interest of these results, recent work has also highlighted their potential pathophysiological implications. For instance, in the pilocarpine/hippocampal culture model of temporal lobe epilepsy, Di Maio et al. (2011) have shown that protracted exposure of hippocampal neurons to pilocarpine induced cellular thiol oxidation, intracellular calcium increase (ascribed to NMDA receptor activation), and resistance to further glutamate application. One plausible explanation for the latter result was that NMDA receptors were rendered resistant to glutamate because of cysteine oxidation. In support of this hypothesis, NMDA currents could be restored partially by the antioxidant NAC and more completely by the reducing agent tris(2-carboxyethyl)phosphine. These results complement earlier work using a different model of epileptiform activity elicited in hippocampal slices by low magnesium concentrations. Under these conditions, epileptiform activity appeared to be suppressed by the oxidant DNTB and restored by DTT (Sanchez et al. 2000). Therefore, in the context of epilepsy described above, it appears likely that acute reducing modulation enhances ictal activity, which in turn induces protracted oxidative phenomena and subsequent suppression of NMDA currents.

A different approach using the lipid peroxidation product 4-HNE (1 μM, a concentration in the lower range of those achieved by in vitro oxidative conditions) showed a biphasic effect with an initial stimulation of NMDA currents, which resolved within 3 h and was replaced by a protracted decrease. This effect paralleled an increase in the phosphorylation levels of NR1 and NR2A subunits and a decrease in ATP levels, which were thought to underlie, respectively, the increase and decrease in NMDA currents: okadaic acid, a phosphatase inhibitor, increased NR1 and NR2A phosphorylation and accordingly enhanced NMDA function, while the mitochondrial toxin rotenone, which depletes cellular ATP levels, induced a decrease in NMDA currents. Interestingly, the authors could not demonstrate the formation of adducts between 4-HNE and NR1 or NR2A subunits, while such adducts existed for the AMPA receptor subunits GluR1-4, although the AMPA current was left unchanged under the same conditions (Lu et al. 2001).

Another well-known modulatory site—beside the redox site—of the NMDA receptor is the binding site for the obligate coagonists glycine or D-serine. The formation of the latter product depends on the enzyme serine racemase, which has

been suggested in association studies and preclinical models to contribute to schizophrenia pathophysiology (Labrie et al. 2009; Ma et al. 2012; Morita et al. 2007). Recently, it has been shown that inflammatory, oxidative, and nitrative conditions increased the formation of covalent dimers of the enzyme, which was associated with decreased activity. Indeed, the nitric oxide donor SIN-1 induced a dose-dependent decrease in serine racemase function, at concentrations actually lower than those necessary to achieve cross-linking (Wang and Barger 2012), which suggests intramolecular events distinct from dimerization per se.

3.3 NMDA Receptor Subunits Levels

The cellular localization and transcriptional regulation of glutamate receptors have shown varying response patterns to oxidative status across receptors, across subunits, and across experimental conditions.

Available evidence suggests a robust upregulation of the NR1 subunit in response to pro-oxidative conditions. Ischemia/reperfusion paradigms lead to an early increase in NR1 and NR2A/B expression (Won et al. 2001).

In a different context, it has been shown, in cultured cortical neurons, that neurotoxicity induced by neurotrophin-4/5 involved upregulation of NR1 and, more prominently, NR2A (Choi et al. 2004). This response seemed to be under the dependence of the redox-sensitive transcription factor early growth response protein 1 (Egr-1) (Gao et al. 2009). Although, in this case, normalization of redox status by inhibitors of NOS or NOX2 did not prevent NR2A upregulation, no data were presented on an eventual normalization of NR1 by the same agents. Hypoxic conditions designed to mimic the effects of high altitude for 3,7, or 14 days induced unequivocal evidence of increased oxidative markers and a transcriptional activation and upregulation of the NR1 subunit, while the GluR2 subunit tended to decrease (Hota et al. 2008). Even a shorter (4 h) hypoxic treatment induced a strong increase in NR1 immunoreactivity in *nucleus tractus solitarius* neurons, which was partially prevented by α-tocopherol and ascorbic acid. These two antioxidants were also able to decrease NR1 levels during normal development, suggesting that some tonic level of oxidative conditions contributes to basal expression of NR1 (Wu et al. 2011). Later work by the same group demonstrated that the upregulation of NR1 was under the dependency of the transcription factor specificity protein 1 (Sp1) and could be prevented by the antioxidant acetyl-l-carnitine (Hota et al. 2010).

Exposure of hippocampal neurons to pilocarpine for 24 h upregulated NR1 and NR2B subunits by some 40 %, an effect that could be reversed by the antioxidant NAC and the NOX inhibitors apocynin and 6-aminonicotinamide (Di Maio et al. 2011). However, other work has rather documented a downregulation of NR2 subunits by oxidative conditions: an 8 weeks exposure to the diabetes mimic streptozotocin induced a decrease in hippocampal NR2A and NR2B levels, in parallel with a well-known disruption of oxidative status (Piotrowski 2003). Polyunsaturated fatty acids normalized malondialdehyde levels and partially restored NR2A/B levels

(Delibas et al. 2004). As well, repeated injections of the psychotomimetic NMDA antagonist ketamine induced a decrease in NR2A expression in the prefrontal and cingulate cortices of wild-type but not NOX2-KO mice, while the effects of ketamine on NR2B appeared independent of NOX2 (Sorce et al. 2010).

Caracciolo et al. (2011) have provided an extensive characterization of glutamate receptor subunit response to injections of kainate in wild-type or cyclooxygenase-2 (COX2) knockout mice. Indirect markers of oxidative/inflammatory status such as NFκB and iNOS levels were increased in the KA/COX2-KO group. As regards NMDA receptor subunits (NR1, NR2A-D, NR3A/B), there was a general decrease, for the same group, in the cortical and hippocampal transcript levels, with the exception of increased hippocampal NR3A mRNA. As for AMPA and KA receptors (GluR1-7 and KA1/2), there was also a general trend for decreased transcripts levels in the hippocampus, while cortical expression was generally unaffected. Social isolation increases oxidative stress and has been used to mimic some features of Alzheimer's disease (among other conditions) (Hsiao et al. 2012); in this case, social isolation was associated with a decrease in the density of GluR1 and GluR2 subunits associated with the cell membrane, while the overall cellular complement of the same subunits was unchanged, and NAC was able to reverse these changes. Conversely, in the work cited earlier (Hota et al. 2010), the GluR2 subunit level was also increased by hypoxia but resisted normalization by acetyl-l-carnitine.

Overall, there appears to be consistent evidence that NR1 expression increases in response to oxidative conditions, which could be driven by at least three types of redox-sensitive transcription factors, Sp1, Egr-1, and nuclear respiratory factor 1 (NRF1) (Dhar and Wong-Riley 2009). Results for other subunits are scarcer and less consistent.

4 Glutamate Modulates Cellular Redox Status

4.1 Demonstration

The implication of ROS in the cellular effects of glutamate was initially studied in the context of excitotoxic phenomena. Coyle and Puttfarcken (1993) put forward formal criteria for such involvement.

The demonstration of ROS production after NMDA engagement was initially obtained in cultured cerebellar granule cells: NMDA increased levels of the superoxide radical $\cdot O_2^-$ in a rapid (10 min) and transient (resolution within 40 min) manner; this response was duly abolished by the NMDA receptor antagonist MK-801, was partially calcium dependent, and was not mimicked, in this system, by KA (Lafon-Cazal et al. 1993). Similarly, NMDA increased ROS levels (as monitored by dichlorofluorescein fluorescence) in cultured cortical neurons (Reynolds and Hastings 1995). Evidence of increased O_2^- production (as evidenced by dihydroethidium fluorescence) after NMDA, KA, and AMPA administration was obtained in cultured hippocampal neurons and ex vivo slices (Bindokas et al. 1996).

The demonstration of significant oxidative damage to cellular components, another criterion for ROS involvement in glutamate effects, has been obtained for different macromolecules. Evidence of increased lipid peroxidation has been by far the most documented, notably through malondialdehyde and 4-HNE levels; all three glutamate receptor types appear to give rise to increased lipid peroxidation (Agostinho et al. 1996; Bae et al. 2002; Bruce and Baudry 1995), although some systems have yielded conflicting results (Yang et al. 2003). Similarly, all three types of receptors have been shown to induce some degree of protein carbonylation, a widely used index of protein oxidative modification (Mueller-Burke et al. 2008; Gluck et al. 2000; Tateno et al. 2004). Lastly, glutamate damage to deoxyribonucleic acid (DNA) was also demonstrated in early work (Didier et al. 1996) and could play role in excitotoxic cell death, but appears to elicit efficient repair mechanisms under milder conditions (Yang et al. 2010).

The last criterion of ROS involvement in glutamate action is the prevention/reversal of (some) toxic effects of glutamate by antioxidants or ROS scavengers. Such examples abound both in vitro and in vivo and indeed constitute the test generally used to ascertain the role of ROS in biological phenomena, but have generally—and notably—not translated well in clinical applications (Isaac et al. 2008; Muir 2006).

4.2 Effectors of Glutamate Redox Modulation

Although the place of glutamate as an inducer of oxidative stress has been overwhelmingly confirmed, the precise cellular origin of ROS (or at least the relative contribution of different cellular sources) is still a matter of debate, mostly on methodological grounds, which will not be addressed here (Brennan et al. 2009; Alekseenko et al. 2012).

4.2.1 Mitochondrial Involvement

It has been suggested that up to 50 % of CNS ROS originate from mitochondria, and more precisely from the reverse electron transport (Kudin et al. 2008), and early as well as more recent work has tried to unravel the interaction of glutamate (either as a neurotransmitter or as a metabolic substrate) with the complex mitochondrial dynamics.

One well-established mechanism relates to calcium homeostasis, in line with the robust calcium dependency of glutamate oxidative phenomena. Excessive cytoplasmic calcium concentrations are progressively transferred (through the calcium uniporter) to the mitochondrial matrix, exceeding the homeostatic possibilities of mitochondrial calcium cycling and reaching a threshold of calcium overload. The latter, associated with other triggering signals such as increases in inorganic phosphate concentration, ATP depletion, and oxidative stress, provokes the opening of the permeability transition pore with subsequent diffusion of large molecules and disruption

of the tricarboxylic acid cycle (Crompton 1999). Glutamate-dependent ROS production as such has been suggested to depend on dissipation of mitochondrial membrane potential (Scanlon and Reynolds 1998), uncoupling of respiration from ATP production (Panov et al. 2009), increased respiration (even at maximal uncoupling; Kumari et al. 2012), specific enzymatic sources such as the α-ketoglutarate dehydrogenase complex (Chinopoulos and Adam-Vizi 2006), or lifting of oxaloacetate inhibition of complex II succinate dehydrogenase (Panov et al. 2009). Glutamate has also been shown to profoundly affect the dynamics of mitochondria, notably by promoting mitochondrial fragmentation and autophagy, by upregulating mitochondrial fission markers and promoters dynamin-related protein 1 (Drp1) and Fis1 (Kumari et al. 2012; Grohm et al. 2010).

4.2.2 NOX2 Activation

Nicotinamide adenine dinucleotide phosphate oxidases (abbreviated to NOX) are a family of transmembrane which catalyze the reduction of molecular oxygen O_2 to the superoxide anion O_2^- and have been initially described as giving rise to the "phagocytic oxidative burst." The most widely studied form, in general and in the context of glutamate toxicity, is NOX2 (previously called $gp91^{phox}$). NOX2 is in obligatory and stabilizing interaction with $p22^{phox}$; upon phosphorylation and subsequent confirmation changes, a third subunit, the "organizer" $p47^{phox}$, associates with the membrane-bound NOX2/$p22^{phox}$ complex and recruits to it a number of cytoplasmic factors, among which the "activator" p67phox, $p40^{phox}$, and the GTPase Rac. The active NOX2 complex catalyzes the reduction of NADPH, the result of which is a transmembrane electron transfer with subsequent release of superoxide O_2^- in the luminal or extracellular space. NOX2 is an inducible enzyme, and its promoter bears binding sites for multiple redox-sensitive transcription factors. The cellular functions of NOX2 can be probed by a number of pharmacological inhibitors such as diphenylene iodonium, whose specificity is weak, or apocynin, a prodrug which must be activated by peroxidases and prevents the translocation of cytoplasmic components (Bedard and Krause 2007). More recently, knockout NOX2 models have provided clear results to some its functions, including in the context of glutamate-induced oxidative phenomena. Cortical neurons loaded with the redox-sensitive probe dihydroethidium showed a strong increase in fluorescence levels upon NMDA application, which was restored to control levels by MK-801, apocynin, or an inhibitor of NADPH production. Oxidative phenomena (4-HNE-positive neurons) induced by NMDA were also prevented by NOX2 inhibition. To more completely assess the respective contribution of mitochondria versus NOX2, the authors used the fact that mitochondria can use pyruvate to sustain ATP levels and ROS production, while NOX2 superoxide production is dependent on glucose: providing neurons with pyruvate (at the exclusion of glucose), in the presence of NMDA, strongly decreased the number of ethidium-positive neurons, while the level of superoxide production was unaffected by providing only glucose (at the exclusion of pyruvate); on the basis of the metabolic requirements of the two

pathways, it was thus concluded that in this system, superoxide production induced by NMDA relied mostly on NOX2. Moreover, in vivo, NMDA-induced neuronal death was prevented in $p47^{phox-/-}$ cells. Hippocampus neuronal degeneration induced by NMDA was also prevented by some 60 % in $p47^{phox-/-}$ mice, as was 4-HNE induction. Subtype-specific peptide inhibitors of protein kinase C moreover suggested that NMDA activation of NOX2 relied on PKCζ activation (Brennan et al. 2009). Similarly, Girouard et al. showed that ROS increases elicited by NMDA (as assessed by dihydroethidium fluorescence) were attenuated in NOX2-KO mice. Pharmacological assessments in cultured neurons suggested a signaling pathway between NMDA and NOX2, consisting of NO increase (mediated by the neuronal nitric oxide synthase nNOS), guanylate cyclase activation, and subsequent activation of protein kinase G (PKG) by cyclic guanosine monophosphate (cGMP) (Girouard et al. 2009). These effects have been extended, beyond cortical regions, to the striatum. After striatal glutamate injection, apocynin or NOX2 knockout decreased cell death, ROS production, and protein nitration by ≈50–60 %, a significant but partial rescue. A direct assay of NADPH oxidase activity confirmed the stimulating effect of glutamate and its prevention in NOX2-KO and apocynin-treated animals. Pharmacological analysis also showed a significant stimulating effect of non-NMDA ionotropic receptors and metabotropic M1 (and possibly M5) receptors on NADPH oxidase activity (Guemez-Gamboa et al. 2011).

4.2.3 Nitrative Phenomena

The implication of increased NO production, beside ROS, has been proposed early (Lafon-Cazal et al. 1993) to account for some of the detrimental or, for that matter, neuroplastic effects of glutamate and has received ample experimental confirmation (Ishikawa et al. 1999). NO derivatives can induce S-nitrosylation (originating from NO^- singlets) and react (NO^- triplets) with superoxide anion $O2^-$ to form peroxynitrite ONOO. There appears to be a significant interplay with the mitochondrial aspects of ROS production and consequences (Crompton 1999; Almeida and Bolanos 2001) with possible feedforward amplifying phenomena. Interestingly, it has also been shown that NO-dependent events could link NMDA receptor engagement to NOX2 activation; therefore, beside its own interaction with ROS, NO is also linked mechanistically to the two main glutamatergic oxidant sources.

4.2.4 Cystine Uptake

Cystine is transported into neurons and glia by the X_{AG} system (cysteine permeable glutamate transporter), but also, in immature neurons, oligodendrocytes and some cell lines by the X_C^- cystine glutamate exchanger. Intracellular cystine can be converted back to cysteine and incorporated in the endogenous antioxidant glutathione. Cystine and glutamate are the two preferred substrates of this system, which normally extrudes glutamate. Increasing extracellular glutamate concentrations (which

competes with cystine for cellular entry) or decreasing extracellular cystine levels have been shown to decrease glutathione levels and induce toxicity (Murphy et al. 1989), through an original mechanism of oxidative stress that does not involve excessive ROS production but decreased antioxidant mechanisms. Interestingly, the same system is also involved in the protective effects of preconditioning: mixed neuron/glia cell culture exposed to oxygen glucose deprivation, or mice exposed to 15 min carotid artery ligature, reacted by upregulating X_{CT} (a subunit of the X_C^- transport system) and the catalytic subunit of glutamate cysteine ligase (cGCL), involved in the synthesis of glutathione. This neuroprotective glial coordinated response is under the dependence of the transcription factor NRF2 (Bell et al. 2011a, b).

4.3 Antioxidant Effects of Glutamate Stimulation

Most of the data regarding glutamate toxicity tried to mimic "catastrophic" events such as ischemia/reperfusion, apoptotic cell death, or generally neurodegenerative events, at the risk of overlooking milder effects (Yang et al. 2010) or bona fide neuroprotective actions of glutamate, which are often related to synaptic (vs. extrasynaptic) NMDA receptor engagement (Hardingham and Bading 2010). These have been recently reviewed and include activation of the protective AKT/GSK-3β pathway, suppression of "prodeath" FOXO transcriptional activity, and engagement of cAMP-response element binding protein, among others (Hardingham 2009). These neuroprotective mechanisms enhanced by synaptic activity also included (1) transcriptional suppression of the thioredoxin-interacting protein (which itself inhibits thioredoxin and is therefore pro-oxidative in a FOXO dependent fashion) and (2) transcriptional activation of sestrin2 and sulfiredoxin, which can reduce oxidized forms of sulfiredoxin back to their active form (Papadia et al. 2008).

5 Concluding Remarks

The investigation of the redox modulatory effects of glutamate neurotransmission enters its fourth decade and remains a very active field.

5.1 Empirical Confirmation of the "Vicious Circle" Model

The idea that glutamatergic transmission could give rise to positive feedforward (e.g., increased NMDA subunit expression) or feedback (e.g., decreased uptake) phenomena was proposed relatively early to account for the anti-homeostatic, "catastrophic" behavior of this system (Coyle and Puttfarcken 1993). In the more restricted field of glutamate-induced oxidative stress, mitochondrial disruption and NOX2 activation are attractive partners to engage in such feedbacks, but this

conceptual framework has received scant empirical confirmation in spite of its attractiveness. Recent work has indeed explored the interaction between mitochondrial dynamics and NMDA transmission (Nguyen et al. 2011). The optical atrophy protein 1 (OPA1) is an obligate step of mitochondrial fusion and is mutated (deletion) in a frequent form of optic neuropathy, associated with the loss of retinal ganglion cells through a likely excitotoxic process partially rescued by the NMDA antagonist memantine. In mice heterozygous for the mutated OAP1, mitochondria were, as expected, both shorter and more numerous, indicating that fission was favored over fusion. Antioxidant status was compromised by decreased expression of superoxide dismutase; most notably, there was significant upregulation of NMDA subunits NR1, NR2A, and NR2B, a likely consequence of the *primum movens*, disrupted mitochondrial dynamics, but also a potential amplifying factor through increased oxidative stress and upregulation of fission promoters dynamin-related protein 1 (Drp1) and Fis1 (Kumari et al. 2012; Grohm et al. 2010), as stated above.

5.2 Relevance to Psychiatric Disorders Pathophysiology

While the results detailed in this chapter have been used to gain a better understanding of neurological conditions such as stroke, epilepsy, or neurodegenerative disorders, their relevance for the pathophysiology of psychiatric disorders is also becoming obvious. The most salient example, the phencyclidine model of schizophrenia and its relation to GAD67 downregulation, where NOX2 implication has been formally demonstrated by M. Behrens' work, will be discussed in chapter "The Impact of Oxidative Stress on GAD67 Levels and Parvalbumin-Positive Neurons" in the present volume. In a different perspective, NOX2 has recently been implied as well in the effects of social isolation, a chronic stressor mimicking, in rodents, some aspects of diverse conditions such as depression, anxiety, suicidality, or schizophrenia, among others. The authors have taken advantage of a spontaneous mutation in rats (threonine/methionine substitution at position 153) of the NADPH oxidase organizer subunit $p47^{phox}$, which reduces oxidative burst capacity by 40 %. During social isolation for at least 4 weeks, animals developed increased locomotor activity, loss of discriminating capacities in the novel object recognition test, increased glutamate levels, and loss of physiological NR2A increase. There was also a time-dependent decrease in GABAergic markers GAD67 and parvalbumin and an upregulation of NOX2 and $p47^{phox}$ that was restricted to pyramidal neurons. Accordingly, oxidative markers also increased in a time-dependent fashion. Interestingly, the loss of function mutation of $p47^{phox}$ was protective against behavioral abnormalities (locomotor activity), NR2A, and parvalbumin decrease. In another set of experiments, the authors went on to show a preventive effect of apocynin administered from weeks 4 to 7, vis-à-vis the development of behavioral abnormalities induced by social isolation.

Therefore, normalization of redox status was instrumental in attenuating some of the neurochemical and behavioral abnormalities induced by social isolation, which

shows that interrupting the feedback mechanisms between glutamatergic neurotransmission and oxidative stress could be of paramount interest in the understanding and treatment of psychiatric disorders.

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Mitochondrial Dysfunction in Psychiatric Disorders

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Abbreviations

ADP	Adenosine diphosphate
ASD	Autism spectrum disorder
ATP	Adenosine triphosphate
CNS	Central nervous system
ETC	Electron transport chain
$FADH_2$	Flavin adenine dinucleotide
GSH	Glutathione
MD	Mitochondrial disorder
MELAS	Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes
mtDNA	Mitochondrial DNA
NAC	*N*-acetylcysteine
NADH	Nicotinamide adenine dinucleotide
nDNA	Nuclear DNA
OCD	Obsessive-compulsive disorder
POLG1	Polymerase gamma-1
ROS	Reactive oxygen species
TCA	Tricarboxylic acid

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1 Background

Psychiatric disorders represent a large class of medical disorders with unclear etiologies and limited effective treatments. A simple single gene or chromosomal abnormality has not been found to explain most psychiatric disorders. Although linkage studies have identified many candidate regions of certain chromosomes that could be associated with many psychiatric disorders, these findings have been inconsistent across studies. For example, recent studies have identified genetic polymorphisms associated with increased susceptibility to psychiatric disorders such as schizophrenia, but most polymorphisms identified are in the noncoding regions of the genome, making the understanding of how these genetic changes contribute to psychiatric disorders opaque (Harrison and Weinberger 2005; Kleinman et al. 2011). Some research studies have started to investigate gene-environment interactions and epigenetic factors in psychiatric disorders, rather than fixed genetic defects. These studies may lead to a better understanding of how interactions between genetic polymorphisms and the environment contribute to the development of psychiatric disorders and also provide a deeper understanding of the pathophysiological mechanisms that cause these disorders. Other research studies examining the etiology of psychiatric disorders have embraced the study of pathophysiological mechanisms that could more directly result in cellular dysfunction and the subsequent development of psychiatric disorders. Pathophysiological mechanisms identified in some psychiatric disorders include immune dysregulation, inflammation, impaired detoxification, environmental toxicant exposures, redox regulation/oxidative stress, and mitochondrial dysfunction (Burke and Miller 2011; Dantzer et al. 2008; Ng et al. 2008; Shao et al. 2008). The focus of this chapter is on mitochondrial dysfunction in common psychiatric disorders.

2 Mitochondria and Their Physiological Function

Mitochondria are distinct cellular organelles that generate adenosine triphosphate (ATP), the energy carrier in most mammalian cells, from adenosine diphosphate (ADP) by oxidizing glucose and fatty acids (Haas et al. 2007). Acetyl-CoA is a key intermediate generated from the oxidation of glucose and fatty acids that is further metabolized by the tricarboxylic acid (TCA) cycle. The TCA cycle produces flavin adenine dinucleotide ($FADH_2$) and nicotinamide adenine dinucleotide (NADH). NADH and $FADH_2$ transport energy to the mitochondrial electron transport chain (ETC), a series of reactions known as oxidative phosphorylation. Mitochondria contain two plasma membranes, an inner and an outer membrane. The ETC is located in the inner mitochondrial membrane and consists of five multi-subunit enzyme complexes (complexes I through V) and two electron carriers (ubiquinone, also known as coenzyme Q10, and cytochrome *c*) (Zeviani et al. 1996).

Mitochondria are the only organelle in mammalian cells with their own genome. The ETC is coded by both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) (Zeviani et al. 1996). mtDNA contains 37 genes that code for 13 subunits of complexes I, III, IV, and V, as well as the machinery required to translate and transcribe the mtDNA genes into ETC complex subunits. The rest of the ETC complex subunits are coded by over 850 nDNA genes (Cotter et al. 2004). nDNA also codes for mitochondrial enzymes that participate in carbohydrate and fatty-acid oxidation. Thus, mutations in either genome can impair mitochondrial function and cause ETC complex deficiencies (DiMauro and Schon 2003).

The ETC complexes, particular complexes I and III, are the source as well as the major target of reactive oxygen species (ROS) (Fernandez-Checa et al. 1998; Trushina and McMurray 2007). The ETC is protected from damage caused by ROS by a mitochondrial specific superoxide dismutase and by antioxidants such as glutathione (GSH) (Fernandez-Checa et al. 1998) as well as by uncoupling proteins (Lambert and Brand 2004). Mitochondria lack the enzymes to synthesize GSH and therefore are dependent on cytosolic GSH production (Enns 2003; James et al. 2009b). The depletion of GSH in mitochondria makes cells more vulnerable to oxidative stress and damage from ROS originating from the mitochondria (Fernandez-Checa et al. 1997). Additionally, factors that increase ROS production (such as environmental toxicants, infections, and autoimmune disease) can directly and indirectly lead to impairments in ETC activity (Anderson et al. 2008; Calabrese et al. 2005; Munnich and Rustin 2001), deplete GSH (Calabrese et al. 2005), and activate mitochondrial- and non-mitochondrial-dependent biochemical cascades that result in programmed cell death (apoptosis) (Roberts et al. 2009).

The number of mitochondria in each cell depends on the cellular energy demands. For example, low-energy cells, such as skin cells, have fewer mitochondria, while cells that require high energy demands, such as muscle, liver, brain, cerebrovascular endothelium, and GI cells, have many mitochondria. Neural synapses are areas of high energy consumption (Ames 2000) and are therefore especially dependent on mitochondrial function (Mattson and Liu 2002). Mitochondria are concentrated in the dendritic and axonal termini where they play an important role in ATP production, calcium homeostasis, synaptic plasticity (Chen and Chan 2009; Li et al. 2004), as well as neurotransmitter release (Vos et al. 2010). Mitochondria help to regulate neuroplasticity, and abnormalities in mitochondrial function can play a role in psychiatric and neurodegenerative disorders (Mattson 2007). Therefore, central nervous system (CNS) manifestations are common in patients with mitochondrial disorders (MD) (Finsterer 2006).

Studies of healthy individuals have revealed a decrease in brain mitochondrial function associated with healthy aging (Forester et al. 2010) and brain mtDNA mutations are generally more common in elderly subjects compared to younger individuals (Lin et al. 2002). Mitochondrial dysfunction is particularly interesting to study as it has been implicated in a wide variety of diseases including psychiatric disorders (Anglin et al. 2012b; Jou et al. 2009; Manji et al. 2012; Marazziti et al. 2011, 2012; Rezin et al. 2009; Scaglia 2010; Shao et al. 2008) such as schizophrenia

(Clay et al. 2011; Kato et al. 2011; Scaglia 2010; Verge et al. 2011), bipolar disorder (Clay et al. 2011; Kato et al. 2011; Scaglia 2010), depression (Kato et al. 2010; Scaglia 2010), and autism spectrum disorder (Frye and Rossignol 2011; Rossignol and Bradstreet 2008; Rossignol and Frye 2011) as well as neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Federico et al. 2012). Mitochondrial dysfunction has also been reported in genetic syndromes associated with neurodevelopmental delays such as Rett syndrome (Condie et al. 2010; Gibson et al. 2010; Grosser et al. 2012), PTEN abnormalities (Napoli et al. 2012), Phelan-McDermid syndrome (Frye 2012a), 15q11-q13 duplication syndrome (Filipek et al. 2003; Frye 2009), Angelman syndrome (Su et al. 2011), Septo-optic dysplasia (Schuelke et al. 2002), and Down syndrome (Pagano and Castello 2012; Pallardo et al. 2010) along with a wide variety of medical disorders such as persistent systemic inflammation (Cox 2012), cardiac disease (Dai et al. 2012), and diabetes (Naudi et al. 2012). Mitochondria are also intimately involved in programmed cell death (apoptosis), calcium homeostasis, synaptic plasticity, and neurotransmitter release (Anderson et al. 2008; Roberts et al. 2009). In fact, mitochondrial dysfunction may be one of the common pathways in the development of pathology associated with a wide variety of diseases, especially since mitochondrial dysfunction can cause profound dysfunction in many organ systems, particularly high-energy organs such as the nervous and immune systems and the gastrointestinal tract (Rossignol and Frye 2011). The identification of mitochondrial dysfunction in psychiatric conditions could lead to the development of better medications for these conditions (Manji et al. 2012). Given the importance of mitochondria in CNS function, we review the involvement of mitochondria and MD in patients with psychiatric disorders.

3 Psychiatric Disorders in Patients with Mitochondrial Disease

Psychiatric disorders appear to be relatively common in patients with MD. For example, in a study of 36 adults with MD, 54 % had major depression, 17 % had bipolar disorder, and 11 % had a panic disorder (Fattal et al. 2007). In one study of 24 Italian patients with MD, psychiatric conditions were more common (60 %) than in the general population and included agoraphobia, panic disorder, anxiety disorders, and psychotic syndromes (Mancuso et al. 2013). As a group, fourteen adolescents and young adults with MD self-reported significant depression and anxiety on the Behavior Assessment System for Children (Schreiber 2012) and in another study, 14 % of children with MD developed symptoms of major depression before the MD diagnosis (Koene et al. 2009). Dementia has also been reported in multiple types of MD (Finsterer 2009).

Some patients with mtDNA mutations have been reported to have psychiatric symptoms. For example, one study reported that 19 adults with mtDNA mutations had more depressive symptoms and mood disorders compared to 10 controls (Inczedy-Farkas et al. 2012). Progressive psychiatric disturbance and dementia

along with neurological disturbances have been reported in a 57-year-old woman (Young et al. 2010) and a 27-year-old man (Salsano et al. 2011) with mitochondrial transfer RNA mutations. One study reported on a family with multiple deletions in mtDNA; this family contained multiple generations of psychiatric problems, including bipolar disorder, schizophrenia, and depression (Mancuso et al. 2008). Dementia (Hopkins et al. 2010) and psychiatric problems (Komulainen et al. 2010) have also been reported in the polymerase gamma-1 (POLG1) mutation. In one study of 50 patients with MD, of whom 52 % had mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), the most common psychiatric diagnoses were mood disorders, psychosis, cognitive deterioration, and anxiety (Anglin et al. 2012a). Confusion, aggressive behaviors, hallucinations, and paranoid delusions can also occur along with nonconvulsive status epilepticus and recurrent complex partial seizures in MELAS (Kaufman et al. 2010). In addition, MELAS has been associated with depression (Ju Seok et al. 2009) and obsessive-compulsive disorder (Lacey and Salzberg 2008).

Biomarkers of MD have been found to be abnormal in some patients with psychiatric disorders. For example, some hospitalized psychiatric patients have been reported to have low carnitine levels (Cuturic et al. 2011). Treatment with vitamins and minerals which improve mitochondrial function might help with certain psychiatric symptoms. One case report revealed that coenzyme Q10 improved psychiatric symptoms in a patient with MELAS (Shinkai et al. 2000). Another case report demonstrated that riboflavin treatment normalized behavior in a patient with MD and psychiatric illness (Triggs et al. 1992).

4 Schizophrenia

Several studies have reported genetic findings that might affect mitochondrial function in patients with schizophrenia. For example, one study reported that postmortem analysis of brains in patients with schizophrenia revealed a global downregulation of genes encoding mitochondrial elements, such as the electron transport chain (Iwamoto et al. 2005). Another study reported that postmortem brain samples from the dorsolateral prefrontal cortex revealed base pair substitutions in the mtDNA genome that was more common in individuals with schizophrenia compared to controls (Rollins et al. 2009). Finally, one study of 100 patients with schizophrenia found evidence of mtDNA inheritance (Verge et al. 2012).

Several studies have examined mitochondrial function in patients with schizophrenia. One postmortem study reported a 63 % reduction in complex IV activity in the nucleus caudatus and a 43 % reduction in the cortex gyrus frontalis in patients with schizophrenia compared to controls (Cavelier et al. 1995). Decreased hippocampal neuron gene expression affecting mitochondrial function was reported in 22 patients with schizophrenia compared to 24 controls (Altar et al. 2005). One study reported a patient with MELAS who had paranoid delusions, confusion, hallucinations, and aggressive behaviors (Kaufman et al. 2010). Finally, one study reported

improvements in 15 patients with schizophrenia using normobaric hyperoxia (40 % inspired oxygen) compared to room air; the investigators suggested that the increased oxygen may have improved mitochondrial function by augmenting oxygen delivery to mitochondria (Bloch et al. 2012).

5 Major Depression

One case reported discussed a 17-year-old girl with MELAS and reported depressed mood, loss of interest, and catatonia that improved with medication (Ju Seok et al. 2009). Another study reported on 35 children with MD; five of these children had major depression prior to the diagnosis of MD (Koene et al. 2009). Finally, one study reported symptoms of depression and anxiety in students with MD (Schreiber 2012).

6 Bipolar Disorder

Several studies have reported genetic findings in individuals with MD that might contribute to bipolar disorder. For example, one study reported that postmortem analysis of brains in patients with bipolar disorder revealed a global downregulation of genes encoding mitochondrial elements, such as the electron transport chain (Iwamoto et al. 2005). Another study reported that 2 of 35 patients had a mitochondrial DNA deletion which was not found in any of 29 normal controls (Kato and Takahashi 1996). Finally, one study reported an increased level of common deletions in mtDNA in the dorsolateral prefrontal cortex in patients with bipolar disorder compared to controls (Sequeira et al. 2012).

Some studies have also reported depressed mitochondrial function in patients with bipolar disorder. In one postmortem study of 15 patients with bipolar disorder, complex I activity in the prefrontal cortex was significantly depressed compared to 15 patients with depression and 15 patients with schizophrenia (Andreazza et al. 2010). In another study, 32 patients with bipolar disorder exhibited gray matter increases in lactic acid levels on brain spectroscopy imaging compared to controls, suggesting mitochondrial dysfunction (Dager et al. 2004). Finally, one study of 10 older adults with bipolar disorder reported a decrease in depressive symptoms with coenzyme Q10 (400–1,200 mg/day) in an open-label study (Forester et al. 2012).

7 Personality/Mood Disorders

One study of 238 healthy Japanese volunteers reported that a mitochondrial DNA polymorphism (C5178A) was associated with increased extraversion compared to those with the 5178C polymorphism (Kato et al. 2004). Another study reported two

individuals with obsessive-compulsive disorder who also had MELAS; the response to standard treatments was relatively poor (Lacey and Salzberg 2008). Finally, one study reported that genetic variants in mitochondrial proteins were associated with oxidative stress in patients with obsessive-compulsive disorder (OCD) (Orhan et al. 2012).

8 Alzheimer's Disease

In an animal model of Alzheimer's disease, decreased activity of complex IV was observed compared to control mice and was related to the production of beta-amyloid (Manczak et al. 2006). Two studies reported changes in TCA cycle enzyme activities in postmortem brain samples in patients with Alzheimer's disease which were consistent with mitochondrial dysfunction (Bubber et al. 2005, 2011). A study of 17 patients with Alzheimer's disease reported increased free radical production and decreased ATP production in brain samples compared to controls (Lin et al. 2002). Beta-amyloid production was associated with changes in mitochondrial structure including fragmentation in another study (Wang et al. 2008). Finally, in one study, relatives of patients who had Alzheimer's disease and who were at increased risk for Alzheimer's disease had evidence of reduced cerebral metabolism (Small et al. 1995).

9 Autism

Several review articles have reported mitochondrial dysfunction in individuals with autism (Frye and Rossignol 2011; Haas 2010; Rossignol and Frye 2011). Autism spectrum disorder (ASD) has also been reported in 120 cases of MD in 21 studies (Castro-Gago et al. 2008; Chauhan et al. 2010; Correia et al. 2006; Ezugha et al. 2010; Filiano et al. 2002; Filipek et al. 2003; Frye 2012a, b; Frye and Naviaux 2011; Gargus and Imtiaz 2008; Graf et al. 2000; Laszlo et al. 1994; Marin-Garcia et al. 1999; Nissenkorn et al. 2000; Oliveira et al. 2005, 2007; Pancrudo et al. 2007; Poling et al. 2006; Pons et al. 2004; Scaglia et al. 2009; Shoffner et al. 2010; Tsao and Mendell 2007; Weissman et al. 2008). One article reported that out of 153 studies examining various aspects of mitochondrial dysfunction in individuals with autism, 145 (95 %) implicated mitochondrial dysfunction in ASD (Rossignol and Frye 2012).

Several studies have suggested that treatment with mitochondrial cofactor supplementation, including antioxidants, coenzyme Q10, carnitine, and B vitamins, may improve mitochondrial function and behavior in some children with ASD (Rossignol and Frye 2011). L-carnitine may be particularly helpful in children with ASD since carnitine deficiency has been implicated in ASD (Filipek et al. 2004; Mostafa et al. 2005) and some studies have reported improvements with the use of L-carnitine in ASD (Ezugha et al. 2010; Filipek et al. 2003; Gargus and Imtiaz 2008; Gargus and Lerner 1997; Pastural et al. 2009; Poling et al. 2006). One double-blind, placebo-controlled study reported improvements in children with ASD using L-carnitine

(50 mg/kg/day), including hand muscle strength and cognition (Geier et al. 2011). A second double-blind, placebo-controlled study of L-carnitine (100 mg/kg/day) reported significant improvements over 6 months of treatment in ASD symptoms compared to placebo (Fahmy et al. 2013). Two double-blind, placebo-controlled studies using a multivitamin containing B vitamins, antioxidants, vitamin E, and coenzyme Q10 reported various improvements in ASD symptoms compared to placebo (Adams et al. 2011; Adams and Holloway 2004). Treatments for oxidative stress have also been shown to be beneficial for some children with ASD. For example, methylcobalamin and folinic acid have been reported to significantly increase glutathione concentrations in children with ASD and appear to improve certain autistic behaviors (James et al. 2004, 2009a). A recent study has demonstrated that *N*-acetylcysteine (NAC) improves irritability in children with ASD compared to placebo (Hardan et al. 2012). Several other antioxidants (Rossignol 2009), including vitamin C (Dolske et al. 1993) and carnosine (Chez et al. 2002), have also been reported to significantly improve autistic behaviors. Finally, one study reported improvements in ASD symptoms using NADH and D-ribose (Freedenfeld et al. 2011).

10 Conclusions

Evidence has started to accumulate that mitochondrial dysfunction plays a role in the development of many psychiatric disorders. The number of studies published to date which have examined mitochondrial function in these disorders is small. Additional studies are needed to evaluate mitochondrial function in psychiatric disorder in order to identify the burden that mitochondrial dysfunction plays. Studies examining prevalence, severity, laboratory testing, and treatments of mitochondrial dysfunction in psychiatric disorders are warranted.

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The Kynurenine Pathway at the Interface Between Neuroinflammation, Oxidative Stress, and Neurochemical Disturbances: Emphasis in Schizophrenia

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Abbreviations

3-HANA	3-hydroxyanthranilic acid
3-HAO	3-hydroxyanthranilic acid 3, 4-dioxygenase
3-HK	3-hydroxykynurenine
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BBB	Blood brain barrier
CNS	Central nervous system
DA	Dopamine
GABA	Gamma-aminobutyric acid
Glu	Glutamate

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H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase-1
IDO	Indoleamine 2,3-dioxygenase
KATs	Kynurenine aminotransferases
KMO	Kynurenine 3-monooxygenase
KP	Kynurenine pathway
KYN	Kynurenine
KYNA	Kynurenic acid
LDL	Low-density lipoprotein
MAPKs	Mitogen-activated protein kinases
NF-κB	Factor nuclear factor-kappa B
NOS	Nitric oxide synthase
PFC	Prefrontal cortex
QUIN	Quinolinic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SP	Schizophrenia
TDO	Tryptophan 2,3-dioxygenase
Trp	Tryptophan
XA	Xanthurenic acid
α7nAChR	α7 nicotine acetylcholine receptor

1 Oxidative Stress and Inflammation in the Brain

Free radical formation is part of the physiological processes of aerobic metabolism. In this manner, cellular metabolism produces free radicals under physiological conditions that are involved in critical functions during neuronal development, differentiation, and signal transduction (Garthwaite et al. 1988; Matsumoto et al. 1993). Oxidative stress is a cytotoxic condition taking place in different tissues when antioxidant mechanisms are overwhelmed by reactive oxygen species (ROS) (Halliwell 2006). Thus, oxidative stress is a threshold phenomenon characterized by a major increase in the amount of oxidized cellular components. Overproduction of ROS results in oxidative damage, including lipid peroxidation, protein oxidation, and DNA damage, which can lead to cell death (Floyd 1999; Love 1999; Phillis 1994). Furthermore, ROS can activate diverse downstream signaling pathways, such as mitogen-activated protein kinases (MAPKs) or the transcription factor nuclear factor-kappa B (NF-κB). Actually, the role of ROS in inflammatory modulation involves NF-κB, since this factor becomes more transcriptionally active in response to the degradation of IκB by ROS, IκB being the inhibitory partner of nuclear factor κB that sequesters it in the cytosolic domain (Hayden and Ghosh 2004), thereby regulating the expression of genes encoding for a variety of proinflammatory proteins. The consequences of excessive inflammatory responses comprise secretion of high levels of proinflammatory cytokines and chemokines and production of more free radicals

causing oxidative stress, which cannot only damage neurons through the downregulation of neurotrophins and their receptors but also by blocking neurogenesis.

Moreover, the brain is particularly susceptible to the damage caused by oxidative stress, due to the high rate of oxidative metabolic activity to support its normal functions, high polyunsaturated fatty acid contents, relatively low antioxidant capacity, and inadequate neuronal cell repair activity (Traystman et al. 1991). Indeed, intracellular oxidative stress is highly associated with the development of neurodegenerative diseases and brain aging (Emerit et al. 2012; Cui et al. 2012), suggesting that the CNS is an important target for oxidative stress. Inflammatory processes could favor proinflammatory molecules from the periphery to invade the CNS, increasing cytokines, and activating glial cells to produce an amplified response. Thus, factors like cytokines, cyclooxygenases, and prostaglandins may act as extracellular signals to generate additional ROS that are associated with decreased neuronal function or glial/neuronal interactions (Rosenman et al. 1995; Schipper 1996; Steffen et al. 1996; Stella et al. 1997; Woodroofe 1995). In this context, metabolites from the kynurenine pathway are implicated in different neurodegenerative disorders because they can be modulated by both proinflammatory cytokines and free radicals.

2 Kynurenine Pathway (KP)

The kynurenine pathway (KP) represents a major route for the catabolism of tryptophan (Trp) in mammals. The human body is unable to synthesize Trp; for this reason, this amino acid is obtained from external sources (Chen and Guillemin 2009). Trp can only be transported across the blood–brain barrier (BBB) in its free form by the competitive and nonspecific L-type amino acid transporter (Hargreaves and Pardridge 1988). The result of KP is to use Trp to produce the essential pyridine nucleotide end product, NAD^+ (Magni et al. 1999), which plays a key role in several biochemical and biological processes (Fig. 1).

In the first step of this metabolic process, Trp is oxidized by cleavage of the indole ring by two dioxygenases: indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), to further produce N-formylkynurenine. TDO was long thought to be exclusively localized in the liver, but now is known to be also expressed in the brain (Haber et al. 1993) and can be induced by corticosteroids (Salter and Pogson 1985). In turn IDO is present in two isoforms (Ball et al. 2009), it predominates extrahepatically and can be expressed in various cell types throughout the body, including fibroblasts, dendritic cells, monocytes, macrophages, and microglia. IDO can be induced by a number of cytokines such as IFN-α and TNF-α (Guillemin et al. 2001, 2005; Robinson et al. 2005). This enzyme is a major immunomodulator, showing increased activity and expression in the brain in association with macrophage infiltration and microglial activation (Saito et al. 1993). Of note, interferon gamma (IFN-γ) is able to induce both gene expression and enzymatic activity of IDO-1 (Dai and Gupta 1990; Hassanain et al. 1993; Babcock and Carlin 2000). IDO is also unique in regard to its known property of using superoxide anion

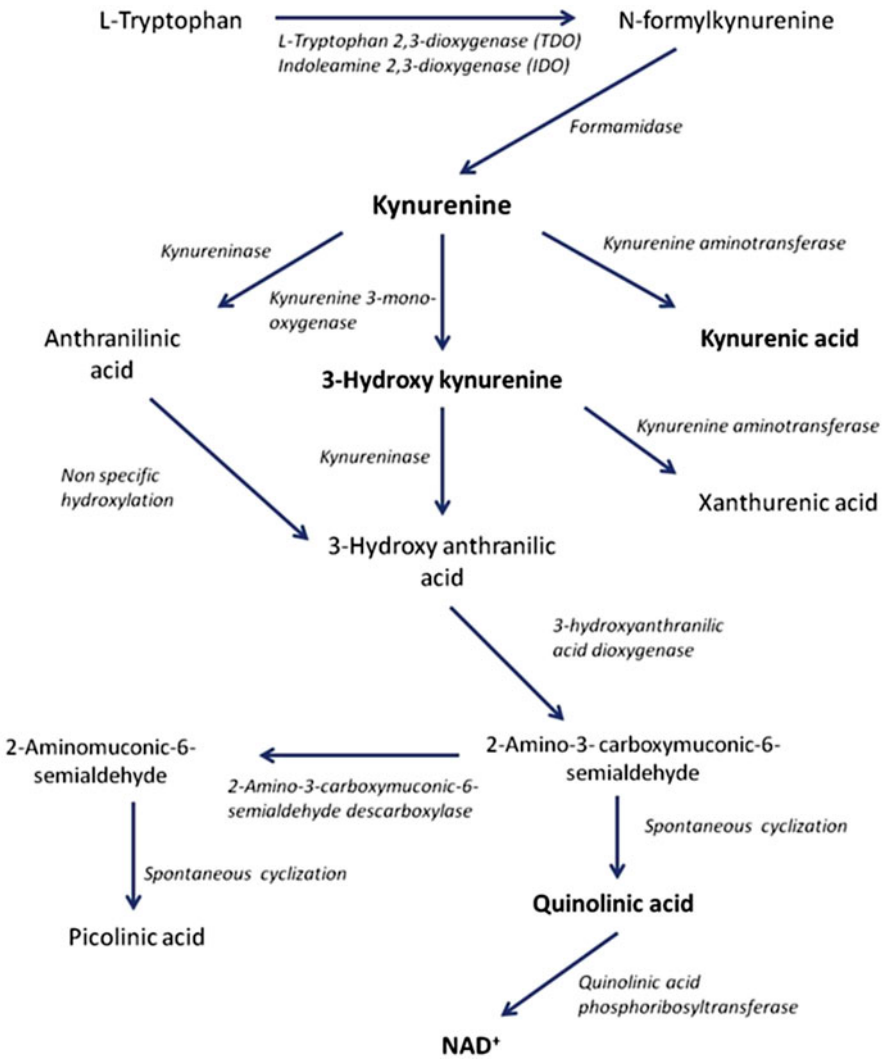

Fig. 1 Schematic representation of the tryptophan metabolism pathway known as kynurenine pathway

radical as substrate and cofactor (Thomas and Stocker 1999), thus requiring the presence of radical generating systems such as ascorbate and xanthine-xanthine oxidase. In addition, the enzyme is known to be inhibited both by superoxide dismutase (SOD) (Hirata and Hayaishi 1971) and nitric oxide (Thomas et al. 1994).

The Trp catabolite N-formylkynurenine is then hydrolyzed to form the first stable metabolite kynurenine (KYN) by the action of kynurenine formamidase. In the brain, KYN gives rise to two physically segregated branches of the pathway, producing 3-hydroxykynurenine (3-HK) and its corresponding downstream metabolites 3-hydroxyanthranilic acid (3-HANA) and quinolinic acid (QUIN) in microglial

cells, as well as kynurenic acid (KYNA) in astrocytes. Thus, KYN is metabolized by three enzymes: (1) kynurenine 3-monooxygenase (KMO), a flavin-containing monooxygenase requiring the presence of NADPH as an electron donor (Charconnet-Harding et al. 1953; Stevens and Henderson 1959) to catalyze the hydroxylation of KYN to 3-HK; (2) kynurenine aminotrasferases (KATs), which catalyze the transamination of KYN to KYNA—although several of these enzymes may participate in cerebral KYNA biosynthesis under physiological and physiopathological conditions, it appears that the pool of KYNA that can be most readily mobilized in the brain is largely provided by KAT II (Amori et al. 2009); and (3) kynureninase, which catalyzes the degradation of KYN to anthranilic acid (AA).

Mammalian kynureninase is a pyridoxal phosphate-dependent enzyme that preferentially recognizes 3-HK over kynurenine, catalyzing the formation of 3-HANA (Kawai et al. 1988). Of note, AA is a better precursor for 3-HANA within the brain than 3-HK (Baran and Schwarcz 1990). KAT II—and possibly other KATs—converts 3-HK into xanthurenic acid (XA). 3-HANA is the substrate for 3-hydroxyanthranilic acid 3,4-dioxygenase (3-HAO), which is present with relative abundance in the brain and is known to be inhibited by several metals ions (Foster et al. 1986), thereby forming 2-amino-3-carboxymuconic-6-semialdehyde. Under physiological conditions, 2-amino-3-carboxymuconic-6-semialdehyde spontaneously rearranges to form QUIN. Notably, the brain seems to contain very little 2-amino-3-carboxymuconic-6-semialdehyde decarboxylase, an enzyme that deflects the metabolic cascade towards the production of picolinic acid (PIC) (Pucci et al. 2007). The cerebral activity of the QUIN's degradative enzyme, quinolinate phosphoribosyltransferase, is very low (Foster and Schwarcz 1985), making this enzyme one of the gatekeepers for the synthesis of NAD^+.

Excessive formation of 3-HK, QUIN, and/or KYNA could play a significant role in brain pathology since these metabolites have been shown to exhibit either neurotoxic or neuroprotective properties, as well as antioxidant or pro-oxidant effects. Therefore, metabolites have been implicated in different neurologic and psychiatric disorders (Moroni 1999; Müller and Schwarz 2007; Németh et al. 2006; Oxenkrug 2011; Ruddick et al. 2006; Schwarcz and Pellicciari 2002).

3 KP Metabolites with Pro- and Antioxidant Properties Can Modulate Oxidative Stress

The CNS plays a key role in the maintenance of homeostasis and physiological functions in mammals. However, its biochemical and cytological characteristics make it vulnerable to the action of different cytotoxic agents. Among the mechanisms leading to neurodegeneration and cell death, ROS-induced oxidative stress plays a pivotal role. Oxidative stress occurs when cellular antioxidant defense mechanisms fail to counterbalance and control endogenous ROS and reactive nitrogen species (RNS) generated either from normal oxidative metabolism or from pro-oxidant conditions (Kohen and Nyska 2002; Berg et al. 2004). ROS/RNS are also

Table 1 Metabolites from KP with pro-oxidative and scavenger properties

Metabolite	Species produced	Species scavenged
Trp		H_2O_2
KYN		$OH^{\bullet}$, $ONOO^{-}$, H_2O_2
3-HK	H_2O_2	$OH^{\bullet}$, NO
3-HANA	$O_2^{\bullet-}$, H_2O_2	$ROO^{\bullet}$, NO
QUIN	$OH^{\bullet}$, $ONOO^{-}$	
XA		$ROO^{\bullet}$, $O_2^{\bullet-}$, $OH^{\bullet}$
AA		H_2O_2
KYNA		$O_2^{\bullet-}$, $OH^{\bullet}$, $ONOO^{-}$

known to modulate inflammation. There is a close relation between oxidative stress and the pathogenesis of neurodegenerative diseases. In this context, KP generates metabolites exhibiting antioxidant and pro-oxidant properties (Table 1), which production can be modulated by the prevailing redox status in cells; the imbalance in these metabolites is implicated in different pathologies of the CNS.

Under physiological conditions, KP modulates glucose metabolism: while ATP and 3-HANA formed from this pathway activate glycolysis—through which glycogen is stored in the cells to be used in case of energy stress or glucose depletion—QUIN inhibits gluconeogenesis (Lardy 1971). Several KP metabolites participate in complex pro- and antioxidative processes in the brain (Giles et al. 2003). In particular, 3-HK and 3-HANA readily autooxidize under physiological conditions, producing in the process hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radicals (Goldstein et al. 2000). However, these effects are currently balanced by the antioxidant capacity of KYNA and XA due they can scavenging radicals (Lugo-Huitrón et al. 2011a; Christen et al. 1990).

3-HK is present in the brain of mammals at nanomolar concentrations (Pearson and Reynolds 1992). This metabolite undergoes autooxidation and can be converted into quinonimines with the accompanying generation of ROS (Hiraku et al. 1995). The ability of 3-HK to generate ROS seems to be the mechanism by which it causes neurotoxicity, given that cell damage induced by this metabolite is prevented by coadministration of metal chelating agents and free radical scavengers (Chiarugi et al. 2001; Eastman and Guilarte 1990; Goldstein et al. 2000; Nakagami et al. 1996; Okuda et al. 1996). 3-HK uptake into cells is required for neurotoxicity, as its inhibition by competing large neutral amino acids prevents this damage. In addition, 3-HK toxicity depends on the cellular type because cortical and striatal cells were more vulnerable to cerebellar neurons (Okuda et al. 1998). The levels of 3-HK are increased in the brains of mice following immune activation or administration of interferon-γ (Saito et al. 1992). It is likely that some of the deleterious actions attributed to 3-HK are actually due to its metabolite 3-HANA, since the later readily undergoes autooxidation with the formation of superoxide anions (Dykens et al. 1987, 1989). Toxic pro-oxidant effects of 3-HK and 3-HANA were mainly observed in neuronal cell cultures exposed for long periods and high concentrations (100–200 mM) of these compounds (Lee et al. 2001, 2004). Furthermore, 3-HK potentiates QUIN toxicity; intrastriatal co-injection of these agents in low doses, which

alone cause only minimal or no neurodegeneration, results in substantial neuronal loss (Guidetti and Schwarcz 2003). Nevertheless, antioxidants such as N-acetylcysteine can attenuate the damage produced by 3-HK in vivo, whereas catalase and glutathione can prevent the toxicity evoked by this metabolite in neural hybrid cell line N18-RE-105. Our group has recently collected experimental evidence showing that 3-HK can also act as a peroxynitrite scavenger, partially preventing ROS formation in rat brain homogenates exposed to $FeSO_4$ (unpublished data). This evidence is in agreement with previous reports describing 3-HANA and 3-HK as potent radical scavengers since they can protect B-phycoerythrin from peroxyl radical-mediated oxidation for longer periods of time at equimolar concentrations of ascorbic acid and a water-soluble analogue of vitamin E (Christen et al. 1990). These two metabolites also inhibited spontaneous lipid peroxidation in the brain, protecting cerebral cortex against oxidative damage even in the presence of Fe III or Fe II, which stimulate auto-oxidation of these metabolites and hydroxyl radical formation, respectively. 3-HK is also able to scavenge hydroxyl radicals because it reduces 2-deoxy-D-ribose oxidation (Leipnitz et al. 2007). Hence, it is conceivable that under conditions in which 3-HK acts as antioxidant, the autooxidation or hydroxyl formation did not occur or was insufficient to overcome the antioxidant properties of this metabolite.

3-HANA has also been shown to generate hydrogen peroxide and superoxide in the presence of transition metal ions such as copper (Goldstein et al. 2000). However, 3-HANA can also act as an antioxidant, scavenging peroxyl radicals more effectively than equimolar concentrations of either ascorbic acid or Trolox (Christen et al. 1990). 3-HANA was highly effective in inducing in astrocytes the expression of heme oxygenase-1 (HO-1), an antioxidant enzyme with anti-inflammatory and cytoprotective properties in human glial cells (Krause et al. 2011). Additionally, 3-HK and 3-HANA are also efficient NO scavengers (Backhaus et al. 2008), and 3-HANA also prevented the spontaneous oxidation of GSH (Leipnitz et al. 2007). It has been observed that 3-HANA acts as a co-antioxidant for the low-density lipoprotein (LDL), preventing lipid peroxidation. It was then postulated that 3-HANA regenerates α-tocopherol, which is the endogenous antioxidant for LDL, by reducing the α-tocopheroxyl radical (Christen et al. 1994; Thomas et al. 1996).

On the other hand, the toxic actions of QUIN are primarily linked to N-methyl-D-aspartate receptor (NMDAr) overactivation through excitotoxic events (Stone 1993; Susel et al. 1989). More recently, evidence involving oxidative stress as an integral part of the toxic model induced by QUIN has appeared (Rodríguez-Martínez et al. 2000; Behan et al. 1999). Some studies suggest that QUIN stimulates lipid peroxidation in brain tissue (Ríos and Santamaría 1991), and this effect is likely to be mostly dependent on NMDAr overactivation since this marker of oxidative stress is attenuated by NMDAr antagonists like KYNA and MK-801 (Santamaría and Ríos 1993). QUIN has also shown to induce peroxynitrite formation through a concerted inhibition of SOD activity and increased activity of nitric oxide synthase (NOS) (Pérez-de la Cruz et al. 2005). Noteworthy, it seems that only a small fraction of this damage corresponds to an NMDAr-independent component (Santamaría et al. 2011a; Behan et al. 1999; Stone et al. 2000), and this is probably due to

the ability of this metabolite to form complexes with iron (II) (Stipek et al. 1997). Once these complexes are autooxidized, they yield hydroxyl radical formation through the Fenton reaction (Pláteník et al. 2001; Santamaría et al. 2011b). Therefore, QUIN is a prototypical molecule combining excitotoxic and pro-oxidant properties.

XA has been shown to act as a peroxyl radical scavenger in vitro, but its function as an antioxidant in vivo has been considered unlikely because the concentrations that were found in the tissue that has been studied (mouse lung) were in the low micromolar range (Christen et al. 1990). In the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) system, XA scavenged superoxide anions (Zsizsik and Hardeland 1999a). A recent study evaluated the antioxidant action of XA using heme and iron as promoters of radical formation: in this model, XA proved to be a powerful antioxidant, inhibiting lipid peroxidation induced both by heme and iron in a pH-dependent manner (Lima et al. 2012).

In regard to KYNA, some studies have shown that this metabolite scavenges hydroxyl radicals, efficiently protecting 2-deoxyribose when hydroxyl radicals were generated photolytically from N-hydroxy-2-pyridinethione (Zsizsik and Hardeland 1999b, 2001). KYNA also prevented the ROS production and lipid peroxidation induced by $FeSO_4$ and 3-nitropropionic acid in rat brain homogenates and decreased the hydroxyl radical production in vivo, independently of its activity on NMDAr and nicotinic receptors (Lugo-Huitrón et al. 2011a). We have collected recent evidence demonstrating that KYN, the direct precursor of KYNA, exerts stronger scavenger properties since it was able to scavenge hydroxyl radicals and peroxynitrite in synthetic medium and reduced ROS formation in rat brain homogenates exposed to $FeSO_4$ and peroxynitrite (Ugalde-Muñiz et al. 2012). Additionally, upon controlled conditions, peroxynitrite is capable of promoting KYNA production using L- and D-KYN as substrates (Lugo-Huitrón et al. 2011b). These data correlated with the study conducted by Zsizsik and Hardeland (2001) in which the incubation of KYN with H_2O_2 yields KYNA formation, a reaction that was enhanced in the presence of peroxidase. However, KYNA strongly potentiated the pro-oxidant behavior of δ-aminolevulinic acid, generating the degradation of 2-deoxyribose (Coto-Montes et al. 2001). Altogether, this evidence suggests that metabolites of KP exert both antioxidant and pro-oxidant properties, depending on the prevailing redox status.

4 Inflammation

Psychiatric disorders are associated with mild proinflammatory events. There is evidence demonstrating that KP is upregulated in inflammatory states, with activated macrophages and microglial cells producing QUIN together with other cytotoxins (Espey et al. 1997; Myint 2012). During inflammatory processes, the increased degradation of Trp and the peripheral amounts of KYN are propitious for KP metabolism in the brain, given that KYN can be transported through the BBB. Also, during inflammatory processes, KYN metabolism is increased. Most of KP metabolites contribute to homeostasis in the brain through their modulatory actions on neurotransmitters and redox status. Up to date, the unbalance in KP metabolites has

been implicated in a variety of disorders of the CNS, including the AIDS-dementia complex, Alzheimer's disease, schizophrenia, Huntington's disease, amyotrophic lateral sclerosis, etc. (Guillemin et al. 2005; Beal et al. 1990). Furthermore, during the occurrence of neuroinflammatory processes, when KP is activated in microglial cells and/or when invading macrophages infiltrate the brain, the concentrations of kynurenines may increase dramatically, reaching the micromolar range within the brain. In this regard, it is known that IFN-α can induce IDO, KMO, and 3-HAO. When IDO is induced by IFN-α, it yields a substantial increase in KYNA concentrations and other tryptophan metabolites.

The inflammatory cytokines IL-1 and TNF-α, and lipopolysaccharide (LPS), act synergistically with IFN-α to induce IDO (Robinson et al. 2005; O'Connor et al. 2009). Human microglia, blood macrophages, and mixed cultures of human fetal brain cells can ordinarily convert tryptophan, kynurenine, or 3-HK into QUIN even if there is no immune stimulation (Heyes et al. 1992). Human macrophages stimulated with TNF-α or IFN-γ yielded large amounts of QUIN (Pemberton et al. 1997). Kappa opioid receptors modulate the release of QUIN from microglial cells in culture (Chao et al. 2000). Interestingly, the amount of QUIN in the brain after immune stimulation can be prevented either by inhibitors of Trp metabolism or by compounds able to suppress the activation of immune-competent cells (Saito et al. 1994). 3-HANA and QUIN induce selective apoptosis of HT1 cell through the activation of caspase-8 and the release of cytochrome c from mitochondria (Fallarino et al. 2002) as well as by mean of processes mediated by oxygen-derived free radicals (Grohmann et al. 2003). Additionally, QUIN has been shown to induce the expression of chemokines and chemokine receptors in astrocytes, thereby leading to a possible amplification of brain inflammation (Guillemin et al. 2003). The synaptic and neuronal damage initiated by the QUIN-induced activation of microglia eventually leads to apoptotic cell death of oligodendrocytes and microglia, together with a loss of GFAP positive astrocytes (Dihné et al. 2001).

Loss of 3-HANA may have important consequences for the immune system. 3-HANA inhibits the proliferation of $CD8^{+}$T cells (Weber et al. 2006). It can also suppress the responses of T cells to allogeneic stimuli (Terness et al. 2002), acting primarily on Th1 rather than Th2 cells (Fallarino et al. 2002). At molecular level, it has been demonstrated that 3-HANA can suppress the activation of the proinflammatory transcription factor NFκB (Hayashi et al. 2007; Sekkaï et al. 1997) as well as inhibiting nitric oxide synthase (Sekkaï et al. 1997; Oh et al. 2004). This evidence suggests that 3-HANA seems to be protective, limiting the inflammatory response—including the activation of microglia, which is thought to contribute to brain damage following stroke. In addition, AA interacts with copper to form an anti-inflammatory complex able to remove highly injurious ROS (Miche et al. 1997; Halova-Lajoie et al. 2006).

It has been shown that inflammation plays a key role in the pathological onset of depression, and since cell-mediated immune activation induces IDO, this effect would lead to an increase in the Trp metabolism, reducing its levels in plasma, increasing the formation of KP metabolites, and decreasing serotonin synthesis. Altogether, these effects could explain the lower levels of this neurotransmitter and hypoactivation of its receptors observed in pathological conditions (Maes and Meltzer 1995). Additionally, generation of oxidative and nitrosative stress is an important mechanism

contributing to toxicity in inflammation and depression (Maes and Meltzer 1995; Maes et al. 2011), and because IDO employs superoxide anion as oxidant factor (Sun 1989), its activity could be even more augmented.

Recently, KYNA was identified as a ligand of GPR35 (Wang et al. 2006). Among immune cells, GPR35 is highly expressed in human $CD14^+$ monocytes, T cells, neutrophils, and dendritic cells, with lower expression levels in B cells, eosinophils, basophils, and iNKT cells; in the nervous system, it is mainly expressed in the dorsal root ganglia (Wang et al. 2006; Fallarini et al. 2010). The discovery that KYNA is an endogenous ligand for GPR35 further highlighted the importance of KP in regulating immune functions since the activation of GPR35 inhibits TNF-α release by macrophages under inflammatory conditions induced by LPS; in this context, KYNA might exert an anti-inflammatory effect (Wang et al. 2006). Additionally, GPR35 decreases intracellular Ca^{2+} probably by inhibiting its entrance (Oshiro et al. 2008); therefore, KYNA probably exerts an effect on the release of inflammatory mediators and excitatory amino acids from glial cells. Nevertheless, this action still remains unclear since KYNA activates the receptor at relatively high concentrations (10–100 μM), and so, it does not exert influence on extracellular neurotransmitters levels (Moroni et al. 2012).

The ligand-activated transcription factor aryl hydrocarbon (AHR) is also activated by KYNA. Considered as a xenobiotic receptor, AHR regulates the expression of different inflammatory intermediates and can facilitate carcinogenesis (DiNatale et al. 2010; Moroni et al. 2012). However, KYNA is not the only metabolite from KP that activates this receptor as kynurenine has been shown to act as agonist on AHR; actually, kynurenine seems to be more active than KYNA in this effect (Nguyen et al. 2010; Optiz et al. 2011), and it has been hypothesized that AHR can be activated by other KP metabolites, which in turn means a contribution of KP to the immunosuppressant action of T cells in carcinogenic processes (Mezrich et al. 2010; Moroni et al. 2012).

Another KP metabolite, PIC, is an unselective metal ion chelator (Aggett et al. 1989) that activates macrophages via induction of macrophage inhibitory proteins MIP-1α and MIP-1β (Bosco et al. 2000). Its effect is potentiated by simultaneous IFN-α treatment (Pais and Appelberg 2000). It possesses both extracellular and intramacrophage antimicrobial activity (Abe et al. 2004).

5 Neurochemical Modulation by KYNA

Inflammatory reactions and enhanced oxidative stress are recognized as two important factors associated with KP under both physiologic and pathologic conditions. Importantly, the imbalance in KP metabolites formation has a direct effect on neurotransmission, as they can modulate the release of glutamate (Glu), dopamine (DA), gamma-aminobutyric acid (GABA), and acetylcholine.

The major KP metabolite considered as a neuronal inhibitor is KYNA, which is synthesized and released by astrocytes and antagonizes NMDAr (Kessler et al. 1989)

and α7 nicotine acetylcholine receptor (α7nAChR) (Hilmas et al. 2001). As previously described, KYNA synthesis is mediated by KATs. Four KAT isoforms have been described so far (KAT I–IV), from which KAT I and KAT II are the most studied.

Activation of α7nAChR facilitates the release of multiple neurotransmitters, thereby providing multiple opportunities for modulation of synaptic communication. Stimulation of presynaptic α7 receptors directly facilitates Glu and GABA release (Wonnacott et al. 2006; Dani and Bertrand 2007). Indeed, DA, norepinephrine, and serotonin are indirectly modulated by α7 receptor-induced facilitation of Glu and GABA release in various brain regions (Kaiser and Wonnacott 2000; Wonnacott et al. 2006; Dani and Bertrand 2007; Sher et al. 2004; Gotti et al. 2006). At a functional level, enhanced KYNA in the brain has been demonstrated to cause cognitive deficits in animals (Shepard et al. 2003; Erhardt et al. 2004; Chess et al. 2009). Interestingly, reductions in brain KYNA levels cause significant cognitive improvements, which can be demonstrated both in behavioral paradigms and using electrophysiological outcome measures (Potter et al. 2010). Decreased KYNA levels lead to enhanced extracellular concentrations of Glu and acetylcholine, indicating that endogenous KYNA might function as a bidirectional modulator of glutamatergic and cholinergic neurotransmissions (Konradsson-Geuken et al. 2010; Wu et al. 2010; Zmarowski et al. 2009).

The fact that KYNA can directly influence neurotransmission is quite relevant as this metabolite can influence neuronal excitability but is limited to cross the BBB and can enter the brain only under certain circumstances. The ability of KYNA to enter the CNS can be augmented when the BBB is compromised. Modest elevations or reductions in KYNA levels reduce or facilitate extracellular DA and Glu release, respectively (Rassoulpour et al. 2005; Kaiser and Wonnacott 2000; Wu et al. 2007; Carpenedo et al. 2001; Alkondon et al. 2004). Accordingly, dysregulation of endogenous KYNA may contribute to the physiopathology of several neuropsychiatric disorders, including schizophrenia (SP). Elevated KYNA levels have been found in both cerebral spinal fluid (Erhardt et al. 2001) and *postmortem* brain tissue of schizophrenic patients (Schwarcz et al. 2001). Thus, a disruption between KYNA, Glu, and DA levels may exacerbate dysfunctional cortical and subcortical communication, contributing to inappropriate information processing in neuropsychiatric disorders like SP.

6 Schizophrenia and KYNA

Psychiatric disorders are associated with a mild proinflammatory state. Proinflammatory mediators could activate the Trp breakdown, causing dysregulation of KP, which results in hyper- or hypofunction of active metabolites. In turn, these changes are associated with neurodegenerative and other neurological disorders, as well as with psychiatric diseases such as schizophrenia (SP) (Schwarcz et al. 2012).

SP is one of the main psychiatric disorders reported and has been described as a psychotic disease characterized by impaired cognition and accompanied by emotional and behavioral alterations. Major symptoms are auditive hallucinations, para-

noid or bizarre delusions, or disorganized speech and thinking with significant social or occupational dysfunction (Myint 2012). Dysfunctional interactions between neurotransmitter systems and brain regions are implicated in SP. Cognitive impairments in SP are now hypothesized to be due to primary neuronal dysfunctions rather than chronicity or neurodegeneration (Hoff et al. 1999; Rajkowska et al. 1998). The neurochemistry of cognitive impairment in SP invokes distinct interdependent changes in major neurotransmitter systems within the prefrontal cortex (PFC). Namely, changes in cholinergic, glutamatergic, dopaminergic, and GABAergic functions are critically involved in the physiopathology of SP (Sarter et al. 2005; Lewis and Moghaddam 2006). Recent studies suggests that KYNA, the only endogenous NMDAr antagonist identified up to now—and also an antagonist for the nicotinergic acetylcholine receptor—might be involved in prefrontal dysfunctions in SP. Since its levels are elevated in the PFC of individuals with this disorder, with Brodmann areas 9 and 10 increasing by 46.8 % and 83.4 %, respectively, versus control (Sathyasaikumar et al. 2011), thereby leading to the concept that changes in KYNA concentrations might contribute to cognitive dysfunction associated with this disorder. Despite the fact that it has been argued that the physiological levels of KYNA could be below those levels needed to exert antagonism on glutamatergic receptors (K_D~ 8 μM; Ganong and Cotman 1986; Kessler et al. 1989), in some specific places of synapses, KYNA levels could be sufficient to exert responses in nerve tissue (Scharfman et al. 2000). Experiments in rodents have demonstrated that even relatively minor elevations in KYNA levels in the PFC cause a decrease in the extracellular levels of Glu, acetylcholine, and DA known to be associated with cognitive dysfunctions. Interestingly, these effects are bidirectional since a selective reduction in KYNA formation substantially enhances the extracellular presence of these classic neurotransmitters (Wu et al. 2007, 2010; Zmarowski et al. 2009).

Several other studies have shown that increasing endogenous KYNA concentrations induced by L-KYN administration results in spatial and contextual learning deficits in rats (Chess et al. 2007; 2009) as well as impaired sensory gating, prepulse inhibition, and attention in adult rats (Shepard et al. 2003; Erhardt et al. 2004; Chess and Bucci 2006). Noteworthy, when L-KYN is administered to young adult rats (equivalent to adolescence, a critical period for brain development), the increase in KYNA concentrations impact cognitive functions in adulthood and exhibited deficits in contextual fear memory, while impaired on a novel object recognition memory task. Recently, it was also showed that prolonged KYN treatment during prenatal and early postnatal development in rats increased the KYNA levels, which was accompanied by a reduction in basal levels of extracellular glutamate in adult rats. Additionally, it was observed impaired performance in passive avoidance and the Morris water maze (Pocivavsek et al. 2012). The implications of these findings lie in the fact that exposure to high levels of KYNA results in inhibition of NMDAr and/or α7nAChR during critical stages of the development, thereby exerting lasting impacts on brain morphology and/or cognitive functions during adulthood, contributing to cognitive deficits typically observed in SP (Akagbosu et al. 2012).

In this context, epidemiological evidence indicates that microbial pathogens and parasitic infections may contribute to cognitive impairments in patients with

SP. However, the precise mechanisms whereby the parasite impacts cognition remain poorly understood. Infection during pregnancy in mothers of offspring later developing SP has been repeatedly described (Mednick et al. 1988; Brown et al. 2004; Buka et al. 2001). In a follow-up study of children who had suffered from bacterial meningitis from age 0 to 5 years during an epidemic in Brazil, a fivefold increased risk for developing psychoses later on was observed (Gattaz et al. 2004). Since the development of the brain is not finalized at birth, but is still ongoing for the first years of life, an infection during early childhood is still in accordance with the assumption that an infection-triggered disturbance in brain development plays a pivotal role in SP (Muller and Schwarz 2006). Considerable body of evidence links *Toxoplasma gondii* infection to an increased incidence of schizophrenia (Dickerson et al. 2007; Mortensen et al. 2007; Hinze-Selch et al. 2007; Schwarcz and Hunter 2007). An interesting study measured antibody titers against infectious agents not only in the serum but also in the cerebrospinal fluid of individuals with recent onset of SP. Titers against cytomegalovirus and *T. gondii* were significantly increased (Leweke et al. 2004). The link between *T. gondii* and changes in glutamatergic neurotransmission remains poorly studied, but KYNA has already been hypothesized to be a pathogenic link between *T. gondii* infection and cognitive impairment in SP (Schwarcz and Hunter 2007). Experimental studies have shown that diminishing elevated KYNA levels is predicted to ameliorate cognitive deficits. Knockout mice with deletion of the enzyme that converts kynurenine into KYNA, KAT II, express lower levels of KYNA and perform better in cognitive test when compared to control mice (Potter et al. 2010). Because rodents infected with *T. gondii* and patients with SP exhibit increased KYNA levels in the brain (Schwarcz and Hunter 2007; Kannan and Pletnikov 2012), one could predict that reduction of levels of this NMDAr antagonist may have therapeutic effects.

A disruption of the immune response is associated with an altered balance in KP metabolism as well as oxidative stress. Clinical and preclinical investigations of the actions of antioxidative defense systems in the brain suggest several ways in which ongoing oxidative stress might impact the occurrence and course of SP. A recent meta-analysis indicated that there is an increase in the levels of lipid peroxidation products and NO in SP, while SOD activity was found to be significantly decreased in this disorder (Zhang et al. 2010). These findings show an increase of superoxide and other ROS and correlated with an increased expression of TDO compared to IDO in SP patients (Miller et al. 2004). Interestingly, TDO2 mRNA is elevated in the brain of individuals with SP, and a concomitant increased density of TDO2-immunopositive astroglial cells is seen in white matter of these patients (Miller et al. 2004). Because TDO is one of the upstream enzymes responsible for the biosynthesis of KYNA, this enhanced expression could conceivably lead to an elevation of KYNA levels in the diseased brain, therefore playing a part in the pathophysiology of this disorder.

Further evidence favors the concept that high levels of KYNA are implicated in SP: a recent study revealed distinct abnormalities in KP enzymes in BA9 and BA10 cortical regions (Sathyasaikumar et al. 2011). While the activity of KATII was in the normal range, a significant decrease in KMO activity in the PFC of individuals with

SP was observed. Of note, this reduction was not accompanied by decreased kynureninase activity. The activity of 3-HAO, which catalyzes the formation of QUIN from 3-HANA, was found to be reduced in the PCF. Decreased 3-HAO activity might account for the elevation in tissue levels of 3-HANA in SP, which was recently demonstrated in the anterior cingulate cortex (Miller et al. 2008) and might affect the redox status of neurons and glial cells in the area. This KMO downregulation provides an explanation for the increased levels of KYNA consistently found in *postmortem* brain tissue (Schwarcz et al. 2001) as well as in the cerebrospinal fluid of individuals with SP (Nilsson et al. 2005).

Altogether, this body of evidence suggests an impact of KYNA levels on cognitive deficit in SP; however, the routes by which KYNA production is increased in SP remain unclear since the "canonic" pathway involving KATII activity is not altered. In this regard, some studies have shown that KYNA can be formed by the nonenzymatic oxidation of kynurenine and Trp via indole-3-pyruvic acid (Politi et al. 1991), a reaction which is increased by oxidative stress. Increased levels of nitric oxide have been noticed after brain injury, and this can inhibit SOD. The resulting increase in superoxide anions could, in turn, oxidize indolepyruvate to KYNA, consistently with reports that nitric oxide donors increase KYNA production (Luchowski and Urbanska 2007). The close correlation between inflammation, oxidative stress, and KP and the impact that these components exert in neurotransmission are likely to be involved in the pathogenesis of SP.

7 Concluding Remarks

In recent years, different groups have investigated the impact of KP metabolites on SP—especially KYNA—and its role on the hypoglutamatergic function observed in patients with this disorder. Notably, the upregulation of KYNA levels in SP is often accompanied by increased tissue levels of kynurenine, the immediate KYNA bioprecursor (Schwarcz et al. 2001). Different mechanisms could be accounting for KYNA formation in SP: (1) increased TDO activity, (2) decreased KMO activity, (3) early infectious/inflammatory events affecting the brain, and (4) altered redox status. Taken together, these changes would serve to hypothesize the following order of events (summarized in Fig. 2), potentially leading to the pathological status involved in SP: First, an early inflammatory process probably due to an infectious origin would trigger metabolic alterations in peripheral and central KP, thus increasing the Trp and kynurenine availability in the brain, together with increased TDO and IDO activities and a concurrent KMO activity. The scenario produced by these changes would also imply increased levels of KYNA apparently produced by mechanisms other than KATs activation, i.e., via ROS formation and oxidative modifications, whose origins are either Trp conversion into 3-indole-pyruvic acid—further leading to KYNA when reacting with ROS—or kynurenine conversion—which, in the presence of H_2O_2 and a peroxidase, yields KYNA formation. In addition, if kynurenine

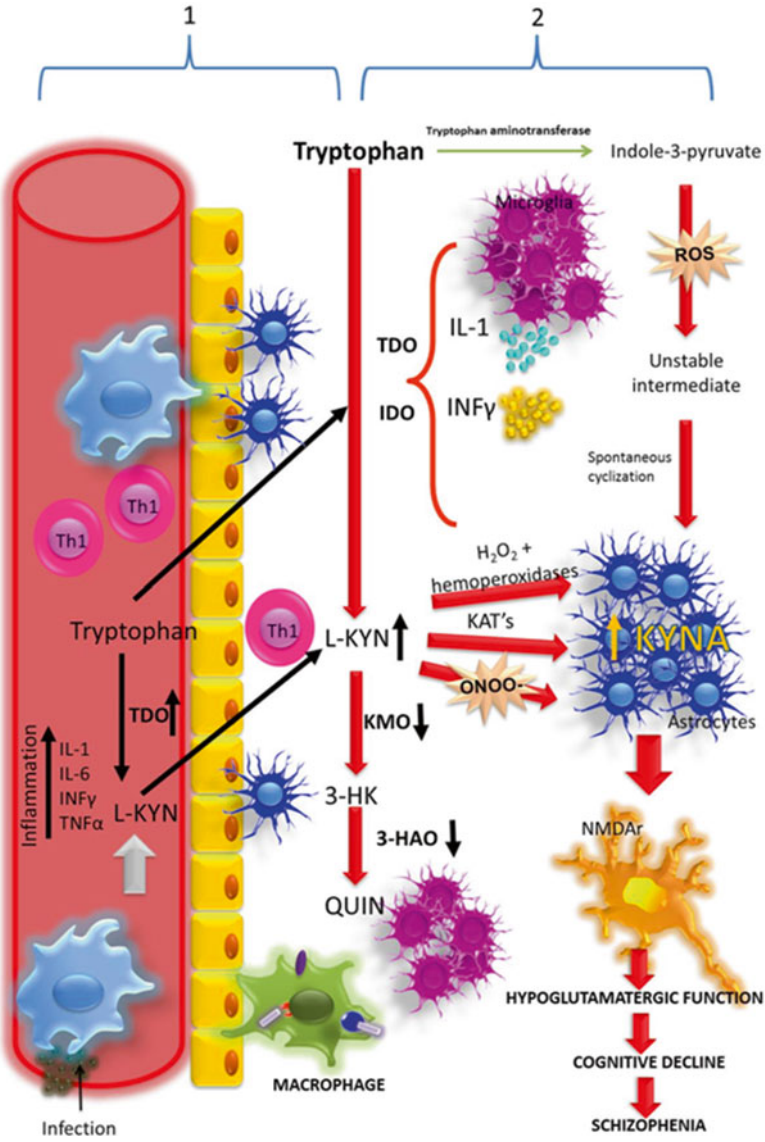

Fig. 2 Schematic representation of the mechanism underlying the events involved in the increases of KYNA levels in SP. In step 1, an inflammatory process due to possible infection or stress favors KP and its vulnerable brain barrier allowing passage of metabolites formed in the periphery to the CNS, in such events possibly early impact modified in later stages (2), in which the increase in KYNA levels seems to be the key of cognitive impairment present in patients with SP

actions recruit scavenger properties, as already reported (Ugalde-Muñiz et al. 2012), then kynurenine oxidation itself could account for KYNA formation (Lugo-Huitrón et al. 2011b). The latter would, in turn, explain why, during the early stages of SP, the levels of kynurenine and KYNA are both substantially increased, which also

matches with a hypoglutamatergic function typically observed in cognitive decline seen in SP patients. The precise degree of involvement of these events on the onset of SP constitutes a fertile line of research to explore in the next years. In the meantime, it is clear that KYNA hypothesis in SP is a promising tool to develop therapeutic designs for this and other psychiatric disorders.

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Dysregulation of Glutathione Synthesis in Psychiatric Disorders

Elżbieta Lorenc-Koci

1 Introduction

There is a growing body of evidence implicating oxidative stress mechanisms and the impaired redox regulation in the pathophysiology of diverse psychiatric disorders (Do et al. 2009; Steckert et al. 2010; Bitanihirwe and Woo 2011; Yao and Keshavan 2011). Oxidative stress defined in accordance with the free radical hypothesis refers to the cytopathological consequences of an imbalance between free radical production on the one side and deficiency of the antioxidant defense system on the other side. Brain cells are particularly vulnerable to oxidative damage due to relatively low to moderate activity of antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx) when compared to the liver or kidney, high levels of lipids and polyunsaturated fatty acids, high metal content, and high oxygen utilization (Dringen 2000; Valko et al. 2007). Hence, the free radical-mediated damage of important cellular molecules, like lipids, proteins, and DNA, leading to the impairment of cell function and vitality is currently considered as one of the main mechanisms in the pathophysiology of both neurodegenerative and psychiatric disorders (Bains and Shaw 1997; Valko et al. 2007; Ng et al. 2008; Steckert et al. 2010; Bitanihirwe and Woo 2011).

On the other hand, as the clinical trials with supplementation of free radical scavenging antioxidants show little benefit in humans, a complementary hypothesis for oxidative stress has been postulated (Jones 2006, 2008). This hypothesis, which is termed the "redox hypothesis" to facilitate its distinction from the "free radical hypothesis," assumes that disruption of the redox states of thiol systems which

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normally function in cell signaling and physiological regulations is the most central feature of oxidative stress. Three main thiol systems represented by thiol/disulfide redox couples exist in biological systems: (i) reduced glutathione (GSH) and its disulfide (GSSG), GSH/GSSG couple; (ii) cysteine (Cys) and its disulfide, cystine (CySS), Cys/CySS couple; and (iii) reduced and oxidized thioredoxins (Trx), Trx^{red}/Trx^{ox} couple (Kemp et al. 2008). All these systems are responsible for the maintenance of the appropriate redox potential in different cellular compartments (Jones 2006, 2008). The "redox hypothesis" draws attention to non-radical mechanisms of oxidative stress. It has been demonstrated that biological systems generate significantly more non-radical oxidants than free radicals (Jones 2008). Hydrogen peroxide (H_2O_2), the most abundant non-radical oxidant, is formed in many enzymatic reactions. For instance, H_2O_2 is the predominant product in the reaction catalyzed by xanthine oxidase although the superoxide radical anion ($O_2^{-\bullet}$) is also produced. Moreover, $O_2^{-\bullet}$ is converted into oxidant H_2O_2 in the reaction catalyzed by the antioxidant enzyme SOD. The free radicals $NO^{\bullet}$ and $O_2^{-\bullet}$ react to generate peroxynitrite ($ONOO^-$) that is a powerful oxidizing and nitrating non-radical intermediate (Thomas et al. 2006). Apart from the abovementioned oxidants, biological systems produce other oxidants, such as hydroperoxyfatty acids, aldehydes, quinones, epoxides, and disulfides. All these oxidants, albeit not free radicals, contribute importantly to the regulation of cellular redox state by modulation of the thiol-containing proteins that play important roles in cell-to-cell signaling, macromolecular trafficking, and physiological regulation (Jones 2008). Redox elements represented by sulfhydryl (–SH) residues of cysteine and thioether groups of methionine, found in the active site of many proteins, are susceptible to two-electron oxidants. Hence, the two-electron oxidation can be considered to be a component of oxidative stress that is distinct from free radical-mediated macromolecular damage. The function of these thiol-containing proteins is controlled by thiol antioxidants, GSH, Cys, and Trx^{red}, which are able to prevent the two-electron oxidation. Therefore, the appropriate levels of these antioxidants are of great importance for the maintenance and regulation of the thiol redox status of the cells.

The scope of this chapter is to review the available literature referring to GSH synthesis and its multiple functions in the central nervous system. Particular attention will be focused on GSH deficit and dysregulation of redox state in psychiatric disorders such as schizophrenia and bipolar disorder. Oxidative stress-mediated alterations in psychiatric patients will be compared to those observed in animal models of GSH deficiency. Finally, a treatment restoring the redox balance will be discussed in the context of therapy of psychiatric disorders.

2 Functions of Glutathione

Glutathione (γ-glutamyl-L-cysteinylglycine, GSH), a cysteine-containing tripeptide and the most abundant nonprotein thiol, is present in the mammalian brain with an average concentration of 2–3 mM, but there are marked differences in its content between particular cell types (Dringen 2000). Neurons have much less GSH than

glial cells. Among glial cells, microglia contains the highest amount of GSH, while oligodendrocytes, which are affected in schizophrenia, the lowest level. Astrocytes, like microglial cells, are characterized by a relatively high level of GSH (Dringen 2000). Glutathione exists predominantly in the thiol, i.e., reduced (GSH) form (99 %), while the disulfide, i.e., oxidized form (GSSG), represents less than 1 % of the total glutathione pool under physiological conditions.

2.1 The Main Functions of Glutathione

In the mammalian cells, GSH plays a lot of diverse functions including (1) scavenging of free radicals; (2) detoxification of xenobiotics; (3) maintenance of the redox state of proteins; (4) providing a nontoxic storage form of cysteine; (5) modulation of critical cellular processes, such as DNA synthesis and repair, cell proliferation, and redox signaling; (6) regulation of nitric oxide homeostasis; and (7) modulation of the activity of glutamate receptors in the central nervous system (Jánaky et al. 1998, 1999; Oja et al. 2000). GSH serves as an endogenous NO reservoir to form S-nitrosoglutathione (GSNO) (Hogg 2002). In the brain, GSNO can also elicit neuroprotective effect under oxidative stress conditions (Rauhala et al. 1998).

Thus, given many critical processes that are affected by GSH, it is not surprising that disturbances in its homeostasis have been implicated in the etiology and/or progression of a number of human diseases, including neurodegenerative and neuropsychiatric diseases (Ballatori et al. 2009; Do et al. 2009).

2.2 Antioxidant Activity of Glutathione

As an antioxidant, GSH scavenges reactive oxygen species (ROS) generated during electron transport and cellular metabolism of endo- and exogenous compounds. GSH is also involved in the disposal of hydrogen peroxide (H_2O_2) and hydroperoxides, which are non-radical oxidants produced during different cellular processes, strongly affecting redox state of cells. The detoxification of ROS and peroxides is associated with two types of reaction. Firstly, GSH reacts directly and nonenzymatically with such radicals as superoxide radical anion ($O_2^{-\bullet}$), nitric oxide ($NO^{\bullet}$), or hydroxyl radical ($HO^{\bullet}$) (Hogg 2002; Winterbourn and Metodiewa 1994). The removal of $HO^{\bullet}$ via this route is one of the most important functions of GSH in the nervous system:

$$2\,GSH + 2\,HO^{\bullet} \longrightarrow GSSG + 2\,H_2O$$

Secondly, GSH is an electron donor in the reduction of peroxides, mainly H_2O_2 in the reaction catalyzed by glutathione peroxidase (GPx):

$$H_2O_2 + 2\,GSH \xrightarrow{GPx} GSSG + 2H_2O$$

Final products of the GPx-mediated reduction of H_2O_2 comprise water and glutathione disulfide (GSSG). Also, catalase can reduce H_2O_2, but the brain has relatively low level of this enzyme as compared with that of GPx (Dringen and Hamprecht 1997). GSSG is then reduced back to GSH by glutathione reductase (GR):

$$\mathrm{GSSG} + \mathrm{NADPH} + \mathrm{H}^{+} \xrightarrow{\mathrm{GR}} 2\,\mathrm{GSH} + \mathrm{NADP}^{+}$$

This enzyme transfers electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to GSSG, thereby regenerating GSH. Hence, the detoxification of peroxides depends on the availability and regeneration of NADPH. Organic peroxides can be reduced by GPx and glutathione S-transferase (GST). During the reactions catalyzed by GPx and GR, glutathione is not consumed but recycled. The relative ratio of the reduced to oxidized glutathione (GSH/GSSG) serves as an indicator of the cellular redox environment. The maintenance of high GSH/GSSG ratio requires energy expenditure. The adult brain relies almost exclusively on the glucose oxidation to meet its energy requirements. The pentose phosphate pathway is present in the brain, especially in astrocytes, but only 3–5 % of glucose is converted via this pathway, while the rest is oxidized via the tricarboxylic acid cycle. Nevertheless, the pentose phosphate pathway is important in the brain as a means of providing NADPH for the GSSG reduction to GSH (Dringen 2000). Consistently, it has been demonstrated that the pentose phosphate pathway was strongly activated in cultured astrocytes during the detoxification of H_2O_2 (Ben-Yoseph et al. 1996). On the other hand, glucose deprivation of astrocyte cultures significantly reduced astrocyte ability to remove H_2O_2 (Dringen and Hamprecht 1997) and prolonged regeneration time of GSH from GSSG after treatment with hydroxyperoxides (Dringen et al 1998).

The third important role of GSH is associated with the maintenance of intracellular redox homeostasis. Protein S-glutathionylation, the reversible formation of mixed disulfides between glutathione and low-pKa cysteinyl residues of proteins, is thought to be a regulatory and antioxidant mechanism (Dalle-Donne et al. 2007; Mieyal et al. 2008). The binding of glutathione molecules to proteins to form mixed disulfides protects protein –SH groups against irreversible oxidation to $–SO_2H$ and $–SO_3H$. Hence, protein S-glutathionylation is an important mechanism for a dynamic, posttranslational modification of a variety of regulatory, structural, and metabolic proteins as well as for the regulation of signaling routes and metabolic pathways (Dalle-Donne et al. 2007; Mieyal et al. 2008). This modulation of proteins is not only a cellular response to mild oxidative/nitrosative stress but also occurs under physiological conditions.

2.3 Detoxifying Function of Glutathione

GSH reacts with various endogenous compounds and xenobiotics in the reaction catalyzed by glutathione S-transferase (GST) to form glutathione S-conjugates which are exported to the outside of the cell (Commandeur et al. 1995; Salinas

and Wong 1999). There is only one enzyme, γ-glutamyl transpeptidase (γ-GT), localized on the outer side of the plasma membrane of certain cell types that is able to hydrolyze GSH conjugates to cysteinyl-glycine conjugates. The cysteinyl-glycine bond is then cleaved by dipeptidase, resulting in a cysteinyl conjugate that following N-acetylation is further metabolized to mercapturic acid.

Lipid peroxidation induced by ROS leads to the conversion of polyunsaturated fatty acids to highly reactive aldehydes, such as 4-hydroxynoneal (4-HNE), that inactivate proteins required for cell viability (Esterbauer et al. 1991). Therefore, rapid and efficient removal of these compounds is necessary to maintain cell function. GSH can react with 4-HNE via the action of GST to form GSH-HNE conjugates (Xie et al. 1998) which are then exported from the cells via transport-mediated efflux (Berhane et al. 1994). This process plays an important role in cellular detoxification. Furthermore, in the brain, GST detoxifies quinones that are formed during the oxidation of dopamine and other catecholamines (Baez et al. 1997; Dagnino-Subiabre et al. 2000). The latter reactions irreversibly consumes intracellular GSH, and without supplementation of GSH stores, the formation of glutathione S-conjugates can rapidly compromise cellular antioxidant capacity, finally leading to the enhanced production of ROS and disruption of the cellular redox balance.

2.4 Specific Function of Extracellular Glutathione in the Brain

The presence of GSH in the extracellular space has been confirmed using microdialysis method (Yang et al. 1994; Lada and Kennedy 1997). Experiments performed on brain slices demonstrated that GSH was released by depolarization induced by high K^+ concentration in a Ca^{2+} dependent manner, which indicates its origin from a neuronal compartment (Zängerle et al. 1992). The mechanism of this release is unknown; however, the fact that it is Ca^{2+} dependent suggests that GSH is released by a vesicular mechanism similar to that of classical neurotransmitters or its efflux is under the control of a released neurotransmitter. In the rat brain slices, the most prominent release of GSH was observed in the mesodiencephalon, cortex, hippocampus, and striatum and lowest in the pons-medulla and cerebellum (Zängerle et al. 1992). On the other hand, studies performed in cell culture have shown that astrocytes are mainly involved in GSH release and together with γ-GT affect its extracellular level (Sagara et al. 1996; Dringen et al. 1997). Consistently, it has been calculated that cultured astrocytes release about 10 % of the intracellular pool of GSH within one hour (Dringen et al. 1997). So, the astrocyte-mediated release of GSH is the process consuming the largest amount of this peptide.

Although GSH plays a crucial role in many cellular processes, its extracellular functions are less known. However, in the nervous system, besides these generally known functions, GSH may serve additionally as a modulator of ionotropic glutamate receptors or as a new neurotransmitter (Jánaky et al. 1998, 1999, 2000; Ogita et al. 1998; Pasqualotto et al. 1998). So, it has been demonstrated that GSH binds via γ-glutamyl moiety to ionotropic glutamate receptors, preferentially AMPA

and NMDA (Janáky et al. 1999). At micromolar concentrations, GSH displaces excitatory agonists from their binding sites, acting to halt their physiological actions on target neurons (Janáky et al. 1999; Oja et al. 2000). Since AMPA and NMDA receptors are colocalized and cooperate at postsynaptic membranes, the co-release of glutamate and GSH from nerve endings (Hjelle et al. 1998) may have profound consequences in synaptic transmission. According to the model of GSH synaptic actions proposed by Janáky et al. (1999), this peptide may inhibit the fast depolarization evoked by glutamate via AMPA receptors and thus inhibit the voltage-dependent opening of NMDA receptor ionophores. Hence, the co-release of glutamate and GSH would lead to a cascade of events enabling the receptors to be reactivated within the short time (Janáky et al. 1999; Oja et al. 2000). Moreover, GSH (at millimolar concentrations) acting via its cysteinyl thiol group can modulate the redox site of NMDA receptors (Janáky et al. 1999; Oja et al. 2000). As such modulation has been shown to increase NMDA receptor ion channel currents, this action may play a significant role in normal and abnormal synaptic activity. Finally, it has been demonstrated that GSH at nanomolar to micromolar range binds to at least two populations of binding sites that are distinct from any known glutamate receptor subtypes. It is believed that these binding sites represent a unique population of GSH receptors. GSH binds to these receptors via cysteinyl moiety and is not displaceable by glutamatergic agonists or antagonists (Shaw et al. 1996; Janáky et al. 1999, 2000). The application of GSH to cortical slices elicits a fast depolarizing potential that is markedly larger than that produced by NMDA and AMPA (Shaw et al. 1996). The GSH current appears to be linked to sodium ionophores as it was blocked by the absence of sodium ions but not by lowering of calcium or NMDA or AMPA antagonists (Shaw et al. 1996; Janáky et al. 1999, 2000). These reports suggest that GSH receptors may be a key component of cortical excitatory neurotransmission (Shaw et al. 1996).

3 Synthesis of GSH

3.1 Biosynthesis of GSH in the Brain

GSH is synthesized from its constituent amino acids, i.e., glutamate, cysteine, and glycine, in the cytosol of all mammalian cells by the consecutive action of two enzymes requiring adenosine triphosphate (ATP) as a co-substrate (Dringen 2000; Lu 2009):

$$\text{L-glutamate} + \text{L-cysteine} + \text{ATP} \rightarrow \gamma\text{-glutamyl-L-cysteine} + \text{ADP} + \text{Pi} \quad (1)$$

$$\gamma\text{-glutamyl} + \text{L-cysteine} + \text{L-glycine} + \text{ATP} \rightarrow \text{GSH} + \text{ADP} + \text{Pi} \quad (2)$$

In the first step of GSH biosynthesis, glutamate cysteine ligase (GCL, EC 6.3.2.2; formerly γ-glutamylcysteine synthetase) catalyzes the formation of the dipeptide γ-glutamyl-L-cysteine (γ-GluCys) from glutamate and cysteine (Reaction 1).

This reaction exhibits absolute requirement not only for ATP but also for Mg^{2+} or Mn^{2+} (Franklin et al. 2009). The second step of GSH biosynthesis is catalyzed by GSH synthase (GS, EC 6.3.2.3; formerly known as GSH synthetase) which ligates glycine to γ-GluCys, thus forming GSH (Reaction 2).

Cysteine is the rate-limiting substrate for GSH synthesis (Dringen et al. 1999). In the brain, mature neurons use exclusively cysteine for GSH synthesis, while astrocytes utilize both cystine and cysteine (Dringen et al. 1999; Dringen 2000). Cystine is taken up by astrocytes and microglial cells via cystine/glutamate exchanger also known as system xc^- (Shih et al. 2006). Only immature neurons express the xc(−) transport system (Murphy et al. 1990), while mature ones do not possess this transporter and therefore are unable to take up cystine for GSH synthesis. In the mature brain, neurons rely mainly on cysteine derived from GSH released by astrocytes into the extracellular space, where GSH is cleaved by sequentially acting the membrane-bound enzymes γ-GT and dipeptidase to constituent amino acids (Dringen et al. 1999; Dringen 2000). In addition to cysteine, neurons can utilize the cysteine-containing dipeptides γ-GluCys or cysteinylglycine (CysGly) for GSH synthesis (Dringen et al. 1999), although it is unclear how these dipeptides are taken up into neurons. Neural uptake of cysteine is mediated primarily by sodium-dependent excitatory amino acid transporter (EAAT) systems, known as excitatory amino acid carrier 1 (EAAC1 also termed EAAT3) (Shanker et al. 2001; Chen and Swanson 2003; Himi et al. 2003). EAAC1-deficient mice showed 30–40 % decreases in brain GSH levels, increased vulnerability to oxidative stress, as well as developed brain atrophy and behavioral abnormalities (Aoyama et al. 2006). The abovementioned data clearly indicate that transport of cysteine by EAAC1 system is also a rate-limiting factor for GSH synthesis in neurons.

3.2 Regulation of GSH Synthesis

Under physiological conditions, the rate of GSH synthesis depends on the expression and catalytic activity of GCL (Dalton et al. 2004; Dickinson et al. 2004). GCL is a heterodimeric protein composed of a heavy or catalytic (GCLC, Mr ~ 73,000) and a light or modifier (GCLM, Mr ~ 30,000) subunit, which are encoded in humans and rodents by different genes (Gipp et al. 1992; Dalton et al. 2004; Franklin et al. 2009). Only GCLC possesses all the enzymatic activity and is a subject of feedback inhibition by the end product GSH (Richman and Meister 1975; Seelig et al. 1984). GCLM is enzymatically inactive but plays an important regulatory function by increasing the V(max) and K(cat) of GCLC, by lowering the K(m) of GCL for glutamate and ATP, and by raising the K(i) for GSH-mediated feedback inhibition of GCL (Chen et al. 2005; Lu 2009; Yang et al. 2007). Thus, the holoenzyme is catalytically more efficient and less susceptible to inhibition by GSH than GCLC alone. GCLC-knockout mice showed embryonic lethality, demonstrating that the gene encoding this subunit was critical for development (Dalton et al. 2000, 2004). In turn, GCLM-knockout ($Gclm^{-/-}$) mice are viable, but in the absence of this subunit,

GCLC activity is insufficient, leading to a decrease of GSH level (Yang et al. 2002; Dalton et al. 2004). Alterations in GCL activity can result from regulation at multiple levels affecting only catalytic (GCLC) or both catalytic and modifier (GCLM) subunits of this enzyme. Many studies have focused on transcriptional regulation of GCL at the promoter level (Lu 2009).

GSH synthase (GS), the second enzyme participating in GSH biosynthesis, is composed of two identical subunits and has a Mr of approximately 118,000 Da. Mapping studies of the GS substrate binding sites indicate that the regions of the active site that bind glycine and the cysteinyl moiety of γ-GluCys are highly specific, while the γ-glutamyl moiety can be replaced by a variety of analogs. However, in contrast to GCL, GS is not subject to feedback inhibition by GSH. Moreover, GS overexpression had no effect on GSH level, whereas GCL overexpression markedly increased GSH level (Grant et al. 1997). Hence GCL, but not GS, is considered to be the rate-limiting enzyme in the GSH synthesis.

4 Oxidative Stress and GSH Synthesis in Schizophrenia and Bipolar Disorder

4.1 Glutathione Deficiency as a Marker of Oxidative Stress in Schizophrenia

Several studies have shown that the level of GSH, the major antioxidant and redox regulator, is decreased in a cerebrospinal fluid and medial frontal cortex (by 27 % and 52 % of control level, respectively) of drug-naïve schizophrenic patients (Do et al. 2000) as well as in the postmortem striatum (by 40 % of control) (Yao et al. 2006) and prefrontal cortex of those treated earlier with antipsychotic drugs (Gawryluk et al. 2011a). Moreover, there was a significant negative correlation between GSH levels and the severity of negative symptoms in schizophrenic patients (Matsuzawa et al 2008). In periphery, significantly lower levels of GSH were found in erythrocytes (Altuntas et al. 2000; Raffa et al. 2009, 2011; Micó et al. 2011) and plasma (Zhang et al. 2007; Dietrich-Muszalska et al. 2009; Raffa et al. 2009) of antipsychotic-free and chronically medicated schizophrenic patients in comparison to healthy control. As indicated by some genetic studies, the GSH deficit in schizophrenia seems to be linked to polymorphisms of genes encoding both catalytic (GCLC) and modifier (GCLM) subunits of glutamate cysteine ligase (GCL), an enzyme responsible for the biosynthesis of this tripeptide (Tosic et al. 2006; Gysin et al. 2007, 2009, 2011).

In addition, the activity of glutathione peroxidase (GPx), a key antioxidant enzyme involved in the elimination of hydrogen peroxide and lipid peroxides, was found to be unchanged (Mukerjee et al. 1996; Yao et al. 1998; Evans et al. 2003; Raffa et al. 2009), increased (Kuloglu et al. 2002; Raffa et al. 2011; Micó et al. 2011), or decreased (Abdalla et al. 1986; Altuntas et al. 2000) in erythrocytes of drug-naïve and antipsychotic-free schizophrenic patients when compared to

controls. In a majority of chronically medicated schizophrenic patients treated with typical or atypical antipsychotic drugs, GPx activity in erythrocytes was found to be decreased (Altuntas et al. 2000; Ranjekar et al. 2003; Zhang et al. 2006; Ben Othmen et al. 2008; Raffa et al. 2009), while only in a few studies it was unchanged (Reddy et al. 1991; Evans et al. 2003) or increased (Herken et al. 2001; Akyol et al. 2002). Apart from the cytosolic form of GPx, there exists a related enzyme, called human plasma glutathione peroxidase (hpGPx), that is localized exclusively extracellularly. The level of this enzyme was significantly and positively correlated with the psychosis rating scores in schizophrenic patients both on and off haloperidol treatment (Yao et al. 1999). Decreased levels of GPx and glutathione reductase (GR), suggesting attenuated antioxidant functions of these two enzymes, were also found in the caudate region of brains from schizophrenic patients (Yao et al. 2006). Furthermore, a significantly lower level of Mu class of GST isoenzyme in the prefrontal cortex of schizophrenic patients than in nonpsychiatric controls has also been reported (Gawryluk et al. 2011b). The decreased level of GST Mu indicates that these patients had lesser ability to remove xenobiotics and also to detoxify endogenous substances such as quinines and lipid peroxidation products.

All the above-described results clearly indicate that due to a deficit of GSH and the decreased scavenging ability of GSH-related antioxidant enzymes, the redox balance of GSH/GSSG couple in peripheral tissues and in the brain cells of schizophrenic patients has to be shifted in favor of oxidative processes.

4.2 Changes in GSH Redox Status in Bipolar Disorder

Although the exact mechanisms underlying bipolar disorder (BD) are not completely understood, some studies suggest an involvement of oxidative stress and alterations in GSH redox status in the pathophysiology of the disease (Andreazza et al. 2007; Kuloglu et al 2002; Ranjekar et al. 2003; Machado-Vieira et al. 2007). In patients with BD, like in schizophrenic patients, oxidative stress was assessed indirectly by measuring the activities of antioxidant enzymes (glutathione peroxidase, GPx; superoxide dismutase, SOD; and catalase, CAT) in erythrocytes and serum as well as by determination of the content of thiobarbituric acid reactive substances (TBARS) as an index of lipid peroxidation. These studies, although less numerous than in schizophrenia, demonstrated that enzymatic activities of GPx, SOD, and CAT were impaired in BD (Kuloglu et al. 2002; Ranjekar et al. 2003; Ozcan et al. 2004; Andreazza et al. 2007; Kunz et al. 2008; Machado-Vieira et al. 2007) and lipid peroxidation was significantly enhanced when compared to nonpsychiatric control subjects (Kuloglu et al. 2002; Ranjekar et al. 2003; Ozcan et al. 2004; Machado-Vieira et al. 2007; Andreazza et al. 2007, 2008; Kunz et al. 2008). In line with studies on antioxidant enzymes in blood, a postmortem study of the hippocampus from BD patients demonstrated a lowered gene expression for several antioxidant enzymes in that structure, including GPx, CAT, SOD, and glutathione S-transferase (GST) (Benes et al. 2006). Moreover, recently diminished level of

both reduced (GSH) and oxidized (GSSG) forms of glutathione in the prefrontal cortex (Gawryluk et al. 2011a) as well as oxidative damage to mitochondrial proteins (Andreazza et al. 2010) in that structure, increased lipid peroxidation in the cingulate cortex, and RNA oxidation in the hippocampus (Wang et al. 2009; Che et al. 2010) have been reported in postmortem brain from BD patients.

In addition, in patients not medicated with mood stabilizers, the level of class Mu of GST was decreased, while in BD patients treated with these drugs, the level of this isoenzyme was not different from that observed in controls (Gawryluk et al. 2011b). The latter effect is consistent with previous studies which demonstrated that mood stabilizers such as lithium, lamotrigine, and olanzapine increased GST expression and activity in primary cultured rat cerebral cortical cells (Shao et al. 2008; Bakare et al. 2009). Furthermore, chronic treatment with lithium and valproate increased the glutamate cysteine ligase (GCL) expression and GSH levels in these cultures (Cui et al. 2007). Since GST conjugates GSH with a variety of oxidized compounds to form nontoxic products, it has been suggested that lithium and valproate selectively target GST isoenzymes in order to produce neuroprotective effects against oxidative stress (Cui et al. 2007; Shao et al. 2008).

Summing up, all these studies suggest that oxidative stress and disturbances in GSH homeostasis play a significant role in the etiology of other psychiatric illnesses besides schizophrenia, e.g., BD, and the drugs increasing GSH content exert beneficial therapeutic effects in the treatment of this disease.

5 GSH-Deficient Animal Models of Schizophrenia

In general, the lowering of GSH level may result either from genetically determined alterations in the activities of GSH synthesizing enzymes (Tosic et al. 2006; Gysin et al. 2007, 2009) or from the limited availability of cysteine, a substrate for GSH synthesis (Dringen 2000). In experimental animals, the tissue GSH content can be decreased by inhibition of GCL enzymatic activity using specific compounds (Broquist 1992), by modulation of genes encoding catalytic or modifier subunits of this enzyme (Yang et al. 2002; Dalton et al. 2000, 2004), or by GSH depletion evoked by different endogenous or exogenous compounds that oxidize or conjugate the thiol group of this tripeptide (Masukawa et al. 1989). All these models have been used to check whether GSH deficit can lead to behavioral, morphological, and biochemical anomalies similar to those observed in schizophrenic patients.

5.1 Behavioral Effects of Glutathione Deficiency in Animals

The consequences of brain GSH deficit on cognitive functions were examined in the abovementioned animal models. It has been demonstrated that the GSH deficit induced in adult rats by intracerebra, chronic administration of L-buthionine-(S,R)-sulfoximine (BSO) combined with intracerebral injection of dopamine (DA),

induced psychomotor (Shukitt-Hale et al. 1997) and spatial memory deficits in the water maze test (Shukitt-Hale et al. 1998).

According to the neurodevelopmental theory of schizophrenia, unknown gestational or perinatal events can impair brain development, leading to the establishment of an abnormal cerebral connectivity and detrimental effects of which may appear in adolescence, hence the consequences of GSH deficit during development were also studied in animal models (Rougemont et al. 2002; Castagné et al. 2004a, b; Cabungcal et al. 2007). It was found that rats treated with BSO between postnatal days 5 and 16 developed a strong ~50 % GSH deficit in various brain structures including the cortex (Rougemont et al. 2002). It is worth to mention that in the rat, the peak GSH concentration on postnatal day 7 is critical for brain development, as it occurs during a period of intensive synaptogenesis and may play the neuroprotective role during that time (Nanda et al. 1996). Rodents may compensate for GSH deficit by increasing ascorbic acid synthesis, as demonstrated in some studies on the Osteogenic Disorder Shionogi (ODS) mutant rats, which, like humans, cannot synthesize ascorbic acid (Castagné et al. 2004a, b; Cabungcal et al. 2007). Moreover, as the dysfunction of dopaminergic system is associated with schizophrenia, ODS rats were treated in the early postnatal period (between days 5 and 16) with BSO or the dopamine uptake inhibitor GBR 12909, alone and in combination, and later on in juvenile and adult rats, the object recognition test was performed (Castagné et al. 2004b). The object recognition test is based on the spontaneous tendency of rats to investigate objects and to favor novel objects versus familiar ones. In the latter study, it has been demonstrated that ODS rats receiving BSO and GBR 12909 failed to discriminate between familiar and novel objects, while ODS rats treated with either BSO or GBR 12909 alone had normal behavior in this test (Castagné et al. 2004b). Since after the combined treatment these rats preserved basic motor and sensory skills, the alterations observed in the test of object recognition can be attributed to cognitive impairment. The fact that ODS rats treated with BSO and GBR 12909 did not investigate more intensively the novel object than the familiar one suggests that increased dopaminergic tone coinciding with GSH deficiency during development can result in the long-term cognitive deficit observed in adult rats (Castagné et al. 2004b). Hence, the observed disturbances in the object recognition test in ODS rats treated with BSO and GBR 12909 are in line with the decreased object recognition capacity of schizophrenic patients as compared to healthy control subjects (Danion et al. 1999; Doniger et al. 2001; Heckers et al. 2000).

On the other hand, in the genetic model of GSH deficit, that is, in GCLM-knockout ($Gclm^{-/-}$) mice, some subtle alterations in behavior of animals were observed (Steullet et al. 2010). In particular, $Gclm^{-/-}$ mice (4–6 months old) displayed an increased novelty-induced exploration, altered behavior during the object recognition task, altered emotion and stress-related behaviors, and lower response to delayed fear conditioning but had intact spatial learning and spatial memory (Steullet et al. 2010). The authors of the latter study revealed that genetically compromised GSH synthesis in $Gclm^{-/-}$ mice affected the morphological and functional integrity of hippocampal parvalbumin-immunoreactive (PV-ir) fast-spiking interneurons (FSIs), which are known to be altered in schizophrenia. In that study, it was demonstrated that the decreased GSH level in $Gclm^{-/-}$ mice caused a selective reduction of PV-ir

interneurons in CA3 and dentate gyrus of the ventral hippocampus (VH) but not the dorsal hippocampus (DH) and a concomitant reduction of β/γ oscillations (Steullet et al. 2010). According to those authors, the altered behavior of Gclm$^{-/-}$ mice was associated with the functional disruption of the VH. Therefore, the hippocampus-dependent behaviors, known to implicate differentially the VH and DH, observed in Gclm$^{-/-}$ mice were discussed on the background of other literature data, in comparative manner. Mice with functional disruption of PV-ir FSI in the whole hippocampus had deficit in recognition of novel spatial arrangement of familiar objects and in novel object recognition (Fuchs et al. 2007). Gclm$^{-/-}$ mice that had dysfunctional only the VH recognized changes in spatial arrangement of objects, a task that requires functional DH (Gaskin et al. 2009), but explored novel and familiar objects with the same intensity. Thus, Gclm$^{-/-}$ mice recognized spatially displaced objects but showed altered behavior during an object recognition task. Moreover, mice with functional disruption of PV-ir FSI in the whole hippocampus had also impaired spatial working memory (Fuchs et al. 2007), while Gclm$^{-/-}$ mice that had normally functioning DH did not show such deficit. Consequently, mice with functional disruption of PV-ir FSI in the whole hippocampus were hypoactive, while Gclm$^{-/-}$ mice showed potent novelty-induced exploration. Steullet et al. (2010) suggest that hyperactivity in Gclm$^{-/-}$ mice could be induced by a decreased GABA inhibition in the VH. Finally, altered emotion and stress-related behaviors in Gclm$^{-/-}$ mice were in line with specific disruption of the VH but not the DH. It should be mentioned here that there is a growing evidence of structural and functional anomalies of the anterior hippocampus, a region of human brain that corresponds to the VH in rodents, in schizophrenic patients (Goldman and Mitchell 2004).

Moreover, in the most recent study, Kulak et al. (2012) using a different package of behavioral tests demonstrated that Gclm$^{-/-}$ mice displayed hyperlocomotion in the open field and forced swimming test but normal activity in home cage, suggesting that hyperlocomotion was selective to environmental novelty and mildly stressful situations. In the study by Kulak et al. (2012), similarly as in that performed by Steullet et al. (2010), spatial working memory in Gclm$^{-/-}$ mice remained unaffected. Gclm$^{-/-}$ mice showed a potentiated hyperlocomotor response to an acute amphetamine injection, impaired sensorimotor gating (prepulse inhibition), and altered social behavior when compared to wild-type mice (Kulak et al. 2012).

Altogether, the experimental data from different animal models of GSH deficiency reported above show that low level of this antioxidant and redox regulator can induce behavioral alterations that are relevant to those observed in schizophrenia.

5.2 Biochemical Consequences of Glutathione Deficiency in In Vitro and In Vivo Models

Glutathione deficiency seems to be related to the dysfunction of central dopaminergic, glutamatergic, and GABAergic neurotransmissions that are known to be implicated in the pathogenesis of schizophrenia (Laruelle et al. 2003; Moghaddam 2003; Cabungcal et al. 2006).

5.2.1 The Effect of Glutathione Deficit on Dopaminergic System Function

According to the dopaminergic hypothesis of the disease, a decrease in dopamine (DA) release in the prefrontal cortex (PFC) has been associated with negative symptoms, particularly cognitive deficits, while disinhibition of DA release in the dorsal striatum (nucleus accumbens) with the manifestation of positive symptoms, such as delusions and hallucinations. Evidence for dopaminergic dysfunction in schizophrenia is in majority indirect and is mainly based on the fact that most antipsychotic drugs being antagonists of DA D_2 receptors alleviate some symptoms of this disease (Seeman et al. 1975; Kane and Marder 1993), while DA-releasing stimulants, such as amphetamine, induce psychosis (Janowsky and Risch 1979). Although the mechanisms underlying dopaminergic dysfunction in schizophrenia have not been elucidated yet, the hypothesis of GSH deficiency creates possibility to explain, at least, some of its aspects.

The Potential Role of Glutathione Deficiency in the Loss of Dendritic Spines

Metabolism of DA is closely linked with both intracellular and extracellular levels of GSH. DA is a major source of ROS in the mammalian brain, as an excess of this neurotransmitter can easily auto-oxidize to produce DA quinones that have potent oxidizing properties (Baez et al. 1997; Dagnino-Subiabre et al. 2000). Moreover, DA, via monoamine oxidase activity or DA quinones, through redox cycling, can induce the formation of H_2O_2 and $O_2^{\bullet -}$, which are known to cause lipid peroxidation, DNA modification, and protein oxidation (Baez et al. 1997; Bains and Shaw 1997; Rabinovic and Hastings 1998). Under physiological conditions, GSH detoxified DA quinones and H_2O_2 via reactions catalyzed by GST and GPx, respectively (see Sects. 2.2 and 2.3). However, under conditions of GSH deficiency, an excess of reactive intermediates of DA can disrupt cellular functions (Grima et al. 2003; Hastings et al 1996; Hirrlinger et al. 2002a), and this could contribute to the pathogenesis of schizophrenia. Although schizophrenia is not considered to be a neurodegenerative disease (Harrison 1997; Stevens and Casanova 1988; Garey 2010), low level of GSH in the PFC (Do et al. 2000; Gawryluk et al. 2011a) can sensitize neurons to DA-mediated dendritic degeneration (Hastings et al. 1996; Rabinovic and Hastings 1998; Grima et al. 2003). Hence, peroxidation reactions can lead locally to microlesions, affecting the synaptic contacts on dendritic spines of cortical pyramidal neurons, where excitatory glutamatergic terminals converge with dopaminergic ones (Goldman-Rakic et al. 1989). This may lead to the reduction of neuropil, mainly dendritic spines density, reported in postmortem histological studies of the PFC of schizophrenic patients (Selemon and Goldman-Rakic 1999; Glanz and Lewis 2000; Garey 2010; Glausier and Lewis 2012). As a degeneration of spines with their synaptic contacts may lead to abnormal cortico-cortical connectivity, these postmortem findings imply that neuronal integrity is compromised in schizophrenia. Thus, abnormal connectivity in the PFC may be responsible for part of symptoms, particularly those involving cognitive and perceptive functions (Garey 2010).

In line with the "reduced neuropil hypothesis" by Selemon and Goldman-Rakic (1999), it has been demonstrated that the application of DA under conditions of GSH deficiency into cultures of primary mouse cortical neurons induced a significant decrease in the number of neuronal processes which are considered to be spines analogous (Grima et al. 2003). Also, a deficit in brain GSH combined with a DA uptake inhibition during rat postnatal development caused a decrease in the number of dendritic spines of pyramidal neurons in the PFC (Do et al. 2004). So, morphological changes found in in vitro and in vivo studies could be related to morphological alteration reported earlier in the PFC of schizophrenic patients. Moreover, abnormal connectivity in the PFC was suggested by in vivo nuclear magnetic resonance imaging (NMR) studies in which it was found that N-acetyl aspartate (NAA), a marker of neuronal integrity, was decreased in schizophrenic patients (Callicott et al. 2000; Deicken et al. 2000; Yamasue et al. 2002). Interestingly, GSH deficiency decreases NAA level in the rat brain (Heales et al. 1995; Jain et al. 1991). Thus, it is likely that GSH deficiency and neuronal impairment are functionally linked in schizophrenia.

The Potential Role of Glutathione Deficiency in the Amphetamine-Induced DA Release in Subcortical Regions of the Brain

Besides the possible role of GSH deficiency in the loss of dendritic spines in the PFC (Grima et al. 2003; Do et al. 2004), it is believed that pathological low level of this antioxidant could cause disturbances in the dopaminergic neurotransmission (Jacobsen et al. 2005). The effect of a short-lasting GSH deficiency induced by the GSH synthesis blocker, BSO, on extracellular DA level in the nucleus accumbens of mice receiving amphetamine was investigated using a microdialysis method (Jacobsen et al. 2005). The latter study revealed that extracellular DA release after amphetamine (5 mg/kg) was increased twofold in the nucleus accumbens of GSH-deficient mice as compared to control mice with normal GSH level (Jacobsen et al. 2005). GSH deficiency per se did not change basal extracellular level of DA in the examined brain structure. These results indicate that GSH-deficient mice may experience accumbal hyperdopaminergia when DA transmission is considerably enhanced. The exacerbated amphetamine-induced DA release in the mouse model of GSH deficiency is consistent with the elevated amphetamine-induced DA release in the striatum of schizophrenic patients demonstrated by means of a single photon emission computed tomography (SPECT) and positron-emission tomography (PET) methods (Laruelle et al. 1996; Breier et al. 1997). Moreover, Laruelle et al. (1996) have shown that the elevated amphetamine effect in schizophrenic patients was associated with emergence or worsening of positive psychotic symptoms. These results suggest that psychotic symptoms in schizophrenia are related with exaggerated stimulation of dopaminergic transmission.

The mechanism underlying the interplay between GSH and DA after amphetamine administration in conditions of GSH deficiency is difficult to explain. However, based on previous reports on the general properties of GSH and DA, plausible explanations for these findings can be suggested. It is well known that GSH

directly conjugates DA in vitro (Baez et al. 1997; Dagnino-Subiabre et al. 2000) and in vivo (Rabinovic and Hastings 1998). Since extracellular concentration of GSH is relatively high (1–2 μM; Lada and Kennedy 1997) and astrocytes permanently release this antioxidant into extracellular space (Dringen et al. 1997; Hirrlinger et al. 2002b), it is likely that GSH could scavenge the released DA. The amount of GSH released by astrocytes is correlated with the intracellular GSH content (Sagara et al. 1996). Therefore, deficit in intracellular GSH may lead to a concomitant decrease in extracellular pool of this antioxidant. Since GSH directly affects DA transmission by extracellular conjugation, deficit in its extracellular level could impair this mechanism that keeps the released DA under control. However, whether DA-GSH conjugation has functional significance remains to be established.

The Potential Role of Glutathione Deficiency in the DA-Mediated Modulation of Glutamatergic Transmission in the Prefrontal Cortex

GSH plays an important role in the redox control of various signal transduction pathways and gene expression (Sen 2000; Esposito et al. 2004). Thus, GSH deficit can alter the function of redox-sensitive proteins implicated in neurotransmission and synaptic plasticity such as NMDA receptors (Köhr et al. 1994; Choi et al. 2001), $GABA_A$ receptors (Amato et al. 1999), and ryanodine receptors (Bull et al. 2003) as well as calcium-activated K^+ channels (DiChiara and Reinhart 1997) and L-type calcium channels (Campbell et al. 1996). These redox-sensitive proteins could affect neurotransmitter systems, i.e., dopaminergic, glutamatergic, and GABAergic, that are known to be dysfunctional in schizophrenia. In this section, a potential role of GSH deficiency in the DA-mediated modulation of glutamatergic transmission in the prefrontal cortex (PFC) will be discussed.

In the PFC, DA plays an important role in cognitive functions including working memory, reword, and attention. DA-containing neurons are localized in the ventral tegmental area and project to the PFC. A critical function of DA in this brain region is a modulation of glutamatergic transmission. NMDA responses are known to be modulated by DA via the activation of D_1 and D_2 receptors through multiple pathways acting on different targets, including NMDA receptors and voltage-gated calcium channels (Tseng and O'Donnell 2004). In brain slices obtained from developmentally matured rats, it was demonstrated that the application of NMDA and D_1 agonist SKF38393 induced concentration-dependent excitability increases measured by whole-cell patch clamp technique, whereas the application of the D_2 receptor agonist quinpirole induced concentration-dependent excitability decrease. The NMDA-mediated responses were potentiated by a D_1 agonist while they were attenuated by a D_2 agonist (Tseng and O'Donnell 2004).

The hypofunction of NMDA receptors reported in schizophrenia (Laruelle et al. 2003; Moghaddam 2003; Labrie and Roder 2010) could be evoked by pathologically low level of GSH in the brain. Therefore, it was interesting to check whether GSH deficiency could change DA receptor-mediated signaling in the PFC. Consistently, Steullet et al. (2008) examined, in cultures of embryonic cortical

mouse neurons treated with BSO, how GSH deficiency influenced intracellular pathways implicated in DA signaling, namely, DA modulation of calcium responses to NMDA. In this study, it was shown that in the absence of DA, calcium responses evoked by NMDA were significantly larger in GSH-deficient neurons than in control ones (Steullet et al. 2008). In further experiments, it was established that the increased calcium responses to NMDA were due to the increased function of both L-type calcium channels and ryanodine receptors (RyRs), but not NMDA receptors. So, caffeine, an agonist of RyRs, induced significantly larger calcium release from internal stores in BSO-treated neurons than in control ones, confirming in this way the enhanced function of RyRs in the GSH-deficient neurons (Steullet et al. 2008). Moreover, the increase in the function of RyRs in neurons with low GSH content was in line with the finding that RyRs were redox sensitive, with oxidative conditions enhancing their function (Bull et al. 2003).

DA administration decreased calcium responses evoked by NMDA in GSH-deficient neurons but enhanced them in control ones. To exclude unspecific effects of BSO, it was evidenced that replenishing GSH levels by administration a membrane-permeable GSH analog abolished DA-mediated decrease in calcium responses to NMDA in the cultured GSH-deficient neurons. Since the blockade of DA D_2 receptor by sulpiride caused a significant increase in calcium responses in GSH-deficient neurons but not in control ones, it was concluded that DA acting via DA D_2 receptors decreased calcium responses evoked by NMDA under conditions of GSH deficiency (Steullet et al. 2008). In contrast, the blockade of DA D_1 receptors with SCH23290 did not have any significant effect on DA-mediated modulation of calcium responses in GSH-deficient neurons while tending to decrease them in control neurons. The latter effect suggested that the activation of DA D_1 receptors was involved in DA-induced increase of calcium responses evoked by NMDA only in control neurons.

So, the above-described results showed that a GSH deficit changed DA modulation of calcium responses evoked by NMDA. In cultured cortical neurons, NMDA-evoked calcium responses resulted from an initial calcium influx via the activation of NMDA receptors followed by secondary calcium influxes, through voltage-gated calcium channels (L-type channels) and calcium release from intracellular stores via the activation of RyRs (Hayashi et al. 1997). Steullet et al. (2008) examined which of these calcium sources contributing to the total response evoked by NMDA were altered by DA modulation under conditions of GSH deficiency. Consequently, it was demonstrated that DA decreased the calcium influx through L-type channels in GSH-deficient neurons but enhanced it in control ones. Such an effect of a GSH deficit on L-type channels was also observed when these channels were activated by either KCl or by specific agonist, BAY-K8644. DA is known to either increase the function of L-type channels via the activation of DA D_1 receptors or decrease it via the activation of DA D_2 receptors (Tseng and O'Donnell 2004). Hence, the results presented by Steullet et al. (2008) suggested that a GSH deficit strengthened DA D_2 receptor-mediated decrease in the function of L-type calcium channels, occluding DA D_1 receptor-mediated increase in the function of these channels.

Regarding internal source of calcium, Steullet et al. (2008) have found that the alteration of DA signaling in GSH-deficient neurons required the redox-sensitive RyRs. Because of the enhanced function of RyRs under condition of oxidative stress, DA evoked a larger release of calcium from intracellular stores in neurons containing a low level of GSH than in normal control. This, in turn, promoted in GSH-deficient neurons a decrease in the function of L-type channels via DA D_2 receptor-mediated calcium-dependent pathway, whereas in control neurons, the function of these channels was enhanced. So, the deficit of GSH affected DA modulation of L-type channels but not the other calcium sources implicated in the responses to NMDA. As a consequence of the specific alteration of DA modulation of L-type channels, DA decreased NMDA responses in GSH-deficient neurons but increased them in normal neurons. So, GSH deficit, as that observed in some groups of schizophrenic patients (Do et al 2000; Yao et al. 2006; Gawryluk et al. 2011a), could play a significant role in the pathophysiology of this disease via dysregulation of dopaminergic and glutamatergic transmissions.

5.2.2 The Effect of Glutathione Deficiency on Glutamatergic System Function

In addition to dopaminergic abnormalities, also glutamatergic dysfunction has been associated with the pathogenesis of schizophrenia (Laruelle et al. 2003; Moghaddam 2003; Labrie and Roder 2010). Consistent with this view, reduced NMDA receptor function has been proposed as a cause of schizophrenia, because noncompetitive NMDA receptor antagonists like phencyclidine and ketamine induce psychotic and cognitive symptoms in healthy humans (Moghaddam 2003; Kantrowitz and Javitt 2010) and exacerbate symptoms in schizophrenic patients (Javitt and Zukin 1991; Krystal et al. 1994, 2005). Moreover, a loss of dendritic spines from cortical and hippocampal pyramidal neurons may be combined with the glutamatergic hypothesis of schizophrenia as NMDA receptors are present on their dendrites and probably dendritic spines. It is well documented that the vast majority of excitatory synapses (80–95 %) in the central nervous system are formed onto dendritic spines (Wilson 2007) and, as such, the spines perform a significant role in regulating neuronal excitability. In mature neuronal systems, pharmacological blockade of AMPA receptors or surgical deafferentation of glutamatergic inputs resulted in decreased spine density (Smart and Halpain 2000; Jacobs et al. 2003). Additionally, two recent studies showed that a constitutive reduction in NMDA receptor activity results in decreased spine density and cortical volume in the PFC and sensory cortex (Balu et al. 2012; DeVito et al. 2011). Although the loss of dendritic spines in schizophrenia was reported earlier (Glausier and Lewis 2012), the cause of NMDA receptor hypofunction in this disease has not been established as yet. However, an increasing number of experimental data suggest that GSH deficit may be an important factor contributing to this phenomenon (Steullet et al. 2006, 2010; Do et al. 2009). The potential role of DA in the loss of dendritic spines in condition of GSH deficiency

has been presented in section "The Potential Role of Glutathione Deficiency in the Amphetamine-Induced DA Release in Subcortical Regions of the Brain", whereas the role of this antioxidant in the regulation of NMDA and AMPA receptor functions is presented in Sect. 2.4.

GSH can affect NMDA receptor function via binding to its regulatory, redox-sensitive site and to glutamate recognition site. Redox sites of NMDA receptor are unusually sensitive to the oxidizing potential of the extracellular environment (Aizenman et al. 1989). Hence, oxidizing agents diminish NMDA receptor function, while reducing compounds, including GSH, enhance it (Köhr et al. 1994; Choi et al. 2001). Since GSH is the main regulator of the brain redox systems, it was assumed that GSH deficiency of the same magnitude as in schizophrenic patients (about 50 % of the control level) could lead to the dysfunction of NMDA receptors. A low GSH level could also alter NMDA receptor function via non-redox mechanisms because GSH can bind via its γ-glutamyl moiety to the glutamate recognition sites of NMDA and AMPA receptors and in this way modulate their function (Varga et al. 1997; see Sect. 2.4). To check experimentally whether GSH deficit could be a causal factor for NMDA hypofunction reported in schizophrenia, Steullet et al. (2006) examined in the CA1 region of the rat hippocampus how GSH deficit, induced by BSO administration, altered basal neurotransmission, cell excitability, and short-term and long-term plasticity. Using electrophysiological techniques, it was demonstrated that in hippocampal slices with low GSH level, the basal excitatory synaptic transmission that mostly depends on the AMPA receptor activation was not changed but NMDA receptor function was markedly depressed (Steullet et al. 2006). An extracellular level of GSH depends on its intracellular content and on the rate of GSH release from the glial compartment (Sagara et al. 1996; Hirrlinger et al. 2002b). Therefore, a deficit in intracellular GSH may result in a concomitant decrease in the extracellular content of this antioxidant. Consequently, the GSH deficit could lead to an excessive oxidation of the extracellular redox-sensitive site of NMDA receptors and to subsequent attenuation of their function. Results by Steullet et al. (2006) partially confirmed this view, as DTNB (5,5'-dithiobis(2-nitrobensoic acid)), a membrane-impermeable thiol-oxidizing compound, diminished pharmacologically isolated NMDA receptor-mediated field excitatory postsynaptic potentials (fEPSPs) in control, but not in BSO-treated slices. In turn, TCEP (tris(carboxyethyl)phosphine hydrochloride), a membrane-impermeable disulfide-reducing agent, increased NMDA responses more distinctly in BSO-treated slices than in control. These data indicate that under experimental conditions, the extracellular redox sites of NMDA receptors were fully oxidized in BSO-treated slices but were partially reduced in the control ones. Hence, the hypofunction of NMDA receptors under conditions of GSH deficit can be explained at least in part by an excessive oxidation of the extracellular redox-sensitive sites of NMDA receptors. In the above-reported study, it was also found that NMDA receptor-dependent long-term potentiation induced by high-frequency stimulation was impaired in GSH-depleted slices. The impairment of such synaptic plasticity could have adverse effects on normal brain functioning, including cognitive processing.

5.2.3 The Effect of Glutathione Deficiency on GABAergic System Function

An alteration of the GABAergic system in the prefrontal cortex (Lewis et al. 2005) and hippocampus (Zhang and Reynolds 2002) is a characteristic feature of the pathology of schizophrenia. Postmortem studies of these brain tissues have provided strong evidence that the GABAergic system is impaired in schizophrenia. These studies showed decreases in the concentration of cortical GABA (Perry et al 1979); in the activity of glutamic acid decarboxylase 67 (GAD-67), the GABA-synthesizing enzyme (Akbarian et al 1995; Hashimoto et al. 2003); and in the content of the calcium-binding protein parvalbumin (PV) in the fast-spiking interneurons (FSIs) of the prefrontal cortex and hippocampus (Hashimoto et al. 2003; Reynolds et al. 2004; Torrey et al. 2005). The existence of GABAergic deficit in schizophrenia was supported by in vivo studies using noninvasive methods. GABA measured in the human brain by magnetic resonance spectroscopy was shown to be decreased in schizophrenic patients (Rosso et al. 2006). Moreover, GABAergic inhibitory activity, as measured by transcranial magnetic stimulation (Daskalakis et al. 2002), was reduced.

NMDA receptor hypofunction could contribute to these abnormalities in the GABAergic system because the administration of NMDA receptor antagonists can cause the loss of parvalbumin and GAD-67 (Keilhoff et al. 2004; Kinney et al. 2006), alter GABA-mediated inhibitory control of cortical neurons (Homayoun and Moghaddam 2007), and disrupt the development of GABAergic neurons (Abekawa et al. 2007). Hence, the hypofunction of NMDA receptors induced by GSH deficiency (Sect. 5.2.2) could also affect the functioning of the GABAergic system in an indirect way. In line with this assumption, in the pharmacological (ODS rats treated with BSO + GBR during early postnatal development; see Sect. 5.1) and genetic ($Gclm^{-/-}$ mice) models of GSH deficiency, it was demonstrated that low level of this antioxidant caused a selective decrease of PV-ir interneurons in the rat prefrontal cortex (Cabungcal et al. 2006) and in the mouse dorsal hippocampus (Steullet et al. 2010). In the latter structure, a concomitant reduction of γ oscillations was also documented. Interestingly, γ oscillations were reduced in schizophrenic patients during impaired performance in cognitive tasks (Cho et al. 2006; Uhlhaas et al. 2008).

The decline of PV-ir FSIs has functional consequences because the activity of cortical pyramidal neurons is regulated by FSIs. These interneurons are necessary for the generation of γ neuronal synchrony that facilitates information processing and transfer within and between brain regions during cognitive tasks (Bartos et al. 2007; Sohal et al. 2009). Chronic GSH deficit in $Gclm^{-/-}$ mice affected the structural and functional integrity of PV-ir FSIs (Steullet et al. 2010), impairing information processing in the VH and leading to specific behavioral alterations, such as enhanced novelty-induced exploration and inadequate responses to stress described in more detail in Sect. 5.1. In conclusion, the alterations observed in the GABAergic system in animal models of GSH deficiency are consistent with that found in schizophrenic patients. Therefore, the participation of GSH deficiency in the pathogenesis of schizophrenia seems to be more and more convincing.

6 N-Acetylcysteine in the Treatment of Psychiatric Disorders

Considering GSH deficiency in the context of characteristic symptoms of schizophrenia, Matsuzawa et al. (2008) described the existence of a negative correlation between the brain GSH levels and the severity of negative symptoms of this disease. This observation suggested that agents increasing GSH levels could be potential therapeutic drugs for the treatment of negative symptoms of schizophrenia (Matsuzawa et al. 2008). The best thiol compound that fulfills such criterion seems to be N-acetylcysteine (NAC), as it acts as a precursor for GSH synthesis by supplying cysteine. NAC has been shown to penetrate successfully the blood-brain barrier and raise brain GSH levels in animal models (Farr et al. 2003). It enters the cell readily (Mazor et al 1996) and is then deacetylated to form L-cysteine. In addition to providing cysteine for GSH production, NAC acts as a direct antioxidant, although with less potency than that of GSH (Aruoma et al. 1989; Hussain et al. 1996).

For more than 30 years, NAC has been used for the treatment of paracetamol overdose, but now it is widely used as a mucolytic agent and in the treatment of HIV infection. As more information comes to light about NAC mode of action, its clinical applications are extending. Currently, potential application of NAC in the treatment of psychiatric disorders particularly in schizophrenia and bipolar disorder is being considered. Recently, in the double-blind, placebo-controlled study, it has been demonstrated that NAC addition (1 g twice daily over 24-week period) to antipsychotic therapy alleviated the negative symptoms, measured on the Positive and Negative Syndrom Scale. Furthermore, improvements in global functioning and reduction of abnormal movements, particularly akathisia, were also found in patients with chronic schizophrenia (Berk et al. 2008a). In addition, NAC relieved the depressive symptoms of bipolar disorder (BD) patients (Berk et al. 2008b). In another clinical study, Lavoie et al. (2008) reported that NAC application (1 g two times daily for 60 days) in schizophrenic patients mitigated an impaired mismatch negativity, which is an auditory evoked potential component related to NMDA receptor function. The abovementioned studies suggest that NAC has the potential to become a therapeutic drug for negative symptoms in schizophrenia and depressive symptoms in BD. These findings are particularly interesting because currently used antipsychotic drugs are rather ineffective against negative symptoms of schizophrenia.

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The Role of Nitric Oxide and Nitrosative Stress in Schizophrenia

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Abbreviations

·OH	Hydroxyl radical
ADMA	Asymmetric dimethylarginine
ADP	Adenosine diphosphate
AMPA	Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate
AMPAR	AMPA receptor
ATP	Adenosine triphosphate
CAPON	Carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein
cGMP	Cyclic guanosine 3′,5′-monophosphate
cNOS	Cellular NOS
CNQX	Cyano-nitroquinoxaline-dione
CNS	Central nervous system
$CO_3^{\cdot-}$	Carbonate radical anion
DA	Dopamine

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DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of 32 kDa
DNA	Deoxyribonucleic acid
ECT	Electroconvulsive therapy
eNOS (or NOS-3)	Endothelial nitric oxide synthase
GABA	Gamma-aminobutyric acid
GluR	Glutamate receptor
GRIP	Glucocorticoid receptor-interacting protein
H_2O_2	Hydrogen peroxide
HOONO	Peroxynitrous acid
HPA	Hypothalamic–pituitary–adrenal axis
HSP	Heat-shock protein
IFN	Interferon
IL	Interleukin
iNOS (or NOS-2)	Cytokine-inducible nitric oxide synthase
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha
LTD	Long-term synaptic depression
LTP	Long-term potentiation
mGlu-1	Type 1 metabotropic glutamate
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
N_2O_3	Dinitrogen trioxide
NAD^+	Nicotinamide adenine dinucleotide cation
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NADPH-d	Nicotinamide-adenine dinucleotide phosphate-diaphorase
nDNA	Neuronal DNA
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
nNOS (or NOS-1)	Neuronal nitric oxide synthase
NO	Nitric oxide
NO^+	Nitrosonium cation
NO^-	Nitroxyl anion
$NO^·$	Nitric oxide radical
NO_2^+	Nitrogen dioxide cation
$NO_2^·$	Nitrogen dioxide radical
NOS	Nitric oxide synthase
NOS1AP	Nitric oxide synthase 1 adaptor protein
O_2	Molecular oxygen
$O_2^{·-}$	Superoxide anion radical.
$ONOO^-$	Peroxynitrite
PANSS	Positive and Negative Syndrome Scale
PARS	Poly(ADP-ribose) synthetase or PARP – poly(ADP-ribose) polymerase
PCR	Polymerase chain reaction

PGH	Prostaglandin H
PMN	Polymorphonuclear leukocytes
PSD93	Postsynaptic density protein-93
PSD95	Postsynaptic density protein-95
REM	Rapid eye movement
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCID	Structured Clinical Interview for DSM Disorders
SCN	Suprachiasmatic nucleus
SOD	Superoxide dismutase
SR	Serine racemase
TNF	Tumor necrosis factor

1 Introduction

Schizophrenia is one of the most severe and chronic forms of mental disorders, but the etiopathogenesis of this illness has not been clarified yet, due to heterogeneity of patient population, different symptoms of schizophrenia, and difficulties in making a diagnosis particularly in the early stage of the disease. Various etiopathological hypotheses for schizophrenia have been proposed, such as the following: neurodevelopmental (Lewis and Levitt 2002; Murray and Lewis 1987; Weinberger 1987), neurodegenerative (Lieberman 1999; Rund 2009), immunological (Kinney et al. 2010; Kliushnik et al. 2009), inflammatory (Covelli et al. 2003; Hanson and Gottesman 2005), infectious (Babulas et al. 2006; Brown 2009; Yolken et al. 2000), and membrane phospholipids (Horrobin et al. 1994; Horrobin 1998), among others.

Neurons are particularly vulnerable to damage by free radicals or reactive oxygen and nitrogen species (ROS and RNS), due to the consumption of large amounts of oxygen, lipid content, and high capacity of the brain and nervous system to initiate free radical reactions (Halliwell and Jenner 1998). ROS/RNS cause oxidative modifications of phospholipids, neuronal DNA (nDNA), mitochondrial DNA (mtDNA), and proteins; the changed function of these molecules may lead to pathological processes in schizophrenia. In addition to their pathological role, free radicals and other ROS and RNS fulfill physiological functions in neurodevelopment and signal transduction, which may be modified in schizophrenia. The development of new investigative techniques, especially neuroimaging, and studies of apoptotic pathway seem to confirm neurodegenerative and neurodevelopmental theories; and oxidative/nitrative damage may integrate and support basic mechanisms of neurodevelopmental and neurodegenerative processes in schizophrenia. Therefore, biochemical alterations in the brain, particularly the dopamine (DA) system with free radical production and oxidative and nitrative damage to brain structures, might be involved in pathogenesis of this heterogeneous disorder.

The increase in nitric oxide (NO) generation in the brain may have putative impact on synaptogenesis and synaptic remodeling, impaired receptor expression and/or functioning, release of neurotransmitters, mitochondrial pathology,

oligodendrocyte injury, and impaired myelination of outer membrane in schizophrenic patients. The implication of NO in schizophrenia is well documented although it is not yet clear whether net over- or underproduction of NO is typical of this disease (Bitanihirwe and Woo 2011). There is experimental evidence that NO is functionally linked to dopaminergic and glutamatergic neurotransmission. Both inhibitory and facilitatory effects of NO on DA released in the striatum and prefrontal cortex have been reported indicating that NO is an important modulator of dopaminergic activity. Further, NO seems to be involved in attentional deficits produced by DA hyperfunction (Gourgiotis et al. 2012).

2 Reactive Nitrogen Species in the Central Nervous System

NO is a small molecule of profound importance in intercellular signaling. It is the main vasodilating agent responsible for lowering blood pressure. It also inhibits platelet aggregation. At high concentrations, it has cytocidal properties and is produced by macrophages for defensive purposes against pathogens (Chakravortty and Hensel 2003; Chen et al. 2008). This molecule has also been identified as a widespread and multifunctional biological messenger molecule in the central nervous system (CNS) and functions as a retrograde neurotransmitter playing roles in neurodevelopment, cell migration, and neurotransmission including long-term potentiation (LTP), neurosecretion, formation of synapses, synaptic plasticity, and release of other neurotransmitters. Nitric oxide is especially important as a second messenger of the *N*-methyl-D-aspartate (NMDA) receptor activation, which interacts with both dopaminergic and serotonergic pathways (Bitanihirwe and Woo 2011). It also has many other physiological functions, including regulation of cardiac function and peristalsis and sexual arousal in males and females (White et al. 2010). It is important in tissue injury in various neuropsychiatric disorders, including schizophrenia (Bernstein et al. 2005a; Kleppisch and Feil 2009; Yao and Keshavan 2011).

Nitric oxide is produced mainly enzymatically by nitric oxide synthases (NOS; EC 1.14.13.39) from L-arginine in the presence of NADPH, tetrahydrobiopterin, and flavin adenine nucleotides as cofactors (Fig. 1). There are three isoforms of NOS. Two of them are constitutive: neuronal (nNOS, NOS-1) and endothelial (eNOS or

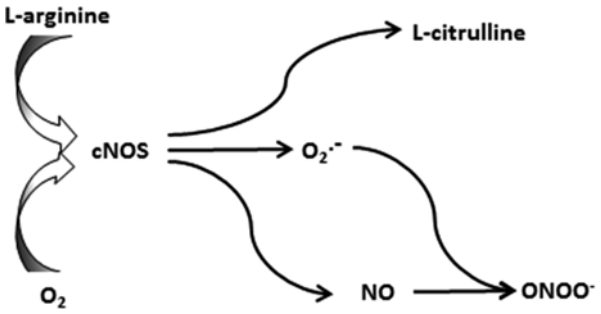

Fig. 1 Nitric oxide (NO) and peroxynitrite ($ONOO^-$) synthesis; cNOS – cellular NOS (e-NOS, nNOS, iNOS)

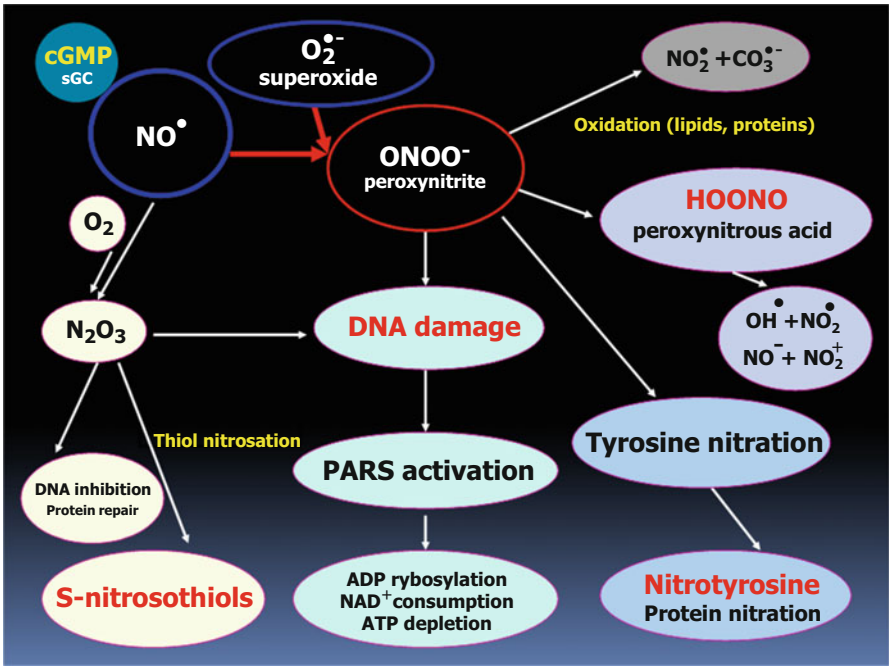

Fig. 2 Reactivity of nitric oxide, peroxynitrite formation in the reaction of superoxide anion with nitric oxide, and other intermediates. *ADP* adenosine diphosphate, *ATP* adenosine triphosphate, *cGMP* cyclic guanosine 3′,5′-monophosphate, $CO_3\cdot$ carbonate radical anion, *DNA* deoxyribonucleic acid, N_2O_3 dinitrogen trioxide, $\cdot OH$ hydroxyl radical, O_2 molecular oxygen, NAD^+ nicotinamide adenine dinucleotide cation, $NO\cdot$ nitric oxide radical, NO^- nitroxyl anion, NO_2^+ nitrogen dioxide cation, $NO_2\cdot$ nitrogen dioxide radical, $ONOO^-$ peroxynitrite, *HOONO* peroxynitrous acid, *PARS* poly(ADP-ribose) synthase, *sGC* soluble guanylate cyclase, *O2*·- superoxide anion radical

NOS-3). They are calcium dependent, since they are activated by increase in cytosolic calcium. The third one (iNOS or NOS-2) is calcium independent and cytokine inducible.

The primary target for NO produced by NOS-1 and NOS-3 is the soluble guanylate cyclase. Binding of NO to the heme group of this enzyme enhances its enzymatic activity and increases the concentration of cyclic GMP (cGMP), an important intracellular signaling molecule; many secondary targets of NO have also been identified. Another mode of NO action is S-nitrosylation (binding to thiol groups of cysteine residues).

The NMDA–NO–cyclic GMP pathway has been shown to modulate the release of neurotransmitters such as glutamate and dopamine. NO can be converted into a variety of different reactive nitrogen species (RNS) including nitrosonium cation (NO^+), nitroxyl anion (NO^-), peroxynitrite ($ONOO^-$), and S-nitrosocysteine or S-nitrosoglutathione (Bartosz 1996) (Fig. 2). NO in the postsynaptic region, where it is synthesized, may influence the activation of transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and during the excessive activation of NMDA receptors and excessive release of NO, it may be involved in free radical damage to DNA.

The primary target for NO produced by NOS-1 and NOS-3 is the soluble guanylate cyclase, which as activated increases the production of cGMP. Activation of cGMP-dependent protein kinase by cGMP is a major pathway of cellular communication. The NMDA–NO–cyclic GMP pathway has been shown to modulate the release of neurotransmitters such as glutamate and dopamine. Nitric oxide belongs to the scarce group of gas intracellular and intercellular neurotransmitters. In the CNS, NO is released under physiological condition by stimulation of postsynaptic NMDA receptors, it provides information to the presynaptic nerve terminals and takes part together with cGMP in the LTP in the hippocampus and long-term synaptic depression (LTD) in the cerebellum, and it may play a role in the mechanisms of learning and memory. Nitric oxide also activates the processes of S-nitrosylation and ADP-ribosylation of proteins (Fig. 2).

Cyclic GMP can control the pre- and postsynaptic processes including the release of neurotransmitters such as glutamate and can regulate the intracellular calcium ion concentration [Ca^{2+}], as well as cause modulation of calcium and potassium channel functions and expression of early genes in cells (Garthwaite and Boulton 1995; Dawson and Dawson 1996) Cyclic GMP, like NO, is involved in LTP and LTD processes. Nitric oxide acts as a retrograde messenger, influencing the presynaptic terminals; as a result of the guanylate cyclase or ADP-ribosyltransferase activation, there is the increased glutamate release (Dawson et al. 1992).

NOS-1 is involved in the development of the nervous system. In the mammalian central nervous system, NOS-1 is the major NOS isoform accounting for about 90 % of the overall NO production in the brain (Hara et al. 1996). In the CNS, the 160 kDa NOS-1 is the predominant splice variant of the enzyme. It contains an N-terminal postsynaptic density (PSD)/Disc-large/ZO-1 homologous (PDZ)-binding domain, which anchors this complex in the vicinity of the NMDA receptor (Mungrue and Bredt 2004).

In the human brain, the highest levels of NOS-1 were found in the substantia innominata, septal area, cerebellar cortex, nucleus accumbens, hypothalamus, and subthalamus, while the lowest levels were found in the corpus callosum, thalamus, occipital cortex, and cerebellar dentate nucleus. A similar distribution pattern has been revealed by immunocytochemical studies. The density of NOS-1-expressing neurons is region dependent. In the cerebral cortex, NOS-1 is localized in about 2 % of the neurons, whereas in the human hypothalamic paraventricular nucleus, up to 20 % of the neurons express the enzyme. Apart from being present in neurons, NOS-1 can also be found in certain glial cells and brain blood vessels (Bernstein et al. 2005a).

Nitric oxide is also involved in sleep regulation. Both NOS-1 inhibition and an NO scavenger were shown to prevent recovery sleep induction, while application of an NO donor during the spontaneous sleep–wake cycle increased sleep, indicating that NO is necessary and sufficient for the induction of recovery sleep (Kalinchuk et al. 2006).

2.1 Neurotoxic Mechanism of Nitric Oxide Activity in the Brain

NO, when synthesized excessively, can be a cytotoxic compound, which can damage DNA possibly by free radical processes and activate the transcription factor NF-κB leading to death of the neuron. The stimulation of NMDA receptors and excessive release of NO can cause neuronal death (Dawson et al. 1992). However, the administration of NO can also be neuroprotective by the inhibition of NMDA receptor (Lei et al. 1992). NO can cause S-nitrosylation of thiol groups of the NMDA receptor and inhibit the receptor functions (Manzoni et al. 1992; Manzoni and Bockaert 1993). The reaction of NO with superoxide radical results in formation of the peroxynitrite anion (Fig. 1) and subsequent formation of nitrogen dioxide radical ($NO_2^{\bullet}$) and hydroxyl radical ($^{\bullet}OH$), which are probably responsible for the peroxynitrite toxicity (Beckman 1994; Lipton et al. 1993). Superoxide dismutase, which removes superoxide anions, reduces the formation of $OONO^-$. NO may disturb mitochondrial electron transport chain (Bolanos et al. 1997; Knowles 1997); NO also inhibits glyceraldehyde-3-phosphate dehydrogenase and lowers intracellular glutathione level (Almeida et al. 1998).

DNA damage, which can be induced by NO, results in the activation of poly(ADP-ribose) synthetase (PARS), a nuclear enzyme that attaches ADP-ribose to the nuclear proteins such as histones or the PARS itself (Zhang et al. 1994) (Fig. 2).

PARS activation can lead to cell death due to depletion of NAD and ATP (Dawson and Synder 1994). NO can be cytotoxic by the direct inhibition of enzymes that catalyze vital cellular functions as energy metabolism, DNA synthesis. All compounds that inhibit the activity of NOS function prevent NMDA-induced neurotoxicity (Dawson et al. 1994).

However, it is believed that cGMP plays an insignificant role in NO toxicity except for retinal neurons; it also is unexplained whether there is a possible neuroprotective effect of cGMP suggested by Farinelli et al. (1996). Guanylate cyclase inhibitor or cGMP analogs had no effect on the toxicity induced by NMDA receptor stimulation and NO (Lusting et al. 1992).

Excessive NO levels are cytotoxic and may be responsible for the death of neurons as a result of the proposed sequence which includes the activation of free radical-dependent processes and the transcription factor NF-κB and damage to DNA.

DNA damage, in turn, activates poly(ADP-ribose) synthetase (PARS), a nuclear enzyme, and its overstimulation leads to NAD and ATP depletion and cell death (Fig. 2).

A common feature of numerous pathophysiological conditions including schizophrenia is the generation of excess reactive oxygen intermediates in particular superoxide and hydrogen peroxide. Superoxide anion either reacts rapidly with nitric oxide forming peroxynitrite or can be converted into hydrogen peroxide (H_2O_2) by the action of superoxide dismutase (SOD). Both SOD and NO compete for "scavenging" the anion, but the rate constant of the reaction of transformation

into the H_2O_2 anion catalyzed by SOD is only one-third of the rate constant of reaction of NO with superoxide (Bartosz 1996).

Nitric oxide may play a dual role, both neuroprotective and neurotoxic. Its damaging effects are largely due to its reaction with superoxide radical and formation of peroxynitrite (Pryor and Squadrito 1995, Bartosz 1996). Peroxynitrite ($ONOO^-$), anion of unstable peroxynitrous acid, is a relatively long-lived cytotoxic oxidant that may be implicated in schizophrenia. It reacts with a wide range of biological molecules, damaging proteins, amino acids, nucleic acids, and nucleotides, initiating lipid peroxidation, and depleting cellular antioxidants, such as glutathione and ascorbate. It may initiate lipid peroxidation in schizophrenic patients' membrane; it can react directly with sulfhydryl residues in cell membranes. These reactions may lead to enzyme inhibition and, what may be especially relevant for psychiatric diseases, to autoxidation of the neurotransmitter dopamine (Antunes et al. 2005). The molecular footprint of peroxynitrite is nitration of tyrosine residues in proteins to form 3-nitrotyrosine (and also of tryptophan and histidine residues, and other compounds can also be nitrated, albeit with lower efficiency) (Olas et al. 2004, Radi et al. 2004).

Paradoxically, the reaction of superoxide anion and nitric oxide may also function as a defense against oxidative stress by reducing intracellular levels of these reactive intermediates.

The condition that occurs when the excessive production of reactive nitrogen species, such as nitric oxide and peroxynitrite, exceeds the ability of cells/organism to neutralize and eliminate them is called nitrosative stress. Biomarkers of nitrosative stress are enhanced content of products of nitric oxide metabolism (nitrite + nitrate), increased protein nitrosylation, and increased protein nitration (especially increased content of 3-nitrotyrosine residues) (White et al. 2010; Tao et al. 2012).

There have been contradictory reports on the levels of NO in schizophrenia: some of them suggested an increase of the NO-mediated neurotransmission, while others supported a decrease (Oliveira et al. 2008). Contrary to the report of Zoroglu et al. (2002) who demonstrated significantly higher level of NO and adrenomedullin in schizophrenia, Akiibinu et al. showed significantly decreased NO content in acute schizophrenia. Diversion of the nitric oxide to the peroxynitrite pathway mediated by high level of peroxides could be responsible for the lower plasma level of NO in schizophrenic patients. Since L-arginine is the precursor of NO, inadequate intake of L-arginine (malnutrition) could also cause impaired synthesis and lower level of NO in schizophrenic patients (Akiibinu et al. 2012).

NOS activity was found to be significantly higher in platelets of drug-naïve schizophrenic patients compared to controls, drug-treated schizophrenics and panic disorder subjects. Apparently, there is an imbalance of the calcium-induced L-arginine–nitric oxide pathway in platelets of schizophrenic subjects that may be modified by treatment with antipsychotics (Das et al. 1995) (see 4). However, other authors found a decreased level of plasma metabolites of nitric oxide (17.8 vs. 24.7 μM in controls) (Akiibinu et al. 2012). Nitrite content in the polymorphonuclear leukocytes (PMN) was reduced to 68 %, while plasma and platelet nitrite content in schizophrenic patients was not significantly changed in comparison to controls, indicating a significant decrease in NO synthesis (Srivastava et al. 2001).

However, a recent meta-analysis of 10 studies of nitrite + nitrate determinations in the blood of patients with schizophrenia leads to the conclusion that there are no differences in the level of nitric oxide metabolites between patients and controls. In contrast, patients under antipsychotic treatment were found to have higher levels of NO metabolites than controls.

Interestingly, it has been demonstrated that nitric oxide metabolites are significantly elevated in the plasma of patients following a suicide attempt (Kim et al. 2006; Lee et al. 2006).

Accumulating evidence suggests that mitochondrial dysfunction and oxidative stress contribute to the pathogenesis of bipolar disorder and schizophrenia. Protein nitration, as measured by the level of 3-nitrotyrosine, was increased in the prefrontal cortex of patients with schizophrenia and bipolar disorder groups (Andreazza et al. 2010).

Increased protein nitration in the central nervous system is reflected by changes detectable in the blood. Increased level of 3-nitrotyrosine was found in the blood plasma proteins (Dietrich-Muszalska et al. 2009; Dietrich-Muszalska et al. 2012) and blood platelet proteins (Dietrich-Muszalska and Olas 2009) of patients with schizophrenia. This clear-cut increase of the marker of peroxynitrite formation may seem astounding when confronted with the apparent lack of changes in the level of nitric oxide metabolites in the blood. It should be remembered, however, that the rate of peroxynitrite formation is a function of superoxide available for reaction with nitric oxide. Oxidative stress occurring in schizophrenia – including lowered activity of superoxide dismutase found in the blood of patients (Dietrich-Muszalska et al. 2005) – leaves a higher fraction of superoxide not scavenged, which may promote enhanced peroxynitrite formation even in the absence of significant elevation in the concentration of nitric oxide (Beckman and Koppenol 1996).

Apparently, this controversial area requires further studies, which should both bring deeper understanding of the role of reactive nitrogen species in the pathogenesis of schizophrenia and perhaps provide analytical tools to monitor the course of the disease and its therapy.

3 Nitric Oxide and Nitric Oxide Synthase in the Brain and Schizophrenia

3.1 Synthesis of Nitric Oxide in Schizophrenia

Multiple studies have suggested that NO and NOS-1 are involved in the dorsolateral prefrontal cortex dysfunction in schizophrenia. It was shown that neurons characterized by histochemical staining for the enzyme nicotinamide-adenine dinucleotide phosphate-diaphorase (NADPH-d) (a marker for NOS activity) migrate improperly in the prefrontal cortex (as well as in other brain regions, e.g., in the hippocampal formation) of schizophrenic patients (Akbarian et al. 1993a). In the normal brains, these neurons are found in highest numbers in the white matter immediately deep to layer VI of the cortex where they remain from the subplate, an early formed but

transitory structure that plays a key role in cortical development and connection formation. The dorsolateral prefrontal area of schizophrenics showed a significant decline in NADPH-d neurons in the superficial white matter and in the overlying cortex but a significant increase in these neurons in the white matter deeper than 3 mm from the cortex (Akbarian et al. 1993a, b). However, interpretation of these results was limited due to the histochemical distribution and quantification of NADPH-d neurons (Akbarian et al. 1993a). For example, it is unknown whether the alterations in the density of NADPH-d neurons were due to changes in the expression of three NOS isoforms or a change in NOS activities. Moreover, all three NOS isoforms contain a recognition site for NADPH, and NADPH-d histochemistry and NOS immunochemistry label identical populations of neural cells. Therefore, a change in NADPH-d neuron density does not allow the differentiation between the calcium-dependent NOS and the calcium-independent (NOS-2) activities (Xing et al. 2002). It was also reported that schizophrenic patients have decreased NOS-1 activity (Xing et al. 2002), but also have elevated NOS-1 mRNA levels (Baba et al. 2004; Silberberg et al. 2010) in the prefrontal cortex.

3.2 Nitric Oxide Metabolites and Nitric Oxide Synthases in Brain Tissues and Schizophrenia

Elevated level of nitric oxide metabolites such as nitrite + nitrate, has been found in the caudate region of postmortem brain tissues from patients with schizophrenia, compared to control subjects without schizophrenia (241 vs.142 and 125 pmol/mg dry weight, respectively) (Yao et al. 2004).

Findings concerning the cerebellum are rather inconsistent. Bernstein et al. reported an increased number of NOS-immunoreactive cerebellar Purkinje and dentate nucleus cells in schizophrenia (Bernstein et al. 2010a, 2011), while Doyle and Slater were unable to find differences in the expression of NOS in cerebellar granule cells between schizophrenics and controls (Doyle and Slater 1995). Increased content of nitric oxide synthase was also reported in the cerebellum and brainstem tegmentum by Karson et al. (unfortunately, the histological staining employed did not allow to identify the NOS isotype (Karson et al. 1996)). However, other findings suggest a reduction of NOS mRNA in cerebellar neurons in schizophrenia (Bullock et al. 2008). The abnormal cellular expression of NOS observed in the cerebellum might be part of a disturbed (glutamatergic and/or GABAergic) circuitry in schizophrenia. Recently, NOS1AP protein levels were found to be altered in the cerebellum of patients with schizophrenia (Bernstein et al. 2001).

A great number of studies in the literature suggest that there is a competition between arginase (amidinohydrolase, EC 3.5.3.1) and NOS and that they control each other's level (Quirié et al. 2013). Because arginase and NOS compete for L-arginine, arginase plays a crucial role in the modulation of NO production. Arginase is known as the last enzyme in the urea cycle in the liver, but it is also pres-

ent in extrahepatic tissues. Arginase hydrolyzes L-arginine to L-ornithine and urea, and its biochemical and physiological role varies depending on the organism and tissue. Besides its participation in ammonia detoxification, arginase is involved in the synthesis of polyamines (crucial for the proper course of many metabolic processes), proline (an important component of connective tissue proteins), and glutamate (an amino acid important in the nervous system and also a substrate for protein synthesis). When NOS-3-mediated NO generation is high (bacterial infection, etc.), arginase activity is usually low. Conversely, NO production can be significantly lowered when arginase expression increases in the same cell.

Some studies suggest that peripheral NO metabolites can be used as a marker of CNS-dependent NO changes. However, the relationship of plasma arginase to brain arginase activity remains unclear (Yanik et al. 2003). Recently, Quirié et al. reported that changes in arginase expression were not restricted to inflammatory cells but also affected activated astrocytes and neurons. Evidence of a similar pattern of arginase 1 and brain-derived neurotrophic factor expression in astrocytes opens new possibilities for a potential link between arginase and neuroplasticity (Quirié et al. 2013).

3.3 Polymorphisms in the NOS: Gene and Schizophrenia

Abundant evidence indicates that NOS-1 genomic locus (chromosome 12q24) is genetically linked with schizophrenia (Holmans et al. 2009; Cui et al. 2010). A number of genetic association studies have reported that single nucleotide polymorphisms in the NOS-1 gene were associated with schizophrenia (Reif et al. 2006; Tang et al. 2008; Wratten et al. 2009; Cui et al. 2010), although some results did not confirm such associations (Okumura et al. 2009). Cui et al. suggested that genetic and functional data for NOS-1 revealed an association between a putative cis-acting polymorphism in the NOS-1 gene and decreased protein NOS-1 expression in the prefrontal cortex of patients with schizophrenia. Moreover, these authors also showed that the age of schizophrenia onset was earlier in patients carrying the cis-acting polymorphism in the NOS-1 gene (Cui et al. 2010).

Significant linkage disequilibrium has been identified between schizophrenia and markers within the gene encoding nitric oxide synthase 1 adaptor protein [NOS1AP, also termed carboxyl-terminal PDZ ligand of nNOS (CAPON)]. Expression of the short form of the NOS1AP gene (NOS1AP-S) is significantly increased in patients with schizophrenia and bipolar disorder. A physiological role of the short form would likely be limited to the competitive inhibition of binding of other ligands to the PDZ domains of nNOS and postsynaptic density protein-93 (PSD93) or PSD95. This isoform can disrupt the binding of nNOS to PSD95 through competitive inhibition and remove nNOS from the NMDAR complex, thereby decoupling NO generation from NMDAR activation. This could produce a picture consistent with the NMDAR hypofunction hypothesis of schizophrenia (Xu et al. 2005).

The allele frequencies of the polymorphism in exon 29 of the NOS1 gene (single nucleotide polymorphism (SNP: a C→T substitution located 276 base pairs downstream from the translation termination site) of the human NOS1 gene, which is located in chromosome 12q24) differed significantly between patients with schizophrenia and control group (relative risk = 1.92). These results suggest that the NOS1 gene polymorphism may confer increased susceptibility to schizophrenia (Shinkai et al. 2002). Additionally, many rare structural variants in multiple genes in NOS signaling are disrupted in patients with schizophrenia. Several downstream effectors in nNOS signaling, such as CAPON and serine racemase (Morita et al. 2007), have also been reportedly associated with schizophrenia. However, a study of a Japanese population and a recent study of a Chinese population did not support a significant association between NOS1 gene polymorphisms and schizophrenia (Okumura et al. 2009; Wang et al. 2012).

3.4 The ADMA, NOS Inhibition, and Regulation of Nitric Oxide Generation

Asymmetric dimethylarginine (ADMA) has been demonstrated to be an endogenous inhibitor of NOS and to regulate nitric oxide generation in numerous diseases. There was a significant increase in plasma ADMA concentrations in patients with schizophrenia when compared to healthy controls. There were no significant correlations between the plasma concentrations of ADMA and scores of psychiatric rating scales (e.g., PANSS). In the multiple episode schizophrenia subgroup, the mean plasma ADMA concentration was significantly higher than in the first episode schizophrenia subgroup (Celik et al. 2011).

The role of nNOS in the regulation of behavior is also suggested by studies of nNOS knockout mice. These animals showed hyperlocomotor activity in a novel environment, increased social interaction in their home cage, decreased depression-related behavior, and impaired spatial memory retention. In striatal slices from nNOS KO mice, the effects of a dopamine D1 receptor agonist, SKF81297, on the phosphorylation of DARPP-32 and AMPA receptor subunit GluR1 at protein kinase A sites were enhanced. These data indicate that knockout of nNOS KO upregulates dopamine D1 receptor signaling and may serve as a unique animal model of psychiatric disorders, including schizophrenia (Tanda et al. 2009).

L-lysine, an amino acid that interferes with NO production, was found to have positive effects on patients with schizophrenia. A four-week L-lysine treatment of 6 g/day caused a significant decrease in positive symptoms as assessed by PANSS in addition to self-reported symptom improvement by three patients (Wass et al. 2011).

Serine racemase (SR) generates D-serine, a coagonist with glutamate at NMDA receptors. SR is physiologically S-nitrosylated leading to marked inhibition of enzyme activity. NMDA receptor physiologically enhances SR S-nitrosylation by activating neuronal nitric oxide synthase (nNOS). These findings support a model

whereby postsynaptic stimulation of nitric oxide (NO) formation feeds back to presynaptic cells to S-nitrosylated SR and decreases D-serine availability to postsynaptic NMDA receptors (Mustafa et al. 2007).

Multivariate logistic regression analyses identified the expression of inducible isoforms of nitric oxide synthase and cyclooxygenase in PMBC and homocysteine plasma levels as the most reliable potential risk factors and the inhibitor of the inflammatory transcription factor NF-κB, IκBα, and the anti-inflammatory prostaglandin 15d-PGJ_2 as potential protection factors (García-Bueno et al. 2013).

NOS-1 and NOS-2 are soluble and found predominantly in the cytosol, while NOS-3 is membrane associated. NO produced by NOS-3 has been shown to be a vasodilator identical to the endothelium-derived relaxing factor, produced in response to shear from increased blood flow in arteries, which dilates blood vessels by relaxing smooth muscles in their linings (White et al. 2010). NO generation by NOS-2 is controlled by several factors, including calcium-activated calmodulin, caveolins 1 and 3, bradykinin B2 receptors, angiotensin AT1 receptors, steroid hormones, heat-shock protein (HSP) 90, and plasma membrane ionotropic receptors (Fleming and Busse 2003). NOS-3 plays a central role in the inflammatory reactions that follow infection, disease, and tissue damage. In the brain, NOS-3 is mainly expressed in microglia, brain endothelial cells, infiltrating macrophages, and T lymphocytes, but may also be induced in neurons and glial cells (Bernstein et al. 2005a).

The inducible isoform NOS-2 produces large amounts of NO as a defense mechanism. It is synthesized by many cell types in response to cytokines, and it is an important factor in the body response to attack from parasites, bacterial infection, and tumor growth. It is also the cause of septic shock and may play a role in diseases with an autoimmune etiology (Chatterjee et al. 2008; Fitzpatrick et al. 2008; Zhou and Zhu 2009; Förstermann and Sessa 2012).

In addition to nNOS in the brain, various immunomodulators, such as lipopolysaccharide and interferon, can induce the production of NO by the inducible NOS (Lorsbach et al. 1993; Lowenstein et al. 1993). Elevated plasma levels of IL-6 in patients with schizophrenia have been reported (Shintani et al. 1991; Ganguli et al. 1994; Maes et al. 1995; Lin et al. 1998; van Kammen et al. 1999). It seems that the increased production of NO in the brain of patients with schizophrenia can occur involving induction of IL-6.

3.5 The Ionotropic Glutamate Receptors, Nitric Oxide, and Schizophrenia

One of the key neurotransmitters thought to be involved in schizophrenia is glutamate, and there is the support for the involvement of hypoactivity of NMDA glutamate receptors in the pathogenesis of schizophrenia. NMDA receptors are most densely concentrated in the cerebral cortex (hippocampus, particularly the CA1 region), amygdala, and basal ganglia. Nitric oxide can act as neuromodulator of

NMDA receptor functions, while glutamate stimulation of NMDA receptors results in nitric oxide synthesis and release, enhancing neurotransmitter release from adjacent synapses. Granule cells of the dentate gyrus of the hippocampus are rich in NOS-1. Proper expression and regulation of NMDA receptors in the brain are critical for learning and memory processes as well as cortical plasticity and maturation. Furthermore, mutations in many of the known genetic risk factors for schizophrenia suggest that NMDA receptor hypofunction is a convergence point for schizophrenia (Snyder and Gao 2013).

In one of the first studies of NMDA receptor subunits in schizophrenia, Akbarian et al. presented data that suggested a regional deficit in NMDA subunit density in brain tissue of schizophrenic patients. These authors suggested that in patients with schizophrenia, the alterations in expression of NR2 subunit mRNA in the prefrontal cortex were potential indicators of deficits in NMDA receptor-mediated neurotransmission accompanying functional hypoactivity of the frontal lobes in the cerebral cortex (Akbarian et al. 1996).

Meador-Woodruff et al. demonstrated that the expression of NR1 and NR2C subunit transcripts was decreased in the thalamus in schizophrenia. Interestingly, three intracellular postsynaptic density molecules that link the NMDA receptor with signal transduction pathways are also abnormally expressed (Meador-Woodruff et al. 2003). Clinton et al. observed increased protein expression of the NR2B NMDA receptor subunit and its associated intracellular protein, PSD95, in the dorsomedial thalamus of patients with schizophrenia. These data provide additional evidence of thalamic neurochemical abnormalities, particularly in thalamic nuclei which project to limbic regions of the brain (Clinton et al. 2006). Li et al. suggested that an impairment in NR1 subunit phosphorylation of NMDA receptors produces glutamatergic hypofunction that can contribute to behavioral deficits related to psychiatric disorders (Li et al. 2009). However, these findings indicate that phosphorylation of NMDAR subunits by protein kinases is also subject to regulation by redox and free radicals. Phosphorylation of NMDAR subunits by protein kinases is also free radical associated. In addition, redox-mediated activation of NMDA receptors induces a series of further redox-associated free radical signaling processes, such as NADPH oxidase activity, NOS-1 activity, mitochondrial enzyme activity, and induction of the arachidonic acid cascade, phospholipase A, and prostaglandin H (PGH) synthase (Bókkon and Antal 2011). Recently, Weickert et al. found that reduction in NR1 and NR2C in the dorsolateral prefrontal cortex of patients with schizophrenia may lead to altered NMDAR stoichiometry and provides compelling evidence for an endogenous NMDAR deficit in schizophrenia. Genetic variation in the NR2B gene predicts reduced levels of the obligatory NR1 subunit, suggesting a novel mechanism by which the NR2B single nucleotide polymorphism may negatively influence other NMDAR subunit expressions and reasoning abilities in schizophrenia (Weickert et al. 2012).

Giegling et al. first provided evidence for the NMDA/glutamatergic theory of schizophrenia including calcium processes (Giegling et al. 2010). The activation of NMDA receptors by glutamate results in calcium influx into the cell. In the cytosol, calcium binds to calmodulin and stimulates the neuronal nitric oxide synthase (nNOS).

Nitric oxide activates guanylate cyclase, which increases the production of cyclic GMP. This NMDA–NO–cyclic GMP pathway has been shown to modulate the release of neurotransmitters such as glutamate and dopamine. Oliveira et al. suggested that this pathway has been consistently implicated in schizophrenia (Oliveira et al. 2011).

Although the hypofunction of the NMDA receptor results in reduced calcium flow through these channels, the resultant effect is an increased level of free intracellular calcium in large neuronal populations. This is because deactivation of NMDA receptors on GABAergic interneurons removes the inhibition of major excitatory pathways that innervate primary neurons of cerebrocortical and limbic brain regions. These pathways are, then, able to induce an abnormal increase in free intracellular calcium in their targets by activating non-NMDA glutamate-gated ion channels as well as G protein-coupled receptors, which are capable of mobilizing calcium from internal stores and subsequently triggering oxidative damage (Bitanihirwe and Woo 2011).

West et al. demonstrated that dopamine potently modulated NMDA-dependent NOS activity and NO production in striatal neurons (West et al. 2002). NOS activity is upregulated by D1 receptor activation through a direct effect on nNOS neurons in the striatum and downregulated by activation of D2 receptors through still unknown inhibitory mechanism. Activation of D2 receptors has been shown to block the D1-mediated facilitation of NOS activity, most likely through an indirect mechanism involving activation of presynaptic D2 receptors and consequent inhibition of glutamate NMDA-induced effects on NOS activity (Gourgiotis et al. 2012). However, D2-mediated effects may also modulate NOS activity via action on other mechanisms, for example, GABA (Robello et al. 1996). It could be speculated that enhancing NO activity may oppose the inhibitory effects of D2 receptor activation on D1 facilitation of NOS activity. On the other hand, apomorphine increases cGMP content in the brain (Biggio et al. 1977); in analogy to what was observed with phencyclidine (noncompetitive inhibitor of the NMDA receptor), it could be imagined that inhibiting NOS activity may decrease cGMP and abolish memory deficit (Fejgin et al. 2008). Thus, the beneficial effects of stimulation or inhibition of NO signaling on cognitive deficits may be due to their actions on two functionally opposing systems.

Several studies have demonstrated a potential pathological link between NMDA hypofunction, enhanced neuronal production of IL-6, and oxidative stress, which may in turn be associated with the GABAergic dysfunction often observed in the brain of patients with schizophrenia. Taking hypoactivity of NMDA ionotropic glutamate receptors in the pathogenesis of schizophrenia into account. Bernstein et al. suggested that increased NO generation via activated NMDA receptors was unlikely (Bernstein et al. 2005a).

The role of receptor dependent on alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) in NO generation might be of relevance because of the demonstrated increased expression of this receptor in schizophrenia (Okada et al. 2004; Bernstein et al. 2005a; Dracheva et al. 2005). Dracheva et al. measured mRNA expression and abundance of AMPAR subunits (GluR1-4) and several AMPAR-

binding proteins (SAP97, PICK1, GRIP, ABP) in the dorsolateral prefrontal cortex and the occipital cortex of elderly schizophrenic patients and matched normal controls by quantitative real-time PCR. The mRNA expression of GluR1, GluR4, and GRIP in the dorsolateral prefrontal cortex and expression of the GluR4, GRIP, and ABP in the occipital cortex were significantly elevated in schizophrenics (Dracheva et al. 2005). Okada et al. visualized NO production mediated by NMDA, AMPA, and type 1 metabotropic glutamate (mGlu-1) receptors in rat cerebellar slices and granule cells in culture, with an NO-specific fluorescent indicator, diaminofluorescein-2. AMPA receptor, but not NMDA or mGlu-1 receptor, was responsible for NO production at parallel fiber terminals, which was blocked by CNQX, tetrodotoxin, or voltage-dependent calcium channel blockers. In the granule cell layer, activation of AMPA or mGlu-1 receptor produced NO uniformly, while NMDA receptor activation produced NO in discontinuous areas of this layer. These findings suggested that distinct glutamate receptors, including non-NMDA receptors, govern occurrence and level of NO production in a layer-specific manner (Okada et al. 2004). Zhou et al. showed that AMPA agonism might occur upstream to nNOS upregulation in inhibitory interneurons of layer I of the pyriform cortex. Moreover, results of these authors indicate that transsynaptic neuronal degeneration in the limbic cortex involves complex AMPA–glutamatergic and nitrinergic signaling events (Zhou et al. 2006). Yamada and Nabeshima suggested that AMPA may induce NO production through an NOS-independent pathway although NMDA receptor-mediated NO production is dependent on NOS activity in the rat cerebellum in vivo (Yamada and Nabeshima 1997).

Decreased activity of receptors sensitive to NO has also been reported in schizophrenia. The cholinergic receptors (e.g., α7 nicotinic acetylcholine receptor) known to be sensitive to NO toxicity were decreased in both the blood and cortex of patients with schizophrenia (Mathew et al. 2007). Some neuropsychological tests performed in patients with schizophrenia as well as in healthy controls indicated that nNOS was associated with functions of the prefrontal cortex, including cognition. Cognitive deficits (including attention, memory, learning, executive and motor functions, and speech) are found in more than 85 % of patients with schizophrenia, and they occur before the onset of the illness and are present at early stage and during the remission phases. It suggests that nNOS is involved in the etiopathogenesis of schizophrenia and, more specifically, in specific cognitive function (Reif et al. 2006).

NO acts as a second messenger of NMDA receptor activation, which further interacts with both dopaminergic and serotonergic pathways. Glutamatergic system is modulated by a number of neurotransmitter systems including the serotonergic system (Maura et al. 1995; Hoyt et al. 1992). Serotonergic 5-HT receptor activation may be responsible for the weakening of signal transduction dependent on NO and cGMP released by NMDA receptor activation as well as on arachidonic acid. Becquet et al. observed that NMDA receptor activation stimulates the release of serotonin, and this effect is modulated by glycine (Becquet et al. 1993). Serotonin via the 5-HT2 receptor reduces the cholinergic receptor-dependent increase in the release of IPs and the mobilization of intracellular calcium (Samochocki and Strosznajder 1995). It seems that serotonin in this way can prevent certain adverse processes caused by excessive activation of the NMDA receptors. Studies by Ross et al. also showed the interaction between the serotonergic and glutamatergic system (Ross et al. 1992). Prehn et al. described that the 5-HT1A receptor agonist was

capable of reducing damage to hippocampal neurons in culture subjected to hypoxia and action of glutamate dependent on the dose (Prehn et al. 1994). These studies suggest that the release of serotonin and agonist action at relevant serotonergic receptors can prevent the excessive release of neurotransmitters associated with ischemic brain damage (Prehn et al. 1993; Poderoso et al. 1996).

3.6 The Hypothalamus, Nitric Oxide, and Schizophrenia

Hypothalamic abnormalities in schizophrenia have been associated with endocrine dysfunctions and stress response. The hypothalamus is involved in several pathways found disrupted in schizophrenia (e.g., hypothalamic–pituitary–adrenal axis, HPA axis). Recently, a study using a 3-Tesla magnetic resonance imaging (MRI) scanner found abnormally increased size of the hypothalamus and the mammillary bodies in schizophrenia. The size of the mammillary bodies was inversely correlated with negative symptoms and directly correlated with anxiety. Moreover, mammillary body volumes were associated with negative symptoms and anxiety (Tognin et al. 2012). Hypothalamic paraventricular neurons are believed to trigger these processes by aberrant generation and/or release of corticotropin-releasing hormone, oxytocin, vasopressin, and nitric oxide (NO). The suprachiasmatic nucleus (SCN), which in part controls the cellular activity of paraventricular neurons, is involved in affective disorder. Bernstein and colleagues using stereological analysis showed that SCN-derived NO may be a relevant pathophysiological factor in neuropsychiatric disorders (Bernstein et al. 2005b).

Furthermore, in NOS-1 knockout mice, REM sleep is substantially reduced. Thus, reduced cellular expression of NOS-1 in the SCN neurons in schizophrenia might be part of a disturbed signaling cascade finally leading to the profound sleep disturbances. Other hypothalamic factors with known effects on sleep architecture as hypocretin-1 are apparently normal in patients with schizophrenia (Bernstein et al. 2010b). Interestingly, it has been postulated that the successful treatment of major depression and other psychiatric disorders by electroconvulsive therapy (ECT) might be due to the endocrine effects of hypothalamic NO (Hui et al. 2010). Virtually every neurotransmitter system is affected in ECT. Rosen et al. described the involvement of NO in long-term potentiation, the NMDA receptor activity, regulation of cerebral blood flow, and the hypothalamic–pituitary axis and proposed that this involvement is critical in ECT's efficiency, treatment refractoriness, and neuropsychological sequelae by its influences on these systems (Rosen et al. 2003).

4 Nitrosative Stress in Schizophrenia

Reaction of nitric oxide with superoxide radical anion produces peroxynitrite. Reactions of peroxynitrite may lead to enzyme inhibition and, what may be especially relevant for psychiatric diseases, to autoxidation of the neurotransmitter dopamine (Antunes et al. 2005).

A condition that occurs when the excessive production of reactive nitrogen species, such as nitric oxide and peroxynitrite, exceed the ability of cells/organism to neutralize and eliminate them is called nitrosative stress. Biomarkers of nitrosative stress are enhanced content of products of nitric oxide metabolism (nitrite + nitrate), increased protein nitrosylation, and increased protein nitration (especially increased content of 3-nitrotyrosine residues) (White et al. 2010; Tao et al. 2012).

There have been conflicting reports on the levels of NO in schizophrenia: some of them suggesting an increase of the NO-mediated neurotransmission and another part supporting a decrease (Oliveira et al. 2008). Contrary to the report of Zoroglu et al. (2002) who demonstrated significantly higher level of NO and adrenomedullin in schizophrenia, Akiibinu et al. show significantly decreased NO content in acute schizophrenia. Diversion of the nitric oxide to the peroxynitrite pathway mediated by high level of peroxides (i.e., TPP) could be responsible for the lower plasma level of NO in schizophrenic patients. Since L-arginine is the precursor of NO, inadequate intake of L-arginine (malnutrition) could also cause impaired synthesis and lower level of NO in these schizophrenic patients (Akiibinu et al. 2012).

Elevated level of nitric oxide metabolites in the blood plasma of the patients was found by Zhang et al. In their study, risperidone and haloperidol did not reduce the elevated plasma NO levels in the patients (Zhang et al. 2012).

NOS activity was found to be significantly higher in platelets of drug-naïve schizophrenic subjects compared to controls, drug-treated schizophrenics and panic disorder subjects. Apparently, the impairment of the calcium-induced L-arginine–nitric oxide pathway in platelets of schizophrenic subjects may be caused by antipsychotics treatment (Das et al. 1995). A significant increase of erythrocyte NO level was found in patients with schizophrenia treated with neuroleptic drugs (Herken et al. 2001). However, other authors found a decreased level of plasma metabolites of nitric oxide (17.8 vs. 24.7 μM in controls) (Akiibinu et al. 2012). Nitrite content in the PMN was reduced to 68 %, while plasma and platelet nitrite content in schizophrenic patients was not significantly changed in comparison to controls, indicating a significant decrease in NO synthesis (Srivastava et al. 2001). Another study concludes that it is unclear whether nitric oxide is related to the severity of schizophrenia because nitrate levels are also affected by antipsychotic treatment. Patients with schizophrenia presented higher nitrate levels than controls, but subjects under olanzapine had lower nitrate levels than those treated with risperidone or haloperidol. Nitrate levels were correlated with PANSS total score but not with SCID II scores (Minutolo et al. 2012).

However, recent meta-analysis of results of 10 studies of nitrite + nitrate determinations in the blood of patients with schizophrenia lead to a conclusion that there are no differences in the level of nitric oxide metabolites between the patients and the healthy controls. In contrast, patients under antipsychotic treatment were found to have higher levels of NO metabolites than controls. The study revealed also a positive correlation between the duration of disease and levels of NO, which could be explained both by pathophysiological differences between the acute and chronic phases of schizophrenia and by the use of medications in the course of the disease (Oliveira et al. 2012).

Interestingly, it has been demonstrated that nitric oxide metabolites are significantly elevated in the plasma of patients following a suicide attempt (Kim et al. 2006; Lee et al. 2006). Thus, one might speculate a role of microglial cytokines or iNOS in triggering suicide (Steiner et al. 2008).

Accumulating evidence suggests that mitochondrial dysfunction and oxidative stress contribute to the pathogenesis of bipolar disorder and schizophrenia. Protein nitration, as measured by the level of 3-nitrotyrosine, was increased in the prefrontal cortex of patients with bipolar disorder and schizophrenia groups (Andreazza et al. 2010).

Increased protein nitration in the central nervous system is reflected by changes detectable in the blood. Increased level of 3-nitrotyrosine was found in the blood plasma proteins (Dietrich-Muszalska et al. 2009, 2012) and blood platelet proteins (Dietrich-Muszalska and Olas 2009) of patients with schizophrenia. This clear-cut increase of the marker of peroxynitrite formation may seem astounding when confronted with the apparent lack of changes in the level of nitric oxide metabolites in the blood. It should be remembered, however, that the rate of peroxynitrite formation is the function of superoxide available for reaction with nitric oxide. Oxidative stress occurring in schizophrenia, including lowered activity of superoxide dismutase found in the blood of the patients (Dietrich-Muszalska et al. 2005) leaving a higher fraction of superoxide not scavenged, may promote enhanced peroxynitrite formation even in the absence of significant elevation in the concentration of nitric oxide (Beckman and Koppenol 1996).

Apparently, this controversial area requires further studies which should both bring deeper understanding of the role of reactive nitrogen species in the pathogenesis of schizophrenia and perhaps provide analytical tools to monitor the course of the disease and its therapy.

5 Nitric Oxide and Antipsychotics in Schizophrenia

Nitric oxide plays a role in the pathophysiology of schizophrenia, but it is unclear how antipsychotics may affect NO generation and its metabolic pathways.

In various authors' studies, contradictory results were presented. Das et al. (1996) showed that the plasma nitrate level was significantly lower in first episode drug-naïve patients with schizophrenia than in normal control subjects. In humans, the level of platelet NOS was significantly higher in drug-naïve patients with schizophrenia than in normal control subjects. It appears that treatment with chlorpromazine, haloperidol, or clozapine normalizes platelet NOS in schizophrenic patients (Das et al. 1995). Arnaiz et al. described that haloperidol reduced the production of NO in the submitochondrial fraction of mice, suggesting inhibited mitochondrial electron transfer with increased production of superoxide anion and hydrogen peroxide (Arnaiz et al. 1999). This result is consistent with the result obtained by Rengassamy and Johns (1993), who found inhibition of NO synthase by superoxide anion-generating system.

In schizophrenic patients, both before and after the treatment, NO metabolite levels in plasma were significantly lower than in normal control subjects (Lee et al. 2006). Lee and Kim suggested that the improvement of psychiatric symptoms can lead to partially normalize a deficiency of NO after the treatment in schizophrenic patients (Lee and Kim 2008).

In contrast to that, Herken et al. showed a significant increase in the level of NO in erythrocytes in patients with schizophrenia treated with antipsychotics (Herken et al. 2001). Similarly, patients with schizophrenia presented higher nitrate levels than controls, but subjects under olanzapine had lower nitrate levels than those treated with risperidone or haloperidol. Moreover, nitrate levels were correlated with PANSS total score (Minutolo et al. 2012).

Elevated level of nitric oxide metabolites in the blood plasma of patients was described by Zhang et al. In their study, risperidone and haloperidol did not reduce the elevated plasma NO levels in the patients (Zhang et al. 2012).

Some studies have shown an increase in cyclic GMP levels in the cerebrospinal fluid in patients with schizophrenia after treatment with antipsychotics (Oliveira et al. 2011). Stimulation of soluble guanylate cyclase by NO leads to an increased synthesis of cGMP, a secondary messenger, which in turn has effects on the activity of kinase cascades, mRNA stability, translocation, transcription factors, and primary gene products in target cells (Oliveira et al. 2012). Presumably an increase of cyclic GMP found in those studies might be associated with stimulation of the NMDA–NO–cGMP pathway by antipsychotic drugs. Oliveira et al. revealed also a positive correlation between the duration of disease and levels of NO, which could be explained both by pathophysiological differences between the acute and chronic phases of schizophrenia and by the use of antipsychotics in the course of the disease (Oliveira et al. 2012).

Kato et al. have studied the effects of risperidone on the nitric oxide production, inducible NO synthase (iNOS) expression, and inflammatory cytokines: interleukin (IL)-1beta, IL-6, and tumor necrosis factor (TNF)-alpha by IFN-gamma-activated microglia (Kato et al. 2007). Risperidone significantly inhibited the generation of NO and proinflammatory cytokines by microglial activation, in comparison with haloperidol. The levels of iNOS in risperidone-treated cells were significantly lower than those in the haloperidol-treated ones.

It suggests that antipsychotics, especially risperidone, may have an anti-inflammatory effect via the inhibition of microglial activation, which is not only directly toxic to neurons but also has an inhibitory effect on neurogenesis and oligodendrogenesis; both of which have been reported to play a crucial role in the pathology of schizophrenia (Eastwood and Harrison 2005; Vostrikov et al. 2007; Segal et al. 2007).

The immunological mechanisms via such as interferon (IFN)-gamma and cytokines might be relevant to the pathophysiology of schizophrenia in accordance with the results of numerous recent studies (Schuld et al. 2004; Müller and Schwarz 2006; Craddock et al. 2007; Potvin et al. 2008). Antipsychotics used in the treatment of schizophrenia may have different effects on NO generation. In further studies, the role of oxidative/nitrosative stress and nitric oxide generation in schizophrenic patients together with their clinical symptomatology and use of antipsychotics should be taken into account.

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Blood Platelet as a Peripheral Cell in Oxidative Stress in Psychiatric Disorders

Barbara Wachowicz

Abbreviations

ADAM	A disintegrin and metalloprotease
ADP	Adenosine diphosphate
AMPAR	Glutamate receptor
cAMP	Cyclic adenosine monophosphate
CGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
DAG	Diacylglycerol
DAT	Dopamine transporter
DTS	Dense tubular system
EAAT	Excitatory amino acid transporter
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GP	Glycoprotein
GPCRs	G protein-coupled receptors
GPX	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidized glutathione
HMWK	High molecular weight kininogen
IL	Interleukin
LIGHT	Cytokine
LOX	Lipoxygenase

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MAO	Monoamine oxidase
MDA	Malonyldialdehyde
MMP	Matrix metalloproteinase
MP	Microparticle
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
OCS	Open canalicular system
PAF	Platelet-activating factor
PAR	Protease-activated receptor
PC	Phosphatidylserine
PDGF	Platelet-derived growth factor
PE	Phosphatidylethanolamine
PF_4	Platelet factor 4
PGH_2	Prostaglandin H_2
PKC	Protein kinase C
PKG	Protein kinase G
PLA_2	Phospholipase A_2
PLC	Phospholipase C
RANTES	Regulated on activation, normal T-cell expressed and secreted
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSNOs	S-nitrosothiols
SOD	Superoxide dismutase
SSRI	Selective serotonin reuptake inhibitor
TBARS	Thiobarbituric acid reactive substance
TGF	Transforming growth factor
TIMPs	Tissue inhibitor of metalloproteinases
TNF	Tumor necrosis factor
TP	Thromboxane receptor
TXA_2	Thromboxane A_2
Tyr	Tyrosine
VASP	Vasodilator-stimulated phosphoprotein

1 Introduction

Excessive oxidative stress occurring in the brain may be reflected by abnormalities in oxidative processes in peripheral cells from patients with psychiatric disorders, and blood platelets may be useful as diagnostic markers and indicators of the progression of the disease and as a tool to develop therapeutic approaches monitoring the therapeutic efficacy (Bakken et al. 2006; Camacho and Dimsdale 2000; Da et al. 1988; Fisar and Raboch 2008; Freedman 2008; Plein and Berk 2001; Stahl 1977).

Although the brain is the major organ affected by psychiatric disorders, research indicates that other cell types in the body show changes in these diseases, and

studying the more readily accessible cell could provide important information about the disease progression.

Important pathogenic mechanisms are likely common to psychiatric disorders, and oxidative stress appears to be a trigger in the complex chain of events leading to psychiatric diseases (Ng et al. 2008). Enhanced production of reactive oxygen/nitrogen species (ROS/RNS) in aging and neurological or psychiatric diseases is not restricted to the brain, but can also be seen in several peripheral tissues. Oxidative/nitrative changes are not confined to the CNS but also occur in peripheral cells, especially in circulating blood platelets. Moreover, impaired cognitions are associated with oxidative changes in platelet constituents or altered platelet function. Excessive oxidative processes and oxidative stress, hallmark features of the brain, have also been shown to occur in other peripheral blood cells such as lymphocytes or red blood cells. Since the oxidative stress is a global phenomenon and the markers of oxidative cell injury in the CNS correlate with the markers in peripheral non-CNS material in animal as well as in humans, it has become a common practice to assess the extent of cellular oxidative damage in the CNS from analyses of peripheral indices of oxidative stress and resultant cell injury.

Peripheral lymphocytes are more difficult to purify compared to platelets or erythrocytes. The preparation of lymphocytes from anticoagulated blood requires several steps and contamination with other cell types, e.g., platelets in the range of 1–5 % cannot be excluded. Isolation and purification of small platelets from anticoagulated samples of the blood is much easier than lymphocytes, and in the isolation from blood platelet suspension, the contamination with leukocytes is less than one leukocyte in 10^6 platelets. Cultured skin fibroblast from patients is also used to provide information about psychiatric disorders especially in signal transduction (Mahadik and Mukherjee 1996). They are limited model since they lack nucleus and mitochondria used to determine the changes in membrane lipids or activities of antioxidative enzymes.

Platelets contain several receptors, intracellular signaling cascade components, and have long been utilized as peripheral model of receptor-mediated signal transduction mechanisms in the central nervous system (Stahl 1977; Mangano and Schwarcz 1981; Da et al. 1988; Barradas and Mikhailidis 1993; Plain and Burk 2001; Bakken et al. 2006; Camacho and Dimsdale 2000). Platelets are useful for discovering mechanisms that underlie the multiple changes in cell signaling pathways that accompany psychiatric disorders. Oxidative stress seems to be a convergence factor that leads to many psychiatric disorder-related changes (Plein and Berk 2001; Mahadik et al. 2001; Ng et al. 2008; Yao et al. 2004, 2006).

2 Blood Platelet Activation

Blood platelets derived from megakaryocytes are the smallest anucleated cells in the blood which are essential elements of primary hemostasis. They play important roles in several diverse processes beyond hemostasis, malignancy, and infection and by promoting inflammatory and immune response. They maintain vascular integrity

and contribute to wound healing (Brydon et al. 2006; McNicol and Israels 2008; Smyth et al. 2009; Horstman et al. 2010; Nurden 2011).

Individual platelets vary in terms of volume, density, and reactivity. Resting platelets are discoid with a diameter which averages 1–2 μm and a mean cell volume of around 5–6 fl. The normal platelet count is in the range of $150–350 \times 10^9$/L. Under normal conditions human platelets circulate in the bloodstream for approximately 8–10 days. They respond immediately to vascular injury and interact with exposed elements of the underlying connective tissue and rapidly change from discoid shapes to active round forms with filopodia and lamellipodia. Platelet cytoskeleton composed of actin and tubulin polymers maintains the shape of the resting and activated platelets. The shape change represents one of the earliest events (< 5 s) after platelet stimulation. In platelet cytoplasm there are few mitochondria and different granules, large amounts of glycogen as a source of energy and a complex membranous system consisting of an open canalicular system (OCS) which allows connections between cytosol and surrounding medium, and the dense tubular system (DTS) which stores metabolic enzymes and together with mitochondria is involved in metabolic processes and controls the cytosolic calcium (Blockmans et al. 1995; Kamath et al. 2001).

The platelet possesses three types of specific granules: alpha granules, dense granules, and lysosomes. The granules store numerous substances which are released after activation of platelets induced by different agonists (Blair and Flaumenhaft 2009; Horstman et al. 2010).

Activation of platelets is a process controlled via a multitude of biochemical events, ranging from receptor stimulation, intracellular signal transduction to platelet response: shape change, adhesion, aggregation, and secretion of active compounds stored in platelet granules (Li et al. 2010; Rivera et al. 2009).

The controlled platelet granule exocytosis is an essential part of platelet function leading to secondary amplification of ongoing platelet activation. Platelets are activated at sites of vascular injury by the combined effects of a number of molecules, including collagen, thrombin, ADP, serotonin, epinephrine, and thromboxane A_2 (TX A_2) that interact with specific platelet glycoprotein receptors. Platelet response to agonist includes reorganization of the actin cytoskeleton, secretion of compounds stored in platelet granules, exposure of integrin fibrinogen receptors (GPIIb/IIIa) on platelet surface membranes, and aggregation. Activated platelets alter the composition of their membranes resulting in the expression of P-selectin derived from the alpha granule membrane on the surface of the platelet. The exposure of P-selectin is especially important for platelet–leukocyte interaction. During platelet activation, microparticles (MPs) are released and catalyze the coagulation cascade. MPs may play an important role in hemostasis, neuroinflammation, and neurodegenerative diseases (Horstman et al. 2010). They express most of the platelet membrane proteins (proteomic studies revealed about 600 proteins) and can directly activate and bind to leukocytes. Proteomic has became a promising technology for platelet research. It is a method for analysis of the rapid changes in platelet protein organization during activation and aggregation (Zahedi et al. 2006). The initial steps of platelet activation are regulated by protein phosphorylation events. Phosphorylation of amino acid residues is mostly transient and can change rapidly upon activation or inactivation of phosphatases and

kinases by specific stimuli like cyclic adenosine monophosphate (cAMP) and Ca^{2+} (Coles et al. 2002; Nagai et al. 1994). Malfunctions of these enzymes can lead to pathological events. The phosphorylation of serine and tyrosine residues is a very important switch in the signaling. The level of phosphorylated proteins depends on the activities of kinases and phosphatases (Zahedi et al. 2006). The human platelet membrane proteome reveals several new membrane proteins acting as signal receptors, mediators, or enhancers (Moebius et al. 2005). The platelet possesses different receptors. G protein-coupled receptors (GPCRs) on platelet membranes are present with only a few hundred copies per cell, whereas the platelet integrin αIIbβ3 (GPIIb/IIIa) receptors are present with 500,000 copies per platelet. On activated platelets P-selectin is present with about 10,000 copies per cell (Moebius et al. 2005) and activation can change the level of receptor expression on platelets.

In activated platelets anionic phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) become exposed on the platelet surface and render the membrane procoagulant. In vivo, the most important aggregating agents (agonists) are collagen in the vessel wall, ADP from injured cells (red cells) or released from the platelet dense granules, and thromboxane A_2 formed from arachidonic acid by stimulated platelets and thrombin. Other agonists such as serotonin, epinephrine, norepinephrine, platelet-activating factor (PAF), and vasopressin may contribute to the aggregation and activation. Serotonin is a weak agonist and involves $5\text{-}HT_{2A}$ receptors which differ from the sites responsible for serotonin uptake. Platelets carry all the serotonin in the blood in their dense granules.

In the signaling pathways induced by a platelet agonist, the known effectors include phospholipase C (PLC), phospholipase A_2 (PLA_2), and phosphoinositide 3-kinase. PLC hydrolyses membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to form 1,4,5-IP3 and diacylglycerol (DAG), which in turn raise the cytosolic free Ca^{2+} concentration and activate protein kinase C (PKC) isoforms. In platelets PLA_2 mobilizes arachidonic acid mainly from PC and PE, membrane phospholipids containing over 70 % arachidonic acid in the cell. Free arachidonic acid is converted via cyclooxygenase (COX) pathway to active thromboxane A_2 (Hamberg et al. 1975). Both TXA_2 (half-lives of 30 s) and its immediate precursor PGH_2 are labile potent stimulators of platelet activation and act on TP receptor (Armstrong 1996). Activated platelets acutely generate docosahexaenoic acid-containing phospholipids via lipoxygenase (12-LOX) pathway (Morgan et al. 2010).

Thrombin, within seconds, causes the increase in the cytosolic Ca^{2+} concentration triggering downstream Ca-dependent events, including activation of PLA_2. The activation of Ras superfamily members (Rc, Rao, Rap1b) takes place, leading to rearrangement of the actin cytoskeleton and shape change. Thrombin, like ADP or epinephrine, is able to inhibit adenylyl cyclase activity causing the decrease in cAMP level. Suppression of cAMP formation induced by other agonists plays an important role in platelet activation. The increase of cAMP (after prostacyclin or adenosine) inhibits platelet activation. cGMP as a second messenger activates PKG with phosphorylation of intracellular targets and inhibition of Ca mobilization, integrin GPIIb/GPIIIa activation, cytoskeleton rearrangement, secretion, and phosphoinositide 3-kinase (Radomski et al. 1987b, 1990; Gordge and Xiao 2010).

To respond to extracellular signals, platelets possess unexpectedly a large variety of surface receptors for stimulatory and inhibitory ligands, and platelet activation can change the level of receptor expression. There are three groups of platelet surface receptors with specific properties: G protein-linked receptors, enzyme-linked receptors, and ion channel-linked receptors. Thrombin activates platelets by proteolytic cleavage of protease-activated receptors (PARs). Human platelets express members of the Gq,G_{12}, and Gs family of G proteins and four of the Gi family, G_{11}, G_{12}, G_{13}, and Gz. Gq is the primary link to PLC beta activation. Gs and the three Gi family members stimulate and inhibit adenylyl cyclase activity in platelets. The activation of adenylyl cyclase by Gs is counterbalanced by the inhibitory protein Gi. Thrombin, ADP, epinephrine, and PAF stimulate platelets and at the same time lower cAMP concentration by activating G_1.

Thrombin interacts with platelet via a specific proteolysis of the extracellular N-terminal of PAR (proteinase-activated receptor), which leads to the exposure of a new tethered ligand, binding to the receptor and initiating signal transduction.

In platelets stimulated by strong agonists, arachidonic acid is released from membrane phospholipids by active $cPLA_2$ and rapidly converted by cyclooxygenase (COX) pathway into active TXA_2, a potent platelet agonist. The activity of $cPLA_2$ is regulated by at least two major mechanisms: translocation of $cPLA_2$ from cytosol and/or phosphorylation of Ser505 mediated by ERK 1/2. Aspirin inhibits platelet activation by inhibition of COX activity and reducing level of TXA_2. Thromboxane B_2, the stable metabolite of short-lived TXA_2, is the marker of platelet activation.

Different lipid mediators are generated during platelet activation: resolvins derived from omega-3 fatty acids via LOX pathway, PAF, eicosanoids and endocannabinoids. Endocannabinoids which include anandamide (*N*-arachidonoylethanolamine) and 2-arachidonoylglycerol (2-AG) are released from activated platelets and may activate these cells through endocannabinoid receptors present in platelets (Signorello et al. 2011). Human platelets express endocannabinoid receptors CB_1 and, to a lesser extent, CB_2 belonging to the superfamily of G protein-coupled receptors (Catani et al. 2010). 2-AG can be consider a new physiological platelet agonist. Anandamide at low concentrations, through PI3/AKT pathway activation, stimulates eNOS activity and increases NO level in platelets (Signorello et al. 2003). The ability of activated platelets to release 2-AG suggests that a chronic over-release of 2-AG by platelets may be a causal factor in the cognitive deficits associated with negative symptom in schizophrenia, and increased platelet activation may lead to the changes of endogenous cannabinoid level in the brain (Pryor 2000; Pandey et al. 2010).

Adenosine is an important regulatory metabolite and an inhibitor of platelet activation via binding of A_2 platelet receptor and the elevation of intracellular cAMP. The expression of A_2 adenosine receptor is induced by oxidative stress (Johnston-Cox and Ravid 2011).

Platelets synthesize proteins (COX, integrin, Fyn) and they have developed extranuclear mechanisms to process and efficiently translate mRNA into protein (Weyrich et al. 2009; Hattori et al. 2009). Moreover, the transcriptomic profile of platelets has begun to be studied. They contain NF-kB – a transcription factor which controls the gene expression of inflammatory mediators. The latter may be involved in platelet function (Beaulieu and Freedman 2009).

3 Blood Platelets in Inflammation

The traditional role of platelets as mediators of hemostasis and thrombosis is well documented. Increasing evidence suggests that activated platelets are involved in and may promote inflammation. They help maintain and modulate inflammation and are a major source of proinflammatory molecules such as P-selectin, CD40L, tissue factor, and matrix metalloproteinases (MMP_5) (Smyth et al. 2009; Horstman et al. 2010; Nurden 2011). Activated platelets release and express different inflammatory mediators. The adhesion of circulating platelets to the vascular endothelium is a key element of the proinflammatory and prothrombogenic states and is associated with oxidative stress: a variety of mechanisms are involved (Cooper et al. 2002; Urbich et al. 2002; Chakrabarti et al. 2005; Stokes et al. 2009).

Platelets as inflammatory mediators possess the major classes of factors active in inflammatory states. Platelet–leukocyte aggregation links hemostasis to inflammation. Horstman et al. (2010) suggest that platelets are active partners with leukocytes in the entry to the CNS, and PAF derived from platelets would facilitate opening of the blood–brain barrier in the microenvironment since PAF is involved in disruption of endothelial cell junctions.

The initial platelet–leukocyte contact is mediated by P-selectin which in activated platelets is rapidly translocated to the surface. In addition to P-selectin, platelets contribute to platelet–leukocyte aggregation by releasing microparticles. During platelet–leukocyte aggregation MMPs are liberated and present on the cell surface. Interaction between platelets and leukocytes can occur via P-selectin on the surface of activated platelets and PSGL-1 (P-selectin GP ligand-1, CD162) on leukocytes. High molecular weight kininogen (HMWK) can also form the bridges between GPIbα on the platelet and CD11b/CD18 (Mac 1) on leukocytes (Chavakis et al. 2003). From alpha granules, upon platelet activation, platelet factor 4 (PF_4, chemokine $CXCL_4$) in abundance other chemokines including RANTES and a variety of growth factors, PDGF, FGF, EGF, TGF-beta, VEGF, and IL-1, IL-8, IL-6, are released in abundance. Platelets after stimulation express on their surface CD40L, a member of the tumor necrosis factor (TNF) superfamily which is then cleaved and circulates as soluble sCD40L (Inwald et al. 2003; Santilli et al. 2007). The binding of sCD40L to platelet CD40 results in cell activation, expression of P-selectin on platelets, and subsequent binding of platelets to leukocytes (Stokes et al. 2009). Platelets are the main source of CD40L in the circulation (Pignatelli et al. 2004). LIGHT is another transmembrane protein belonging to the TNF family associated with platelets and released after activation. Platelet-derived LIGHT induces inflammatory responses in endothelial cells and monocytes (Otterdal et al. 2006).

Matrix metalloproteinases (MMPs) have been recognized as major factors participating in disruption of the blood–brain barrier (Horstman et al. 2010). MMPs comprise a family of zinc-dependent endopeptidases with differential proteolytic activity against various proteins of the extracellular matrix. In platelets five different MMPs such as MMP-1, MMP-2, MMP-3, MMP-9, and MMP-14 have been identified (Chung et al. 2004). ADAM-10, ADAM-17 (a disintegrin and metalloprotease), ADAMTS13 (ADAM with thrombospondin domain), and TIMPs (tissue inhibitor of metalloproteinases) are present. MMP-1 and MMP-2 may prime

platelets for adhesion and aggregation (Chung et al. 2004). The release and action of platelet MMPs are regulated by nitrite oxide (NO) (Martinez-Cuesta et al. 2001). MMPs play an important role in the migration of immune cells to sites of inflammation by degrading basement membranes and extracellular matrix components. During platelet–leukocyte aggregation NO and free radicals are released (Cooper et al. 2002). NO and MMP-1, MMP-2, MMP-3, and MMP-9 play an important role in the regulation of PAR agonist-induced platelet–leukocyte aggregation (Chung et al. 2004).

Thornton et al. (2010) have identified platelets as a key source of IL-1α. It indicates that platelet activation and platelet-derived IL-1α are major contributors to inflammation-mediated injury in the brain.

4 Changes of Platelet Structure and Function in Psychiatric Disorders

The diversity of platelet components is implicated in psychopathological states, and platelets are important tools for psychopharmacological research since they offer an interesting vantage point for understanding the neurophysiology processes of various psychiatric disorders as well as their association with cardiovascular diseases. Several data support the role of oxidative stress in diverse psychiatric disorders such as bipolar disorder, depression, autism, schizophrenia, and anxiety disorders; however, the broadest data regarding oxidative stress mechanisms have been derived from studies in schizophrenia (Table 1).

Similarities between platelets and neurons are particularly important with respect to serotonin metabolism (Lesch et al. 1993). Since serotonergic neurons and platelets express serotonin-related enzymes, receptors, and transporters, the alterations in the CNS are likely to be reflected in platelet levels of the same molecules. Abnormalities of the platelet serotonergic system have been found in major depression, schizophrenia, seasonal affective disorder, obsessive–compulsive disorder, posttraumatic stress disorder, panic disorder, eating disorders, aggressive behavior, substance use, autism, and Alzheimer's or Parkinson disease (Mikuni et al. 1991; Yao et al. 1996; Yamawaki et al. 1998; Khait et al. 2002; Ljubicic et al. 2007; Chen 2009; Nemeroff and Owens 2009; Safai-Kutti et al. 1985). Moreover, various parameters of the serotonergic system in platelets undergo seasonal fluctuation (Khait et al. 2002; Ljubicic et al. 2007).

Platelet 5-HT_{2A} receptors are coupled to PLC through Gq proteins, and their stimulation induces generation of IP_3 and DAG. The platelet 5-HT_{2A} receptor (a serotonin postsynaptic receptor in the brain) is a common object of study (Kovacic et al. 2008; Velayudhan et al. 1999). 5-HT level is elevated in platelets of autistic individuals. Platelet hyperserotonemia has been detected in 25–60 % of autistic children, and a significantly higher level of 5-HT_{2A} receptor mRNA in platelets has been revealed which could suggest serotonin system dysregulation (Hranilovic et al. 2009; Kazek et al. 2010). Serotonin is involved in many of the same processes affected by cannabinoids (Janusonis 2008; Zvetkova et al. 2010). 5-HT uptake by

Table 1 Markers of oxidative stress in blood platelets and changed platelet reactivity in patients with some psychiatric disorders

Schizophrenia	Decreased activities of antioxidant defense system	Reddy et al. (1991); Dietrich-Muszalska et al. (2005)
	Platelet lipid peroxidation, platelet protein nitration/carbonylation, decreased thiol and GSH level	(Dietrich-Muszalska and Olas 2009a)
	Reduced cAMP in platelets	Kafka et al. (2009)
	Increased activity of platelet PLA2	Gattaz et al. (1995)
	Overactivity of COX	Das and Khan (1998)
	Alteration in platelet function: increased aggregation, secretion	Yao et al. (1992, 1994); Dietrich-Muszalska and Olas 2009b; Dietrich-Muszalska 2008)
	Alteration in membrane composition	du Bois et al. (2005)
Drug-naive, first-episode schizophrenic patients	Elevated expression of platelet integrin receptors	Walsh et al. (2002)
	Supersensitive platelet glutamate receptors	Berk et al. (1999, 2000); Baier et al. (2009)
Depression disorder	Ultrastructural changes in platelets	Palmar et al. (1997)
	Increase platelet reactivity and alterations in platelet function	Bruce and Musselman (2005); Celano and Huffman (2011); Wittstein (2010)
	Increased level of PGE2, TXA2	Lieb et al. (1983); Zafar et al. (2010)
	Increased platelet reactivity	Celano and Huffman (2011); Chen (2009)
	Increased platelet aggregation, P-selectin expression	Mendoza-Sotelo et al. (2010)
	Supersensitive glutamate receptors	Berk et al. (2001)
Bipolar disorder	Changes in glutamate uptake	Do Nascimento et al. (2006)
	Decreased PKC changes in platelet signaling	Pandey et al. (2008)

platelets was significantly increased in a group of chronic marijuana smokers suffering impairment of cognitive function, and the activity of 5-HT transporters was affected by cannabinoids at high concentrations. It seems that a lowered 5-HT uptake may reflect gender-related differences in effects of psychoactive cannabinoids (Velenovska and Fisar 2007).

Depressed patients appear to have more platelet receptors compared with healthy volunteers and exhibit higher platelet activation and enhanced procoagulant properties than healthy control (Palmar et al. 1997; Nemeroff and Owens 2009). Abnormalities in inositol phosphate signaling system in platelets can be used as diagnostic marker for psychiatric disorders including major depression (Dwivedi and Pandey 2009; Panday et al. 2010).

Platelets possess some key components of functional dopaminergic system including the dopamine transporter (DAT) and dopamine receptor D_2 (Frankhauser

et al. 2006). Dopamine can potentiate ADP-induced platelet adhesion. Platelets store acetylcholine, and acetylcholine esterase is present indicating platelet cholinergic system. Platelet monoamine oxidase (MAO) activity has been evaluated in several neuropsychiatric disorders indicating the higher activity in parkinsonian and demented patients but lower in alcoholics. Platelet MAO is solely of B type (Magos 2002).

In the etiology of various psychiatric diseases such as depression, schizophrenia, ADHD, and addiction, monoaminergic dysfunction is involved, and platelets may well serve as an easy accessible peripheral system to study monoaminergic neurotransmission that is controlled by rapid and selective reuptake of neurotransmitters by specific transport proteins: DAT, SERT, and NED (Frankhauser et al. 2006; Zalsman et al. 2011).

Glutamate metabolism is also modified in platelets from patients with psychiatric disorders (Berk et al. 1999, 2000, 2001; Do Nascimento et al. 2006; Baier et al. 2009). Platelets express glutamate uptake transporters (EAATs) to clear glutamate from the blood where its concentration is relatively high and vesicular glutamate (VGLUT) transporter to load glutamate into platelet dense granules. Platelets express both mRNA and proteins for the three major glutamate transporters, namely, EAAT1, EAAT2, and EAAT3 (Zoia et al. 2004). Platelets store and release glutamate during platelet activation (Berk et al. 2000; Berk et al. 2001; Begni et al. 2005; Do Nascimento et al. 2006; Morrell et al. 2008). Platelets express glutamate receptors AMPARs that have a functional role in regulating platelet agonist response. Morrell et al. (2008) demonstrated the importance of glutamate as a modulator of platelet function. In platelets the presence of AMPAR subunit protein $GluR_1$ has been described (Chen 2009). This type of AMPA receptor could play a role in comorbid depression and cardiovascular disease. NMDA receptor signaling is involved in the regulation of platelet production from megakaryocytes. Platelet glutamate receptors are supersensitive in schizophrenia and depression with psychotic disorders. Glutamate uptake by platelets may be modified in parallel with mood changes in the subjects. Platelets from patients with bipolar I disorders with manic episodes have an increased uptake of glutamate compared to platelets from control subjects. The glutamatergic system is modulated by oxidative stress induced by heavy metals (Borges et al. 2007).

In platelets from schizophrenic patients, reduced level of cAMP (Kafka et al. 1979; Kaiya et al. 1990), overactivity of COX (Das and Khan 1998), elevated expression of integrin (Walsh et al. 2002), increased phosphatidylinositol pathway (Yao et al. 1992), and platelet activation (Yao et al. 1994) are described. The increased activity of platelet PLA_2 (Gattaz et al. 1995), changed membrane lipids (Horobin 1996), depressed expression of protein signaling (Hattori et al. 2009), and changed antioxidant defense with oxidative alteration in platelet molecules, especially membrane lipid peroxidation (Mahadik et al. 2001; Dietrich-Muszalska et al. 2005, Dietrich-Muszalska and Olas 2009a), are also present. Oxidative modification of platelet membrane phospholipid composition may lead to the alterations in neurotransmitter systems in psychiatric disorders, especially in schizophrenia,

where omega-3 and omega-6 polyunsaturated fatty acid levels are reduced (Horrobin 1996; Du Bois et al. 2005). Numerous neurotransmitter systems are sensitive to ROS (Sah et al. 2002; Nakamura and Lipton 2011).

Several lines of evidence support the hypothesis that imbalance between the production of ROS and the detoxification of reactive intermediates is a feature of psychiatric disorders, and oxidative stress may play a functional role in these disorders. Psychiatric disorders are characterized by a lower antioxidative defense system, higher free radical production, and improvement of some symptoms after antioxidant administration (Ng et al. 2008; Tsaluchidu et al. 2008; Schedel et al. 2010). Oxidative stress leads to the modulation of vascular homeostasis (Zho et al. 1999). In psychiatric disorders, when excess of ROS/RNS production together with oxidative stress is observed, the biomolecules (lipids, proteins, nucleic acids) present in plasma and blood cells are modified. Measurement of levels of markers of oxidative stress reflects a status of increased oxidative stress. Alterations in oxidative processes in the brains of patients with psychiatric disorders may also occur in non-neuronal tissues including fluids such as cerebrospinal fluid, plasma, urine, and blood cells. In platelets from schizophrenic patients, oxidative damage was present (Reddy et al. 1991; Yao et al. 2001). In platelets from patients with schizophrenia of paranoid type, a significantly low thiol level in platelet proteins was observed (Dietrich-Muszalska and Olas 2009a), and oxidative stress in platelets from schizophrenic patients seems to be associated with the oxidation of free protein thiols to disulfides. It may be the consequence of oxidation/nitration process (Essex and Li 2003; Kalyanaraman 2004).

Modifications caused by ROS/RNS in platelet proteins from schizophrenic patients include not only oxidation of thiol groups but also protein carbonylation and nitration of tyrosine. Nitric oxide and its metabolites may have a role in the pathophysiology of schizophrenia (Bernstein et al. 2005). Peroxynitrite generated from NO and superoxide anion can induce oxidation of proteins measured by the level of carbonyl groups and nitration of some amino acid residues, particularly tyrosine. A stable product of tyrosine nitration is measured as a biomarker of protein modifications caused by peroxynitrite or other RNS. Nitration of tyrosine residues in platelet proteins results in the alteration of protein structure and function and, usually, inhibition of activity of enzymes. Moreover, nitration of tyrosine may directly inhibit the tyrosine phosphorylation involved in signal transduction pathways in platelets (Ischiropoulos 1996). Dietrich-Muszalska and Olas (2009a) provide evidence that in platelet proteins from patients with schizophrenia, in acute period of psychosis, a high level of 3-nitrotyrosine is present. The measurements of abnormalities in oxidative processes in peripheral cells such as platelets from patients have the potential to be useful as diagnostic markers, as indicators of the disease progression, as a tool to develop therapeutic approaches, and as monitors of therapeutic efficacy. Platelets are also useful for discovering mechanisms that underlie the multiple changes in cell signaling pathways that accompany psychiatric diseases and lead to the alteration of platelet function, mainly hyperactivation of platelets and cardiovascular diseases.

5 Modification of Platelet Function Induced by ROS and RNS

Platelets are influenced by ROS in multiple types of pathology based on inflammation, endothelial cell damage, or thrombosis (Radomski et al. 1987b; Cooper et al. 2002; Olas and Wachowicz 2007; Freedman 2008; Forstermann 2010). The platelet activation cascade is a complex process with different cellular signaling pathways, and ROS produced mostly intracellularly are involved in cellular signaling and may act as second messengers (Krotz et al. 2004; Begonja et al. 2006; Essex and Li 2006; Essex 2009; Savini et al. 2010; Sill et al. 2007; Manickam et al. 2011; Shamova et al. 2011). ROS and RNS play a very important role in platelet activation, since they may modulate the signal transduction in various and sometimes opposite ways affecting different platelet metabolic pathways (Blackmore 2011; Handin et al. 1977; Morrel 2008; Radomski et al. 1987a; Rodrigues et al. 2010; Patel et al. 1999; Krotz et al. 2004; Freedman 2008). ROS may regulate platelet function by reducing $^{\cdot}$NO bioavailability because ROS scavenge platelet or endothelium-derived $^{\cdot}$NO (Hirata et al. 1995; Matsubara et al. 2003; Munzel et al. 2003). Rapid reaction between $^{\cdot}$NO and superoxide anion leads to the generation of peroxynitrite which is a potent nitrating and oxidizing agent and may modify platelet structure and function (Beckman and Koppenol 1996; Bermejo et al. 2005; Moro et al. 1994; Practico and Violi 1997; Olas and Wachowicz 2007; Wachowicz et al. 2008). Even small increase in these radicals, particularly superoxide anion ($O_2^{\cdot-}$), may cause a remarkable peroxynitrite generation (Huie and Padmaja 1993; Pryor and Squadrito 1995). $^{\cdot}$NO produced in endothelial cells or in platelets interacts with $O_2^{\cdot-}$, leading to the reduction of vasorelaxation and platelet activation. Increased concentration of $O_2^{\cdot-}$, especially during inflammation, is one of the factors controlling the half-life of $^{\cdot}$NO. Platelets themselves can generate several ROS/RNS including superoxide anion (Jahn and Hansch 1990; Wachowicz et al. 2002), hydrogen peroxide (H_2O_2) and hydroxyl radical (Pratico et al. 1999), nitric oxide (Radomski et al. 1987b; 1990), and peroxynitrite (Olas and Wachowicz 2007).

In stimulated platelets the aggregation of platelets is accompanied by the burst of H_2O_2 (Maresca et al. 1992; Iuliano et al. 1994, 1997; Pignatelli et al. 1998; Hedin and Fowler 1999). H_2O_2 is involved in platelet activation cascade (Del Principe et al. 1985, 1991; Salvemini and Botting 1993; Ambrosio et al. 1994). ROS regulate tyrosine phosphorylation in integrin subunit responsible for aggregation (Irani et al. 1998; Hernandez-Hernandez et al. 1999).

Blood platelets synthesize $^{\cdot}$NO (Signorello et al. 2003; Leoncini et al. 2005), and platelet NO synthase (NOS) has been described (Muruganandam and Mutus 1994; Mehta et al. 1995; Sase and Michel 1995). Platelet activation leads to stimulation of NOS which in turn generates NO (Freedman et al. 1997). Protein kinase C may regulate NOS activity by direct serine/threonine phosphorylation (Hirata et al. 1995; Matsubara et al. 2003). In platelets $^{\cdot}$NO activates the soluble guanylyl cyclase and thereby increases intracellular cGMP (Munzel et al. 2003; Lohmann and Walter 2005; Marjanovic et al. 2005; Begonja et al. 2006; Marcondes et al. 2006). Inhibition

of integrin GPIIb/GPIIIa enhances the release of NO (Chakrabarti et al. 2004). NO inhibits platelet activation and the NO/cGMP pathway is a well-established mechanism of platelet inhibition. The inhibition of ROS production in platelets might lead to increase of cGMP and VASP (vasodilator-stimulated phosphoprotein) serine phosphorylation when more NO is present (Butt et al. 1994). Nitration of VASP, a protein critical for actin cytoskeletal rearrangement, may be important in the regulation of platelet aggregation (Sabetkar et al. 2002, 2008). Platelet superoxide anion can be converted to H_2O_2 by platelet superoxide dismutase SOD. H_2O_2 serves as a substrate for the production of hypochlorous acid by means of neutrophil myeloperoxidase.

The biological events triggered by signaling stimuli involve ROS, but it is not clear exactly what the molecular targets of ROS in signal transduction mechanisms are. Brill et al. (2009) hypothesize that oxidative damage could directly suppress platelet function by loss of platelet glycoprotein receptors (GPIbα, GPV). Oxidative stress activates tumor necrosis factor alpha (TNF_a)-converting enzyme (TACE/$ADAM_{17}$) and induces shedding of GPIbα and GPV on platelets. TACE activation is dependent on p38 mitogen-activated protein kinase signaling (Brill et al. 2009). Targets of ROS are G alpha proteins (Nishida et al. 2000).

After stimulation platelets produce superoxide anion mainly via NAD(P)H oxidase (Krotz et al. 2002; Begonja et al. 2006) and xanthine oxidase (Miller et al. 1993) which participates in signaling leading to integrin $\alpha_{IIb}\beta_3$ receptor activation and may act by other mechanisms than scavenging $^{\cdot}NO$. Ligand–receptor activation triggers a signaling cascade leading to ROS production which in turn enhances expression and activity of Ecto-NOX1. NAD(P)H oxidase-derived $O_2^{\cdot-}$ flux is in the nanomolar range and is similar to the flux that is present in endothelium cells, but less than <1 % of the amount of $O_2^{\cdot-}$ from activated neutrophils. Thrombin and collagen are thought to act in part via NAD(P)H^- oxidase-dependent intraplatelet oxygen radical generation, and the $O_2^{\cdot-}$ generated seems to be a particular link to integrin activation. Significant amounts of $O_2^{\cdot-}$ aside from NAD(P)H oxidase are generated during arachidonate cascade (Jahn and Hansch 1990). Since platelet-derived O_2 is a functionally relevant scavenger of platelet-derived $^{\cdot}NO$, it is possible that intraplatelet iron via iron-dependent oxidants would provide the redox conditions for the generation of further radicals such as hydroxyl radicals from H_2O_2 and lipid peroxides (Olas and Wachowicz 2005). Hydrogen peroxide does not evoke the activation of integrin receptor for fibrinogen, although both H_2O_2 and $O_2^{\cdot-}$ are produced within activated platelets. Hydrogen peroxide acts as a signaling molecule, and serine phosphorylation of VASP induced by H_2O_2 has been observed (Sabetkar et al. 2008). It induces platelet Ca mobilization, PKC activation, and phosphorylation of tyrosine (Juliano et al. 1994; Hedin and Fowler 1999; Gopalakrishna and Jaken 2000). Collagen stimulates intraplatelet H_2O_2 production which serves as an intraplatelet second messenger but alone is incapable to activate the integrin receptor (Del Principe et al. 1985, 1991; Pignatelli et al. 1998).

ROS participate in platelet activation (Maresca et al. 1992; Salvemini and Botting 1993; Ambrosio et al. 1994; Iuliano et al. 1997; Komiya et al. 1999; Olas et al. 2009; Pratico et al. 1999). On the other hand, platelets represent a relevant target for the action of exogenous ROS derived from the vascular wall. Enhanced ROS release

from the vessel wall can indirectly affect platelets (Forstermann 2010). Under inflammatory conditions platelets are also exposed to phagocyte-dependent production of high quantities of ROS. There are enzymes responsible for the reduction of free radicals and ROS. H_2O_2 is relatively stable and diffuses through membranes. In vitro exogenous H_2O_2 inhibits ADP-induced platelet aggregation but enhances platelet activation induced by collagen or arachidonate. Plasma possesses a variety of antioxidants that have effects on the level of different mainly vessel wall-derived ROS. Plasma redox state is altered by several plasma thiols such as cysteine and homocysteine and endogenous and exogenous antioxidants derived from diet. Homocysteine may change the redox state in platelets (Olas et al. 2008). Endothelial dysfunction or damage by oxidants is associated with enhanced risk for platelet activation (Cooper et al. 2002; Forstermann 2010).

In platelets NO is produced in the cytosol, in about 100 fmol NO/mg of platelet proteins. In unstimulated platelets $^{\cdot}$NO generation is about 4–7 pmol/min/10^8 platelets and increased to 11–21 pmol/min/10^8 platelets after activation (Mehta et al. 1995; Radomski et al. 1996; Zhou et al. 1995; Krotz et al. 2004). $^{\cdot}$NO-mediated reactivity depends mostly on the formation of secondary intermediates such as peroxynitrite and nitrogen dioxide than $^{\cdot}$NO per se. Peroxynitrite is a transient species with a biological half-life of 1–10 ms, shorter than of NO (1–30 s) (Radi 2004).

An elevated production of NO for prolonged periods of time in many pathological states, including psychiatric disorders, contributes to oxidative damage of different cellular macromolecules. NO is produced in stimulated platelets (Mehta et al. 1995; Zhou et al. 1995; Pignatelli et al. 2006), but collagen may decrease NO synthesis (Leoncini et al. 2005). A key oxidant and nitrating agent responsible for oxidation and nitration of platelet biomolecules leading to their altered function is peroxynitrite. It is an inflammatory mediator and its amount depends on the competition for superoxide dismutase and NO (Bartosz 1996). NO effectively competes with SOD for scavenging of superoxide. Platelets are very sensitive to low amounts of peroxynitrite which increases the levels of 3-nitrotyrosine in platelet proteins (Ischiropoulos and Gow 2005; Ischiropoulos 1998; Naseem et al. 2000; Reiter et al. 2000), oxidizes tryptophan in proteins (Kato et al. 1997), and alters protein structure and function (Ischiropoulos and Al-Mehdi 1995; Mondoro et al. 1997; Ducrocq et al. 1999; Bruckdorfer 2001; Low et al. 2002; Ischiropoulos 2003; Ischiropoulos and Gow 2005). Its effects may be also linked to platelet eicosanoid formation (Zhou et al. 1995; Boulos et al. 2000) because peroxynitrite inhibits cyclooxygenase via nitration of tyrosine residue (Tyr 385) and reduces synthesis of eicosanoids, especially TXA_2, a potent lipid mediator of platelet activation and vessel constriction. Platelets contain high level of CO_2 (~1 mM) and thiols (5 mM) which react with peroxynitrite. The reaction of glutathione with peroxynitrite produces a relatively stable S-nitroglutathione (Lufrano and Balazy 2003), whereas carboxynitrite is highly unstable. Peroxynitrite appears to possess a dual effect on platelet activation: it can inhibit or stimulate this process (Brown et al. 1998; Ducrocq et al. 1999; Olas et al. 2004a, b; Nowak and Wachowicz 2001a, b, 2002). Its inhibitory effect is probably caused by the formation of S-nitrosothiols (Radomski et al. 1992; Mayer et al. 1995; Gaston 1999; Crane et al. 2002; Gordge and Xiao 2010). Peroxynitrite is a

potent oxidative and nitrative species, and due to its ability to nitrate tyrosine, it affects cellular processes in the platelet dependent on tyrosine phosphorylation. The altered signaling cascades in platelets may be dependent on the complex effects of peroxynitrite on the activity of various kinases and phosphatases (Liaudet et al. 2009; Minetti et al. 2002).

Platelets express antioxidant enzymes, superoxide dismutase SOD, glutathione peroxidase GPx, and catalase that can not only prevent cytotoxic effects of ROS but also regulate by ROS signaling pathways in platelets. In addition to its antioxidant properties, GPx enhances the bioavailability of NO by catalyzing its liberation from S-nitrosothiols and reducing lipid peroxide.

Platelet ROS generation estimated by means of specific markers of oxidative stress such as 3-nitrotyrosine (Khan et al. 1998), lipid peroxidation (measured usually as TBARS – thiobarbituric acid reactive substances, or level of malonyldialdehyde MDA), free thiols, GSH, and carbonyl groups in protein (Dalle-Donne et al. 2003a, b; Wong et al. 2010) could potentially be used as a marker and a predictor for the progression of psychiatric diseases.

The defense mechanisms against RNS, especially peroxynitrite, are crucial for platelet function. Plant antioxidants (polyphenols) present in human diet or selenocompounds may protect blood platelets against toxicity of peroxynitrite (Arteel et al. 1999; Klotz and Sies 2003; Olas et al. 1999, 2004, 2006).

Numerous transmitter systems are sensitive to ROS. Exogenous ROS may interact with receptors and transporters and alter ligand–receptor interaction or ion transport indirectly via actions within the lipid environment of the platelet membranes. ROS are raised in both physiological and pathological processes (psychiatric disorders), but efficient mechanisms have evolved for their detoxification. Increased oxidative stress was described in platelets from chronic smokers (Takajo et al. 2001). Platelet redox state may be influenced by the alteration of vascular redox state, the presence of endogenous and exogenous antioxidants, and the formation of ROS and RNS that the defense mechanisms against RNS/ROS are unable to counterbalance. ROS, by directly affecting the redox state, modulate platelet function.

5.1 The Effects of ROS/RNS on Platelet Thiols

The oxidation of the protein thiols to mixed disulfides is an early cellular response to oxidative stress. S-nitrosothiols (RSNOs) are compounds produced by the S-nitrosation of thiols, usually cysteine (Nakamura and Lipton 2011). They act as NO donor agent via cGMP or cGMP-independent mechanisms of their action, including prevention of TXA_2 synthesis, nitration of alpha actinin, and inhibition of ADP receptors ($P2Y_{12}$). RSNOs regulate protein disulfide isomerase (Gordge and Xiao 2010).

ROS/RNS, especially peroxynitrite, oxidize GSH producing oxidized glutathione (GSSG) and decrease the equilibrium between reduced and oxidized glutathione (GSH–GSSG ratio) in the platelet (Quijano et al. 1997; Nowak et al. 2003). This ratio is very important for the redox regulation of protein thiols (Giustarini et al. 2000;

Schafer and Buettner 2001; Essex 2009). The majority of protein thiols exist in reduced form, when the GSH–GSSH ratio is high. *N*-acetyl-L-cysteine is able to restore intraplatelet free thiols and shifts the redox balance favor of GSH concomitant with the inhibition of platelet aggregation. The GSH–GSSG ratio in platelets seems to be one of the potentially important regulators of platelet signaling. *N*-acetylcysteine a glutathione precursor reduces ROS generation associated with the increase of intraplatelet GSH (Berk et al. 2008; Dean et al. 2011; Gibson et al. 2009).

Several glycoprotein receptors in platelets such as GPIb and GPIIb/GPIIIa contain thiol groups which are extracellular redox-sensitive sites. Various forms of redox modulations of thiols or disulfides in platelet glycoprotein receptors exist. These include modification by low molecular weight thiols such as reduced glutathione or homocysteine and oxidized glutathione or by NO derived from S-nitrosothiols. Levels of these redox compounds are changed in various disease states, and in some cases physiological concentration of these compounds may modify platelet responsiveness (Essex and Li 2003, 2006). Moreover, platelets themselves contain a trans-plasma membrane redox system capable of reducing extracellular disulfide bounds. In the blood a redox homeostasis exists and redox environment is controlled. Changes in the extracellular redox state induced by diseases or pharmacological agents that modify the platelet redox environment will modify platelet function (Schafer and Buettner 2001; Crane et al. 2002). Redox-sensitive sites in the platelet such as vicinal thiols in GPIIb/GPIIIa involved in platelet aggregation can be regulated from the extracellular or cytoplasmic environment (Manickam et al. 2011). Extracellular redox state can also induce changes of the cell surface glycocalyx (Shamova et al. 2011). Since many biological processes are regulated by redox reactions that involve surface thiols, the extracellular redox state can have an important influence on disease status and may be a target for therapeutic interventions. Specific nitrosative or oxidative modifications of thiols in platelets may modulate platelet function (Minetti et al. 2002). Thiol-based reactions occur in proteins involved in platelet function, especially in extracellular platelet proteins, not only integrin receptors for fibrinogen but also in collagen receptors (integrin $\alpha_2\beta_1$) on platelets. They are regulated by protein disulfide isomerase and thiol metabolism. In the blood, low molecular thiols regulate redox state by converting disulfide bonds to thiols (Essex and Li 2003; 2009).

Blood platelets contain several organelles and proteins necessary for apoptotic processes and are able to undergo apoptotic events. Moreover, a number of apoptotic markers of nucleated cells have also been recognized in platelets, including markers of both receptor and mitochondrial pathways with executioner caspases, suggesting that anucleated platelets may undergo programmed cell death (Lopez et al. 2007) and ROS generated in the platelet are involved in apoptotic events. Platelets can display phosphatidylserine on their surface, produce microparticles, induce mitochondrial membrane depolarization, and activate caspases. Platelets modified oxidatively/nitratively by peroxynitrite can undergo apoptosis (Wachowicz et al. 2008). Peroxynitrite causes the activation of caspase-3; induces depolarization of mitochondrial membrane potential, microparticle formation, and PS exposure; and may be responsible for the activation of intrinsic pathways of apoptosis in platelets.

6 Platelet Activation and Cardiovascular Events in Psychiatric Disorders

There is considerable epidemiological evidence supporting the association between depression and coronary heart disease where blood platelets play the central role in both acute and chronic coronary syndromes (Maes et al. 1996; Bruce and Musselman 2005; Wittstein 2010).

Depression is associated with increased platelet reactivity. Platelets share many biochemical similarities with neuronal monoamine system, particularly in the uptake, storage, and metabolism of serotonin. Serotonin participates in hemostasis by inducing platelet aggregation; therefore, therapy with selective 5-HT reuptake inhibitors (SSRIs) which modulate platelet activation and may protect platelets from hyperaggregability seems to be responsible for the reduction of cardiovascular mortality in major depression, where hyperaggregability of platelets is observed. Citalopram (SSRI) specifically inhibits collagen-induced platelet aggregation, secretory process, and expression of P-selectin on platelet membrane (Tseng et al. 2010). Platelets chronically medicated with SSRI exhibit lower platelet 5-HT content and reduced platelet aggregation induced by ADP, collagen, and epinephrine, but not arachidonic acid. This may explain the increased bleeding risk associated with SSRI treatment as well as beneficial effect of SSRIs in the prevention of myocardial infarction. It appears that SSRI may modulate platelet reactivity by an independent pathway different from GPIIb/GPIIIa inhibitors and antioxidants (Serebruany et al. 2001). SSRIs may represent an optimal class of dual agents treating depression and simultaneously inhibiting platelet reactivity Bismuth-Evenzal et al. (2012).

It has been proposed that serotonin-mediated platelet activation may be a key pathogenic link between depression and coronary heart disease. Platelets from patients with depression release from their granules PF4, B-thromboglobulin and P-selectin (Mendoza-Sotelo et al. 2010; Fisar and Raboch 2008). The activated integrin receptors for fibrinogen and the generation of thromboxane A_2 have also been demonstrated (Lieb et al. 1983). Zafar et al. 2010) studied the effects of both depression and anxiety on serotonin- and epinephrine-mediated platelet reactivity in a patient population with stable coronary artery disease. The authors have suggested that anxiety may be a better predictor of platelet reactivity than depression; however, the combination of depression and anxiety that frequently coexists resulted in greater serotonin-mediated platelet activation than depression alone (Zafar et al. 2010). Larger studies are necessary to determine whether depression and anxiety have independent effects on platelet reactivity.

Anorexia nervosa is a serious psychiatric disorder associated with significant cardiovascular mortality. Pereira et al. (2010) observed that aggregability of platelets from adolescents with anorexia nervosa was unchanged, whereas platelet NOS activity was reduced and cGMP unchanged. They hypothesized that alteration of L-arginine–NO–cGMP pathway in platelets may be early predictors of the incidence of cardiovascular disease in adult life.

In cardiovascular patients under platelet therapy (clopidogrel, aspirin), close monitoring of platelet function is recommended, especially when they are also under treatment with antipsychotic drugs.

7 Conclusion

Psychiatric nosology depends primarily on identifying syndromes, and a peripheral marker that reflects a specific disorder would have important clinical implications either diagnostically or as a measure of disease process. It seems that redox states in platelets that partly reflect the oxidative stress in the brain together with the changes of platelet function may predict or correlate with treatment outcome in some psychiatric disorders.

The results indicate the importance of the platelets in obtaining an insight into the changes in neurotransmitter function.

The neuron functions as part of a complex nervous network and it is not directly affected by changes in the blood, whereas the blood platelet has no direct connection with the nervous system, has a relatively short half-life (about 10 days), and is directly influenced by changes in the blood and vessel wall. Moreover, a major advantage in studying the blood platelet lies in its ease of access in patients.

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Mitochondrial Dysfunction in Autism

Abha Chauhan, Feng Gu, and Ved Chauhan

Abbreviations

ADP	Adenosine diphosphate
AGC	Aspartate/glutamate carrier
ASDs	Autism spectrum disorders
ATP	Adenosine triphosphate
ETC	Electron transport chain
$FADH_2$	Dihydrogen flavin adenine dinucleotide
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HEADD	Hypotonia, intractable epilepsy, autism, and developmental delay
MD	Mitochondrial disease
MELAS	Mitochondrial encephalopathy with lactic acidosis and seizures
MMP	Mitochondrial membrane potential
MRS	Magnetic resonance spectroscopy
NADH	Reduced nicotinamide adenine dinucleotide
NO	Nitric oxide
PDHC	Pyruvate dehydrogenase complex
ROS	Reactive oxygen species
SNPs	Single-nucleotide polymorphisms
SOD	Superoxide dismutase
TCA	Tricarboxylic acid

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1 Structure and Function of Mitochondria

Mitochondria, containing an inner and an outer plasma membrane, are very important cellular organelles that generate adenosine triphosphate (ATP), the energy carrier in most mammalian cells, by oxidizing glucose and fatty acids. Acetyl-CoA is a key intermediate generated from the oxidation of glucose and fatty acids, and it enters the tricarboxylic acid (TCA) cycle, also known as the Krebs cycle, in the mitochondrial matrix. The TCA cycle produces reduced flavin adenine dinucleotide ($FADH_2$) and reduced nicotinamide adenine dinucleotide (NADH), which donate electrons to the electron transport chain (ETC) located in the inner mitochondrial membrane. The ETC is an essential part of mitochondria, and generation of energy in the form of ATP is its most important function. More than 90 % of energy needed in the cell to maintain its physiological activities is supplied by the ETC through oxidative phosphorylation (Bertram et al. 2006; Szewczyk and Wojtczak 2002). The ETC consists of five multi-subunits of enzymes, i.e., complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome bc1 complex), complex IV (cytochrome c oxidase), and complex V (ATP synthase) (Boekema and Braun 2007; Dudkina et al. 2005). While complexes I–IV participate in the generation of proton gradient (membrane potential) in the intermembrane space of mitochondria, complex V transports protons from intermembrane space to the mitochondrial matrix (Fig. 1). The generated proton gradient is used by ATP synthase to catalyze the phosphorylation of adenosine diphosphate (ADP) to ATP. Ubiquinone (also known as coenzyme Q10) and cytochrome c are the electron carriers of the ETC and help in the transfer of electrons between ETC complexes.

The ETC is also a main source of free radicals, i.e., reactive oxygen species (ROS), which have important roles in cell signaling and homeostasis (Cadenas and Davies 2000; Lenaz 2001). ROS generation is a by-product of proton cycling between ubiquinone, cytochromes b and c1, and iron–sulfur protein (Sugioka et al. 1988). Complexes I and III are the main sites of mitochondrial superoxide (O_2^-) production (Barja 1999; Muller et al. 2004). While complex I releases superoxide exclusively into the mitochondrial matrix, complex III releases superoxide to both sides of the inner mitochondrial membrane, i.e., to the mitochondrial matrix and the intermembrane space (Muller et al. 2004). The superoxide radicals generated by complexes I and III are neutralized and converted to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (Mn SOD) in the mitochondrial matrix or by copper/zinc (Cu/Zn) SOD in the intermembrane space of mitochondria (Fig. 1). Under normal circumstances, there is a balance between ROS generation and the antioxidant capacity of the cell. However, in some situations (e.g., environmental exposure to air pollutants and toxins), ROS levels can increase dramatically and exceed the antioxidant ability of Mn SOD and Cu/Zn SOD, thus causing oxidative stress and triggering apoptosis. ROS-mediated lipid peroxidation, oxidation of proteins, and DNA damage are well-known outcomes of oxidative stress, leading to cellular damage and ultimately to cell death (Bandyopadhyay et al. 1999; Cadenas and Davies 2000; Lenaz 2001; Polster and Fiskum 2004). The ETC abnormalities may result in inhibition of ATP synthesis and acceleration of ROS generation, leading to

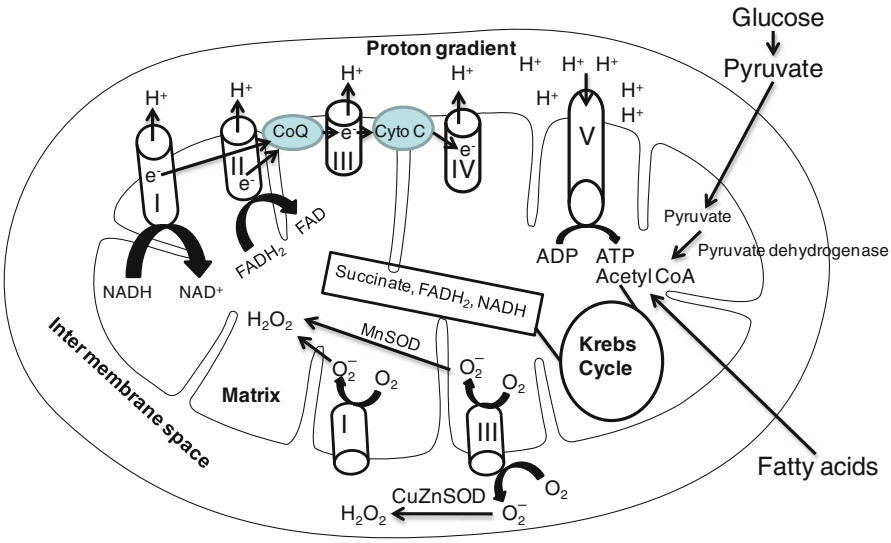

Fig. 1 ATP production and superoxide free radical generation by the electron transport chain (ETC) of mitochondria. Mitochondria have two membranes: an inner and an outer membrane. ETC consists of a series of metalloproteins bound to the inner membrane of the mitochondria, including five enzyme complexes, designated I–V, i.e., complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome bc1 complex), complex IV (cytochrome c oxidase), and complex V (ATP synthase). Electrons are transferred from NADH to O_2 through inner membrane ETC complexes I, III, and IV. Coenzyme Q (CoQ) and cytochrome c. CoQ shuttles electrons from complexes I and II to complex III, and cytochrome c transfers these electrons from complex III to IV. During this process, protons are pumped through the inner mitochondrial membrane to the intermembrane space to establish a proton gradient, which is used by complex V (ATP synthase) to phosphorylate ADP thereby generating ATP. Complexes I and III are also the main sites of mitochondrial free radical superoxide (O_2^-) production. Complex I-dependent O_2^- is exclusively released into mitochondrial matrix, where it is converted to hydrogen peroxide (H_2O_2) by the manganese superoxide dismutase (Mn SOD). On the other hand, superoxide generated at complex III can be released to both sides of the inner mitochondrial membrane. Superoxide released into the mitochondrial intermembrane space is converted to H_2O_2 by Cu/Zn SOD. NADH and $FADH_2$ are produced within the matrix of the mitochondria by the Krebs cycle, also known as the tricarboxylic acid (TCA) cycle. The redox energy from NADH and $FADH_2$ is then transferred to oxygen (O_2) via the ETC of mitochondria

impairment of energy metabolism, elevated oxidative stress, and disruption of mitochondrial functions and subsequently affecting neurons' function and plasticity, which may finally lead to abnormal neurodevelopment.

Although most DNA is packaged in chromosomes within the nucleus, mitochondria also have a small amount of their own DNA, known as mitochondrial DNA (mtDNA). Mitochondrial function is under the dual genetic control of both mtDNA and nuclear DNA (nDNA). mtDNA contains 37 genes and encodes for 13 subunits of complexes I, III, IV, and V (Anderson et al. 1981). The other subunits of the ETC complexes are coded by more than 850 nDNA genes (Cotter et al. 2004). The expression, replication, and maintenance of mtDNA also require the factors encoded by nuclear genes (Shadel 2008). Furthermore, the nuclear-encoded signaling pathway

genes play a role in mediating adaptive functions of mitochondria under altered conditions (Shadel 2008; Shadel and Pan 2009). Hence, mtDNA and/or nDNA genome mutations may lead to deficiencies of ETC complexes and subsequently to mitochondrial dysfunction.

Mitochondria also play a pivotal role in the maintenance of intracellular calcium homeostasis and in amino acid, lipid, and steroid metabolism, thereby regulating developmental processes, including neurite outgrowth, axonal plasticity, and synaptic plasticity (Chinnery and Schon 2003; Fattal et al. 2006; Mattson and Liu 2002; Orth and Schapira 2011; Szewczyk and Wojtczak 2002). The brain has a high demand for energy, and it requires a high content of mitochondria. Neurons are highly dependent upon oxidative phosphorylation as the primary pathway for ATP generation, of which 40–60 % is utilized in the maintenance of ion gradients by ATPases. Neuronal synapses, in particular, are areas of high energy consumption, and therefore, they especially rely on mitochondrial function (Ames 2000; Mattson and Liu 2002). Mitochondria are concentrated in the dendritic and axonal termini, where they are involved in ATP production, calcium homeostasis, and synaptic plasticity (Li et al. 2004). Therefore, neurons' function and plasticity rely mostly on mitochondrial structure and number. Synaptic transmission is affected if there is alteration in the number, morphology, or function of synaptic mitochondria (Polster and Fiskum 2004). Metabolic and mitochondrial defects affect the function and plasticity of neurons, cause neuronal loss, and alter modulation of neurotransmission systems. Therefore, the brain is a prime target of mitochondrial dysfunction (Orth and Schapira 2011).

Mitochondrial dysfunction has been implicated in several human diseases, such as neurodegenerative diseases, neurodevelopmental disorders, and cardiac dysfunction, and it may play a role in the aging process. In addition, mitochondrial abnormalities are also associated with several other medical conditions (Fig. 2).

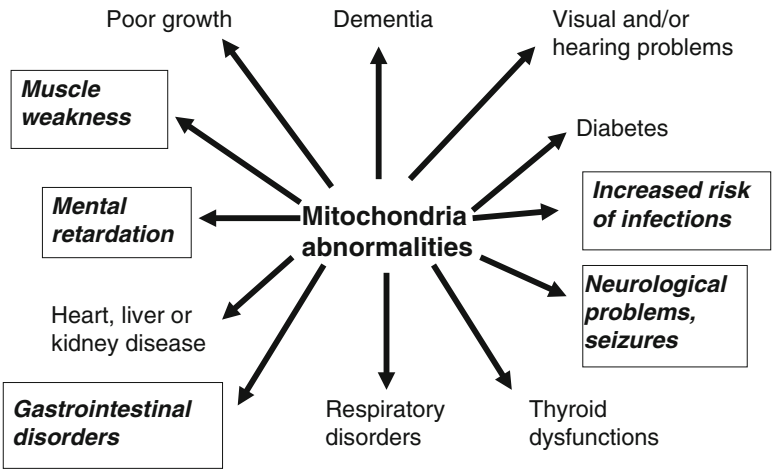

Fig. 2 Various medical conditions suggested to be associated with mitochondrial abnormalities. The medical conditions shown in *italics/bold* inside the boxes are also commonly observed in autism

Depending on how severe the mitochondrial disorder is, the symptoms can range in severity from mild to fatal. Some of the symptoms due to mitochondrial abnormalities shown in boxes in Fig. 2 are frequently observed in individuals with autism.

2 Autism

Autism belongs to a group of neurodevelopmental disorders known as the autism spectrum disorders (ASDs) that also include Asperger syndrome and pervasive developmental disorder-not otherwise specified. Autism is a heterogeneous disorder characterized by impairments in basic social and communicative behaviors such as eye contact, intonation, and facial expressions, as well as by repetitive and stereotyped patterns of behavior (Lord et al. 2000). The symptoms of ASDs are typically present before the age of 3 years. According to a report from the Centers for Disease Control and Prevention, 1 in 68 children in the USA is affected with autism (Wingate et al. 2014). Recently, researchers reported that the prevalence of ASDs in a South Korean community was as high as 3.74 % for boys and 1.47 % for girls among school-age children (Kim et al. 2011). It was 1.89 % in the general population of school-age children from regular schools and 0.75 % in a high-risk group from special education group and disability registry (Kim et al. 2011).

The exact cause of autism is still not known, although roles of genetic and environmental factors, oxidative stress, mitochondrial dysfunction, inflammation, and immune abnormalities have been suggested in ASDs (Chauhan et al. 2009a). While no single gene has been found to be associated with ASDs, multiple genetic components, including mtDNA mutations or deletions, nDNA mutations, and chromosomal defects, have been postulated in the ASDs (Abrahams and Geschwind 2010; El-Fishawy and State 2010; Holt and Monaco 2011; Miles 2011). However, the lack of complete concordance in monozygotic twins and the variation in severity in concordant pairs suggest that nongenetic factors also contribute to the etiology of ASDs. Some gene variants in ASDs may confer altered vulnerability to environmental stressors, and gene–environment interactions may alter the course of development of the central nervous system and lead to behaviorally defined symptoms of ASDs (Herbert 2010). Prenatal or postnatal environmental exposure to prooxidant factors such as metals, viruses, air pollutants, and toxins is known to increase the body burdens and production of ROS, which may trigger oxidative stress and the development of clinical symptoms of ASDs.

3 Mitochondrial Dysfunction in Autism

3.1 Prevalence of Mitochondrial Disease (MD) in Autism

MD is often caused by a gene mutation or deletion and is the most frequent cause of metabolic disease. The diagnosis of MD is complicated and is based on several clinical and laboratory tests. The prevalence of MD is approximately 0.01 % in the

general population (Skladal et al. 2003). However, according to meta-analyses from two large prospective studies (Correia et al. 2006; Oliveira et al. 2005) and one retrospective study (Scaglia et al. 2009), the prevalence of MD in the general population of ASDs is 5.0 % (Rossignol and Frye 2012), which is much higher than its prevalence in the general population. There are many biochemical markers such as lactate, pyruvate, lactate/pyruvate ratio, ubiquinone, alanine, alanine-to-lysine ratio, and acyl-carnitine that may directly suggest mitochondrial dysfunction. Other biomarkers, e.g., creatine kinase, carnitine, aspartate aminotransferase, alanine aminotransferase, and ammonia, may also indirectly suggest mitochondrial dysfunction. However, there is no reliable biomarker to identify all cases of MD (Haas et al. 2007).

3.2 Abnormal Energy Metabolism in Autism

Because of the frequent association of lactic acidosis and carnitine deficiency in autistic subjects, Lombard (1998) presented a hypothesis that mitochondrial dysfunction and defects in neuronal oxidative phosphorylation may be involved in the etiology of autism. Several reviews have recently shed light on mitochondrial dysfunction in autism (Chauhan and Chauhan 2012; Chauhan et al. 2012b, Gargus and Imtiaz 2008; Haas 2010; Palmieri and Persico 2010; Rossignol and Bradstreet 2008; Rossignol and Frye 2012). However, it is not yet known whether mitochondrial dysfunction in ASDs is the primary etiology or pathology secondary to other causes.

Many lines of evidence from biochemical, genetic, anatomical, and neuroradiographical studies indicate a relationship between the dysfunction of brain energy metabolism and autism (Chauhan et al. 2011b; Chugani et al. 1999; Filiano et al. 2002; Guevara-Campos et al. 2010; Lombard 1998; Minshew et al. 1993). In 1993, a ^{31}P-magnetic resonance spectroscopy (MRS) study showed decreased synthesis and increased membrane degradation as well as decreased synthesis of ATP in the dorsal prefrontal cortex of the brain in 11 high-functioning autistic men compared to age-matched control subjects (Minshew et al. 1993). The alterations in brain energy and phospholipid metabolism in autism correlated with the neuropsychologic and language deficits, i.e., the severity of autism symptoms. In 1999, another MRS study showed decreased N-acetyl-aspartate and increased lactate levels in the frontal lobe, temporal lobe, and cerebellum of nine children with autism (Chugani et al. 1999). These studies suggested a disturbance of brain energy metabolism in autism.

In several investigations with blood and/or muscle biopsy samples from individuals with ASD, analysis of biochemical markers of mitochondrial dysfunction showed high lactate, increased lactate to pyruvate ratio, increased alanine levels, and low carnitine levels in autism (Correia et al. 2006; Filipek et al. 2003; Mostafa et al. 2005; Oliveira et al. 2005; Weissman et al. 2008). For example, significantly lower serum carnitine and higher plasma lactate were reported in a study of 30 children with autism compared with the control subjects (Mostafa et al. 2005). The levels of carnitine and lactate correlated with the severity of autism, i.e., individuals with severe autism had significantly lower carnitine and higher lactate concentrations than those with mild or moderate autism.

A population-based survey of children with ASD conducted by Oliveira et al. (2005) showed that 14 of 69 (20.3 %) autistic children had hyperlactacidemia and 5 of 11 autistic children (who also had muscle biopsies) were classified with definite mitochondrial respiratory chain disorder, suggesting that this might be one of the most common disorders associated with autism. However, these investigators did not find any mtDNA mutation and/or deletion associated with known mitochondrial disorders in these children. Another study of 210 autistic subjects reported hyperlactacidemia in 36 subjects (17 %) and elevated lactate/pyruvate ratio in 27 % (53 of 196 subjects) (Correia et al. 2006). MD was also confirmed in 7 of the 30 fully assessed subjects (Correia et al. 2006). The results of a meta-analysis by Rossignol and Frye (2012) showed the prevalence of elevated lactate to be 31.1 % from six studies (Correia et al. 2006; Germanò et al. 2006; Laszlo et al. 1994; Moreno et al. 1992; Mostafa et al. 2005; Oliveira et al. 2005) and of elevated pyruvate to be 13.6 % from two studies (Germanò et al. 2006; Laszlo et al. 1994) in the general population of individuals with ASDs. However, classical MD only occurs in a few autistic individuals and is generally accompanied by genetic abnormalities and defects in the respiratory chain.

3.3 Activities and Expression Defects of Mitochondrial ETC Complexes in Autism

Several case studies showed alterations in the activities or expression levels of ETC complexes in autism. For example, two children with autism had deficiencies in respiratory chain enzymes such as complexes I–III and coenzyme Q_{10} (CoQ) (Tsao and Mendell 2007). In a recent review and meta-analysis, Rossignol and Frye (2012) reported deficiencies of complexes I, III, V, IV, and II in 53 %, 30 %, 23 %, 20 %, and 9 % of children with ASD and concomitant MD, respectively. Multiple complex deficiencies were found in 36 % of the children with ASD/MD.

The onset of autism is gradual in many children. However, in regressive autism, children first show signs of normal social and language development, but they lose these developmental skills at 15–24 months and develop autistic behavior (Ozonoff et al. 2005). This pattern may be different in some children with regressive autism. The reported incidence of regressive autism varies in different studies from 15 to 62 % of cases (Goldberg et al. 2003; Hansen et al. 2008; Stefanatos 2008). A huge reduction of the enzymatic activities of complexes I and III was reported in a 19-month-old autistic girl with developmental regression (Poling et al. 2006). These investigators also performed a retrospective study, which included 159 subjects with autism and 94 age-matched control subjects. They reported increased levels of aspartate aminotransferase and creatine kinase in the serum, suggesting abnormal oxidative phosphorylation in autism (Poling et al. 2006). In another study, Shoffner et al. (2010) reported autistic regression in 61 % (17 of 28) ASD subjects with definite MD and that fever was associated with the onset of regression in 12 of these children.

Weissman et al. (2008) performed a retrospective analysis of cases with autism. In addition to clinical symptoms for autism, these 25 individuals also presented

enzyme- or mutation-defined mitochondrial ETC dysfunction. Complex I activity was decreased in 16 of 25 (64 %) autistic subjects, and this was the most prevalent enzyme defect. It was followed by complex III deficiency, which was affected in 5 of 25 (20 %) autistic subjects. Deficiency of complexes II and IV was reported in 5 % and 4 % of autism cases, respectively. They reported that 40 % of this group demonstrated an unusual pattern of regression (multiple episodes, loss of motor skills, or regression after the age of 3), and six children had the mtDNA mutation (Weissman et al. 2008). Another report on the mitochondrial respiratory chain in the muscle homogenate of a 3-year-old girl with autism also showed a partial deficiency of complex III and a slightly diminished complex IV (Guevara-Campos et al. 2010).

Mitochondrial abnormalities have also been reported in the lymphoblastoid cells and lymphocytes from peripheral blood in autism. Inhibition of complex I was reported in the lymphoblasts from 7 of 9 autistic subjects, and a 40–50 % higher mitochondrial maximal respiratory rate was found in all nine autistic cases compared to lymphoblasts from unaffected subjects (Holtzman 2008). Increased respiratory rate in autism was suggested to be a compensatory response to the partial inhibition of ATP synthesis (Holtzman 2008). We reported that mitochondrial membrane potential (MMP) is reduced, and free radical generation is elevated in the lymphoblasts from autistic subjects (obtained from the Autism Genetic Resource Exchange) compared to lymphoblasts from age-matched control subjects (Chauhan et al. 2009b). In another study, exposure to physiological concentrations of nitric oxide (NO) reduced MMP to a greater extent in the lymphoblasts from autistic subjects than from control subjects (James et al. 2009). Giulivi et al. (2010) examined mitochondrial functions in the lymphocytes from the blood of 10 children with autism and 10 typically developing children (ages 2–5 years). The activities of ETC complexes I–V and pyruvate dehydrogenase complex (PDHC), the mitochondrial rate of H_2O_2 production, and mtDNA copy number were analyzed. PDHC is the critical regulatory enzyme of cell metabolism because it catalyzes oxidative decarboxylation of pyruvate to form acetyl-CoA. They reported reduced activities of complexes I, IV, and V in six, three, and four of ten autistic children, respectively. The activity of PDHC was also significantly reduced, while plasma pyruvate levels and mitochondrial rate of H_2O_2 production increased in children with autism. However, the diagnostic criterion for a definite MD was fulfilled in only one child with autism.

In our recent study, we have reported brain region-specific deficits in the expression levels of mitochondrial ETC complexes in children with autism (Chauhan et al. 2011b). As compared to age-matched control subjects, the levels of complexes III and V in the cerebellum, of complex I in the frontal cortex, and of complexes II, III, and V in the temporal cortex were significantly lower in children with autism (ages 4–10 years) (Chauhan et al. 2011b). Analysis of the scattered plots showed that there was no overlap in the levels of these ETC complexes in the cerebellum and temporal cortex between autistic and control groups. In the frontal cortex, lower levels of ETC complexes were observed in a subset of autism cases, i.e., 60 % (3 of 5) for complexes I, II, and V and 40 % (2 of 5) for complexes III and IV. Interestingly, no change in the levels of any of the five ETC complexes was detected in the parietal and occipital cortices in the children with autism compared to control subjects,

suggesting that the ETC defect in autism is specific to the cerebellum and the frontal and temporal lobes. When adult cases (ages 14–39 years) were examined, no significant difference in the levels of ETC complexes in any brain region was observed between autistic subjects and age-matched control subjects. These results suggest that the expression of ETC complexes is decreased in the cerebellum and the frontal and temporal regions of the brains of children with autism (Chauhan et al. 2011b).

3.4 Mitochondrial Dysfunction in Autistic Subjects with Genetic Abnormalities

Many studies have revealed mitochondria-related gene abnormalities in autism, which may be caused by chromosome depletion, mtDNA mutation or depletion, and/or decreased levels of mRNA. Duplications of the proximal long arm of chromosome 15, including inverted duplication and interstitial duplication, are associated with autism. This abnormality has high frequency in autism, and 1–5 % of individuals with autism carry it (Gillberg 1998; Schroer et al. 1998). Mitochondrial hyperproliferation and deficiency in complex III of ETC were found by muscle biopsies in two autistic children with a chromosome 15q11-q13 inverted duplication (IDIC 15) (Filipek et al. 2003). In another study, two autism cases associated with sudden infant death syndrome showed mild mitochondrial hyperproliferation and a possible complex II defect (Gargus and Imtiaz 2008). The risk of sudden death in individuals with IDIC 15 is approximately 1 % per year.

The results of buccal swab ETC analysis in a 12-year-old boy with autism, epilepsy, and leg weakness showed a severe decrease in complex IV activity and a mild reduction in complex I activity (Ezugha et al. 2010). Chromosomal microarray analysis revealed 1-Mb deletion in the 5q14.3 region in this child. It was suggested that (i) this chromosomal deletion may be related to complex I and IV deficits, thereby manifesting in a mitochondrial disease and autism, and (ii) genes located within the deleted region of 5q14.3 may encode or regulate the expression and/or assembly of complex I or IV subunits (Ezugha et al. 2010).

Several studies have reported mtDNA mutations in autism. In a group of 12 children who presented clinically with hypotonia, intractable epilepsy, autism, and developmental delay (HEADD syndrome), five showed increased levels of large-scale mtDNA deletions that were not related with mitochondrial encephalomyopathies, and three of four children who were further examined had structural mitochondrial abnormalities (Filiano et al. 2002). Activities of mitochondrial respiratory enzymes were reduced in seven of eight children who had muscle biopsy (Filiano et al. 2002). In another report of five children with autism, the mtDNA A3243G mutation was observed in two of these children and in the mothers of two other children (Pons et al. 2004). This mutation is often associated with MELAS syndrome (mitochondrial encephalopathy with lactic acidosis and seizures). Another child in this group had 72 % mtDNA depletion in skeletal muscle and reduction in activities of complexes I, III, and IV to 34 %, 23 %, and 25 % of control

values, respectively (Pons et al. 2004). Another study reported the G8363A mutation in the mtDNA $tRNA^{lys}$ in blood and skeletal muscle from a boy with autism who also showed complex IV defect (Graf et al. 2000).

The protein encoded by the NADH-ubiquinone oxidoreductase 1 alpha subcomplex 5 (NDUFA5) gene is involved in the mitochondrial ETC complex I. In a Japanese case-control study that included 235 subjects with autism and 214 control subjects, Marui et al. (2011) examined three single-nucleotide polymorphisms (SNPs) of this gene and reported a significant association of two SNPs with autism. However, the mRNA level of another subunit of complex I (75-kDa subunit) was similar in the whole blood from autistic children compared to the controls (Taurines et al. 2010).

Normally, each mitochondrion has two to 10 copies of mtDNA (Robin and Wong 1988). This copy number can vary depending on the energy needs of the cells under different physiological conditions (Shay et al. 1990). Using lymphocytes, mtDNA over-replication was reported in five of 10 children with autism (Giulivi et al. 2010). This difference was not lymphocyte-specific. Similar results were also obtained with granulocyte cells. It was suggested that increased copy number of mtDNA in autism may be a compensatory mechanism to the defects of mtDNA replication or repair so that mtDNA can maintain the normal transcript's levels. The defects of mtDNA in autism may be caused by primary gene deficiency or higher oxidative stress of cells.

3.5 Oxidative Stress in Autism

The free radicals are generated endogenously during oxidative metabolism and energy production by mitochondria, and the ETC in mitochondria is a prime source of ROS generation (Cadenas and Davies 2000; Lenaz 2001). ROS can attack vital components of the cell, such as polyunsaturated fatty acids, proteins, and nucleic acid. These reactions can alter intrinsic membrane properties such as fluidity, ion transport, enzyme activities, protein cross-linking, and protein synthesis, ultimately resulting in cell death (Bandyopadhyay et al. 1999). Neuronal cells are very vulnerable to oxidative stress as a result of the high rate of oxygen delivery and consumption in the brain. Extensive evidence from our and other groups suggests a role of oxidative stress in the development and clinical manifestation of autism. Prenatal or postnatal exposure to environmental factors such as air pollution, organophosphates, and heavy metals may act as a trigger to increase the production of ROS, induce oxidative stress, and cause mitochondrial dysfunction, leading to the development of autism in the children (Chauhan and Chauhan 2006; Chauhan et al. 2009a; Herbert 2010).

The levels of oxidative stress markers for lipid peroxidation, protein oxidation, and DNA oxidation are increased in the blood (Chauhan and Chauhan 2006; Chauhan et al. 2004; Mostafa et al. 2010; Zoroglu et al. 2004), urine (Ming et al. 2005) and brains (Chauhan et al. 2011a, b; Chauhan and Chauhan 2012b; Evans et al. 2009; López-Hurtado and Prieto 2008; Muthaiyah et al. 2009; Sajdel-Sulkowska et al. 2011) of individuals with autism as compared to control subjects. In addition, antioxidant defense is decreased in autism. Levels of major antioxidant

proteins, namely, transferrin (iron-binding protein) and ceruloplasmin (copper-binding protein), are decreased in the serum of children with autism, particularly in regressive autism (Chauhan et al. 2004). Glutathione (GSH) is another major endogenous antioxidant produced by the cell, which neutralizes ROS and participates in the detoxification and elimination of environmental toxins. Lower levels of GSH and decreased ratio of GSH/ oxidized glutathione (GSSG) in the blood of individuals with autism have been reported in many clinical studies (Adams et al. 2011; Al-Gadani et al. 2009; Bertoglio et al. 2010; James et al. 2004; Pastural et al. 2009), which could be raised by methyl B12 treatment (Bertoglio et al. 2010) or vitamin/mineral supplementation (Adams et al. 2011). We reported reduced levels of GSH, increased levels of GSSG, and a decrease in the ratio of GSH/GSSG in the cerebellum and temporal cortex of autistic subjects (Chauhan et al. 2012a). James et al. (2009) also reported a decreased ratio of GSH/GSSG in both cytosol and mitochondria in the lymphoblastoid cells from autistic subjects. Glutathione peroxidase (GPx) is an enzyme involved in the direct elimination of ROS. It catalyzes H_2O_2 reduction to H_2O and also reduces lipid hydroperoxides to their corresponding alcohols. Decreased GPx activity of plasma was reported in a group of 44 Egyptian children with autism compared to 44 normal children (Mostafa et al. 2010) and in another group of 20 Egyptian autistic children compared to 25 age-matched control subjects (Meguid et al. 2011). We also reported increased oxidative damage and free radical generation, coupled with reduced activities of antioxidant enzymes in the lymphoblastoid cells from autistic subjects compared to age-matched control subjects (Essa et al. 2009).

3.6 Calcium-Signaling Abnormalities in Autism

Not only do mitochondria play a central role of maintaining Ca^{2+} homeostasis, but intracellular Ca^{2+} levels also modulate mitochondrial activity. Many cellular functions are regulated by intracellular free Ca^{2+} concentration. Calcium is essential for neurotransmitter release, and Ca^{2+} influx is essential for neuronal excitability. Mitochondrial activity and Ca^{2+} signaling have an intense cross talk. Therefore, abnormal calcium signaling can affect normal mitochondrial function and is considered a mitochondrial dysfunction.

Mitochondrial aspartate/glutamate carrier (AGC), which is physiologically activated by calcium, plays an important role in energy metabolism and neuron development by transporting glutamate into mitochondria (Napolioni et al. 2011). AGC transport rates and expression levels of AGC1 protein were significantly higher in the brain of autistic subjects compared with the control subjects (Palmieri et al. 2010). Neocortical calcium levels were also increased in these autistic subjects. Excessive calcium levels were responsible for high AGC1 activity and the activation of mitochondrial metabolism in autism (Napolioni et al. 2011; Palmieri et al. 2010). Furthermore, AGC1-encoding SLC25A12 gene is also reported to be associated with autism (Ramoz et al. 2004; Segurado et al. 2005).

After a stimulus, calcium flows rapidly into neurons through various types of membrane channels, including voltage-dependent and receptor-coupled channels. Intracellular Ca^{2+} concentrations are quickly restored to resting levels, primarily through Ca^{2+}/Mg^{2+} ATPase and Na^{+}/Ca^{2+} exchange. Several studies have reported calcium-signaling abnormalities in autism (Chauhan and Chauhan 2009; Gargus 2009). The L-type voltage-gated Ca^{2+} channel is important for excitation of neurons and activation of various Ca^{2+}-regulated signaling cascades (Catterall et al. 2005). Gain-of-function mutations in the L-type voltage-gated Ca^{2+} channel CaV1.2 (CACNA1C) was revealed in Timothy syndrome, a multisystem disorder including mental retardation and autism (Splawski et al. 2004, 2005). This mutation causes these channels to remain open longer and allow the influx of more Ca^{2+} than wild-type channels, resulting in increased intracellular Ca^{2+}. These studies supported the importance of the L-type voltage-gated Ca^{2+} channel in autism. The mutation in the CACNA1F gene, which encodes the L-type voltage-gated Ca^{2+} channel, CaV1.4, was reported to cause autistic symptoms in a New Zealand family with an X-linked retinal disorder (Hemara-Wahanui et al. 2005; Hope et al. 2005). In addition, the mutation of T-type voltage-gated Ca^{2+} channel was also found in six of 461 individuals with autism (Splawski et al. 2006). The function of T-type channels in the brain is related to the regulation of the oscillatory behavior of neurons in the cortex and thalamus (Perez-Reyes 2003). ASD-associated mutations have also been identified in some genes, which encode ion channels whose activity is directly regulated by Ca^{2+}, such as Ca^{2+}-dependent Na^{+} or K^{+} channel. Several point mutations in SCN1A and SCN2A genes, which encode the voltage-activated K^{+} channels NaV1.1 and NaV1.2, respectively, have been reported in autism (Kamiya et al. 2004; Weiss et al. 2003). Laumonnier et al. (2006) reported functional deficit of Ca^{2+}-activated K^{+} channel (BKCa), a synaptic regulator of neuronal excitability in autism. Disruption of the BKCa gene (KCNMA1) led to the haploinsufficiency and reduced BKCa activity in autism.

Several neurological diseases are caused primarily by malfunctioning of Ca^{2+} channels or Ca^{2+}/Mg^{2+} ATPase (Cooper and Jan 1999; Jacobsen et al. 1999). Ca^{2+}/Mg^{2+} ATPase is a membrane-bound enzyme involved in maintaining the electrochemical gradient of the cells in an energy-dependent manner and the concentration of intracellular calcium by extrusion of calcium from cytosol. We reported increased activity of Ca^{2+}/Mg^{2+} ATPase in the cerebellum of autistic subjects compared with age-matched control subjects (Ji et al. 2009). Increased Ca^{2+}/Mg^{2+} ATPase activity may be a compensatory mechanism in response to increased intracellular calcium levels in autism. Taken together, the studies above suggest that calcium-signaling abnormalities may also contribute to mitochondrial dysfunction and pathophysiology of ASDs.

4 Conclusion

Mitochondria play a vital role in many pathways such as energy generation, developmental metabolism, calcium homeostasis, free radical generation, and cell survival. Mitochondrial dysfunction can be caused by alterations in activities or

expression levels of ETC complexes, genetic abnormality, oxidative stress, or calcium-signaling abnormalities. Collectively, several independent studies have provided evidence of impaired mitochondrial function and, as a result, impaired cellular energy state as one of the mechanisms involved in the pathophysiology of autism. Any abnormal change in the mitochondrial activities may affect the cellular energy production and neurotransmission system and destroy the balance between ROS generation and antioxidant capacity of the cell, thus leading to abnormal neurodevelopment in children with autism.

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Ultrasound and Autism: How Disrupted Redox Homeostasis and Transient Membrane Porosity Confer Risk

Emily L. Williams and Manuel F. Casanova

Abbreviations

AP-1	Activator protein 1
APC	Activated protein C
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CRE	cAMP response element
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
ERK	Extracellular signal-regulated protein kinase
FDA	United States Food and Drug Administration
FGF	Fibroblast growth factor
GMP	Guanosine monophosphate
GSK3β	Glycogen synthase kinase 3β
HAT	Histone acetyltransferases
HDAC	Histone deacetylase
iNOS	Irreducible nitric oxide synthase
JNK	c-Jun N-terminal kinase

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LAMP-1	Lysosome-associated membrane glycoprotein 1
LEF	Lymphoid enhancer factor
LRP	Lipoprotein receptor-related protein
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor κB
NMDA	N-methyl-D-aspartate
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PTEN	Phosphatase and tensin homologue
redox	Reduction-oxidation
ROS	Reactive oxygen species
TCF	T cell factor
US	Ultrasound

1 Introduction

The study of sonic waveforms long predates the invention and application of the current forms of clinical ultrasound (US). As early as 1826, Jean-Daniel Colladon, a Swiss physicist and engineer, and Charles-Francois Sturm, a mathematician, studied hydroacoustics by utilizing the combination of an underwater bell and a simultaneous ignition of gunpowder to compare speed of underwater sound to that of light, thereby estimating the former at 1,435 m/s. While this early study of sonification was of an academic vein, underwater sonar was eventually studied, refined, and popularized for submarine navigation during World War I. The disastrous voyage of the *Titanic* likewise highlighted the need for better means of navigation. By the 1930s, ultrasound had been exapted for such uses as radar and metal flaw detection, the latter largely utilized for detecting flaws within the metal construction in hulls of large ships and plates of battle tanks. Also by this time, high-intensity ultrasound was being recognized as a useful therapeutic tool by the medical industry. Eventually, it evolved into an important tool for neurosurgery. Unfortunately, the 1940s saw an uprise in enthusiasm for ultrasound use, leading to boisterous claims of its cure-all capacity and its utilization in a variety of unwarranted therapies. Simultaneously, a mounting skepticism grew – a skepticism which ultimately retarded research into ultrasound's numerous applications, such as diagnostics.

Following a body of research investigating its bioeffects, ultrasound has slowly overcome the medical community's initial skepticism from the 1940s to 1950s. However, a similar albeit more confined overconfidence in ultrasound's safety has led to its overuse within obstetrics. For the first few decades of ultrasound's use in prenatal care, the FDA strictly regulated absolute intensity levels according to the

specific application. Now, however, risk is assessed by real-time thermal and cavitational indices available on the device itself. This has shifted control away from a regulatory authority dictating absolute exposure levels, to the end user who interprets machine output and adapts usage based on medical experience (Barnett et al. 2000).

To complicate matters, machine reliability is being called into question. Recently, a series of studies assessing ultrasound transducer error rates reveals that of seven manufacturer's equipment tested across 676 different transducers, on average 40 % of those transducers were defective (Mårtensson et al. 2009). All companies tested exhibited a minimum of 20 % error rates, while one company tested as high as 67 %. A separate study by the same research group sampled additional ultrasound transducers in a single hospital setting, finding 81 of 299 actively used ultrasound machines to be defective. Approximately 83 % of these errors were due to delamination of the ultrasound lens or breaks in the cables (Mårtensson et al. 2010). The group stresses the need for thorough and frequent transducer testing, beyond that of annual testing, in part because it is usually very difficult for the sonographer to recognize transducer defects due to a slow degradation of image quality that may not be readily identifiable. However, not only for the purpose of image quality and diagnostics is it imperative that ultrasound machines perform as they are intended: faulty transducers lead one to question whether the safety indices are accurately gauging exposure levels and, if not, what the true range of exposure levels may be. With faulty equipment, there is no way to ensure that exposures are not reaching harmful ranges.

In addition to questionable transducer performance, practitioners and sonographers who routinely utilize ultrasound in their practices appear to be poorly educated in the possible risks of ultrasound exposure. In a survey of 130 end users, 82.3 % failed to demonstrate understanding of the term "thermal index" which gauges temperature levels within exposed tissue, 96.2 % failed to demonstrate understanding of "mechanical index" which gauges cavitational levels within the tissue, and, alarmingly, only 20 % of end users knew where these safety indices were located on the machines (Sheiner et al. 2007). While patient safety is now dependent on machine performance and sonographer judgment, it is clear that earlier FDA deregulations have placed the onus of that safety on the shoulders of faulty equipment and undereducated end users.

Along with deregulated medical practices, ultrasound services are available for private use through businesses which offer additional ultrasonography so that patrons can "start the family photo album early," have 3D image renderings of the baby, and even have videos made. Ultrasound equipment is also available for purchase at websites such as eBay and Amazon, and additional devices like Doppler heart rate monitors can be purchased for daily, medically unsupervised monitoring of the baby's heartbeat. While the FDA has warned against these services and the use of such devices within the home, these warnings have been poorly publicized and the vast majority of the public and practitioners are unaware of their potential dangers.

However, not only are there poor regulations, poor practitioner and public education, and faulty equipment; the understanding of the basic biophysics of ultrasound has been weakly applied across disciplines. There is a considerable body of evidence which illustrates ultrasound's bioeffects on various cell and tissue types; however,

when reviewing the literature, it appears that little of this knowledge has been viewed in light of prenatal care. Instead, articles abound on cardiovascular studies, targeted drug therapy, transmembrane delivery of nonviral genes, and even transcranial stimulation of brain circuitry – each of these illustrating a host of its primary and secondary effects on the cell. It is amazing that prior to now, there has been only a minimal application of this vast body of knowledge to the study of ultrasound exposure and prenatal development (for some of the few examples in the literature, see Dinno et al. 1989; Ang et al. 2006; Schneider-Kolsky et al. 2009).

With the climbing rates of autism, the increasing frequency of ultrasound use and its frightening deregulations, and what we currently know about the basic biophysics of ultrasound, links are being drawn between cellular morphologies characteristic of autism and those which result from ultrasonic exposure. In addition, there are superficial links between their various cell metabolics, including altered rates of reduction-oxidation (redox) reactions at lipid membranes as well as throughout the cell, abnormalities in calcium signaling, upregulation of certain growth factor signaling pathways, and even modifications in key epigenetic patterns. One likely reason why these bioeffects have rarely been studied following prenatal ultrasound is because they are subtle, usually microscopic, and may involve changes in transcription and not the degradation of tissues or the mutagenesis of DNA. It takes the skilled study of microscopic morphology and shifts in molecular networks to gauge how ultrasound functions as a teratogen. Ultimately, there may be some avoidance within the medical and lay communities to disturb the status quo: not only do parents want that first picture of their unborn child, but ultrasound as an industry makes good money. Should prenatal ultrasound ultimately prove unsafe in its current application, it will take the cooperation of research, medicine, business, government, and the public to tighten regulations and ensure, as useful a tool as it is, that the risks do not outweigh the benefits.

2 Two Main Effectors: Transient Membrane Porosity and Disruption of Redox Homeostasis

While most laymen think of obstetric ultrasound as an image, there are distinct differences between sonography and photography. The medium of photography for instance is light or photons; light enters the aperture of the camera and is then recorded on film or an electronic image sensor. While flash bulbs are often employed, from the point of view of the camera, this entire process is fairly passive: the camera measures the dispersed rays of light already present within the environment. An ultrasound, however, not only captures the sound vibrations of a target object; it also actively creates them and focuses them onto a target. Therefore, unlike a camera, the ultrasound is aiming sound waves onto an object then measuring the echo as the sound returns to the device.

Scientific convention has normally separated ultrasound mechanics into thermal and nonthermal means, the latter comprising cavitational and noncavitational mechanisms. Noncavitational mechanisms include forces such as radiation torque, radiation

force, radiation pressure, and acoustic streaming, all reflective of the different types of fluid pressure placed on the cell surface by the ultrasound beam. Cavitation, named for the gaseous cavities induced by ultrasound exposure, includes two subtypes: stable and transient. The former denotes bubbles which have formed; oscillate, creating a shear stress (stress which is applied parallel or tangential to a surface); and remain intact during many acoustic cycles. The latter, transient cavitation, refers to the formation, expansion, and implosion of bubbles during a compression half cycle from which a shock wave of pressure is emitted. This shock wave can rupture the cellular membrane, triggering necrosis or apoptotic pathways, or, at lower intensities, can create transient porosity of the membrane leading to teratogenic yet nonlethal chains of events (Riesz and Kondo 1992, for review).

Though conventional science and medicine have been most concerned with risks of hyperthermia and tissue damage due to shock waves from transient cavitation, we would like to broaden the research and medical communities' focus to include less deleterious but no less teratogenic mechanisms: namely, the transient permeation of the membrane and the redox reactivity resultant from lower intensity cavitation. This includes both cavitational and noncavitational means in the application of mechanical force upon the cell, as well as free radical release from the imploded cavity. It is these elements, we argue, which have the most teratogenic potential because they do not endanger the cell but alter genetic expression and epigenetic patterning, potentially leading to multigenerational cellular effects from a single exposure. For instance, Liebeskind et al. (1981) found that cultured cells expressed changes in their overall morphology following ultrasound exposure, exhibiting abundant numbers of irregular microvilli which are small pseudopod extensions arising from the surface of the cell due to actin polymerization (or growth) of the actin cytoskeleton. These were present as early as three days following exposure and were still present 37 days later, suggesting that ultrasound had led to permanent changes in DNA transcription. Ultrasound may be capable of inducing this actin polymerization via targeting the MAPK pathway, possibly through a series of events triggered by hyperosmotic shock, through redox-triggered phosphorylation of MAPK, and also through direct targeting of the redox-sensitive cytoskeleton itself (Ho 2006; Zhu et al. 2005; Fiaschi et al. 2006; McDonagh et al. 2005).

As mentioned, increased membrane permeability is one of two important biological effectors in ultrasound exposure. Upon exposure, minute gaseous bubbles present in the fluids surrounding and within the cell begin to grow through a process called rectified diffusion, oscillate, and then implode, creating shock waves that assault the cell membrane. Cavities which do not implode but remain for several cycles create shear stress across the membrane. Likewise, noncavitational means can increase membrane permeability simply through radiative forces of water against the cell. When the water molecules comprise large enough clusters, the lipid membrane is disturbed, creating a pore of approximately 1.4 nm in diameter (Minkel 2010, for review). These pores then reseal following the influx of calcium, which triggers fusion of lysosomes with the membrane in a lysosomal-associated membrane protein 1 LAMP-1-dependent manner (Deng et al. 2004; Reddy et al. 2001; Yang et al. 2008). During this transient porosity, a portion of extracellular fluids is taken up into

the cell, triggering a cascade of molecular activity in response to the breakdown of barriers between intracellular and extracellular compartments. To give some perspective, gap junctions, which are direct open funnels between select cells and allow the passage of larger molecules between those cells, are approximately 1.2–2 nm in diameter (Hormuzdi et al. 2004; Bennett and Zukin 2004). This is very similar in size to the 1.4 nm transient pore that is created during ultrasound exposure from noncavitational radiative forces. Such a pore would allow the rapid influx of ions like calcium and sodium into the cell, as well as other external amino acids and cellular metabolites, and the efflux of ions such as potassium (Pébay et al. 2011, for review). This in turn would trigger chains of events related to the breakdown of barriers, such as conduction within neurons, and downstream targeting of CREB, PKC, MAPK, cAMP, cGMP, histone deacetylases (HDACs), and numerous other pathways. For pathways which function as positive feedback loops, or which lead to alterations in epigenetic patterns such as the targeting of HDACs, such transient permeability may permanently alter the phenotype of the cell. In addition, the gap junctions which are vital in the development and maintenance of various stem cell populations may further increase tissue reactivity to transient prenatal exposure by sharing these influxes among all interconnected cells, and in support of these, studies have noted the occurrence of calcium waves during and following ultrasound (Kumon et al. 2007).

Prior to the realization that water pressure alone could alter the fluidity and permeability of cellular membranes, it was considered that the redox reactions, or lipid peroxidation, in the outer membrane might cause the degradation seen therein (e.g., see Juffermans et al. 2006). Under intense redox conditions, this may be possible; however, groups such as Lawrie et al. (2003) have found that the uptake and subsequent expression of nonviral transgenes are not dependent upon redox mechanisms but utilize another means of entry. Koshiyama et al. (2006) found that structural changes of the phospholipid bilayer due to ultrasound-induced jets of water were enough to create transient pores in the membrane. While lipid peroxidation can indeed alter the fluidity and subsequent permeability of the cell, the more immediate effector of porosity appears to be the radiative force of water on the phospholipid bilayer (Zhu et al. 2005; Pohl et al. 1993; Koshiyama et al. 2006).

Redox reactions are, however, responsible for a number of the bioeffects seen following clinical ultrasound and are therefore considered here as a second main effector of exposure. *Redox*, short for reduction-oxidation, refers to the theft of an electron by a free radical from a surrounding compound or molecule, with a new free radical forming in its place. This chain reaction continues like a string of dominoes and can be thousands of reactions long. Unsaturated fats, particularly polyunsaturated fats, within the lipid membrane can be especially vulnerable to this kind of attack due to the weak double-carbon bonds present in these unsaturated lipids. A reactive oxygen species (ROS) can target the multiple carbon double bonds within the polyunsaturated fatty acid, allowing for easy dissociation of the hydrogen atoms present in the lipid. Another free radical can then steal the unpaired electron from the hydrogen linked with the carbon double bond, turning the carbon into a free radical itself. This conjugated diene can then react with oxygen to form a proxy radical, which thus propagates the redox chain reaction, until at which point two free radicals meet and form a covalent bond with one another. Mitochondrial membranes are

likewise vulnerable to redox mechanisms, not only from the surrounding environment but from within the mitochondrion itself through the production of ATP which produces superoxide anions as a by-product. Superoxide can then either be turned into hydrogen peroxide through the intervention of superoxide dismutase or can form a highly reactive ROS, a hydroxyl radical. Proteins, RNA, and DNA are also targets of oxidation; however, they are comparatively less vulnerable than the polyunsaturated lipids in the various cellular membranes, not only due to their molecular composition but also because of their locations within the cell, given that membranes generally feel the first brunt of a free radical attack.

The sonolysis of water produces hydroxyl radicals (OH^-) as one of its main by-products. Of the initial molecular products succeeding this reaction is the formation of hydrogen peroxide (H_2O_2), hydrogen atoms, water, and other oxidized molecules coming from the cellular landscape, such as peroxidized lipids (for review, see Riesz and Kondo 1992). Increased levels of hydrogen peroxide have also been noted in vivo following ultrasound exposure (Juffermans et al. 2006). The production of this and other reactive oxygen species, such as the aforementioned conjugated dienes, lipid hydroperoxides, and malondialdehydes, each exhibits a dose-dependent increase following ultrasound. This in turn is tightly linked with the occurrence of lipid peroxidation within the membrane (Jana et al. 1990). Following exposure, there are changes in membrane fluidity as well as changes in cytoskeletal arrangement mentioned previously (Zhu et al. 2005). Actin polymerization may be triggered through a number of means, each working in concert to create the microvillus extensions seen following ultrasound. The MAPK pathway is a prime potential target for ultrasound-induced oxidation, given its natural sensitivity to redox mechanisms under normal circumstances. It has been shown that if MAPK is inhibited during H_2O_2 exposure, the membrane and cytoskeletal changes reminiscent of ultrasonic exposure which would have otherwise resulted will instead be suppressed (Zhu et al. 2005).

As mentioned, the cytoskeleton is itself a direct target of redox reactions. While oxidation is normally considered as a destructive mechanism, actin oxidation is necessary for the growth of the cytoskeleton (Fiaschi et al. 2006; McDonagh et al. 2005). Cell-to-cell adhesion, however, is also a necessary component in the maintenance of stem cell fate, and loss of that adhesion, such as occurs following oxidation-induced polymerization of the cytoskeleton, can cause cells to differentiate prematurely, creating heterochronies in tissue development (Campos et al. 2004). It has been shown that following US exposure, such loss of adhesion can occur (e.g., see Siegel et al. 1979). For neural stem cell fate, this can alter subpopulation numbers of mature cells and thus lead to changes within the structure and function of the entire cerebral system.

2.1 Secondary Effects Following US Exposure

When discussing secondary effects following low-level ultrasound exposure, one must ultimately ask: What are the general downstream effects of transient membrane porosity and the production of free radicals? A US-induced membrane pore is

similar in diameter to that of a gap junction and allows the influx and efflux of ions, amino acids, and other metabolites which would also flow through gap junctions. In this instance, however, extracellular contents are allowed into the cell while intracellular contents are allowed out, different from a gap junction which allows the exchange of intracellular fluids between cells.

In the case of ions, the creation of a transient pore within the membrane is indiscriminate and the equivalent of briefly opening all ionic channels at once. Sodium, calcium, and chlorine rush into the cell, and potassium rushes out altering their concentration gradients. Electrochemically, this alters the resting membrane potential of the cell and can lead to polarization and thus action potentials (Tyler et al. 2008). In the central nervous system, this triggers chains of events related to vesicular release of neurotransmitters into the synapse, as well as intracellular chains of events following calcium influx, such as signal transduction through G protein-coupled receptors. Ultimately, this activation can lead to events such as neuronal transmission, long-term potentiation (LTP) through the NMDA receptor, and motility, growth, and proliferation in the case of immature cells (Berridge et al. 2000). As can be deduced by these chains of events, ultrasound leads to calcium influx, and calcium influx can subsequently lead to changes in gene expression.

Calcium influx ensues transient membrane porosity. As discussed, a continuation of calcium waves generally follows this permeability, targeting a host of downstream effectors (Kumon et al. 2007). In neural stem cells and progenitors, calcium waves appear to control their proliferation. Transient increases in calcium can also lead to cell differentiation and neuritogenesis of these populations (Pébay et al. 2011). As an intracellular signaling molecule, calcium targets pathways such as MAPK/ERK, calmodulin, and PKA, which all converge onto CREB. CREB then binds DNA sequences called *cAMP response elements* (*CRE*) and, depending on its binding partners, can either agonize or antagonize expression of genes such as *c-fos*, *BDNF*, *corticotropin-releasing hormone, tyrosine hydroxylase*, and *enkephalin* (Zhang et al. 2005). Some of the calcium receptors are likewise vulnerable to redox regulation, having a so-called redox switch in which oxidative mechanisms turn the receptor "on," whereas reduction turns the receptor "off" regardless of upstream activation (Campbell et al. 1996).

As one can see as per example of the calcium receptor, redox-induced chains of events can lead to considerable alterations in phenotypic expression. This is because redox mechanisms regulate many of the same pathways which are triggered by changes in resting membrane potential as seen following transient porosity, such as pathways for cell survival, growth, proliferation, motility, and even apoptosis. In pyramidal neurons, redox mechanisms also regulate NMDA receptor activity through the receptor's various sulfhydryl groups (Lei et al. 1992). Targets under direct or indirect regulatory control from reduction-oxidation include growth factors such as VEGF and EGF; cytokines such as TNF-α, IL-1β, and IL-8; various adhesion molecules such as integrins; the MAPK/ERK pathway; the Akt pathway; the canonical Wnt pathway; the JNK pathway; the NFκB pathways; AP-1; matrix metalloproteinases; the calcium and NMDA receptors mentioned before; and many others (Roy et al. 2008; Pébay et al. 2011; Rahman et al. 2004; Funato et al. 2006;

Buhimschi et al. 2000). Because redox mechanisms are so heavily influenced by environmental effectors, such as food intake, air quality, radiation exposure, and infection, it is speculated that reactivity to reduction-oxidation, particularly by integrating redox mechanisms into its cell signaling pathways, is one way the living organism adapts to the demands of its environment.

Previous studies have found that redox mechanisms regulate proliferation in numerous cell types (Ranjan et al. 2006; Griendling and Ushio-Fukai 1998). Likewise, ultrasound exposure itself has been shown to increase proliferation in populations of fibroblasts, osteoblasts, monocytes, chondrocytes, and endothelial progenitor cells (Doan et al. 1999; Zhang et al. 2003; Young and Dyson 1990a). Unsurprisingly, downstream of redox activity are targets such as *N-Myc*, a gene which acts both as a transcription factor but more importantly as a regulator for a large number of genes by recruiting histone acetyltransferases (HATs) to the DNA, thus altering chromatin structure and the epigenome (Medina et al. 1992; Cotterman et al. 2008). Oxidative stress is also capable of inhibiting histone deacetylase (HDAC) activity (Rahman et al. 2004). Because the hormone, glucocorticoid, functions to recruit HDACs to the site of gene expression, inhibiting HDACs thereby renders the cell insensitive to glucocorticoid activity and vulnerable to chronic inflammation (Adcock et al. 2005).

The inhibition of HDACs likewise can lead to the upregulation of the canonical Wnt pathway, thus leading to increased cellular proliferation (Wiltse 2005). The Wnt ligand binds to the LRP/Frizzled co-receptors which in turn activate the intracellular mediator, Dishevelled. Dishevelled then disrupts the Axin/APC complex which would normally bind and mark the transcription factor, β-catenin, for degradation; instead, β-catenin is freed into the cytosol, is then transported across the nuclear membrane, and subsequently binds and displaces Groucho, thereby activating LEF/TCF transcription and promoting cell cycle progression. If, however, an HDAC is bound to Groucho, this in turn prevents the binding of β-catenin to the complex and prevents cycle progression. The canonical Wnt pathway is subject to various redox controls: Dishevelled, for instance, is vulnerable to reduction mechanisms which suppress it (Funato et al. 2006); Wnt is also downstream from the Akt, mTOR, and ERK pathways through its involvement with GSK3β, and each of these pathways are redox regulated either via direct redox-induced phosphorylation or phosphorylation of one or more of their constituents (Murata et al. 2003; Sarbassov and Sabatini 2005). Given its various redox regulatory mechanisms, as well as its downstream position of pathways such as Akt, mTOR, and MAPK/ERK, it is perhaps unsurprising that Wnt's upregulation has been reported following ultrasound exposure (Olkku et al. 2010).

Ultrasound is known to upregulate production of a variety of products, such as cyclooxygenase-2, basic FGF, prostaglandin E_2, and nitric oxide (NO), although the precise cause, cavitational or noncavitational, is currently unknown (Hsu et al. 2007; Reher et al. 1999, 2002). While NO is certainly a free radical, its production in this instance is more likely due to one of transcription of inducible nitric oxide synthase (iNOS), based on the time course of its presentation following exposure, and not due directly to cavitation (Reher et al. 2002). Instead, its production is more

likely a result of activation of the MAPK/ERK and NFκB pathways (Hou et al. 2009). While NO normally acts as an antioxidant in its capacity to reduce lipid peroxyl radicals, its increased production does, however, raise the risk of itself forming a free radical, peroxynitrite, in the presence of superoxide anion which is produced by the electron transport chain (Hogg and Kalyanaraman 1999). While it can act as an antioxidant in its native form, NO also functions as an intracellular signaling molecule, such as activating the cyclic GMP pathway, and also plays important roles in vascular dilation which can subsequently affect the tissues around which the vasculature forms its niche (Shen et al. 2004).

2.2 Ultrasound and Corticogenic Heterochrony

As with all tissues, the central nervous system follows a coordinated series of developmental events. The development of a given cell is determined not only by intrinsic factors but by extrinsic ones as well, particularly by temporal cues, location, and its orientation relative to the different cells surrounding it and its location in space. In relation to these cues, hydrodynamic forces play important roles in cellular instruction by helping to form chemical gradients. For instance, the flow of CSF within the ventricles, along with the help of ependymal cilia, promotes aspects of the rostral-caudal patterning of the cortex through a gradient distribution of key soluble instructive factors (Götz and Stricker 2006). Hydrodynamic forces and shear stress likewise play vital roles in the development and continued maintenance of the vascular system (Yamamoto et al. 2003), and the vasculature in turn forms a niche surrounding the neural stem cell population, releasing soluble factors which stimulate self-renewal, neurogenesis, and cell differentiation (Shen et al. 2004).

As one might imagine, ultrasound could impose a serious threat to cellular development by providing false navigational cues via hydrodynamics, cavitation, and reduction-oxidation mechanisms, leading to a heterochrony of neural tissues (for review, see Banerjee and Slack 2002; Guirao et al. 2010). In 2006, Ang et al. published work illustrating this phenomenon: mice were exposed in utero on embryonic day 16 (mid-to-late neurogenesis) to ultrasound, with exposure ranging anywhere from 5 to 420 min. Exposed and control mice were collected on postnatal day 10 and analyzed. The researchers found that following ultrasound, mouse cortex exhibited signs of migratory abnormalities as well as periventricular ectopias. While no behavioral studies were performed on these mice, Schneider-Kolsky et al. (2009) also exposed fetal chickens to 1–10 min of ultrasound and found that Doppler ultrasound in particular triggered significant memory and learning impairment in the range of 4–5 min of exposure. Doppler ultrasound is utilized to evaluate blood flow and is generally used in obstetrics for monitoring heart development; however, as a device it is also available for private use and can be purchased by expectant parents for unregulated use within the home.

The migratory and proliferative abnormalities seen in the US-exposed mouse cortices may have similar causal roots because both mitosis and cell motility utilize

overlapping molecular pathways to perform their functions. Pathways such as Akt, MAPK/ERK, mTOR, Wnt, and JNK exhibit a considerable cross talk and overlap of functions in proliferation and migration (for review, see Williams and Casanova 2011). For instance, PTEN is a protein which regulates the Akt pathway; when PTEN is downregulated, Akt is subsequently upregulated. In a *Pten* knockout model of autism and macrocephaly which specifically targets neural stems cells through a *Nestin-cre* combination, this mouse model not only exhibits drastically increased neurogenesis of certain subpopulations, but it also exhibits extreme laminar maldistribution, suggestive of migratory disturbances (Groszer et al. 2001). Mutations in the transcriptional repressors *Hes1*, *Hes3*, and *Hes5* cause combined abnormalities in proliferation and cell migration (Hatakeyama et al. 2004).

Heterochrony is a term used to describe not only variations in homologous tissues between species; it can also refer to variations within a species of a given tissue and whether cell fate is expressed comparatively early or is retarded in its development and remains in a state of immaturity for an extended period of time. Generally, this prolonged immaturity will involve continued symmetric or asymmetric proliferation and thus result in changes in ratios of cell subpopulations (Banerjee and Slack 2002, for review). On a broad scale, evolutionary theorists have proposed that the retarded heterochrony of the initial radial glial progenitor population has led to increases in encephalization across species, i.e., bigger brains. However, Casanova et al. (2002) also propose that such a heterochrony within the human population may underlie aspects of the autism phenotype, in which retarded development of radial glia leads to a greater number of neocortical minicolumns and a subsequent disturbance in overall cerebral connectivity patterns which cause the behavioral syndrome.

Such heterochronies can also be seen in the variety of autism animal models used to study the conditions. For instance, a model for Fragile X syndrome, a genetic condition highly comorbid with autism, exhibits a distinct increase in the number of Tbr1-immunolabeled cells within the infragranular layers of the neocortex, suggesting that the radial glia which produced these cells are held in a state of prolonged proliferation (Tervonen et al. 2009). The tuberous sclerosis model (*Tsc2* knockout), also highly comorbid with autism, conversely exhibits increased numbers of Tbr2-immunolabeled intermediate progenitors within the subventricular zone and a simultaneous decrease in numbers of infragranular cells, although total number of neurons within the cortex does not appear generally disturbed (Way et al. 2009). This model instead suggests that the *Tsc2* knockout's radial glia mature early, produce fewer infragranular neurons, and instead produce more intermediate progenitors, the latter which are subsequently maturationally retarded and held in a proliferative state for a longer period of time. Thus, both of these models display distinct heterochronies which lead to laminar anomalies different from one another and their controls and are reflective of the neocortical development seen in their respective human conditions, thus illustrating how slight changes in relative tissue development can drastically change the function of that tissue, e.g., behavior and cognition. Ultimately, just as with mutations in the *Fmr1* gene in Fragile X or in the *Tsc2* gene in Tuberous sclerosis, ultrasound may not play a teratogenic role in all cases of autism, but it may play an integral one in a portion of them, particularly

individuals who may be more susceptible to its effects either through inherited proclivity, due to differences in exposure, to multiple environmental hits from other agencies, or a combination thereof. In lieu of developmental plasticity and the capacity for development to be guided towards a particular common phenotype through different yet converging pathways, one can see how the molecular activities which result from ultrasound exposure may share commonalities with other causal factors already known to be associated with autism.

2.3 Factors Associated with Autism

Abnormalities in cerebral growth have been a recognized association with autism for a number of years, in particular the increase in head circumference during childhood which generally normalizes by puberty or adulthood (Aylward et al. 2002). Casanova (2004) has noted that this initiation of unusual growth coincides with the time period for myelination, a process which is known to add considerable volume to the cerebral cortex. Instead, he suggests that this increase in volume is due to myelination and is reflective of the earlier corticogenic disturbances which ultimately lead to a rise in total cell numbers within the neocortex and subcortical structures. This retarded growth of the progenitor population is reflected in the total number of minicolumns which comprises the neocortex. In addition, a combination of migratory and proliferative disturbances have been found in the brains of autistic in the form of heterotopias and dysplasias – findings not unlike those produced in the Ang et al. (2006) murine model of prenatal ultrasound exposure (Bailey et al. 1998; Wegiel et al. 2010).

Increases in proliferation following ultrasound exposure can be seen in a number of cell types, such as fibroblasts, osteoblasts, chondrocytes, and monocytes (Doan et al. 1999; Zhang et al. 2003). Unfortunately, little research has been performed on broader cell types because ultrasound is used heavily as a therapeutic tool for bone repair, and therefore, there is less demand for applied research in other areas. Ultrasound effects on the mitogenic factor-releasing capacity of macrophages and their direct effects on proliferative rates of fibroblasts have however been studied, illustrating that indirect bioeffects from cell-to-cell interaction also follow ultrasound exposure (Young and Dyson 1990b). Given the effects ultrasound has on membrane permeability and the cellular growth which generally follows transient cell porosity, it will be interesting to note in future whether these results are replicated across cell types.

Some of the US-induced disturbances in proliferation are reflected in some of the autism genetics research performed to date. PTEN, for instance, is associated with a set of hamartomatous conditions with loss-of-function mutations; it is also significantly associated with autism and combined macrocephaly (McBride et al. 2010). PTEN lies upstream of pathways such as Akt and regulates the phosphorylation of PIP_2 to PIP_3; therefore, any loss of function of this protein leads to abnormal upregulation of Akt and changes in related pathways. MAPK/ERK is another aforemen-

tioned pathway which shares considerable crosstalk and co-regulation with the Akt pathway. *c-Met*, a proto-oncogene whose mutations are associated with nonsyndromic forms of autism, likewise lies upstream of MAPK/ERK and promotes its activity (Campbell et al. 2006). Its activation is also heavily inhibited by PTEN in a complex fashion, providing links through these various interactive pathways to the heterogeneous forms of autism (Abounader et al. 2004).

Ultrasound, like some of the syndromic and nonsyndromic mutations in autism, targets familiar pathways like MAPK/ERK, Akt, NFκB, Wnt, and numerous more (Hou et al. 2009; Olkku et al. 2010). These pathways are targets of pore-induced cellular signaling and activation of calcium-activated pathways, but many of these molecules have phosphorylation sites that may be directly oxidized through US exposure. As discussed earlier, Dishevelled, upon reduction, is downregulated irrespective of Wnt ligand activity, and thus, following ultrasonic oxidation, this pathway's activity may be upregulated. Oxidation can also inhibit HDACs and thereby upregulate canonical Wnt through a transcriptional means which can then be epigenetically inherited for multiple cellular generations. HDAC inhibition has previously been linked with autism, showing that prenatal valproic acid and/or carbamazepine exposure can increase risk of developing the condition (Rasalam et al. 2005). Both of these medications have a variety of secondary effects, but as one of their primary effects, they act as potent HDAC classes I and II inhibitors (Shimshoni et al. 2007). The MAPK/ERK pathways have also been found to regulate HDACs, linking their activity with chromatin remodeling and autism-associated mutations in some of the MAPK genes, such as MAPK3 (Zhou et al. 2000; Sanders et al. 2011). Ultrasound's effects on migration may also be echoed in the *RELN* gene which is associated with some nonsyndromic forms of autism. Reelin is an extracellular matrix protein which is intimately involved in neuronal migration, cell adhesion, and even synaptic plasticity, and any loss-of-function mutation inevitably causes extreme disruption of the cortex (Weeber et al. 2002). Other genes involved in cell adhesion, such as *CNTNAP2* whose loss of function is associated with autism, may provide phenotypic links to the loss of intercellular adhesion seen following US exposure (Pinto et al. 2010; Siegel et al. 1979).

Oxidative stress, like that following ultrasound exposure, has been associated with autism, although understanding in the field of study is still in its infancy. By-products of oxidative stress have been studied in autism at a number of levels, including the membrane level, in serum and urine, and at the level of specific endogenous and dietary antioxidants. For instance, increased levels of 8-hydroxy-2-deoxyguanosine and 8-isoprostane-F2α, both by-products of lipid peroxidation and thus indicators of the activity of this process, have been noted in the serum of autistic children as have lower levels of glutathione which is an important antioxidant and regulator of redox homeostasis (Ming et al. 2005; James et al. 2006). A poverty of production of the binding proteins, transferrin, an iron-binding protein, and ceruloplasmin, a copper-binding protein, has been noted in the conditions as well (Chauhan et al. 2004). Both iron and copper are potential catalysts of oxidation due to the ease with which they lose and gain electrons, and therefore, their binding proteins are important regulators of these catalysts' activities. Ultimately, lower levels of transferrin or ceruloplasmin

in the blood may suggest an increased risk to oxidative stress due to higher concentrations of free iron and copper. From an ecological standpoint, air pollution, particularly pollution due to heavy metals such as mercury, cadmium, and nickel, has also been associated with increased autism risk. These metals, similar to copper and iron, have the capacity to act as free radicals as well as to inactivate glutathione (Windham et al. 2006; Valko et al. 2006, for review). In summary, it is clear that redox homeostasis is disrupted in a portion of cases of autism, although by means unknown. Because redox dysregulation can be a sign of disrupted cellular health, it is difficult to postulate whether oxidative stress is a cause or a result of the conditions.

3 Discussion

While focusing on a few snippets of the larger ever-growing body of autism, research must seem like magnifying a molehill into a mountain and subsequently taking it out of context; instead we have tried to select examples which speak to an underlying theme, namely, one of dysregulation of cellular growth, migration, and differentiation in autism. And in that sense, heterogeneous autism may be a good example of "all roads lead to Rome" such that multiple avenues, including genetic mutation, epigenetic alteration, medication exposure, oxidative stress, diet, ultrasound, or a combination of effectors, may produce a common phenotype. As West-Eberhard (2005) states:

> [it] does not matter, for the form taken by the morphological change, whether the pivotal change . . . was induced by a mutation or by an environmental accident. The particular characteristics of the novel morphology . . . arose via mechanisms of developmental plasticity, not owing to the particular genetic or environmental change that may have induced them (p. 618).

Biological plasticity is reflected at numerous levels. In this chapter, we have focused on its plasticity at the molecular and cellular levels, illustrating that the activity of key pathways, reflected in the genetics of some cases of autism, can be altered through means other than DNA mutation and may instead be largely epigenetic. *Epigenetic* in this context refers to more than the alteration and inheritance of chromatin and methylation patterns but also to modes of inheritance that exist outside the cell, including that of its surrounding ecology. Mutations associated with syndromic and some nonsyndromic cases of autism, while still only comprising a minority of the larger spectrum, can help us pinpoint some of the particular molecular pathways which are involved in the broad spectrum of autism. It is this concept which we have earlier dubbed, "The Lowest Common Denominator of Autism" (Williams and Casanova 2010, 2011, for review). While genetics gives us vital information, it is obvious that autistic genotypes are heterogeneous, and so we must move to a higher level of comparison to identify trends, namely, the activity and timing of key molecular pathways involved in producing a common phenotype. To paraphrase West-Eberhard (2005) in this context, it does not matter *what* causes the alteration in activity of a given pathway but that it is altered *at all*. Given that

molecular pathways are not unlike a string of dominoes, it does not ultimately matter whether you target one domino or another within the grouping; the end result (genetic transcription or transcriptional suppression) is much the same. And the redundancy and cross-regulation of these various pathways likewise underlie the variability of biological plasticity. Therefore, if we apply this concept to prenatal ultrasound exposure, noting the commonalities its bioeffects share with both syndromic and nonsyndromic forms of autism, we can slowly begin to see how ultrasound may play some role in the conditions' etiology.

It has been extremely difficult to pin down ultrasound as a risk factor for autism. For one, funding and interest have been scarce because the idea of ultrasound as a teratogen to most people sounds like a pseudoscience, when in fact our understanding of its biophysical mechanics shows quite clearly that concern is justifiable. In reference to the modicum of work which has already been done on this topic, Grether et al. (2010) performed an epidemiological study which at face value provides evidence which would quell our concerns; however, as we learn more about the deregulation of the method and its variability of exposure in real medical practices, it becomes almost impossible to gauge risk by searching through medical records. In essence, we need well-controlled studies with well-defined parameters, and that includes animal studies which can give us even greater control within the laboratory and provide postmortem materials. Only in these ways can we clarify what role ultrasound plays in development and precisely how safety standards need to be tightened.

At one point in time, X-rays were used to excess in medical practice until eventually researchers realized that they were carcinogenic. This new tool was discovered in 1895 and was rapidly put to prolific and poorly regulated use. It was not until skin damage was linked with exposure that scientists and practitioners slowly began to improve safety regulations. By 1927, several decades later, there was an inkling of awareness of its mutagenic properties when Hermann Muller discovered that X-rays could promote genetic mutations in *Drosophila* (Muller 1927), and by the 1950s research suggested links between prenatal exposure and later development of cancer in childhood (e.g., see Giles et al. 1956). As discussed within the introduction of this chapter, the history of ultrasound shares parallels to X-ray in its early unregulated use, with a subsequent tightening of safety standards after the realization that ultrasound, while not mutagenic, can nevertheless cause tissue damage. Such is human nature to apply a novelty excessively until we discover its side effects.

Unfortunately, the last several decades have experienced a loosening of these regulations while very little of the growing body of research on ultrasound has been applied to its use in obstetrics. Very few scientists who study its effects on targeted drug therapy, on bone and cartilage regeneration, on gene delivery, and on intravascular dilation and imaging and numerous other studies for its application seem to publish any concerns regarding its use in fetal monitoring. In part, this is likely a reflection of the isolation in which each subfield generally resides. Developmental biologists are probably unfamiliar with the relevant literature, and scientists from other fields who do research ultrasound likely have little professional interest in publishing out of their disciplines. And for the developmental biologist, drudging up

literature on "ultrasound" and "safety" usually results in various old reviews citing the same outdated materials and reiterating the same pacifying statements, while in order to find the current science, the developmental biologist must search through foreign literature on gene transfection, delivery of medications to target organs utilizing ultrasound-enhanced microbubbles, or work elucidating bone repair by therapeutic ultrasound. A good deal of the science of ultrasound is known and available to the reader, but it is hidden by search engines and disciplinary isolation, and this will only be rectified once embryologists delve into these texts and apply them to their own field. And therefore it is our hope that this chapter will serve as a cross-disciplinary starting point for those scientists who wish to travel into less familiar territories.

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Animal Model of Autistic Regression: Link to Toxicant-Induced Oxidative Stress

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1 Overview

Autism is a neurodevelopmental disorder characterized by deficits in social interactions, impairment in communication skills, and stereotypic, sometimes self-injurious behaviors. According to the DSM-IV-TR, the deficits in social interactions include failure to develop peer relationships, lack of emotional reciprocity, and impairment in the use of multiple nonverbal behaviors such as eye contact and facial expression. The impairments in communication skills include a delay or total lack of spoken language, inability to initiate or sustain conversation, and stereotyped and repetitive language. The stereotypic behaviors are characterized by restricted patterns of interest or behavior, inflexible adherence to routines, repetitive motor mannerisms such as hand flapping or twisting, and persistent preoccupation with parts of objects (DSM-IV-TR, 2000). In its most severe form, the stereotypic behaviors escalate into repetitive self-injury (Lecavalier 2006).

From its inception, the defining symptom of autism has been the failure of social reciprocity (Kanner 1943a, b; Asperger 1944). Autistic children have fewer social interactions than normally developing children, and these interactions are shorter

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and of reduced quality compared to typical children (Kennedy and Shukla 1995). Children with autism spend less time near their peers and spend more time in non-social playing than the typically developing children (Kennedy and Shukla 1995). They also have a higher incidence of anxiety and, in particular, social anxiety, than typically developing children or even children with language impairments (Gillott et al. 2001).

The prevalence of autism by 8 years of age is now reported to be approximately 1 in 110 (Rice 2009). Compared to estimates of autism made in the 1980s, this recent estimate reflects a prevalence increase averaging nearly 60 % (Rice 2009). Likewise, a recent survey from Britain reported the prevalence of autism spectrum disorders to be more than 110 per 10,000 (Baird et al. 2006), and, again, these numbers are significantly higher than the prevalence estimates reported in the 1980s of around 10 per 10,000 (Kadesjo et al. 1999; Sponheim and Skjeldal 1998). Variables that may account for the recent increase in autism include broader diagnostic criteria and an increased awareness of the disorder. In addition, this recent increase in the incidence of autism may be the result of an increase in exposure to factors mechanistically responsible for alterations in neural development that lead to autism. While the etiology of autism remains unknown, underlying mechanisms are thought to include both toxicant exposure and genetic alterations that render the individual more sensitive to the deleterious effects of these toxicants.

Clearly the increase in prevalence seen in autism over the last three decades cannot be accounted for by genetic alterations occurring in the population. Therefore, using animal models of autism, we and others have been exploring genetic alterations by toxicant exposure interactions, attempting to demonstrate that mice with certain genetic alterations respond with increased sensitivity to the adverse effects of neurotoxicant exposure and that the functional consequences of such exposure effectively resemble autism in children. With this in mind, the objectives of this chapter are to further expand our animal model of autism (Wagner et al. 2006) focusing on toxicant-induced autistic regression and genetic alterations that might lead to increased sensitivity to toxicant-induced oxidative stress and contributing to the understanding of the etiology of autism.

2 Autistic Regression

Some children who are eventually diagnosed with autism appear to develop normally for their first 24–36 months of life after which autism emerges (Rapin 1997). In these children, autism may appear gradually or abruptly, but in both scenarios, typical development is interrupted and there may be a loss of previously acquired social and language skills (Wiggins et al. 2009). Autistic regression is thought to account for about 35–40 % of all cases of autism. The cause of this regression is not known although there are several factors that have been considered. The occurrence of epileptic seizures has been suggested as a trigger of autistic regression, but this possibility remains somewhat controversial because while there are findings of an

association of seizure activity with autistic regression (Hrdlicka et al. 2004), there are also findings with no clear association of seizure activity with autistic regression (Tuchman and Rapin 1997). Likewise, environmental events have been implicated as possible triggers for autistic regression, and these include medical treatment such as immunization (Wakefield et al. 1998; Goldberg et al. 2003), illness, and immunological mechanisms triggered by stress (van Gent et al. 1997). Genetic susceptibility may also play a part in the cause of this regression as it has been shown that parents of children that show autistic regression display autistic features themselves, more so than parents of non-autistic children (Lainhart et al. 2002).

In this regard, we (Yochum and Wagner 2009) have previously observed that Kanner's initial case study of autism (1943b) provides clear descriptions of autistic regression although he does not use the term. Further, we have previously pointed out (Yochum and Wagner 2009) that in case 3, the period of regression appears to follow an adverse reaction to polio vaccination, a point more recently addressed by Wakefield et al. (1998) although with respect to a different vaccination and not without some controversy. Accordingly, our animal model of autism has behavioral regression as a major component for the mechanistic understanding of the neuropathology of autism.

2.1 Animal Model of Autism

Autism is difficult to diagnose in humans and impossible in other species. The best we can hope for is to develop an animal model of autism that lends some insight into the etiology of autism, the treatment of autism, and, perhaps most importantly, the prevention of autism. We have developed a comprehensive animal model of autism and have concentrated on the etiology and prevention of autistic regression. The model assumes that autism is the result of a gene by toxicant exposure interaction with additional factors being the sex (i.e., males are more vulnerable) and age of toxicant exposure (i.e., there appear to be critical windows of sensitivity to toxicant exposure) (Wagner et al. 2006; Yochum et al. 2008). The early foundations for this model were from the field of behavioral toxicology in which the impact of early toxicant exposure on reaching developmental milestones in physical and motor skill maturation was assessed.

To make this broad-sweeping behavioral toxicology approach relevant and specific to autism, we imposed two additional strategies. First, toxicant-induced deficits were classified along an ontogenetic timeline as retardations, regressions, or intrusions. Retardations were defined as failure for a particular skill to appear or mature during critical windows of development. Regressions were defined as occasions where a skill develops during the correct period (window) of development but then, following toxicant exposure, is either lost or fails to further mature. Finally, intrusions were defined as behaviors that appear following toxicant exposure that mask or obscure the more typically appearing behaviors of that developmental window (Wagner et al. 2006). A second strategy of the model imposed upon these

retardations, regressions, and intrusions was that the toxicant-induced functional deficits would be categorized as social, cognitive, emotional, or motoric (Wagner et al. 2006). By carefully selecting toxicants, period of exposure, and skill sets, the model becomes highly relevant to autism. While we have administered a number of toxicants to mice early in life and under various regimens, we have concentrated our efforts on valproic acid (VPA).

2.2 Valproic Acid

VPA is a branched short-chain fatty acid used clinically as an anticonvulsant agent (Chateauvieux et al. 2010) as well as to treat a variety of other disorders including migraine, schizophrenia, and bipolar disorder (Go et al. 2011). VPA inhibits gamma amino butyric acid (GABA) transaminase, which inhibits the degradation of GABA (Fukuchi et al. 2009). It also increases GABA synthesis and decreases GABA turnover rate (Mesdjian et al. 1982).

VPA has been linked to autism through prenatal exposures. A British study found that 8.9 % of the children exposed to VPA in utero met the criteria for autism spectrum disorder (ASD) and that the prevalence among exposed children was eight times that of the general population (Rasalam et al. 2005). Similarly, Bromley et al. (2008) found that 6.3 % of the children exposed in utero were diagnosed with ASD, and this was seven times higher than the control group. Children exposed to VPA in utero have also been described as having "fetal valproate syndrome" characterized by symptoms similar to autism including stereotypic and hyperexcitable behavior, language and communication deficits, and behavioral developmental delays (Moore et al. 2000; Koch et al. 1996). Infants with this fetal valproate syndrome have also been reported to have physical abnormalities such as low myelomeningocele lesion, minor abnormalities of the face and ear, and microcephaly (Moore et al. 2000). Exposure to VPA during a critical period, when the neural tube closure occurs, results in fewer Purkinje cells in the cerebellar vermis and a reduced tissue volume in the cerebellum of rats (Ingram et al. 2000). This is consistent with neurological findings of cerebellar hypoplasia (Hashimoto et al. 1995) and reduced Purkinje cell numbers in all adult cases (Bailey et al. 1998).

The similarities between rodents exposed prenatally to VPA and the clinical observations of autistic humans led to the proposal that early exposure to VPA may function as an animal model of autism (Rodier et al. 1997). In rodents, embryonic day 13 corresponds to the final stages of Purkinje cell generation (Inouye and Murakami 1980). Exposure to VPA at this time point resulted in a developmental retardation in the maturation of behavioral skills (Wagner et al. 2006). Postnatal day 14 (P14) corresponds to the period when hippocampal and striatal differentiation and migration are still occurring in the mouse brain and when critical developmental behaviors mature or first appear (Wagner et al. 2006; Rice and Barone 2000). To be more specific, by P14 a number of basic motor skills have matured in mice to a point where essentially all untreated wild-type controls can perform these tasks.

Administration of VPA on P14 results in a loss in the ability of mice to perform these acquired behavioral skills (Wagner et al. 2006). This severe behavioral regression is accompanied by a highly selective VPA-induced apoptosis in cerebellum and hippocampus, two regions thought to be involved in the neuropathology of autism. The VPA-induced regression occurs only during a critical developmental window, not affecting mature subjects. Further, the VPA-induced regression is more likely to be observed in males (Yochum et al. 2008). We believe that VPA-induced regression is consequent to VPA-induced oxidative stress and, as such, can be prevented with antioxidant pretreatment (see below).

2.3 Etiological Factors

As noted, the etiology of autism is still not known. There are several hypotheses regarding what might mechanistically underlie the neurodevelopmental changes that ultimately lead to autism including genetic, immunological, and environmental events. Of importance, each of these factors appears to lead to a common endpoint of oxidative stress.

With respect to the genetic component, a study of British twins found a high concordance rate of 60 % for narrowly defined autism in monozygotic twins with no concordance for dizygotic twins (Bailey et al. 1996). When the twins were reevaluated with broader criteria that included children with some but not all characteristics of autism including cognitive and social disorders, the rate of concordance for monozygotic twins rose to 92 % and the concordance rate for dizygotic twins rose to 10 % (Bailey et al. 1996). However, a more recent study, with a larger sample of twins from Sweden, reported a concordance rate of only 39 % for monozygotic twins and 15 % for dizygotic twins (Lichtenstein et al. 2010). This study also used a liability model analysis that suggested a heritability of autism of about 80 % (Lichtenstein et al. 2010). Several genes with rare de novo or familial variants or with copy number variants have been identified that may be associated with autism. Genes for synaptic development including neurexins, neuroligins, reelin, integrins, cadherins, and contactins have been identified in association with autism (Yasuda et al. 2011; Etherton et al. 2011; Skaar et al. 2005; Correia et al. 2009; Pagnamenta et al. 2011; Cottrell et al. 2011). Genes that are involved in methylation, transcription, and signaling, as well as neurotrophic genes, have also been identified in relation to autism. These genes include engrailed 2, brain-derived neurotrophic factor, Ca^{2+}-dependent activator-protein for secretion 2, fragile X mental retardation 1, and methyl-CpG-binding protein 2, among others (Benayed et al. 2009; Nishimura et al. 2007; Sadakata et al. 2007; Hagerman et al. 2011; Nagarajan et al. 2008). Genes that are involved in neurotransmission have also been linked to autism, including the serotonin transporter gene and the GABA subunit gene GABRA4 (Kristner-Griffin et al. 2011; Ma et al. 2005). However, of importance, none of these genetic alterations account for more than the smallest fraction of autism cases. Because of this serious limitation and somewhat akin to the broadening diagnostic criteria for

autism, the genetic hypothesis of autism is undergoing its own expansion to include the possibilities that autism is the result of variation in a constellation of genes and/or epigenetic alterations.

Environmental events and exposure to toxicants have been implicated in the etiology of autism. Children with increasing severity of autism were found to have increasing concentrations of lead and mercury in their hair and nail samples (Priya and Geetha 2011). The concentration of these toxic metals was found to be significantly higher in children with autism compared to healthy controls (Priya and Geetha 2011). Baby teeth of children with autism were also found to have 2.1-fold higher levels of mercury compared with that in healthy developing children (Adams et al. 2007). In addition, exposure to pesticides has been associated with autism. For example, organophosphate exposure has been linked to increased risk for autism as have variants in the gene that encode the enzyme, paraoxonase gene (PON1), which detoxifies this most common pesticide (D'Amelio et al. 2005). Likewise, polychlorinated biphenyls (PCBs) and their hydroxyl metabolites are known to inhibit thyroid hormone-dependent dendritic development of Purkinje cells in the cerebellum (Kimura-Kuroda et al. 2007), and rats exposed early in life to PCBs exhibit deficits in social behaviors similar to those observed in autism (Jolous-Jamshidi et al. 2010). In brief, no single toxicant has been identified that leads to autism. Therefore, like the genetic hypothesis, the environmental exposure hypothesis of autism has been expanded to include the possibility that individuals at risk are exposed at increased levels to any of a wide range of toxicants with total dose being the critical factor. The work of Edelson and Cantor (1998, 2000) illustrates this point quite effectively. While the compounds that Edelson and Cantor (1998, 2000) identified as being present in elevated levels in autistic individuals act through varied mechanisms, oxidative stress does emerge as a common endpoint.

3 Oxidative Stress in Autism

Progress in our understanding of the etiology of autism along genetic and environmental factors has been disappointingly slow. No single gene and no single toxicant have been identified that account for autism in any meaningful way. However, as noted, both strategies do lead to toxicant-induced oxidative stress as a potential common mechanism.

The hypothesis that oxidative stress is involved in the etiology of autism stems from several lines of evidence (Chauhan and Chauhan 2006). First, increased excretion of an oxidative stress biomarker, nitric oxide, was found in erythrocytes of patients with autism as compared to age- and sex-matched controls (Sogut et al. 2003). In addition, elevated nitrite levels in autistic individuals along with thiobarbituric acid-reactive substances and xanthine oxidase activity were reported in the plasma and red blood cells (Chauhan et al. 2004; Zoroglu et al. 2003, 2004); their elevation indicates excess generation of free radicals. Lipid peroxidation (assessed by measuring the biomarker malondialdehyde (MDA) in plasma) has been shown to

be increased in autistic children compared to non-autistic siblings (Chauhan et al. 2004). The prooxidant xanthine oxidase that produces superoxide radicals when converting xanthine to uric acid was found to have increased activity in the erythrocytes of autistic patients (Zoroglu et al. 2004); the biomarker of lipid peroxidation, thiobarbituric acid-reactive substances (TBARS), has also been shown to be increased in the red blood cells of autistic individuals compared with age- and sex-matched controls (Zoroglu et al. 2004). In addition, we have previously reported increased urinary excretion of isoprostane in children with autism (Ming et al. 2005). Of interest, in our study, children with autism consistently had higher levels of urinary isoprostane as compared to age- and sex-matched controls, but a subset of children with autism had exceedingly higher levels.

Antioxidant enzymes that protect against oxidative stress are present at altered concentrations in autistic patients. Altered glutathione peroxidase (Sogut et al. 2003; Yorbik et al. 2002; Ming et al. 2010), superoxide oxidase (Yorbik et al. 2002), and catalase (Zoroglu et al. 2004) activities as well as total glutathione and GSH/GSSG and cysteine levels (James et al. 2004; Melnyk et al. 2011) have been reported in autistic individuals as compared to controls. Likewise, plasma levels of antioxidants (including vitamin C, vitamin E, and vitamin A) were found to be reduced in individuals with autism (James et al. 2004), indicating they may be less able to manage toxicant-induce oxidative stress. A biomarker of oxidative stress, 3 nitrotyrosine (3-NT), was detected at increased levels in brain tissue of autistic patients compared with controls (Sajdel-Sulkowska et al 2011). The biomarker 3-NT is specific to protein damage caused by peroxynitrite (Sajdel-Sulkowska et al. 2011).

Finally, evidence of altered oxidative stress in autism is indicated by impaired energy metabolism. Magnetic resonance spectroscopic study of the brains of individuals with autism showed reduced synthesis of ATP (Minshew et al. 1993). In addition, higher lactate (Coleman and Blass 1985; Chugani et al. 1999) and pyruvate (Moreno et al. 1992) levels in autism may suggest mitochondrial dysfunction in autism (Filipek et al. 2003). Levels of mitochondrial electron transport chain (ETC) complexes were shown to be decreased in the cerebellum and temporal cortex of autistic children, and this may result in abnormal energy metabolism (Chauhan et al. 2011). Mitochondrial dysfunction, such as damaged ETC complexes, decreases ATP production and increases the generation of free radicals, which can then have damaging effects on neurons in autism (Chauhan et al. 2011). Consistent with the increased oxidative stress biomarkers, children with autism were found to have increased body burdens of environmental toxicants that may generate oxidative stress (Edelson and Cantor 1998, 2000).

It appears that the toxicity caused by VPA in humans and other animals may be due to oxidative stress. In vitro studies have shown that VPA causes the generation of hydrogen peroxide in the presence of iron-induced rabbit microsomes (Tabatabaei and Abbott 1999). It was suggested that VPA-induced uncoupling of the cytochrome P450 cycle allowed the release of hydrogen peroxide (Tabatabaei and Abbott 1999). The addition of catalase, the enzyme responsible for detoxification of hydrogen peroxide, was found to prevent the VPA-induced lymphocyte toxicity (Tabatabaei and Abbott 1999). VPA was also shown to increase reactive oxygen species levels

in embryoid bodies from murine embryonic stem cells which in turn inhibited cardiomyocyte differentiation (Na et al. 2003). The cardiomyogenic differentiation was restored upon treatment with vitamin E, a free radical scavenger (Na et al. 2003). Vitamin E was also shown to attenuate VPA-induced neural tube defects (Al Deeb et al. 2000). In our animal model, VPA administration on P14 resulted in a regression of previously mastered behavioral skills. However, treatment with antioxidants completely protected the mice against the VPA-induced behavioral regression (Cheh et al. 2010). More recently, in an important replication of these observations, green tea extract administered to mice on postnatal day 13 through day 40 also protected mice against the P14-induced behavioral regression (Banji et al. 2011). The green tea extract protected the mice against VPA-induced performance deficits on the rotarod, the spatial memory water maze assay, as well as negative geotaxis (Banji et al. 2011). Early exposure to VPA has also been shown to result in increased anxiety, as demonstrated using the elevated plus maze, and this corresponds to the increased anxiety seen in autistic individuals (Schneider et al. 2008). Antioxidant treatment (with green tea extract) was found to ameliorate this increased anxiety induced by VPA (Banji et al. 2011). Finally, it was also shown that green tea extract protected the Purkinje cells from VPA-induced injury (Banji et al. 2011). Collectively, these observations indicate that the regression induced by VPA can be prevented by antioxidant pretreatment. In a similar design, we have shown that antioxidants protect mice against the behavioral deficits induced by early exposure to methylmercury (Ming et al. 2008). This prevention strategy may be particularly successful in cases where there is a genetic susceptibility to toxicant-induced oxidative stress.

4 Genetic Factors and Oxidative Stress

Genetic factors appear to contribute to the susceptibility to toxicant-induced oxidative stress found in autism. Glutathione S-transferases (EC 2.5.1.18; GSTs) are a family of enzymes that catalyze the conjugation of glutathione with a number of electrophilic compounds resulting in the detoxification of xenobiotics. The GSTs can catalyze the isomerization of retinoic acid and prostaglandins as well as the reduction of hydroperoxides formed as secondary metabolites during oxidative stress. In addition, GSTs exhibit non-catalytic-binding activities towards a range of lipophilic chemicals including steroid and thyroid hormones, bilirubin, fatty acids, and drugs. GST isozymes can be classified into at least seven distinct classes (alpha, mu, pi, theta, sigma, kappa, and zeta) and three families (cytosolic, mitochondrial, and microsomal). Members within a class have distinctive substrate and inhibitor specificities and similar exon-intron structures and share extensive (greater than 50 %) sequence homology. Cytosolic human GSTs (GSTM1, GSTP1, and GSTT1) exhibit genetic polymorphisms, some of which can increase susceptibility to carcinogenesis and inflammatory diseases (McIlwain et al. 2006). Targeted disruption of murine genes has demonstrated that cytosolic GST isoenzymes (GSTA4, GSTP1

and P2, Ptgds2, zeta GSTZ1) are broadly cytoprotective and belong to the dynamic and interactive defense mechanism that protects against cytotoxic electrophilic chemicals and allows adaptation to oxidative stress (Hayes et al. 2005). GSTM1 and GSTP1 are capable of modulating stress-mediated signals by directly interacting with ASK1 and JNK protein kinases (MAP kinase family), which occurs independent of their well-known glutathione-conjugating activity (Elsby et al. 2003; Ryoo et al. 2004) and suggests additional physiological function in cellular signaling for these enzymes. MAP kinases contribute to a variety of biological process, including stress response, apoptosis, cell survival, and morphogenesis (Davis 2000). Until recently MAP kinases in central nervous system (CNS) have been mainly considered as degenerative signal transducers. The embryonic lethality of JNK1+2 double knockout mice and brain abnormalities of single knockout animals indicate fundamental roles of MAP kinases during CNS development (Gelderblom et al. 2004). The association of GSTM1 genetic polymorphism with autistic disease (Buyske et al. 2006) as well as emerging roles of this enzyme in cytoprotection and regulation of signal transduction mechanisms made it an attractive candidate for an autism susceptibility gene.

Polymorphisms in glutathione s-transferase M1 (GSTM1), a gene involved in glutathione metabolism, have been associated with autism (Buyske et al. 2006). The ALA6 allele for human glutathione peroxidase (GPX1) was found to be undertransmitted in autistic disorder and could therefore be protective for autistic disorder (Ming et al. 2010). There has also been evidence showing several polymorphic variants that are involved in methionine and glutathione metabolism to be increased in autistic children. The increased polymorphic variants include the genes for reduced folate carrier (RFC80G>A), transcobalamin II (TCN2776G>C), catechol-O-methyltransferase (COMT 472G>A), methylenetetrahydrofolate reductase (MTHFR 677C>T and 1298A>C), and GSTM1 (James et al. 2006). A single nucleotide polymorphism (SNP) of the glyoxalase I gene that changes a cytosine to an adenine, causing an alanine to a glutamine change in the Glo enzyme, has been identified as an autism susceptibility factor (Junaid et al. 2004). There is further evidence to suggest that this A-allele causes the activity of the Glo1 enzyme to be reduced, leading to an increase in the substrate for this enzyme in the cell that is toxic to developing neuronal cells (Barua et al. 2011). In our initial study, we focused on GSTM1 (Yochum et al. 2010).

The hypothesis that genetically altered mice might be more sensitive to early exposure to a neurotoxicant was evaluated using mice with a deletion of GSTM1 (Yochum et al. 2010). Male and female knockout mice and their wild-type controls were exposed to VPA on postnatal day 14 (P14). We had previously established that P14 is one of the earliest possible dates on which a sufficient number of behavioral skills have been mastered by the pups and, therefore, toxicant-induced regression can be assessed (Wagner et al. 2006). It was found that the VPA caused apoptosis in granule cells of the hippocampus and cerebellum, two regions thought to be affected in autism. Furthermore, consistent with the hypothesis, there was a genotype by treatment by sex interaction with wild-type females exhibiting significantly fewer apoptotic cells in these regions compared to all other groups. In addition, the VPA-treated

GSTM1 knockout mice exhibited significantly poorer social behaviors compared to saline-treated knockout animals as well as wild-type controls receiving either treatment (Yochum et al. 2010). This strategy brought together (1) a genetic anomaly associated with autism and thought to render the individual more susceptible to toxicant-induced oxidative stress and (2) a neurotoxicant shown to result in autism-like deficits in exposed children and to exert its neurotoxic actions by an excitotoxic-induced oxidative stress. The combination led to cell death exclusively in brain regions associated with autism and to social deficits in the absence of global motor deterioration. This approach yielded outcomes that, collectively, confer the model with a high validity. Nonetheless, the GSTM1 polymorphism accounts for only a small percentage of autism, and likewise, developmental exposure to VPA is extremely limited. Clearly, the question becomes to what degree can one generalize from this model to other toxicants and other genetic alterations?

4.1 Nrf2

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2 or NFE2L2) is a transcription factor that is involved in defense against oxidative stress (Baird and Dinkova-Kostova 2011). Nrf2 is expressed in many tissues throughout the body including brain, liver, kidney, skin, and the gastrointestinal tract (Moi et al. 1994). Nrf2 is a member of the Cap 'n' Collar (CNC) family of regulatory proteins that has a basic leucine zipper DNA-binding domain (Moi et al. 1994). This basic leucine zipper helps to facilitate dimerization and DNA binding (Moi et al. 1994). Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1) is a negative regulator of Nrf2 (Itoh et al. 1999). Keap1 has a BTB protein interaction domain (broad-complex, tramtrack, and bric-a-brac) and a Kelch domain that bind to Nrf2 (Itoh et al. 1999; Baird and Dinkova-Kostova 2011). Under normal conditions, Keap1 sequesters Nrf2 in the cytoplasm, eventually leading to its degradation. The BTB domain interacts with Cullin3 (Cul3)-based E3 ubiquitin ligases as a substrate adaptor allowing for the ubiquitination and degradation of Nrf2 (Kobayashi et al. 2004). One Nrf2 molecule is bound to a dimer of Keap1 with each of the Keap1 Kelch domains binding to one of two Keap1-binding sites, the DLG or ETGE binding motifs within the NEh2 domain of the Nrf2 molecule (Itoh et al. 1999; McMahon et al. 2006). This hinge and latch model suggests that the hinge is the binding at the ETGE motif and the latch is the binding at the DLG motif that holds the Nrf2 allowing for interactions with ubiquitin and degradation during normal conditions (McMahon et al. 2006). Once the cell is exposed to oxidative stress, these inducers react with cysteine residues in Keap1 which releases the Nrf2 at its DLG binding site causing Nrf2 to no longer be a target for ubiquitination (McMahon et al. 2006). The Nrf2 is no longer being degraded; however, it is still attached to Keap1 at its EGTE binding motif, and therefore, any new Nrf2 will accumulate in the cell and be free to translocate into the nucleus and induce transcription of its target genes (McMahon et al. 2006).

This model suggests that Nrf2 does not dissociate from Keap1; however, there is evidence that in response to certain inducers like cadmium and arsenic, that Nrf2 does dissociate, and it is therefore likely that the mechanism of control is different depending on the inducer (He et al. 2006, 2008). Nrf2 may also be able to interact with inducers directly, which suggests a possible Keap1-independent model of Nrf2 control. Nrf2 has putative nuclear import and export (NES) signals, one of which is located in the Neh5 transactivation domain (Li et al. 2006). This NES has been shown to be sensitive to reactive oxygen species and has been suggested to regulate Nrf2 (Li et al. 2006). Under normal conditions, the export signals (NES) overcome the import, and Nrf2 is found in the cytoplasm and not in the nucleus.

Oxidative stress or other inducers cause the export signals to inactivate, and Nrf2 is then able to translocate into the nucleus and induce the transcription of antioxidant genes (Li et al. 2006). This model does however conflict with some previous reports. The concentration of inducers required for the activity of the Nrf2 NES is much higher than those to inactivate Keap1 (McMahon et al. 2003). There is also evidence from Keap1-knockout mice that shows levels of Nrf2 target genes are constitutively upregulated and cannot be induced, providing evidence that Keap1 and not Nrf2 is responsible for inducer sensitivity (Wakabayashi et al. 2003).

Protein kinases have also been suggested to have a part in the regulation of Nrf2. When activated, protein kinase C (PKC) was found to induce antioxidant response element (ARE)-dependent gene expression, and when inhibited, tBHQ-mediated accumulation of Nrf2 was found to be decreased (Huang et al. 2000). It was further shown that S40 of Nrf2 is the target for this phosphorylation and that when S40 is phosphorylated, it promotes the release of Nrf2 from Keap1 allowing for the induction of ARE-containing genes (Huang et al. 2002). PKCδ- knockout cells have been shown to have a reduced accumulation of Nrf2 after exposure to inducers, further providing evidence of a role of protein kinases in the activation of Nrf2 (Li et al. 2004). Another kinase pathway that has been implicated in Nrf2 regulation is the mitogen-activated protein kinase pathway (MAPK), although the evidence suggests a limited role. Inducers of Nrf2 have been shown to modulate MAPK activity (Yu et al. 2000). Inhibition of ERK, MEK, or p38 decreases the amount of ARE-dependent gene expression (Yu et al. 2000). This provides evidence that kinases may have a role in Nrf2 activation. It has also been shown however that p38 negatively regulates Nrf2, which suggests that the MAPK regulation of ARE-dependent gene expression may be inducer and cell type specific (Yu et al. 2000). In addition, mutation of one of the five phosphorylation sites on Nrf2 had no effect on the activity, and mutation of all five sites resulted in only a slight decrease in ARE-dependent gene expression, suggesting only a small role of MAPK in Nrf2 regulation (Sun et al. 2009).

Nrf2 induces enzymes and other proteins that protect against oxidative stress through transcriptional activation of the antioxidant response element of their genes. Oxidative stress is caused by the production of reactive oxygen species (ROS) and electrophiles through endogenous mechanisms or exposure to xenobiotics, heavy metals, or ionizing radiation (Kaspar et al. 2009). ROS are produced in the mitochondria, from cytochrome P450 reactions, through enzymes, such as cyclooxygenase, lipoxygenase, and monoamine oxidase, or through ionizing radiation (Dröge 2002).

Reactive oxygen species include superoxide anion, hydrogen peroxide, and hydroxyl radical, among others (Dröge 2002). The accumulation of ROS and oxidative stress can lead to modifications in DNA that can be mutagenic (Meneghini 1997). ROS can also cause damage to lipids, proteins, and carbohydrates, leading to cell and tissue damage (Kaspar et al. 2009). This damage can eventually lead to a variety of disease states such as rheumatoid arthritis, Parkinson's disease, Alzheimer's disease, cystic fibrosis, cancers, and many others (Dröge 2002). The genes that Nrf2 induces contain the ARE that allows interactions for basal and inducible expression (Rushmore et al. 1991). Nrf2, when activated by oxidative stress, binds to the ARE of these genes and induces their expression (Alam et al. 1999; Nguyen et al. 2005). Nrf2 has been shown to bind to the ARE with a high affinity only when it is in a heterodimer with a small Maf protein (Itoh et al. 1997).

The genes that Nrf2 upregulates include NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1(HO-1), and glutathione S transferase (GST), which are all involved in detoxification of reactive oxygen species (Alam et al. 1999; Nguyen et al. 2005). NQO1 catalyzes the two-electron reduction of quinones, quinone imines, nitroaromatics, and azo dyes (Ross 2005). It uses NADH or NADPH as electron donors. It is extremely inducible in response to electrophilic metabolites and oxidative stress (Ross 2005). In addition to metabolizing xenobiotic quinones, NQO1 also metabolizes endogenous quinones such as ubiquinone and vitamin E quinone. These two quinones in particular need to be reduced in order to provide their potent antioxidant effects (Ross 2005). NQO1 has also been found to function as a direct superoxide-scavenging enzyme (Ross 2005). Heme oxygenase-1 (HO-1) is an isoform of the heme oxygenase enzymes that catalyze the first step in heme catabolism (Alam et al. 1999). This first step involves the oxidative cleavage of b-type heme, which produces iron, carbon monoxide, and biliverdin that is then converted to bilirubin by biliverdin reductase (Alam et al. 1999). HO-1 is induced by several agents that stimulate the production of reactive oxygen species or deplete glutathione levels including, heavy metals, UV irradiation, inflammatory cytokines, and its substrate, heme (Alam et al. 1999). In addition, HO-1 is considered an antioxidant enzyme involved in cellular defense against oxidative stress because its substrate, heme, is a potent prooxidant, and its product bilirubin is a potent antioxidant (Alam et al. 1999). The GST family of enzymes mentioned above catalyzes the conjunction of a number of substrates, such as quinones, α, β-unsaturated carbonyls, arene oxides, and others with glutathione (Hayes et al. 2005). GSTs metabolize cancer chemotherapeutics, carcinogens, insecticides, herbicides, and by-products of oxidative stress (Hayes et al. 2005). GSTs are also involved in the synthesis of leukotrienes, prostaglandins, testosterone, and progesterone (Hayes et al. 2005). The GSTs are involved in the protection against endogenous oxidative stress. GSTs can reduce cholesteryl hydroperoxides and fatty acid hydroperoxides, as well as phospholipid hydroperoxides, to stop the formation of epoxides and reactive carbonyls (Hayes et al. 2005). Oxidation of nucleotides and catecholamines yields toxic products that can then be detoxified by conjugation with glutathione by GSTs (Hayes et al. 2005).

Nrf2 knockout mice have been shown to have enhanced susceptibility to various toxicities due to xenobiotics and the environment. They have been shown to have

reduced basal or lower induced levels of cytoprotective genes in various organs including liver (Itoh et al. 1997; Liu et al. 2010), gastrointestinal tract (Itoh et al. 1997), lung (Ishii et al. 2005), and brain (Innamorato et al. 2010). In Nrf2 knockout mice, induction of many phase II enzymes was found to be near gone in the liver and gastrointestinal tract, indicating that Nrf2 is needed for the induction of phase II enzyme genes (Itoh et al. 1997). This suggests that the Nrf2 knockout mice would be highly susceptible to neoplastic transformation (Itoh et al. 1997). Nrf2 null mice have been shown to have a greater incidence of forestomach tumors and benzo[a]pyrene-induced DNA adducts compared with wild-type control mice (Ramos-Gomez et al. 2003). It has been shown that Nrf2 knockout mice have a greater susceptibility to 1-bromopropane-induced hepatotoxicity due to low expression levels of antioxidant enzymes compared to wild-type mice (Liu et al. 2010). Nrf2 knockout mice are also more susceptible to hepatotoxicity induced by acetaminophen. When exposed to acetaminophen, Nrf2 knockout mice have decreased survival rates, increased serum alanine aminotransferase activity, and increased centrilobular necrosis (Chan et al. 2001). These results are thought to be caused by lower cellular thiol levels and decreased expression of detoxifying enzymes, and Nrf2 certainly plays a key role in regulating the various detoxifying enzymes (Shen and Kong 2009).

Several studies have provided evidence of the importance of Nrf2 in the susceptibility to numerous toxins in the respiratory system. Nrf2 knockout mice were used to study emphysema and were found to be more susceptible to elastase-induced emphysema and have lower levels of antioxidant and antiprotease gene expression in alveolar macrophages compared to wild-type counterparts (Ishii et al. 2005). Nrf2-deficient mice have been found to have increased DNA adduct formation after exposure to diesel exhaust compared to wild-type mice (Aoki et al. 2001). It has also been shown that Nrf2 knockout mice had a 6.1-fold increase in mutation frequency in the lung after exposure to benzo[a]pyrene compared with untreated nrf2+/– mice (Aoki et al. 2007). Nrf2 has been shown to play a significant role in protecting against pulmonary hyperoxic injury in mice. After 72 h hyperoxia exposure, Nrf2 knockout mice had 7.6-fold greater pulmonary hyperpermeability, 47 % more macrophage inflammation, and 43 % greater epithelial injury than did wild-type control mouse with the same exposure (Cho et al. 2002). It was also found that the Nrf2 knockout mice had significantly lower induction of mRNA levels of NAD(P)H: quinone oxidoreductase(NQO1), GST-Ya and Yc subunits, UDP glycosyltransferase (UGT), glutathione peroxidase-2 (GPx2), and HO-1 compared with wild-type controls (Cho et al. 2002).

The Nrf2 knockout mice have been used to study Parkinson's disease and have been shown to be more susceptible to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and to show more inflammation compared to wild-type animals (Innamorato et al. 2010). The neurotoxin 6-hydroxydopamine is a reactive oxidative stressor and complex I inhibitor used to model Parkinson's disease that activates ARE-regulated genes (Jakel et al. 2007). Cultured cells of Nrf2 knockout embryos exposed to 6-hydroxydopamine had increased apoptotic cells compared to controls (Jakel et al. 2007). Furthermore, injections into the striata of Nrf2 knockout mice produced lesions double in size compared to those seen in wild-type mice (Jakel et al. 2007).

Traumatic brain injury-induced secondary brain injury was found to be more severe in Nrf2 knockout mice compared to wild-type mice. After weight-drop impact head injury, the Nrf2 knockout mice had increased severity of neurological deficit, apoptosis, and brain edema 24 h later (Jin et al. 2009). This increased injury was found to be associated with increased mRNA expression and protein expression of inflammatory cytokines and decreased mRNA expression and enzymatic activity of NQO1 and GST-alpha1 compared with wild-type controls (Jin et al. 2009). In a prior review, we suggested that the etiologies of Parkinson's disease and autism have common features, most notably toxicant exposure and enhanced sensitivity to oxidative stress (Yochum et al. 2010). There is also a strong association of antioxidative stress and anti-inflammatory responses regulated by Nrf2 in various in vitro and in vivo models (Hu et al. 2010; Li et al. 2008). With this in mind, we conducted a very preliminary study to determine if Nrf2 knockout mice might show functional abnormalities in their development as compared to wild-type controls.

4.2 Nrf2 and Autism Model

A series of behavioral tests were conducted beginning on postnatal day 5 (P5) on Nrf2 wild-type (WT) and knockout (KO) pups. These tests included surface righting, negative geotaxis, hanging wire grip strength, motor activity, rotarod, Morris water maze, and play behavior which are typical behavioral tests for normal neurological studies. It was found that the KO mice consistently weighed more than their WT counterparts (Fig. 1-upper). No differences between the KO mice and the WT controls were found in surface righting, negative geotaxis, hanging wire grip strength, midair righting, rotarod, or locomotor activity. In the Morris water maze, the KO and WT mice performed similarly when the platform was visible, but the KO mice actually were able to locate the hidden platform significantly faster than the WT mice (Fig. 1-lower, $p<0.034$). Of interest, at about one month of age, the KO mice engaged in fewer social play contacts with other mice, instead engaging in self-grooming behavior (Fig. 2). Furthermore, as we have demonstrated previously using another strain (Yochum and Wagner 2009), treatment with VPA on P14 also resulted in a reduction in play behavior in the Nrf2 KO mice (Fig. 2). These very preliminary observations indicate that the Nrf2 KO mice may be useful in advancing our understanding of the underlying etiology of autistic regression.

There is evidence to suggest a link between VPA and the redox-sensitive transcription factor Nrf2. VPA has been shown to increase levels of reactive oxygen species as previously discussed. This increase in reactive oxygen species induced by VPA has been implicated in the toxicity caused by VPA, including neural tube defects (Defoort et al. 2006). Antioxidants like vitamin E and green tea extract have been shown to protect against the injuries caused by VPA providing further evidence of the role of oxidative stress in the VPA-induced damage (Banji et al. 2011; Al Deeb et al. 2000). Nrf2 is a transcription factor that is induced by oxidative stress. When reactive oxygen species accumulate in a cell, Nrf2 is activated and translocates into the nucleus of the cell (McMahon et al. 2006). Once in the nucleus

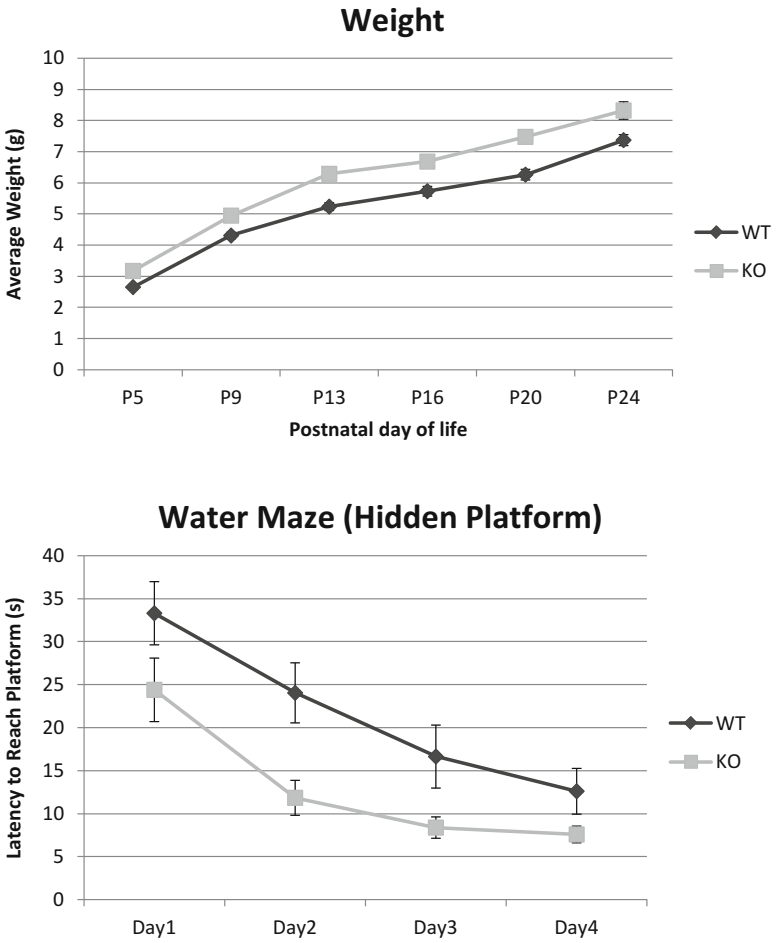

Fig. 1 *Upper*: Body weight of Nrf2 knockout mice (ko; $n=15$) and their wild-type controls (wt; $n=23$) on postnatal days P5–P24. The KO mice weighed significantly more than the WT controls. [$F=(1,180)=17.8$; $p=0002$]. *Lower*: Latency to find the submerged platform for KO and WT mice in the Morris water maze on four successive days of training (P21–P24). The KO mice acquired the task significantly faster than the WT controls. [$F=(1,108)=4.8$; $p=.034$]

Nrf2 binds to the ARE on target gene promoter regions (Rushmore et al. 1991). Thus, Nrf2 induces the expression of genes containing the ARE, including HO-1, NQO1, GST, and Gαi2 (Kawai and Arinze 2006). VPA has been shown to induce gene transcription in an ARE-dependent manner for the genes HO-1, NQO1, and Gαi2 (Kawai and Arinze 2006). Prior reports suggested that VPA induced gene expression by the transcription factor activator-protein-1 (AP1) and its activity at the AP-1 response element in genes (Chen et al. 1999). Kawai and Arinze (2006) provide evidence that ARE is a distinct target for VPA-induced gene expression. The Gαi2 gene does not contain the AP-1 binding motif, but can still be activated by VPA through the Sp1-binding site (Kawai and Arinze 2006). Mutation in the Gαi2

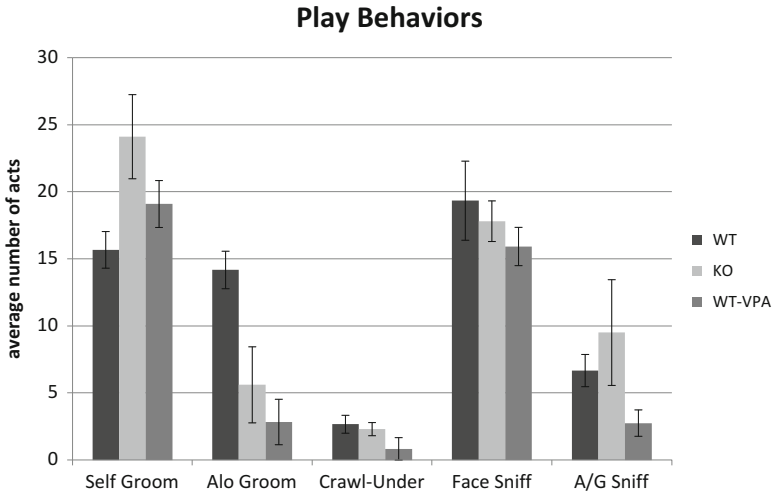

Fig. 2 Play behaviors in 30–40 day-old pairs of mice, matched for sex, genotype, and treatment. WT mice self-groomed significantly less than KO ($p=0.0139$) [$F(2,14)=3.958$, $p=0.0434$]. WT mice groomed other mice significantly more times than KO mice ($p=0.0091$) and WT mice treated with 400 mg/kg VPA on P14 ($p=0.0009$) [$F(2,14)=9.498$, $p=.0025$]. WT: $n=6$ pairs, KO: $n=5$ pairs, WT-VPA: $n=6$ pairs

gene at both ARE and Sp-1 affected activity induced by VPA, but only the mutation at ARE affected activity induced by tBHQ, which shows that the VPA-induced gene promoter activity at ARE is independent of the VPA-induced promoter activity at the Sp-1 binding site (Kawai and Arinze 2006). Furthermore, the VPA-induced transcription was not found to be sensitive to antioxidants in the absence of the ARE sequence (Kawai and Arinze 2006). In a recent study on the primary culture of astrocytes, the histone deacetylase inhibiting activities of VPA has been demonstrated and VPA has restored the Nrf2-antioxidant defense and conferred protection against hydrogen peroxide-induced cell death (Correa et al. 2011, please see attached). This provides evidence of a link between the elevated levels of reactive oxygen species and the activation of the transcription factor Nrf2 and the subsequent induction of ARE-containing genes. Given this information, there should be increased sensitivity of the Nrf2 KO mice to the toxicity induced by VPA. The Nrf2 knockout mice are lacking the ability to upregulate the genes induced by Nrf2 that protect against oxidative stress. VPA administered to these mice should create deficits in behavior as well as increased brain injury caused by increased levels of reactive oxygen species because they lack Nrf2 and the ability to protect them against oxidative stress. Future studies will assess the effects of early administration of VPA on Nrf2 knockout and wild-type mice with the prediction that the knockout mice will be more sensitive, showing a more severe behavioral regression. It will be of interest to determine if antioxidant pretreatment is effective to prevent or ameliorate the behavioral regression in these mice.

Conclusions

In this chapter, we have argued that autistic regression may result from toxicant-induced oxidative stress. The nature of the "toxicant" exposure can range from traditional exogenous toxicants (e.g., valproic acid, methylmercury) to endogenously generated toxicants such as those associated with intense immunological reactions. For some individuals, the timing of exposure and dose may be severe enough as to result in autism. However, for others, the timing and/or dose of toxicant exposure may be in an otherwise tolerable range, but because of a genetic vulnerability, there may be an increased sensitivity, and once again, autism results. In these cases, the genetic vulnerability renders the individual particularly sensitive to the toxicant-induced oxidative stress. Finally, we have demonstrated that autistic regression in mice can be prevented by antioxidant pretreatment just prior to toxicant exposure. Importantly, these observations have now been replicated elsewhere using different antioxidants and dosing regimens.

These observations make it imperative that factors that place a child "at risk" for autistic regression must be identified. Such factors might include male children with autistic siblings or identified genetic variants or seizure disorders or those who have experienced adverse reactions to vaccinations. Individuals at risk for autistic regression may be further identified as being at a greater risk for autistic regression if there are combinations of these risk factors (e.g., male children with autistic siblings and who have had a prior adverse reaction to a vaccination may be considered at risk when they are required to receive additional vaccinations or male children with autistic siblings who experience any severe fever or seizure disorder). The data using this mouse model of autistic regression suggest that antioxidant treatment during the critical periods may prevent autistic regression.

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Genetic Polymorphism Related to Oxidative Stress in Autism

Hee Jeong Yoo

1 Introduction

Autism is a neurodevelopmental disorder that is characterized by impaired communication and reciprocal social interaction and by repetitive behavior and restricted interest. Strong evidence derived from family and twin studies supports the role of genetic factors in the etiology of this complex disorder (Losh et al. 2008). The mode of inheritance is unknown, but the involvement of both common and rare variants is suggested, and several dozen autism spectrum disorder (ASD) susceptibility genes have been identified in the past decade (Geschwind 2011).

The prevalence of autism and ASD has dramatically increased during the past 3–4 decades, from 3 in 10,000 children in 1970 to approximately 20 (autistic disorder)–30 (pervasive developmental disorder, not otherwise specified, PDD NOS) in 10,000 (Fombonne 2009). Although genetic etiology may be the foremost etiological factor of autism, it is not sufficient to account for the overall changes that have occurred within a few decades. Other factors that play an important role in the etiology of ASDs are an increased rate of detection, a widened range of diagnostic criteria, and environmental factors.

Prevailing evidence supports the involvement of genetic, epigenetic, and environmental factors that negatively affect prenatal and postnatal neurologic development (Folstein and Rosen-Sheidley 2001; James et al. 2006). Oxidative stress and the susceptibility of an individual to oxidative stress disorders are proposed as key elements in mediating the influence of environmental factors and genetic predisposition in the development of autism. These influences have been reported in a wide

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variety of other chronic neurological disorders, including schizophrenia, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, HIV-associated dementia, and fetal alcohol syndrome (Bowers et al. 2011; Cohen-Kerem and Koren 2003; Do et al. 2009; Perry et al. 2004; Zawia et al. 2009).

In addition to the metabolic and biological changes associated with oxidative stress and susceptibility genes, the role of specific oxidative stress genes or endogenous antioxidants in the development of autism has been proposed, and research has been focused on understanding the development of mutations and genetic polymorphisms relevant to oxidative pathways and impaired methylation processes.

This chapter will describe the research findings regarding the following: (1) polymorphisms of various genes that are potentially related to oxidative stress and susceptibility genes to autism and Rett's disorder; (2) implications from animal studies using knockout mice; (3) the possible linkage among environmental factors, oxidative stress, and epigenetic factors in the pathophysiology of autism and related developmental disorders; and (4) clinical and treatment implications.

2 Potential Candidate Genes

2.1 Genes from the Glutathione Pathway

The genes that have been most widely studied are closely linked to *folate-dependent methionine transmethylation and transsulfuration cycles* and are involved in the redox status. Oxidative stress during prenatal and early postnatal development results from the abnormal expression of key antioxidant genes that are primarily involved in methionine transmethylation and transsulfuration pathways in the fetus/infant (James et al. 2006). Under oxidative stress, multiple adaptive pathways shift the flux of sulfur resources toward increased de novo synthesis of cysteine-containing tripeptide glutathione (GSH), the primary intracellular antioxidant (Deth et al. 2008).

The first genetic associations reported were based on observations of metabolic abnormalities in autism regarding the methionine transmethylation and transsulfuration pathways. Most of the research was focused on *glutathione* and its metabolic cofactors, which provide the primary defense against oxidative stress, including directly scavenging free radicals and reducing peroxides and conjugations with toxic electrophilic compounds (Maher 2006). The plasma levels of the transsulfuration metabolites are reportedly abnormal in autistic individuals; for example, cysteine, total glutathione, and free reduced glutathione (GSH) are reduced, whereas cystathionine and the oxidized disulfide form of glutathione are increased. Moreover, the ratios of total glutathione and GSH glutathione to oxidized glutathione disulfide (GSSG) (redox ratios) are reduced (Geier and Geier 2006; James et al. 2006).

The genes associated with this phenomena were allelic variations of genes related to the transsulfuration pathway and glutathione metabolism, such as glutathione S-transferase Mu 1 (GSTM1), glutathione S-transferase Pi 1 (GSTP1), and glutathione peroxidase (GPX1) (Buyske et al. 2006; James et al. 2006; Ming et al. 2010; Serajee et al. 2004).

2.1.1 Glutathione S-transferase Gene

Previous data emphasize the importance of the glutathione S-transferases (GST) as protective factors against reactive oxygen species and the products of oxidative stress. Human cytosolic GST is primarily encoded by 5 loci: GSTA, GSTT1, GSTM1, GSTP1, and GSTM3 (Schilter et al. 1993). GSTM1, which conjugates GSH to toxic electrophiles, has three alleles: GSTM1*0, GSTM1*A, and GSTM1*B. The homozygous deletion (0/0), or null genotype, which leads to copy number variations (CNVs) at either the GSTM1 or the GSTT1 locus, resulted in loss of enzyme function. Originally, it was hypothesized that this locus was associated with the susceptibility to autoimmune conditions, such as lupus erythematosus and various forms of cancer, including oral, gastric, colorectal, prostate, bladder, and hepatic cancer and renal cell carcinoma (Economopoulos and Sergentanis 2010; Gronau et al. 2003; Hayes and Pulford 1995; Simic et al. 2009; Zhang et al. 2010, 2011).

Two studies have reported an association between the null allele and autism (Buyske et al. 2006; James et al. 2006), suggesting that GSTM1 contributes to the risk of oxidative stress and autism. The first study, which was a case–control analysis of 358 children with autism and 201 age-matched control children, revealed a marginal increase in allele frequency with borderline significance for the GSTM1 null genotype (OR, 1.37; CI, 0.98 ~ 1.96) (James et al. 2006). A similar but clearer association was observed in a combined case–control family-based study. Although the sample sizes were small (54 complete case–parent trios and 172 controls, 45 with autistic disorders and 9 with PDD NOS), this study was noted for the use of an analytic method of combining both the traditional case–control analysis and the 1-df likelihood ratio test (utilizing controls and family trio data), and the results supported the association of the homozygous GSTM1 deletion genotype with an increased risk of autism ($p = 0.028$ and $p = 0.046$, respectively) (Buyske et al. 2006).

The pi class of GSTs, represented by a single GST (known as GSTP1, GSTP1-1, GSTP, GSTp, and GSTpi) encoded by a gene on chromosome 11q13, are expressed at the highest levels in most extrahepatic tissues (Lev-Ram et al. 1995). A family-based association study of 196 parent–proband trios using the transmission disequilibrium test (TDT) failed to demonstrate a significant genetic association ($p = 1.00$) (Serajee et al. 2004). GSTP1 attracted attention for possessing potential teratogenic alleles, which might contribute to the phenotype of the affected child in the mother during pregnancy. In one study, Williams et al. (2007) genotyped 137 individuals in 49 families with autistic disorders for the GSTP1*G313A and GSTP1*C341T polymorphism using maternal trios, consisting of the mother of an individual with autistic disorder and her parents. The results revealed that the GSTP1*A haplotype was overtransmitted to the case mothers, and the GSTP1*B and GSTP1*C haplotypes were undertransmitted at almost the same rates (OR = 2.67, 95 % CI = 1.39–5.13), while the individual genotypes were not significantly overtransmitted using the TDT ($p = 0.06 \sim 0.36$) (Williams et al. 2007). Despite limitations, including small sample size and the lack of phenotyping the mothers, this study is one of a few studies that explored the maternal genotype of children with autism, proposing the role of hazardous teratogenic allele in the interactions of the intrauterine environment and the susceptibility of the children. Different mechanisms were derived from studies of the

function of GSTP1, including the regulation of kinases, such as mitogen-activated protein kinase (MAPK) and Jun N-terminal kinase (JNK); however, the precise mechanism of teratogenic action has yet to be clarified (Williams et al. 2007).

2.1.2 Glutathione Peroxidase Gene

Glutathione peroxidase (GPX1) has recently been implicated as a candidate gene in autism. GPX is a major enzyme in the glutathione pathway that catalyzes the reduction of free radicals by glutathione and represents a major enzyme for defense against oxidant molecules (Robertson et al. 2007). Altered GPX1 enzymatic activity has been reported in spina bifida, meningomyelocele, idiosyncratic valproate-induced toxicity, fetal valproate syndrome, and autism (Ghanizadeh 2011; Leonard et al. 2008; Williams et al. 2001). A small family-based association study of a GCG repeat polymorphism of a human GPX1 polyalanine repeat (ALA5, ALA6, and ALA7) in 103 family trios of autistic disorder revealed a significant transmission disequilibrium ($p=0.044$) in the overall transmission of the three alleles. The ALA6 allele was undertransmitted ($p=0.017$), suggesting its protective effect for autistic disorder (Ming et al. 2010), although the precise function of ALA6 has not yet been clearly demonstrated, except for evidence showing that the GPX1 ALA6/198Leu polymorphism decreased enzyme activity by 40 %. These results and indirect evidence of an association with fetal valproate syndrome suggest that the GPX1 gene might be one of the plausible candidates in the development of autism (Hamanishi et al. 2004).

2.2 *Genes from Methionine Transmethylation Pathway and Folate Metabolism*

2.2.1 Methylenetetrahydrofolate Reductase

Methylenetetrahydrofolate reductase (MTHFR) is an important methionine transmethylation pathway-related gene. This enzyme catalyzes DNA methylation using a methyl donor from dietary folate and regulates folate availability (Ulrey et al. 2005). Two common functional polymorphisms (677C>T and 1298A>C) are reported to reduce the enzyme activity of MTHFR, and 677TT is associated with a 60 % reduction of enzymatic activity (Frosst et al. 1995; Schmidt et al. 2011). Disrupting the folate metabolic pathway through the reduced enzymatic activity of MTHFR impedes the conversion of homocysteine to methionine, subsequently reduces the formation of the methyl donor S-adenosyl methionine (SAM) for DNA methylation, and leads to hypomethylation (dos Santos et al. 2010). Several studies have reported the proportions and associations of a specific allele of the MTHFR gene in autism across diverse ethnicities, although these researchers did not report consistent results.

MTHFR is the only replicated gene involved in the B vitamin-dependent folate, methionine, and transmethylation pathways. James et al. (2006) observed an increase in the frequency of the MTHFR 677TT over the 677CT genotype in autistic

cases with borderline significance (OR = 1.45 and 1.36 for each) (James et al. 2006). The role of this gene has been explored in small- to moderate-sized case–control and family-based association studies (39 probands ~512 families). Generally, the low-activity MTHFR 677 T allele and 677TT genotype are observed more frequently in cases of autism than in controls with high ($p < 0.01$) or borderline significance ($p = 0.09$) (D'Amelio et al. 2005; Liu et al. 2011; Mohammad et al. 2009; Pasca et al. 2009). Specifically, the role of the MTHFR T allele is critical, as studies have shown an increased risk of autism of approximately threefold in a dose-dependent manner ($P_{trend} < 0.0001$) and an overtransmission in family-based association studies in simplex families (Liu et al. 2011; Mohammad et al. 2009). However, the risk of the T allele and CT genotypes in autism was not evident in cases involving Brazilian children (dos Santos et al. 2010).

In the case of MTHFR 1298AC (another functional polymorphism related to low enzyme activity), the function according to allelic and genotypic variants tends to be increased with regard to the haplotype or co-segregated genotype. For example, the 677T-1298A haplotype and double homozygous 677TT/1298AA genotype are significantly more frequent to affected individuals, and the co-segregation of MTHFR 677T-1298C variant alleles was associated with an 8.11-fold increased risk for autism (95 % CI: 2.84–22.92) as compared with the MTHFR 677CC/1298AA genotype in another study (Liu et al. 2011; Mohammad et al. 2009).

As the autism has a complex and heterogeneous behavioral phenotype, problematic behaviors from 3 principal domains of autism show a wide spectrum. In 25 % of families affected by autism, multiple family members are affected by clinical or subclinical autistic traits, and within this subset of families, the distribution of autistic traits and symptoms appears highly quantitative (Constantino 2011). Understanding the core social abnormality of autism as a quantitative trait rather than as a categorically defined condition has key implications for understanding the underlying genetic and neurobiological mechanisms (Constantino 2011). Moreover, the analysis of phenotypic–genotypic relationship might be a rational approach to exploring the function of this risk allele, assuming the dose-dependent increase of autism risk according to the number of MTHFR 677T alleles (Goin-Kochel et al. 2009).

In a study of 147 stringently phenotyped subjects from the Autism Genetic Resource Exchange (AGRE) collection (94 % white/Caucasians), the authors analyzed the MTHFR 677CT polymorphisms, the "Restricted, Repetitive, and Stereotyped Patterns of Behavior" composite scores, and other behavioral variables consistent with anecdotal reports of behavioral changes among children with autism spectrum disorders who were treated with folate supplementation. An analysis of language, nonverbal cognitive functioning, and adaptive behavior was also included. The results indicated four ADI-R behaviors (direct gaze, current complex body movements, a history of self-injurious behavior, and current overactivity (ORs = 2.72, 2.33, 2.12, 2.47, respectively)) that were more common and problematic (95 % CI) among those with at least one copy of the T allele as compared with homozygous wild-type individuals among the children with autism, although correction of the multiple tests was not applied (Goin-Kochel et al. 2009). Further studies concerning the phenotypic–genotypic interaction of the MHTFR gene need to be replicated with more careful corrections of the population stratification and a direct measure of the variety of autistic symptoms.

MHTFR and other folate metabolism-related genes might have clinical implications regarding the potential treatment and prevention of autism using vitamin supplements. A recent, relatively large cohort study (278 children with autism, 144 with autism spectrum disorder, and 278 typically developing children) was focused on the association of genetic variants and prenatal vitamin intake with autism susceptibility. In the context of gene–environment interactions, the authors examined the association between autism and maternal vitamin supplement intake during conception and prenatal periods, in combination with common functional maternal and infant gene polymorphisms in the folate, methionine, and transmethylation pathways. The results showed a significant association between the prevalence of the maternal MTHFR 677TT genotype and no preconception maternal prenatal vitamin intake (combined OR = 4.5, CI = 1.4 ~ 14.6, interaction $p = 0.04$) (Schmidt et al. 2011). Despite limitations, such as the retrospective reporting of vitamin and supplement information and the fact that only 9 (4 %) children with autism were included in the MTHFR 677TT/no vitamin supplement group, this type of gene–environment interaction study should be expanded and replicated in the future.

2.2.2 Other Genes Within the Pathway

Other functionally important polymorphisms in folate metabolism genes that show a significant association with ASD include a 19-bp deletion in the dihydrofolate reductase gene (DHFR), transcobalamin II (TCN2 776C>G122), catechol-O-methyltransferase (COMT 472G>A122), and reduced folate carrier (RFC1 80A>G), but studies concerning these genes need to be further replicated; currently, there have only been reports of one or two studies for each gene (Adams et al. 2007; James et al. 2006, 2010). Of those polymorphisms, the functional polymorphism RFC1 gene is a potential mediator of oxidative stress–gene interactions; the results from a large-scale study with 529 case–parent trios and 566 neurotypical controls demonstrated a significant increase in the G allele frequency among mothers of children with autism (James et al. 2010).

2.3 Antioxidant Genes Outside the Methionine and Glutathione Pathway

2.3.1 Paraoxigenase Gene

There is evidence for the possible involvement of antioxidant genes outside the methionine transmethylation/transsulfuration and glutathione pathways. *Paraoxonase 1 (PON1)*, which is associated with organophosphate hydrolysis and plays a role in protection against the oxidative modification of low-density lipoprotein, homocysteine-thiolactone, and bacterial endotoxins, has been implicated in

autism (D'Amelio et al. 2005; Herbert 2010; Pasca et al. 2010; Serajee et al. 2004). A significant association of PON1 with autism was demonstrated in Caucasian-American ($p<0.025$), but not Italian, families in which less organophosphate is used (D'Amelio et al. 2005). The genetic association was not significant in another cohort of Romanian children with autism spectrum disorders in which the bioavailability and the catalytic activity of PON1 were significantly impaired (Pasca et al. 2010).

2.3.2 Neuromodulator Genes

There are two different types of inducible enzymes activated by adverse events in the central nervous system. *Nitric oxide synthase IIA (NOS IIA)* is induced in the microglia and astroglia after perinatal hypoxia or intrauterine infection in the central nervous system (Shen et al. 2007). Nitric oxide (NO) is thought to play an important role in neuroinflammation and elevated NO production, and higher plasma NO levels were observed in children with autism as compared with the control children (Sogut et al. 2003; Sweeten et al. 2004). *Cyclooxygenase-2 (Cox-2 or prostaglandin-endoperoxide synthase 2, PTGS2)* is induced by growth factors, cytokines, and proinflammatory molecules. Elevated activities of Cox-2 isoforms at the cellular and subcellular levels in ischemia and neurodegenerative diseases strongly suggest that these enzymes might play important roles in inducing inflammation and oxidative stress associated with neurodegenerative processes (Phillis et al. 2006). A variety of chronic neurological conditions, such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, Creutzfeldt–Jakob disease, and seizure disorders, are associated with increased activities and high levels of Cox-2.

However, few genetic polymorphisms of Cox-2 and NOS IIA in autism have been reported. The results from association studies involving family trios (proband with both biological parents) from 151 Korean children with autism spectrum disorders revealed an SNP from the Cox-2 gene (rs2745557, $p<0.01$) and a haplotype that were significantly associated with ASDs ($p<0.01$) (Yoo et al. 2008). Another study using the same Korean cohort showed weaker but significant associations in 2 SNPs (rs8068149, $p=0.039$ and rs1060826, $p=0.035$) and 2 haplotypes ($p=0.014$ and 0.031, respectively) of NOS IIA gene in ASD (Kim et al. 2009). In both studies, statistically significant associations were demonstrated between each genotype and specific symptom domain scores using ADOS and ADI-R; *communication, qualitative abnormalities in reciprocal social interaction, and overactivity/agitation* were associated with the Cox-2 gene ($p=0.00$), and the *failure to use nonverbal behaviors to regulate social interaction* was associated with the NOS IIA gene (Kim et al. 2009; Yoo et al. 2008). Theoretically, functional polymorphisms in those genes could lead to varying degrees of Cox-2 and NOS IIA production, which could determine the susceptibility of an individual to environmental/endogenous risks for autism. These findings must be replicated using larger and ethnically diverse populations with more genetic markers and genetically informative endophenotypes in the future.

2.3.3 Rett's Syndrome, MeCP Gene, and Oxidative Stress

Rett's syndrome is one of the five conditions included in the category of pervasive developmental disorder and rare form of PDD that exclusively occurs in females. It is characterized by the deceleration of head growth between the ages of 5 and 48 months, the loss of previously acquired purposeful hand skills between the ages of 5 and 30 months with the subsequent development of stereotyped hand movements, the early loss of social engagement, the appearance of poorly coordinated gait or trunk movements, and severely impaired expressive and receptive language development with severe psychomotor retardation following a period of normal development (Percy 2011). In the great majority of cases, Rett's syndrome is an X-linked disorder caused by mutations in the *MeCP2 gene* (Percy 2011). Oxidative stress is suggested as a key modulator of disease expression in Rett's syndrome based on the observation that the MeCP2 mutations that were related to severe phenotypes exhibited higher oxidative stress marker levels than those of milder phenotypes; however, the association of the MeCP2 mutation with oxidative stress remains a major challenge for future research (Leoncini et al. 2011).

3 Single Gene Variation Versus Genetic Pathway

The advent of large protein–protein interaction maps, full genomic expression profiles, and large-scale computing resources, networks, and pathway analyses offers promise for exploring the interaction and connectivity between candidate genes involved in complex autism disorders. For example, recent reports suggest the possibility that the alleles of serpin peptidase inhibitor, clade E (SERPINE), plasminogen activator, urokinase receptor (PLAUR), receptor tyrosine kinase MET (MET), phosphatase and tensin homologue (PTEN), the tuberous sclerosis complex (TSC), fragile X mental retardation 1 (FMR1), and cytoplasmic FMR1 interacting protein 1 (CYFIP1) genes might contribute to autism via epistatic interactions in ASD (Bill and Geschwind 2009). Autism is not a single-gene disease, but rather might be derived from complex interactions between multiple genes; therefore, it would be more informative to obtain more precise information concerning the interactions among the genes involved. However, there has not been many gene–gene interaction studies published regarding autism due to difficulties in experimental methodology concerning the use of genome-wide association data or because of the lack of high-density information concerning certain pathways (Bowers et al. 2011).

While it is possible that the genes involved in the methionine transmethylation/transsulfuration and glutathione pathways might interact among each other, few studies have explored the interrelationship among these candidate genes in the development of autism. One study highlighted gene–gene interactions of MTHFR with borderline significance (OR = 1.78) in the susceptibility of autism, such as homozygous or heterozygous combinations of the RFC1 gene G s and the MTHFR 677T alleles (GA/CT, OR 3.2; GA/TT, OR 4.4; and GG/CT, OR 3.1), MTHFR

677CT/1298AC, and 80G allele of the RFC gene, which encode the enzyme for transporting methylfolate into cells. In addition, the GSTM1 null genotype showed a highly significant interaction with the reduced RFC-1 G allele, rendering a 3.78-fold increase in autism susceptibility in children with combined GSTM1 null and RFC1 heterozygous GA genotypes (James et al. 2006).

However, the comprehensiveness of the genes selected and the density of the markers analyzed limited the interpretation of the results from this study. A recent study reported the occurrence of more systematic multiple gene interactions within the glutathione pathway for 42 genes (308 SNPs) among 1,149 individuals from 318 family trios in the AGRE database (Bowers et al. 2011). This study utilized a carefully designed methodology, such as information-based gene selection, selecting tag SNPs to examine a large amount of genes and flanking regions, acquire standardized genetic data, and evaluate higher-order gene–gene interactions using the logic regression method. A single SNP analysis revealed a significant association ($p < 0.05$) in nine SNPs located in cystathionine gamma lyase (CTH), alcohol dehydrogenase 5 (ADH5), gamma-glutamylcysteine synthetase, catalytic subunit (GCLC), glutaredoxin, and glutaredoxin 3 (GLRX3) genes, which were not previously reported; two SNPs approached nominal statistical significance in independent AGRE samples (rs524553 and rs761141, both located in GCLC). Notably, a three-SNP joint effect was observed for the genotype combinations GLRX3 and CTH (OR = 3.78, 95 % CI: 2.36, 6.04).

Though the function of the associated genes should be validated in future studies, the results indicate that the gene–gene interaction approach might be a promising and more systematic way to explore the contributions of multiple genes to the risk of autism.

4 Functional Genetic Polymorphisms and Animal Model of Autism

Transgenic mouse models are important approaches in the study of human diseases, allowing for the use of a variety of experimental approaches to dissect the contribution of a specific chromosomal or genetic abnormality in human disorders (Robertson and Feng 2011), although a plausible animal model of autism has yet to be established. Most of the attempts at animal models of autism have been targeted to pre-existing monogenic developmental disorders showing autistic-like behaviors and transgenic mouse models that mimic point mutations or null mice for synaptic and signaling molecules (Robertson and Feng 2011). Animal knockout models for genes directly related to oxidative stress have rarely been developed; however, recently, the relationship between the GSTM1 null genotype and autistic behavior was examined using a rodent model based on the hypothesis that genetically altered mice might be more sensitive to toxic exposure early in life, followed by autistic regression (Yochum et al. 2010).

By relating those two ideas, Yochum et al. (2010) examined the sensitivity of the GSTM1 knockout mice to incurring the neural behavioral deficits induced by the postnatal administration of sodium valproate on day 14 (P14). VPA administration induces fetal valproate syndrome, which partially resembles autism due to oxidative stress (Na et al. 2003; Verrotti et al. 2008). Moreover, in this study, VPA treatment resulted in the increase of apoptosis in the hippocampus and cerebellum and social behaviors, such as crawl under and allogrooming, as compared with the saline-treated knockout animals and the wild-type controls, although the effect of the GSTM1 knockout on each social behavior was inconsistent as shown in Fig. 1. A significant genotype x VPA treatment x sex interaction was observed, against VPA-induced neuronal death in female mice (Yochum et al. 2010).

Cerebellar mutant and NOS knockout mice are other proposed models for autism, which are closely related to each other. Behaviorally, the NOS knockout mice showed a significant change in learning behavior through cerebellar long-term depression (Lev-Ram et al. 1997; Yochum et al. 2010). Based on shared cerebellar histopathology and genetic involvement in autism, including cerebellar hypoplasia,

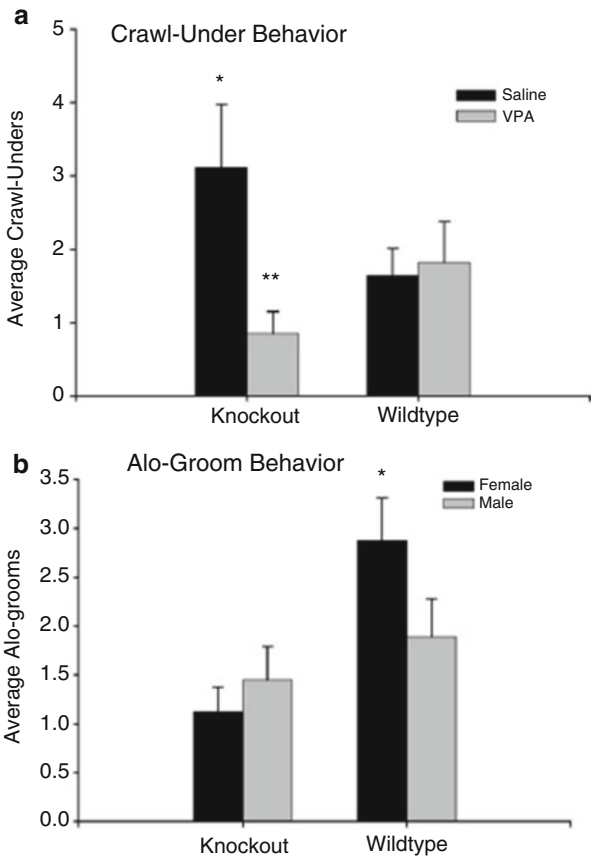

Fig. 1 (**a**) Number of crawl-under behaviors completed by genotype and treatment-matched pairs of GSTM1 wild-type and knockout mice over a 30 min open field trial (run between postnatal days 30–40) following P14 sodium valproate (VPA, 400 mg/kg, s.c.) or saline treatment. (**b**) Number of allogroom behaviors completed by sex and genotype-matched pairs of GSTM1 wild-type and knockout mice over a 30 min open field trial (run between postnatal days 30–40) following P14 sodium valproate (VPA, 400 mg/kg, s.c.) or saline treatment (Reprinted from Yochum et al. 2010, with permission from Elsevier)

Purkinje cell loss or reduced size, decreased blood reelin levels, and the participation of the reelin gene, the cerebellar mutant mice (reeler mice) were proposed as a useful model system to study autism (Lev-Ram et al. 1997; Yochum et al. 2010).

5 What Are the Mechanisms?: Epigenetic Mechanisms and DNA Methylation

Concerning the genetic etiology of autism, the linkage and association studies were rarely replicated, implying that a number of factors including gene–environment interactions and genetic heterogeneity are involved in development of autism, as indicated by the fact that many known genes and genomic regions associated with autism spectrum disorders account for less than 2 % of the cases (Abrahams and Geschwind 2008). Both genetic and environmental factors play causative roles, influencing fetal or early postnatal brain development, directly or via epigenetic modifications (Grafodatskaya et al. 2010). *Epigenetics* is defined as heritable changes that are independent of the genomic sequence, and these changes provide a mechanism for controlling the genome without involving the alteration of the genomic sequence. Epigenetic modifications normally annotate DNA and associated histone proteins and regulate the expression of many genes (Grafodatskaya et al. 2010). The developing mammalian brain is particularly sensitive to epigenetic alterations, and the etiology of a variety of neurodevelopmental disorders are attributed to this process (LaSalle 2011). The epigenetic modulation of gene expression lies at the interface between genes and environmental influences and could potentially provide a molecular explanation for the downregulation of gene expression in the autistic brain (James et al. 2010).

Epigenetics is especially useful to define the molecular mechanism that links environmental effects with gene function in complex diseases, such as schizophrenia, autism, and mental retardation (Zahir and Brown 2011). The interplay among oxidative stress and genetic factors in the pathogenesis of autism could be understood in the context of epigenetic dysregulation, especially in the DNA methylation process (cell-specific gene expression and differentiation). DNA methylation might inhibit gene expression through direct interactions with factors that repress transcription or through the recruitment of methyl-CpG-binding proteins (Grafodatskaya et al. 2010; LaSalle 2011). The functional polymorphisms in genes encoding key enzymes involved in the DNA methylation pathway might disrupt this pathway by decreasing the methyl donors or, conversely, by increasing the demands for more methyl donors (LaSalle 2011). The DNA hypomethylation observed in autism supports the involvement of the DNA methylation pathway in autism (James et al. 2008, 2010).

The roles of oxidative stress and related genetic variants in the development of autism could be explained with two different but biochemically linked processes with regard to DNA methylation dysregulation. The first process involves a decrease in methyl donors in the folate-dependent pathway (one-carbon methylation pathway), involving the remethylation of homocysteine to methionine. This pathway is highly polymorphic, and evidence for the involvement of common functional genetic

polymorphisms of SNPs of MTHFR and related genes in autism supports the hypothesis that alterations in this pathway result in methylation deficits that can cause abnormal brain development (Pasca et al. 2010). As this pathway is dependent on the dietary intake of folic acid and thus gene–nutrient interactions mediated by the dietary intake of folate and vitamin B, amino acid deficiencies and environmental exposure could potentially modify the expression of certain metabolic pathways.

The second process involves the more direct influence of oxidative stress and an increased demand for enhanced glutathione synthesis, which acts as an inhibitor of SAM. When exposed to oxidative stress, the need for glutathione is enhanced to conjugate toxins or as an antioxidant to activate the adaptive response (LaSalle 2011). In autism, if simplified, the functional genetic polymorphisms of the enzymes modulating this pathway, such as the GSTM1 null polymorphism, GST, and GPX, might alter the enzyme activity and inhibit the synthesis of glutathione, rendering genetically susceptible subjects more vulnerable to neuronal damage or developmental aberrations through oxidative stress, especially in the early stages of fetal development. Figure 2 shows a schematic overview of the an integrative genomic model of the major genetic and environmental pathways influencing the human DNA methylation.

In addition, methionine synthase (MS), which converts homocysteine to methionine, is responsive to the cellular oxidative status and is inhibited by oxidative stress (Deplancke and Gaskins 2002). This adaptive process initiates a cascade to increase cysteine availability for glutathione synthesis, while decreasing DNA methylation through a subsequent decrease of SAM as a methyl donor. Thus, the MS gene might be one of the plausible candidate genes related to oxidative stress and neurodevelopmental disorders connecting the two interdependent pathways described above; however, no significant association of MS gene polymorphisms with autism has been reported so far (Adams et al. 2007).

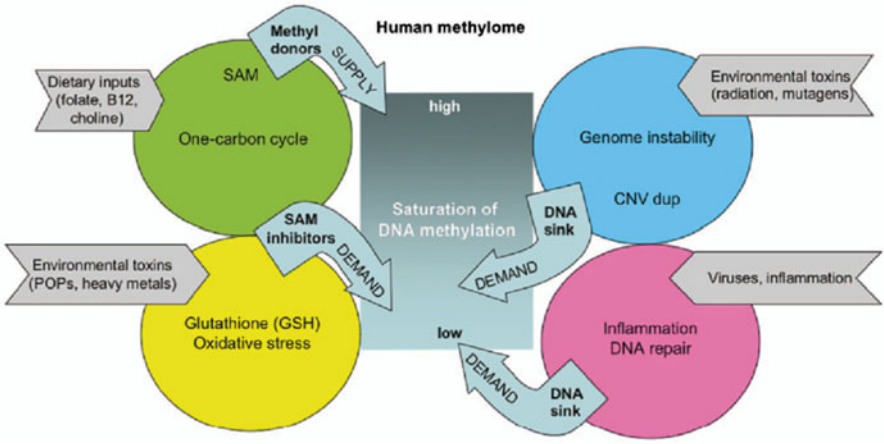

Fig. 2 An integrative genomic model of the major genetic and environmental pathways influencing the human methylome ([Used with permission from LaSalle 2011])

When methionine synthase activity is reduced by oxidative stress, the D4 receptor-mediated dopamine-stimulated phospholipid methylation (PLM) is reduced, as it is absolutely dependent on MS activity (Deth et al. 2008). Impairment in dopamine-dependent PLM limits the frequency-dependent synchronization of the neuronal network, which results in deficits in attention and cognition, which are important features of autistic psychopathology (Deth et al. 2008; Rommelse et al. 2011). As D4 receptor-mediated PLM is synergistic with SNPs affecting dopaminergic functions (e.g., COMT) and/or the neuronal substrates participating in synchronization (e.g., RELN, MET, or NGLN 3 & 4) (Deth et al. 2008) as shown in Fig. 3, all of which are potential candidate genes of autism, it might be presumed that oxidative stress initiates this complex genetic cascade in a variety of interrelated adaptive and developmental mechanisms in autism.

The precise links between oxidative stress and genetic mechanisms other than the DNA methylation process have yet to be explored, especially with regard to inducible neuromodulators, such as Cox-2 or NOS IIA.

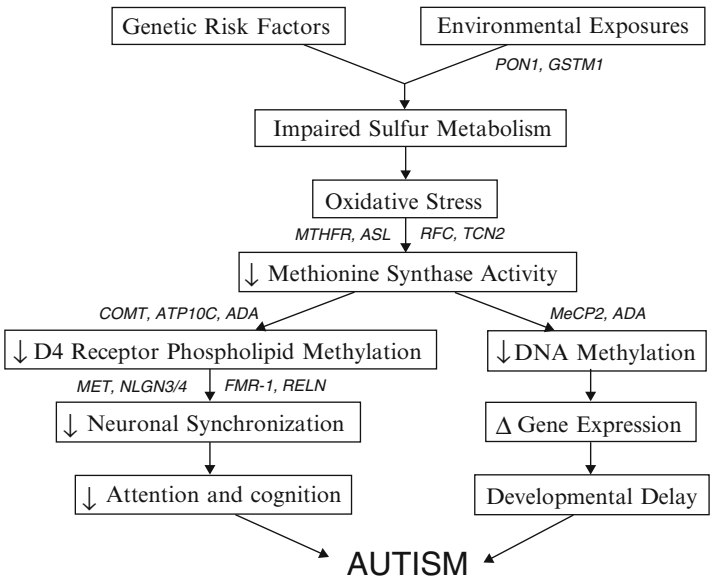

Fig. 3 A redox/methylation hypothesis of autism. Environmental factors (e.g., heavy metals and xenobiotics) can precipitate oxidative stress in a vulnerable subpopulation possessing risk genes (shown in italics), initiating multiple adaptive responses involving sulfur metabolism. Inhibition of methionine synthase broadly reduces methylation activity, with DNA methylation and dopamine-stimulated phospholipid methylation being important examples. Reduced DNA methylation interferes with epigenetic events that are fundamental to normal development. Impairment of dopamine-stimulated phospholipid methylation limits frequency-dependent synchronization of neuronal networks, reflected as deficits in attention and cognition. While all cell types are subject to similar effects, which may be manifested as autism-associated symptoms, neuronal cells exhibit higher sensitivity to oxidative stress (Reprinted from Deth et al. 2008, with permission from Elsevier)

6 Clinical Implications

Many different genes and genetic polymorphisms regarding oxidative stress and human adaptability have been associated with the development of autism. However, in the clinical realm, it is not simple to understand the complex interplay of specific events that cause oxidative stress and genetic susceptibility in the child and the mother. For example, fetal hypoxia, one of the possible causes of oxidative stress, is hardly measurable due to the retrospective nature of perinatal data collection in the proband once diagnosed with autism. Recently, few prospective large cohort studies were conducted to examine the perinatal and prenatal risk factors in autism, but it is difficult to include genetic susceptibility in the analysis (Williams and Marshall 2001).

The second limitation in clinical understanding is that the genes involved in the oxidative pathway are not only associated with autism and similar neurodevelopmental disorders but also with a variety of systemic illnesses, such as cancer, hepatotoxicity, and autoimmune and neurodegenerative disorders. The causative genes implicated in these diseases have similar characteristics as other candidate genes of autism; therefore, the elucidation of the exact roles of these genes in causing autistic psychopathology and differences in the pathophysiological mechanisms of other systemic disorders needs further study.

The treatment of autism is mainly focused on early behavioral intervention. Biological therapies directly involving genetic polymorphisms are not yet available for the treatment of autism. Based on research evidence concerning the abnormalities in methionine and folate metabolism, nutritional interventions, such as supplementation of folic or folinic acid, betaine, and vitamin B6 or B12, have been attempted, but the behavioral outcome after treatment was not fully satisfactory (Main et al. 2010). Even before the direct therapeutic modification of genetic function, the selective supplementation of a certain nutritional element might be attempted; however, the specific function of a gene, the interactions of multiple genes involved, and the specific relationship between environmental factors and genetic polymorphisms have to be further explored to determine the major target of therapeutic intervention.

Another future task is to explore the relationship between specific *endophenotypes* as quantitative traits of autism and genes involved in the oxidative pathway. More stringent and careful phenotyping, including neurocognitive tests and/or brain imaging modalities, and insight from animal knockout studies will help to uncover more powerful and specific phenotypes regarding oxidative stress and related genetic susceptibility.

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Telomere Length in Major Psychiatric Disorders: Is There Any Relationship Between Telomere Length and Oxidative Stress?

Dariusz Nowak

Abbreviations

ALT	Alternative lengthening of telomeres
$ApoE_2$	Apolipoprotein E_2
BD	Bipolar disorder
BD II	Bipolar disorder type II
BD I	Bipolar disorder type I
BMI	Body mass index
CHD	Coronary heart disease
COPD	Chronic obstructive pulmonary disease
DST	Dexamethasone
ECT	Electroconvulsive therapy
ELISA	Enzyme-linked immunosorbent assay
GST mu	Glutathione S-transferase
HAM-A	Hamilton anxiety score
HAM-D	Hamilton depression score
MAOA	Monoamine oxidase A
MD	Major depression
NYHA	New York Heart Association
PCR	Polymerase chain reaction
PCR-RFLP	PCR – restriction fragment length polymorphism

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PTSD	Posttraumatic stress disorder
ROS	Reactive oxygen species
STMN1	Encoding stathmin
SZ	Schizophrenia
TERT	Encoding the catalytic subunit of the telomerase
TL	Telomere length

1 Introduction

Telomeres are located at both ends of each chromosome and are composed of a repetitive DNA sequence (TTAGGG repeats) and associated proteins (shelterin – protein complex). They have a very important vital function for cell biology by preserving the information in their genome. In particular, this consists in protection of the genome from nucleolytic degradation, unnecessary recombination, and interchromosomal fusion (assurance of proper positioning of chromosomes during replication), as well as repair (protection of the chromosome end from being recognized as DNA double-strand breaks by the DNA damage response mechanisms).

During each cell division, a small segment of telomeric DNA is lost leading to the reduction of telomere length (TL). Although this is a normal physiological process, it can result in cell senescence and/or apoptosis when TL reaches a critical limit (Donate and Blasco 2011; Shammas 2011; Martínez and Blasco 2010).

TL can be restored by the enzyme telomerase (ribonucleoprotein reverse transcriptase); however, the majority of cells and tissues (except of stem cells, germ cells, and regenerating tissues) has very low telomerase activities. TL could be maintained by the other alternative mechanism (ALT – alternative lengthening of telomeres) where telomeres use other telomeric DNA as a template for DNA synthesis (Neumann et al. 2013; Muntoni et al. 2009). However, these mechanisms are not efficient at maintaining TL, and consequently telomere erosion occurs with age in numerous somatic tissues. Thus, TL or its shortening could be recognized as a predictive factor of the life span of cells and organisms and the age-related reduction of body's ability to regenerate in response to action of damaging factors (Donate and Blasco 2011; Shammas 2011) and even individual death (Cawthon et al. 2003).

Numerous factors associated with lifestyle, nutrition, and coexistent diseases can predispose to accelerate TL shortening and be responsible for negative effect on individual subject life span and state of health. Among them are lack of exercise, obesity, cigarette smoking, alcohol abuse, exposure to air pollutants, cardiovascular diseases, and diabetes, and these issues have recently been largely reviewed and discussed elsewhere (Donate and Blasco 2011; Paul 2011; Price et al. 2013). On the other hand, plasma levels of vitamin A and D and habitual tea drinking (ingestion of tea polyphenols) were positively associated with TL (Paul 2011). Since venous blood is more easily accessible than most other tissues, majority of these data were derived from cross-sectional studies focused on TL of peripheral blood leukocytes.

Chronic oxidative stress and inflammation are implicated in speeding up the aging process. Numerous studies with cell cultures and animal models proved faster

TL shortening under conditions of oxidative stress. Due to its high content of guanines and inefficient repair of single-strand breaks, telomeric DNA is highly sensitive to damage by reactive oxygen species (Petersen et al. 1998; Oikawa et al. 2001; Oikawa and Kawanishi 1999). Moreover, increased levels of pro-inflammatory cytokines, especially tumor necrosis factor alpha (TNF-α), that are frequently accompanied with oxidative stress can decrease the telomerase activity (Beyne-Rauzy et al. 2004, 2005) and oxidative damage to the bases of telomeric DNA accumulated over the life span of cells (Kawanishi and Oikawa 2004). Therefore, TL could be a biomarker of chronic oxidative stress (Houben et al. 2008).

Systemic and cerebral oxidative stress has been demonstrated in the major psychiatric disorders like schizophrenia (SZ), major depression (MD), and bipolar disorder (BD). Although these issues are discussed elsewhere in this book, it is necessary to mention that patients suffering from SZ, MD, and BD had elevated circulating levels of products deriving from oxidative damage to lipids, proteins, and DNA and decreased plasma antioxidant defense (Kim and Andreazza 2012; Ng et al. 2008; Maes et al. 2011; Yao and Reddy 2011; Bitanihirwe and Woo 2011), and analysis of postmortem obtained brain specimens revealed increased content of oxidative stress markers (Gawryluk et al. 2011a; Michel et al. 2011a; Che et al. 2010; Andreazza et al. 2010; Wang et al. 2009).

Moreover, these groups of patients are prone to diseases associated with aging (e.g., diabetes, cardiovascular diseases) and reveal tendency to have shorter natural life span than the general population. Therefore, researches executed a dozen or so clinical studies (in the vast majority cross-sectional) on blood leukocyte TL in these major psychiatric disorders during the last few years. This chapter will review the results of these studies with special attention to intensity of systemic and brain oxidative stress.

2 Telomere Length in Blood Leukocytes and Brain Samples of Patients with Schizophrenia

Table 1 summarizes the results of cross-sectional studies on TL in patients with SZ. These studies mostly compared TL in DNA isolated from blood leukocytes between SZ patients and matched controls (Kao et al. 2008; Yu et al. 2008; Fernandez-Egea et al. 2009; Mansour et al. 2011). In one of them the additional group of patients with type I bipolar disorder (BD I) was investigated (Mansour et al. 2011). Only one report was based on postmortem brain samples (gray matter of cerebellum) obtained from patients suffering from SZ, BD, MD, and controls where TL was analyzed by quantitative PCR (Zhang et al. 2010). Concerning the TL in blood leukocytes, two reports revealed telomere shortening in SZ subjects (Kao et al. 2008; Fernandez-Egea et al. 2009). One study found this difference limited to SZ patients who were poor responders to the treatment versus healthy matched controls. However, good responders did not differ from controls (Yu et al. 2008).

On the other hand, Mansour et al. on the basis of investigation of relatively large group of subjects (Table 1) did not find any significant differences in the leukocyte TL between SZ group, BD I patients, and controls (Mansour et al. 2011).

Table 1 Cross-sectional studies on telomere length in patients with schizophrenia

Aim of the study	Study description	Main results and [ref]
Do SZ patients have shorter telomeres than unaffected controls?	51 SZ patients, 24 unaffected family members, and 52 unaffected unrelated subjects. TL determined with quantitative PCR in DNA isolated from blood lymphocytes. Analyses were adjusted for age and sex	Patients with SZ had significantly shorter mean TL than controls. Current antipsychotic dose and estimated lifetime antipsychotic dose did not correlate with TL (Kao et al. 2008)
Do SZ patients have shorter TL than controls? Does response to the treatment affect TL in SZ group?	68 SZ patients (34 good responders and 34 poor responders to the treatment) and 76 age-matched healthy controls. DNA isolated from blood and TL measured with Southern blot. Analyses were adjusted for age and sex	Poor responders had shorter TL than good responders and healthy controls. Good responders did not differ from controls (Yu et al. 2008)
Do patients with SZ and other related disorders have shortened TL and increased pulse pressure?	41 subjects with nonaffective psychosis (27 with SZ, 9 schizophreniform disorder, 2 brief psychotic disorder, 2 delusional disorder, 1 psychosis not otherwise specified), 41 demographics-, smoking-, BMI- and resting heart rate-matched control subjects. DNA telomere content (that is highly correlated with TL) was determined in blood leukocytes (DNA hybridization with end-labeled telomere-specific oligonucleotide)	The psychosis group had significantly decreased mean telomere content versus controls. Men and women with nonaffective psychosis had similar telomere content. Pulse pressure was significantly higher in psychosis group than in controls (Fernandez-Egea et al. 2009)
Do SZ and BD I patients have shortened TL? Is TL a mediating factor between inbreeding and increased risk for BD I and SZ?	60 SZ patients, 108 subjects with BD I, and 168 controls. TL determined with quantitative PCR in DNA extracted from blood. The inbreeding coefficient/consanguinity rate estimated from family history data and after genotyping Short Tandem Repeat Polymorphisms among cases and controls. Analyses were adjusted for age and sex	BD I versus controls ($n=114$) and SZ versus controls ($n=60$) – no significant difference in TL. No significant associations between TL and consanguinity estimated with the two methods (Mansour et al. 2011)
Is TL altered in brains of patients with major psychiatric disorders (SZ, BD, and MD)?	DNA extracted from postmortem brain samples (gray matter of cerebellum) of 46 SZ patients, 46 BD, and 15 MD patients and 48 controls for TL determination with quantitative PCR	No difference of TL in the gray matter of cerebellum was noted in SZ, BD, and MD group compared to controls. No differences between psychiatric disorders groups were also found. Age, gender, medication, and drug used had no effect on TL (Zhang et al. 2010)

BD bipolar disorder, *BD I* bipolar disorder type I, *BMI* body mass index, *MD* major depression, *PCR* polymerase chain reaction, *SZ* schizophrenia, *TL* telomere length

In agreement with this report, no difference of TL in the gray matter of cerebellum was noted in SZ, BD, and MD patients compared to controls. Similarly, no differences in brain TL were found between these three groups of psychiatric disorders (Zhang et al. 2010).

In conclusion, studies on the TL in blood leukocytes of SZ patients are not fully conclusive. More studies involving larger patients groups and matched controls are required. Longitudinal studies on telomere shortening in the course of SZ along with the effect of antipsychotic treatment are necessary.

2.1 Discordance Between Telomere Length and Oxidative Stress in Brain Tissue of Patients with Schizophrenia and Other Major Psychiatric Disorders (Bipolar Disorder, Major Depression)

The aforementioned results on TL in brain samples are more than somewhat surprising since numerous studies proved occurrence of oxidative stress in some regions of brain tissue (postmortem samples) in patients with SZ, BD, and MD:

(a) Samples of prefrontal cortex from patients with SZ and BD revealed downregulation of uncoupling protein 2 (involved in controlling the mitochondrial production of ROS) mRNA levels (Gigante et al. 2011).
(b) Samples of prefrontal cortex of patients with SZ, MD, and BD had decreased concentrations of reduced, oxidized, and total glutathione (Gawryluk et al. 2011a). Moreover, the levels of glutathione peroxidase and the mu isoenzyme of glutathione S-transferase (GST mu) were decreased in MD and SZ brain samples evaluated with immunoblotting technique (Gawryluk et al. 2011a, b).
(c) There was an increased activity of prooxidant enzyme xanthine oxidase in the brain tissue (samples of thalamus and putamen) of patients with MD (Michel et al. 2011a). However, decreased activity was noted in SZ brain specimens (Michel et al. 2011b).
(d) Samples of hippocampus of patients with SZ, BD, and MD had increased content of 8-hydroxy-guanosine, a marker of RNA oxidative damage (Che et al. 2010).
(e) Samples of prefrontal cortex of BD patients had increased levels of oxidized proteins (carbonylated proteins) and 3-nitrotyrozine. Elevated 3-nitrotyrosine was also observed in SZ brain tissue (Andreazza et al. 2010).
(f) Anterior cingulate brain sections from BD and SZ subjects (but not from MD patients) had elevated content of 4-hydroxynonenal, a major product of lipid peroxidation (Wang et al. 2009).

Since oxidative stress predisposes to increased telomere erosion, one may expect the shortening of TL in brain specimens of patients with SZ and other major psychiatric disorders (MD, BD). However, brain specimens from patients with SZ, BD, and MD did not reveal telomere shortening in comparison to control samples (Zhang et al. 2010). Similar results (no significant difference in TL) were noted by other studies with postmortem brain specimens (dorsolateral prefrontal cortex) obtained from depressive patients and matched controls (Teyssier et al. 2011). Moreover, there was no difference in expression of genes involved in the oxidative-stress response and repair between brain samples from both studied groups (Teyssier et al. 2011) (Table 3). These findings suggest that telomere erosion did not occur in brain cortex despite the presence of distinct features of brain oxidative stress.

On the other hand, it cannot be excluded that telomere damage and oxidative stress occur in the brain; simply these phenomena could be limited to other brain regions than those studied so far.

Therefore, further studies analyzing additional brain regions in respect of telomere dysfunction, DNA damage, and intensity of oxidative stress in the course of SZ as well as other major psychiatric disorders are necessary.

3 Telomere Length in Blood Leukocytes and Brain Samples of Patients with Major Depression and Bipolar Disorder

Much more cross-sectional studies have been executed on the telomere shortening in MD and BD patients. These involved larger patients groups and were not only focused on comparison of the TL in DNA extracted from blood leukocytes between patients with mood disorders and matched controls (Tables 2 and 3) but also looked for an association between TL and various clinical variables such us disease severity and duration, treatment effect, intensity of oxidative stress, inflammatory response and perceived stress, and the monoamine oxidase A (MAOA) promoter and apolipoprotein E_2 ($ApoE_2$) polymorphism (Lung et al. 2007; Hartmann et al. 2010; Wikgren et al. 2012; Wolkowitz et al. 2011). Out of 7 reports that compared the leukocyte TL in patients with mood disorders (MD and BD) and matched controls, two described telomere shortening in BD patients (Simon et al. 2006; Elvsashagen et al. 2011) and six in MD patients (Lung et al. 2007; Hartmann et al. 2010; Wikgren et al. 2012; Simon et al. 2006; Garcia-Rizo et al. 2012), respectively. Only one study did not report significant shortening of TL in patients with MD (Wolkowitz et al. 2011). However, patient subgroups with cumulative duration of depression $\geq$9.2 years had significantly shorter telomeres than the control subjects (Wolkowitz et al. 2011). TL was inversely correlated with the ratio of plasma F2-isoprostanes (biomarkers of lipid peroxidation) to vitamin C and positively with the circulating vitamin C levels in MD patients (Wolkowitz et al. 2011). TL was also inversely associated with the stress measured with the self-report questionnaire (Wikgren et al. 2012).

However, TL in MD did not correlate with MAOA promoter and $ApoE_2$ polymorphism (Lung et al. 2007) and neither with disease severity, duration of illness, and number of hospital stays (Hartmann et al. 2010) nor with lymphocyte count (Garcia-Rizo et al. 2012). On the other hand, the load of short telomeres in DNA isolated from the blood mononuclear cells positively correlated with the high number of previous depressive episodes in bipolar disorder type II (BD II) patients (Elvsashagen et al. 2011).

These results obtained with well-defined MD and BD patients groups clearly show the occurrence of some processes that are responsible for the telomere shortening in blood DNA of these patients.

Oxidative stress could be responsible for enhanced telomere erosion in the course of MD. However, only one study was devoted to the analysis of the association

Table 2 Cross-sectional studies on telomere length in patients with major depression and bipolar disorder – part I

Aim of the study	Study description	Main results and [ref]
Effect of MAOA promoter and $ApoE_2$ polymorphisms on TL in MD patients	253 unrelated patients with MD, 411 controls with similar age and sex distribution. Southern blot for TL determination in DNA isolated from blood, PCR-RFLP for $ApoE_2$ genotypes, PCR with specific intronic oligonucleotide primers for MAOA promoter polymorphisms. Multiple linear and hierarchical regression analyses, structural equation model	The TL of patients with MD was shorter than that of the controls. No interaction between the MAOA promoter polymorphism, $ApoE_2$ polymorphism, and TL in MD (Lung et al. 2007)
Do MD or BD patients have shorter telomeres than age-matched healthy subjects?	44 patients with chronic mood disorders (15 MD, 14 BD no anxiety, 15 BD plus anxiety), 44 controls with similar age and sex distribution. Southern blot for TL determination in DNA extracted from leukocytes. Linear multiple regression analyses with adjustment for age, gender, and smoking	TL was significantly shorter in the whole group of mood disorders than in the controls. TL did not differ between the three mood disorder subgroups (Simon et al. 2006)
Do disease severity and treatment affect TL in MD patients?	54 patients with MD (20 patients low-dosed, 16 high-dosed, 18 patients additionally treated with ECT) and 20 healthy age-matched controls. HAM-D for evaluation of the disease severity. Southern blot for TL determination in DNA isolated from blood	The mean TL was significantly shorter in MD group. Each subgroup had similar TL but shorter than that of controls. No differences were between smokers and nonsmokers and males and females in MD group
		There was no significant association between TL and the disease severity, duration of illness, and number of hospital stays (Hartmann et al. 2010)
Evaluation of the load of short telomeres and mean TL and their relationships with illness duration and lifetime number of depressive episodes in BD II patients	28 BD II patients and 28 age-, sex-, and education-matched healthy controls. High-throughput quantitative fluorescence in situ hybridization for measurement of short telomeres (percentage of telomeres <3 kb) and mean TL in isolated peripheral blood mononuclear cells. Multiple regression analyses with adjustment for age, body mass index, and smoking	The load of short telomeres was higher in BD II patients than in controls (15.04 % versus 13.48 %, $p=0.04$). Mean TL did not differ significantly between groups. There was a strong association between the load of short telomeres and a high number of previous depressive episodes (Elvsashagen et al. 2011)
Is there any relationship between TL and biological and psychological facets of stress in MD patients and controls?	91 patients with recurrent MD (aged 21–87 years), 451 controls (aged 25–81 years) without dementia, mental retardation, and severe psychiatric disorders. Quantitative PCR for TL determination in DNA from leukocytes. Four self-report questionnaires for assessment of symptoms of depression, anxiety, and perceived stress. Weight-adjusted very-low-dose DST suppression test for assessment biological stress. Multiple linear regression models with adjustment for confounders	TL was shorter in MD patients versus controls. Short TL was associated with a hypocortisolemic state (low post-DST cortisol and high percentage of cortisol reduction after the DST) in both groups. TL was also inversely associated with stress measured with questionnaire (Wikgren et al. 2012)

$ApoE_2$ apolipoprotein E_2, *BD* bipolar disorder, *BD II* bipolar disorder type II, *DST* dexamethasone, *ECT* electroconvulsive therapy, *HAM-D* Hamilton depression score, *MAOA* monoamine oxidase A, *MD* major depression, *PCR* polymerase chain reaction, *PCR-RFLP – PCR* restriction fragment length polymorphism, *TL* telomere length

Table 3 Cross-sectional studies on telomere length in patients with major depression and bipolar disorder – part II

Aim of the study	Study description	Main results and [ref]
Do MD patients have shorter leukocyte TL than age-matched healthy subjects? Is there association between telomere shortening and lifetime depression exposure, intensity of oxidative stress, and inflammation?	18 MD patients, 18 age-, sex- and ethnicity-matched controls. Both groups were free of any medications, acute illnesses, and infections. HAM-D for evaluation of the disease severity. Quantitative PCR for TL determination in DNA extracted from blood. Measurement of circulating vitamin C, F2-isoprostenes, and IL-6. Multiple regression analyses with adjustment for age, sex, body mass index, and smoking	TL did not differ in MD subjects compared to the controls. MD patients ($n=10$) with cumulative duration of depression ≥9.2 years had significantly shorter telomeres than control group. TL inversely correlated with F2-isoprostanes/vitamin C ratio and IL-6 in MD group. Vitamin C positively correlated with TL in MD and control groups (Wolkowitz et al. 2011)
Do depressive patients have shorter TL and increased expression level of nine major genes of the stress response and repair systems in occipital and dorsolateral prefrontal cortex of the brain, respectively?	Total RNA extracted from postmortem dorsolateral prefrontal cortex and DNA from occipital cortex of 24 depressive subjects (13 with MD, 11 with depression associated with psychotic characteristics) and 12 sex-, age-, ethnicity-, and mean brain pH-matched 12 control subjects with no psychiatric disorder. Quantitative PCR for TL, combination of reverse transcription with quantitative PCR for expression (level of transcripts) of genes of superoxide dismutase 1 and 2, catalase, glutathione peroxidase1, 8-oxoguanine DNA glycosylase, nei-like1, methionine sulfoxide reductase A, telomere repeat-binding factor 2 and C-FOS	TL and expression of analyzed genes did not differ between the whole group of depressive subjects and MD subgroup and controls (Teyssier et al. 2011)
Do antidepressant-naïve patients with MD have shorter TL than healthy controls?	15 newly diagnosed, antidepressant-naïve MD patients, 70 matched healthy control subjects. DNA telomere content (that is highly correlated with TL) was determined in blood leukocytes (DNA hybridization with end-labeled telomere-specific oligonucleotide). Two-hour oral glucose tolerance test, blood cell count	MD group had a significantly decreased telomere content, lower lymphocyte count, and greater 2-h glucose concentration, compared with control subjects Telomere content did not correlate with lymphocyte count (Garcia-Rizo et al. 2012)
Is there association between depression and leukocyte TL in a population-based study?	Cohort of 2,225 subjects with depressive current symptoms evaluated with the Center for Epidemiological Studies Depression scale. TL measured with real-time PCR in leukocyte DNA extracted from frozen buffy coat samples. Statistical analysis adjusted for age, sex, body mass index, systolic and diastolic blood pressure, and Framingham risk score	Depressive symptoms, elevated depressive symptoms, and probable depressive disorder were each associated with longer leukocyte TL in unadjusted linear regression models. In all adjusted models depressive symptoms were not significantly associated with TL (Shaffer et al. 2012)
Assessment of the association between TL and psychological well-being in patients with chronic heart failure	890 patients with chronic heart failure (NYHA class II–IV). TL determined with real-time PCR in leukocyte DNA. Psychological well-being measured by set of questionnaires: the RAND-36 (perceived mental health), the Center for Epidemiologic Studies Depression scale (depressive symptoms), and the DS14 (type D personality)	Lower perceived mental health was associated with shorter TL. TL was not associated with depressive symptoms and presence of type D personality. Adjustment for age, sex, the severity of heart failure (NYHA class, left ventricular ejection fraction, estimated glomerular filtration rate), presence of COPD, diabetes, and history of stroke did not change these results (Huzen et al. 2010)

COPD chronic obstructive pulmonary disease, *HAM-D* Hamilton depression score, *MD* major depression, *NYHA* New York Heart Association, *PCR* polymerase chain reaction, *TL* telomere length

between TL and markers of oxidative stress in MD (Wolkowitz et al. 2011). Although this study concluded to a positive correlation between telomere erosion and intensity of oxidative stress, the sample size ($n = 18$) was too low to solve this issue conclusively (Table 3).

Surprisingly, two large cross-sectional studies did not confirm negative effect of depression on TL (Shaffer et al. 2012; Huzen et al. 2010). In a population-based survey that involved 2,225 apparently healthy participants, no association between the leukocyte TL and depressive symptoms as well as TL and probable depressive disorder was found (Shaffer et al. 2012). Similarly, analysis of TL and psychological well-being in 890 patients with chronic heart failure did not reveal any significant association with the depressive symptoms (Huzen et al. 2010). However, no subject had syndromal MD in these studies. Therefore, the intensity and cumulative duration of depressive symptoms could be too low to exert negative effect on the leukocyte TL.

Only one study was executed with postmortem brain samples (dorsolateral prefrontal cortex) of MD patients and matched controls. Neither TL nor the expression of genes involved in the antioxidant defense and repair differed between MD group and controls (Teyssier et al. 2011) (Table 3). These results are analogous to those obtained with postmortem brain samples of patients with SZ discussed in previous subsection.

4 Prospective Studies on Telomere Length in Patients with Mood Disorders

Scanty data exist on association between the TL and the further development of mood disorders and on effect of the current mood disorders on the TL shortening over subsequent time. Moreover, studies reporting these associations involved observation of subjects that did not suffer from MD (and other mood disorders) as the underlying disease (Table 4).

In one study patients suffering from coronary heart disease (CHD) were screened for the presence of coexisting MD, and the TL in their blood leukocytes was measured at baseline and after 5 years (Hoen et al. 2011). Although at baseline CHD patients with current MD had shorter TL than those without MD, the MD group did not reveal a higher rate of telomere shortening over 5-year follow-up. These facts suggest that presence of MD cannot be used as a predictive factor of telomere shortening in patients with CHD (Hoen et al. 2011). As underlined by the authors, this study had some limitations, and the two most important, in my opinion, are the following: groups may differ in the CHD severity, and leukocyte telomerase activity may affect telomere shortening and be responsible for negative results of this study (Hoen et al. 2011). Therefore, it is open to question whether the coexistent MD can accelerate the telomere erosion in CHD patients.

A second study on association between the rate of telomere shortening and the poor mental well-being and poor self-rated health in community-dwelling elderly men also revealed negative results (Rius-Ottenheim et al. 2012) (Table 4).

Table 4 Prospective studies on association between telomere length and mood disorders

Aim of the study	Study description	Main results and [ref]
Association between TL and depression in patients with CHD over a 5-year period	TL measured with quantitative PCR in DNA isolated from blood leukocytes in 952 patients with CHD at baseline and 608 of them after 5-year follow-up. Computerized Diagnostic Interview Schedule used for assessment of presence of MD in CHD patients at baseline. Statistical analyses adjusted various sociodemographic variables	CHD patients with current MD had shorter TL than those without depression at baseline. Current MD did not predict subsequent telomere shortening in CHD patients over 5-year follow-up (Hoen et al. 2011)
Whether accelerated telomere shortening is associated with poor mental well-being and poor self-rated health in community-dwelling elderly men	203 men (mean age 78 years) from Netherlands, 123 men (mean age 84 years) from Greece. Depressive symptoms, dispositional optimism, global cognitive function, feelings of loneliness assessed with battery of tests, and questionnaires. TL measured with quantitative PCR in leukocyte DNA extracted from buffy coat samples. Seven-year follow-up with 75 Dutch subjects. Multivariate models for adjustment for potential confounders (sociodemographic, lifestyle, morbidity parameters)	Leukocyte TL was not associated with measures of mental well-being and self-rated health, neither in the Dutch nor in Greek participants. The rate of leukocyte telomere shortening over 7-year follow-up was not associated with changes in different measures of mental well-being and self-rated health (Rius-Ottenheim et al. 2012)
Is shorter TL a predisposing factor to the development of trauma-related MD and PTSD in rape victims?	64 female rape survivors assessed within 2 weeks from the rape incident (baseline) and after 3 months (follow-up) for resilience or the development of trauma-related MD and PTSD with set of questionnaires and scales. TL measured with quantitative PCR in DNA extracted from blood	No significant association was observed between TL and resilience and the development of MD at both baseline and after 3 months. There was a significant association between TL and PTSD. Victims with PTSD had significantly shorter TL than those without PTSD (Malan et al. 2011)
	Analysis for effect of possible confounding factors (age, ethnicity, and the level of education)	

CHD coronary heart disease, *PCR* polymerase chain reaction, *MD* major depression, *PTSD* posttraumatic stress disorder, *TL* telomere length

However, a third study presented partially positive results. This study involved a group of female rape survivors investigated within 2 weeks from the rape incident and after 3-month follow-up. TL in blood DNA measured at baseline was associated with the development of posttraumatic stress disorder. Victims presenting with posttraumatic stress disorder had shorter TL than those free of this abnormality. On the other hand, TL did not associate with the presence of trauma-induced MD either at baseline or after 3-month follow-up (Malan et al. 2011).

Description of these studies and their results clearly shows that there is a great need of prospective longitudinal studies involving patients suffering from MD, SZ, or BD and matched controls to evaluate the effect of major psychiatric disorders on the rate of telomere shortening.

5 Telomerase Activity and Expression of Genes Involved in DNA Repair in Patients with Schizophrenia and Major Depression

Since the TL in blood leukocytes of patients with SZ and MD was shorter in comparison to the matched healthy controls, one may assume that this may be related to decreased telomerase activity in these cells. Moreover, it cannot be excluded that the rise in telomerase activity may counteract this negative process, perhaps, related to the systemic oxidative stress.

This was investigated in the group of patients with MD and SZ and resulted in opposite outcomes (Table 5). Telomerase activity in subjects with SZ did not differ significantly from that found in the group of their unaffected relatives (Porton et al. 2008) and even was lower when compared to the reference group composed of unaffected relatives and unrelated controls (Porton et al. 2008). This suggests the reduction of telomerase activity in blood lymphocytes in SZ. On the other hand, no correlation between the TL and telomerase activity in lymphocytes of SZ patients was noted (Porton et al. 2008). Similarly, TL did not correlate with the telomerase activity in other immune cells (T lymphocytes, blood mononuclear cells) isolated from healthy subjects (Pan et al. 1997; Iwama et al. 1998). Therefore, it seems that suppression of telomerase activity cannot be a culprit of decreased TL in leukocytes of SZ patients.

In contrast to these results, medication-free MD patients had elevated telomerase activity in blood mononuclear cells in comparison to the unaffected controls (Wolkowitz et al. 2012). No significant correlation was noted between the TL and telomerase activity in these patients likewise to SZ group. Moreover, telomerase activity in mononuclear cells did not correlate with various markers of oxidative stress (plasma concentrations of F2-isoprostanes, 8-hydroxydeoxyguanosine, and vitamin C) and inflammation (interleukin-6 and C-reactive protein levels) both in MD subjects and controls (Wolkowitz et al. 2012). Therefore, it is difficult to judge whether the rise of telomerase activity is a defensive mechanism against the oxidative stress observed in the course of MD.

Another study that involved female MD patients and matched controls studied the expression of the set of gene encoding products that are implicated in and being markers of processes of the telomere dysfunction and repair (STMN1, encoding stathmin; TERT, encoding the catalytic subunit of the telomerase), the aging and senescence ($p16^{ink4a}$ encoded by the CDKN2A locus), the oxidative stress and DNA repair (OGG1 – encoding 8-oxoguanine-DNA glycosylase1), the response to anxiety and psychogenic stress (FOS gene, DUSP-1 gene), and the inflammatory response (Il-6 gene) in blood leukocytes. Although TL did not differ between MD women and controls, there was significant overexpression of OGG1, P16ink4a, and STMN1 genes in the MD group. These results suggest the occurrence of DNA damage and the telomere dysfunction probably due to oxidative stress in leukocytes of female MD patients (51).

While the results seem interesting, the major limitation of these studies is the low number of analyzed patients. Therefore, they are not conclusive and require confirmation in further studies involving larger groups of patients.

Table 5 Studies on telomerase activity and expression of genes involved in telomere dysfunction, DNA repair, and cell senescence in patients with schizophrenia and major depression

Aim of the study	Study description	Main results and [ref]
Comparison of telomerase activity in lymphocytes between patients with SZ and unaffected relatives and unrelated controls	53 patients with SZ, 31 their unaffected first-degree family members, 59 unrelated controls. Telomerase activity measured with real-time PCR-based assay in lymphocytes isolated from peripheral blood	No significant difference in telomerase activity between patients with SZ and their unaffected relatives. Patients with SZ had decreased telomerase activity when compared to all unaffected individuals (control + family) (Porton et al. 2008)
Comparison of telomerase activity in blood mononuclear cells between MD patients and unaffected controls	20 medication-free patients with MD and 18 controls. Plasma oxidative stress and inflammation markers (F2-isoprostanes, 8-hydroxydeoxyguanosine, ascorbic acid, interleukin-6, C-reactive protein) at baseline. Telomerase activity measured with combination of PCR and ELISA (commercial kit) in peripheral blood mononuclear cells at baseline and in 15 MD patients after 8-week treatment (open-label) with sertraline. The HAM-D for assessment of pre- and posttreatment symptom severity Analyses were corrected for age and sex	Baseline telomerase activity was significantly higher in MD patients than in controls. Antidepressant treatment did not affect mean telomerase activity. MD patients with lower pretreatment telomerase activity and with greater increase in telomerase activity during treatment showed superior antidepressant responses. Telomerase activity did not correlate with oxidative stress and inflammation markers in both groups (Wolkowitz et al. 2012)
Comparison of expression of genes implicated in telomere dysfunction, DNA repair, and biological aging between female patients with MD and matched controls	17 female MD patients and 16 control women matched for age, BMI, physical activity, and alcohol consumption. Real-time quantitative PCR for gene expression (p16INK4a, STMN1, OGG1, TERT, FOS, DUSP1, IL-6) and TL in blood leukocytes. HAM-D and HAM-A for assessment of the disease severity. Analyses adjusted for sociodemographic variables	Three genes (OGG1, P16ink4a, STMN1) were significantly overexpressed in MD patients. Expression of p16INK4a and STMN1 correlated with anxiety scores in the MD group. Mean TL did not differ between groups (Teyssier et al. 2012)

BMI body mass index, *ELISA* enzyme-linked immunosorbent assay, *HAM-A* Hamilton anxiety score, *HAM-D* Hamilton depression score, *MD* major depression, *PCR* polymerase chain reaction, *SZ* schizophrenia, *TL* telomere length

6 Concluding Remarks

Patients with MD and BD revealed shortened TL in blood leukocytes as evaluated in cross-sectional studies. Results obtained with the groups of SZ patients are not fully conclusive but also suggest telomere shortening in this disease. Numerous dietary (intake of plant polyphenols and vitamins), demographic, socioeconomic factors, and pathological conditions can affect the rate of telomere erosion in

leukocytes. Therefore, it is very difficult to select homogeneous patient group (with respect to comorbidities and lifestyle) and precisely matched controls. Although the statistical analyses of these studies included the adjustment for some confounding factors, it is not easy to completely eliminate the risk of bias especially when the size of patient group is low.

In the case of blood leukocytes, measurement of the TL reflects their replicative history.

Thus, any factor (e.g., infection, inflammation) that can enhance these cells' turnover will lead to the TL shortening and induce bias. This implicates that apart from current confounding factors, also past confounding factors can affect the results of the TL determination in blood leukocytes.

In light of this, previous reports showing prenatal exposure to influenza virus as a risk factor for adult SZ (Limosin et al. 2003; Izumoto et al. 1999), and the positive association of psychological stress with the number of upper respiratory tract infections in the subjects with chronic fatigue syndrome (Faulkner and Smith 2008), seem interesting.

It should be pointed out that early life stress (childhood adversity) is negatively associated with the TL in adult life (Price et al. 2013; Kiecolt-Glaser et al. 2011; Kananen et al. 2010), which also complicates interpretation of the results of cross-sectional studies.

There are no conclusive data on the association between intensity of systemic oxidative stress and the telomere shortening in blood leukocytes of patients with these three major psychiatric disorders. Moreover, postmortem brain samples of these patients (SZ, MD, BD) did not reveal any TL shortening, although they had elevated markers of oxidative stress. This dissonance between the TL of blood leukocytes and brain tissue is somewhat surprising since in other diseases (e.g., diabetes, autoimmune diseases, cardiovascular diseases, stroke) the reduced leukocyte TL correlated with the telomere shortening in target organs and tissues (Price et al. 2013).

Bearing this in mind, only well-planned longitudinal studies with monitoring of the blood leukocyte TL and the intensity of systemic oxidative stress will definitely solve the question whether SZ, MD, and BD are associated with accelerated telomere shortening and whether the oxidative stress belongs to the main factors responsible for this process.

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The Impact of Oxidative Stress on GAD67 Levels and Parvalbumin-Positive Neurons

Jessica Deslauriers and Sylvain Grignon

Abbreviations

BA	Brodmann area
EEG	Electroencephalographic
GAD	Glutamic acid decarboxylase
LPS	Lipopolysaccharide
MAM	Methylazoxymethanol acetate
NOX	NADPH oxidase
poly IC	Polyinosinic: polycytidylic acid
PV	Parvalbumin
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4

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1 Introduction

There is substantial evidence for dysregulation of GABA neurotransmission in the cortex of patients with schizophrenia. The most replicated finding concerns the decrease in expression levels of the rate-limiting enzyme glutamic acid decarboxylase (GAD). From a functional perspective, these abnormalities have been linked to electrophysiological and cognitive dysfunctions. From a mechanistic point of view, recent evidence has been provided linking GAD abnormalities to glutamatergic dysregulation and suggesting that oxidative status is a key player between glutamatergic phenomena (namely, NMDA hypofunction) and GAD downregulation. Therefore, in parallel with other neurochemical systems, GABA, and more specifically GAD, abnormalities, provide an interesting framework to understand the potential role of oxidative phenomena in the pathogenesis of psychiatric disorders.

2 Overview of GABA Neurotransmission

In GABAergic neurons, the synthesis of GABA (see section "Glutamic acid decarboxylases"), an inhibitory neurotransmitter, occurs in the cytosol, and GABA is transported in the synaptic vesicles by the vesicular GABA transporter (vGAT). At the nerve terminal, an action potential triggers, in a Ca^{2+}-dependent manner, vesicular GABA release (see section "GABA interneurons") (Gonzalez-Burgos et al. 2011). In cortical GABA neurons, released GABA induces effects that are mediated by ionotropic ($GABA_{A/C}$) or metabotropic receptors. The $GABA_A$ receptors are heteropentameric structures composed from a repertoire of 19 subunits that have distinct affinities for GABA and that determine functional properties of the GABA receptor (Uusi-Oukari and Korpi 2010). $GABA_B$ receptors, which are metabotropic receptors coupled to $G_{i/o}$ GTP-binding protein, play a role in the postsynaptic effects of GABA in GABAergic neurons (Olah et al. 2009). Finally, plasma membrane GABA transporters (GATs) reuptake GABA to terminate the effect of GABA. In the central nervous system (CNS), GABA uptake is mainly mediated by GAT1. GAT1 translocates GABA from neuronal cells to glial cells. Other transporters, GAT2 and GAT3, are also found in the brain (Fig. 1) (Gonzalez-Burgos et al. 2011).

2.1 Glutamic Acid Decarboxylases

GABA synthesis, from glutamate, is regulated by the enzyme glutamic acid decarboxylase (GAD). GABA plays a crucial role in the maintenance of excitatory-inhibitory balance of the CNS (Li et al. 2008). GABA is the only neurotransmitter being synthesized by two different enzymes, namely, the two molecular forms of glutamic acid decarboxylase, the 67 kDa (GAD67) and 65 kDa forms of GAD (GAD65).

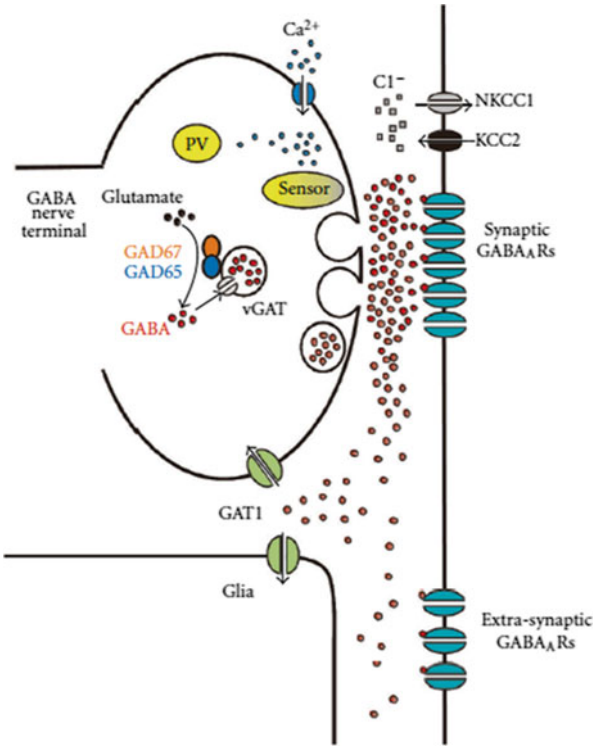

Fig. 1 Scheme of a parvalbumin (PV)-positive GABA neuron after Ca^{2+}-dependent GABA release. GAD65 and GAD67 promote GABA synthesis in the cytosol and synaptic vesicules uptake newly synthesized GABA via vesicular GABA transporter (vGAT). Vesicule fusion with the presynaptic membrane releases GABA and increases GABA concentration in the synaptic cleft. Thus, GABA links and activates postsynaptic $GABA_A$ receptors. Presynaptic GAT1, localized in neuronal and glial membranes, reuptakes GABA, regulating GABA concentration in the synaptic cleft. The transporters KCC2 and NKCC1 uptake and extrude chloride, regulating the chloride current produced by $GABA_A$ receptor activation (Figure from Gonzalez-Burgos et al. 2011)

GAD67 is the main enzyme responsible for most (>90 %) GABA synthesis from glutamate in the central nervous system (Lewis et al. 2005) and is used as a marker for GABA neurons. GAD67 is the product of the GAD1 gene, located on 2q31.1. Knock out of GAD67 provokes, besides a drastic reduction in GABA levels, a cleft palate (which suggests a role in developmental processes besides conventional neurotransmission (Maddox and Condie 2001)) and neonatal lethality.

GAD67 can exist in its native soluble form or bound to membranes, possibly through possible heterodimerization with GAD65 or through other anchoring mechanisms (Kanaani et al. 2010). Cytoplasmic GABA is involved in functions not directly related to neurotransmission, and the different pools of GABA are differentially regulated during resting state, exocytosis, or reversal of membrane uptake processes (Waagepetersen et al. 2001).

Conversely, GAD65 is activity dependent, tightly associated to synaptic vesicles, and synthesizes GABA for exocytotic release (Fukuda et al. 1998; Soghomonian and Martin 1998).

It has been demonstrated that GAD67 mRNA expression increases with the development of CNS (Greif et al. 1991; Thuesen and Lohmann 1992; Lundgren et al. 1997; Hyde et al. 2011) and decreases with aging (Duncan and Wheeler 1999; Gutierrez et al. 1994; Shetty and Turner 1998).

2.2 GABA Interneurons

GABAergic synapses are the key inhibitory synapses within the brain. GABA interneurons are associated with information processing in the cerebral cortex and regulate pyramidal neuron firing rates (McBain and Fisahn 2001). GABA interneurons coexpress different proteins and can be distinguished by expression of these proteins: reelin, parvalbumin (PV), and calretinin (Lieberman et al. 2008), as well as by other morphologic and functional criteria, which have been recently reviewed in the context of neurodevelopmental disorders (Rossignol 2011).

PV-positive basket cells synapse on the perisomatic and proximal dendrite region of their target pyramidal cells. Their electrophysiological properties and divergent projections enable them to provide high-frequency inhibition to their target pyramidal cells. They contribute significantly to the generation of the functionally important fast cortical gamma frequencies.

Chandelier cells are also PV-positive GABA interneurons, able to sustain high-frequency inhibition. They target the axon initial segment, with axoaxonic synapses displaying a characteristic morphology of vertically arranged cartridges. Intriguingly, because of locally high concentration of chloride at the axon initial segment on which they synapse, they have been suggested to trigger depolarization in some, but not all contexts (Woodruff et al. 2010).

Somatostatin-positive interneurons include Martinotti and non-Martinotti cells. They are diversely co-labelled for calretinin and calbindin and have variable morphology and targets. Martinotti cells contact multiple pyramidal cells at the distal dendritic level (a feature they share with the reelin-/calbindin-positive, somatostatin/vasoactive intestinal peptide-negative neurogliaform cells) in adjacent cortical columns, thereby exerting control over dendritic summation (Rossignol 2011).

Reelin, which is a secretory glycoprotein, regulates neural migration and is implicated in synaptic plasticity via its release from GABAergic terminals and binding to integrin receptors. During postnatal development and adulthood, reelin is located in GABAergic interneurons, where it modulates N-methyl-D-aspartate receptor (NMDAR) activity and synaptic plasticity (Beffert et al. 2005). PV and calretinin are calcium-binding proteins that contribute to intracellular calcium-signaling signaling pathways. PV interneurons are implicated in the generation of gamma oscillations, which regulate recall of information for working memory (Bartos et al. 2007). The glutamatergic input from all GABA-releasing neurons in cortex projects to PV interneurons (Lewis et al. 2005; Gulyas et al. 1999). During GABA release (see section “Overview of GABA neurotransmission”), PV (and, for

that matter, other calcium-binding proteins) acts as a Ca^{2+} buffer: it binds residual Ca^{2+} after its entry and activation of the Ca^{2+} sensor (Gonzalez-Burgos et al. 2011), thereby limiting the duration of the exocytotic phase and enabling the fast inhibition typical of PV-containing interneurons, basket, and chandelier cells.

3 GABA Neurotransmission in Neuropathological Conditions

3.1 Postmortem Studies

In 1995, Akbarian et al. published the first report of decreased GAD67 mRNA in the cortex of patients with schizophrenia, with a consistent decrease of ≈30 % across cortical layers III–VI peaking at – 40–50 % in layers I–II (Scottish Schizophrenia Research Group 2000). As of 2006, there were 13 published reports on GAD65/67 levels in schizophrenia or bipolar disorder, 11 of which showed decreased mRNA or protein levels (Akbarian and Huang 2006). Subsequent work has further validated and expanded these observations (Veldic et al. 2005; Bernstein et al. 2007; Woo et al. 2007, 2008; Bullock et al. 2008; Eggan et al. 2008; Hashimoto et al. 2008a, b; Thompson et al. 2009; Curley et al. 2011; Konradi et al. 2011; Thompson Ray et al. 2011; Benes et al. 2007; Huang and Akbarian 2007; Moyer et al. 2012).

There was one report of increased GAD immunoreactive levels in parahippocampal regions (subiculum and parahippocampal gyrus), which correlated with disease duration (Schreiber et al. 2011).

Anatomically, decreased transcript levels have been reported in different cortical regions (prefrontal dorsolateral cortex (Brodmann area (BA) 9) (Guidotti et al. 2000), anterior cingulate cortex, primary motor and visual cortices (Hashimoto et al. 2008b), orbitofrontal cortex (Thompson et al. 2009), primary auditory cortex (Woo et al. 2007), caudate and accumbens nuclei (Thompson et al. 2009), and cerebellum (Guidotti et al. 2000).

Different techniques have provided convergent results: Hashimoto et al. used a combined microarray/reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) approach which yielded convergent (and correlated) decreases of –10 % transcript levels (RT-qPCR), confirming a similar result of the same group, and 1.33-fold decrease (microarray) (Hashimoto et al. 2008a).

In another paper, the same group studied the decrease pattern across four different cortical regions (dorsolateral prefrontal cortex, anterior cingulate cortex, primary motor cortex, and primary visual cortex) and reported a homogeneous decrease in the 20–30 % range (Hashimoto et al. 2008b).

The use of microarrays has provided a much more detailed insight on the signaling network associated with GAD67 transcriptional decrease, e.g., GABA-A receptor subunits, GAT, NMDA receptor subunits, to name a few. In an influential article, Benes et al. combined laser microdissection of hippocampal circonvolutions to provide a comparative analysis of transcription factors and cell cycle molecules in the

stratum oriens of CA2/3 in patients with schizophrenia and bipolar disorder. Indeed, while GAD67 mRNA was decreased in the two conditions, the cell signaling/transcriptional pathways appeared strikingly dissimilar, with an increase in cyclin D2 levels in schizophrenia and a decrease in bipolar disorder, for instance. Although published data often report on mRNA levels (microarray, in situ hybridization, RT-qPCR), decreased protein levels have also been published. Guidotti et al. showed a 40–50 % decrease in GAD67 protein levels (prefrontal cortex Brodmann area 9 and cerebellar hemisphere), with a much more pronounced mRNA decrease in the same cohort. While there was a robust positive correlation between reelin and GAD67 mRNA levels in controls, this correlation was lost in patients with schizophrenia (Guidotti et al. 2000). The results are not always concordant between the two techniques, as exemplified in an earlier report of increased GAD65/67 mRNA levels in the dorsolateral prefrontal and occipital cortices, contrasting with normal protein levels in the same regions (Dracheva et al. 2004).

Although less generally studied, GAD65 signal has also been reported, with a general trend for GAD67 correlation and decreased levels (Bullock et al. 2008; Benes et al. 2007; Fatemi et al. 2005).

Generally, larger decreases are reported when GAD levels are assayed in a more restricted fashion, whether at the anatomical or cellular level. For instance, hippocampal GAD67 levels were found to be severely decreased (ranges −2.8/−9.5-fold) compared to controls when measured on laser microdissected strata, whereas more modest decreases were reported on homogenates of the same region (Benes et al. 2007). Similarly, more pronounced effects were reported in neurons co-expressing PV (Curley et al. 2011) or the NMDA subunit NR2A (Woo et al. 2004, 2008).

Interestingly, complementing previous results showing decreased GAD67 neuronal density in orbitofrontal cortex, it has been recently reported that the density of interstitial white matter neurons expressing GAD65/67 mRNA was indeed increased in adjacent white matter, adding migration abnormalities to the potential mechanisms of altered GAD expression (Joshi et al. 2012).

3.2 Preclinical Models

A significant number of studies have assessed the same neurochemical pathways in preclinical models of schizophrenia or have directly examined more specific mechanistic aspects such as the impact of DNA methylation (or more broadly epigenetic aspects).

One of the first neurodevelopmental models of schizophrenia was obtained after neonatal ventral hippocampus lesion, and, as expected, it gave rise to decreased (50 %) GAD67 mRNA levels (Lipska et al. 2003).

Using the methylazoxymethanol acetate (MAM) gestational injection model, also intended to mimic neurodevelopmental aspects of schizophrenia, a significant decrease in the density of PV-positive neurons has been reported, while GAD67 decreases did not reach statistical significance; interestingly, there was a loss of medial prefrontal cortex theta and gamma frequencies elicited by fear conditioning in the MAM group (Lodge et al. 2009).

A large "family" of animal schizophrenia models relies on the induction of gestational or early postnatal inflammation designed to mimic some well-known epidemiological aspects of the disease such as an excess of winter/spring births, increased prevalence after influenza epidemics, and increased gestational antibody titers retrospectively documented in mothers of future schizophrenic patients. Currently two such models are in wide use and rely, respectively, on the injection of a gram-negative bacterial wall component, lipopolysaccharide (LPS), and polyinosinic:polycytidylic acid (poly IC), a synthetic ribonucleic. Both molecules engage innate immunity: poly IC is a Toll-like receptor 3 (TLR3) and LPS a Toll-like receptor 4 (TLR4) ligand.

Prenatal injection of pregnant female Sprague Dawley rats with LPS induced a decrease of GAD67 immunoreactive cells in the dentate gyrus of the hippocampus at postnatal days 14 and 28 (Nouel et al. 2012). In one of the rare direct comparisons of the two protocols, Harvey and Boksa showed an increase in GAD67 cell number in the ventral stratum oriens of the hippocampal circonvolution CA1 in PD28 male mice prenatally (gestational day 9) treated with LPS and in PD28 female mice prenatally treated with poly IC (Harvey and Boksa 2012).

GAD67 immunoreactivity was, however, decreased by some 40 % by early prenatal (9.5 gestational days) (Soumiya et al. 2011). Other reports have pointed to a decrease in PV levels in the prefrontal cortex in a double hit model of schizophrenia (dominant-negative DISC1 transgenic mice x poly IC) (Ibi et al. 2010) or in hippocampal CA1 region (Ducharme et al. 2012); interestingly, the latter paper also demonstrated a strong reduction in hippocampal theta rhythms. Overall, in spite of somewhat conflicting results, it seems that a number of GABAergic abnormalities can be elicited by the prenatal inflammatory models currently validated and in wide use.

In another perspective, a number of authors have used the GAD67 response, and its robust reproducibility in humans, to directly test the glutamatergic, and more specifically the NMDA hypofunction, hypothesis of schizophrenia.

Postnatal injection of MK-801 to rat pups induced divergent responses with decreased PV levels in the anterior cingulate and decreased GAD67 levels in the somatosensory cortex, where PV levels were unchanged (Turner et al. 2010). In adult rats, daily injections of ketamine for 2 days was sufficient to subacutely decrease GAD67- and PV-immunoreactive cells by *circa* 40 % (Zhang et al. 2008), at odds with the acute effects of MK801, which, however, induced a diffuse decrease of PV levels (Romon et al. 2011). The comparative effects of single versus repeated phencyclidine (PCP) administration was studied in detail by Amitai et al. (Amitai et al. 2012). In all experimental conditions, there was a significant decrease in GAD67 and PV levels, and chronic administration of clozapine provided a partial (GAD) or complete (PV) restoration. Apart from the pharmacological manipulations mentioned above, the most direct test of a relationship between NMDA hypofunction and GAD abnormalities has come from genetic ablation of some components of the NMDA receptor. Belforte et al. achieved selective elimination of the NR1 subunit in cortical and hippocampal interneurons (Belforte et al. 2010). This very specific model mimicked the most salient aspects of schizophrenia pathology including GAD67 and PV reduction in targeted, NR1-deficient, cells. This effect was only observed when the NR1 subunit was ablated at an early age, thereby emphasizing the neurodevelopmental aspects of "hypoglutamatergic" insults.

3.3 Interventional Studies

Most of the subjects with psychiatric diseases whose brains were used in postmortem studies were or had been receiving medication, most often antipsychotic treatment, which raised questions about the origin (endogenous vs iatrogenic) of GAD decreases. To address this problem, some authors reported on animal interventional studies in parallel with their human postmortem results. Bullock et al. (Bullock et al. 2008) showed an augmentation of GAD65/67 levels by clozapine, whereas haloperidol decreased GAD65, but increased GAD67 levels.

In parallel with their postmortem study, Hashimoto et al. (Hashimoto et al. 2008a) treated male macaque monkeys with haloperidol or olanzapine for >12 months, achieving therapeutic blood drug levels. The two drugs were devoid of any effect on GAD67 mRNA levels.

In male Sprague Dawley rats, daily injections of haloperidol or clozapine increased GAD67 mRNA with concomitant protein increase only in the haloperidol group (Chertkow et al. 2006).

Overall, these results have confirmed that GAD abnormalities are indeed related to the underlying pathological process and not to some medication effects.

3.4 Clinical Aspects

The consequences of GABAergic disturbances in schizophrenia, and more relevant to the present chapter, the functional consequences of diffuse GAD downregulation, are generally thought to relate to the well-known cognitive disturbances, which are the defining feature of the disease. A large preclinical literature has consistently shown that GAD67-/PV-expressing interneurons of the cortex were critically involved in the generation of two specific electroencephalographic (EEG) rhythms, the theta and gamma bands (4–7 and 30–80 Hz, respectively). Gamma oscillations are associated with diverse cognitive functions such as perceptual binding, attention, arousal, object recognition, language perception, and executive function, some of which are highly relevant to schizophrenia disturbances (Herrmann et al. 2004). Optogenetic data have confirmed that PV neurons where necessary and sufficient to give rise to gamma rhythms, while the situation appears more complex for the generation of theta rhythms (Royer et al. 2012). While the notion that there is a mere gamma decrease in schizophrenia appears to be an oversimplification, there is little doubt that the power and organization of this spectral band as well as others are disturbed in schizophrenia, another feature being an increase in theta frequencies and a defective theta suppression during sensory gating (Moran and Hong 2011). Overall, it can be hypothesized that GAD/PV disturbances in schizophrenia (and to some extent bipolar affective disorder) disrupt the function of basket and chandelier PV-positive interneurons giving rise to abnormalities in EEG spectra critically associated with proper cognitive functioning. In the absence of sufficiently specific pharmacological interventions, some empirical validation of this model could come from genetic association studies linking GAD polymorphisms to cognitive function

or, more convincingly, to EEG analyses. Indeed, a recent report showed a significant association of GAD1 polymorphisms with schizophrenia, epistasis with the catechol-O-methyl transferase val/met polymorphism (another significant contributor to prefrontal function in schizophrenia), and contribution of a polymorphism in the putative promoter region of the GAD1 gene to GAD67 prefrontal transcript levels (Straub et al. 2007).

4 Role of Oxidative Stress in GABA Neurotransmission

Subanesthetic doses of NMDAR antagonists, like phencyclidine and ketamine administered in adulthood, reproduce positive and negative symptoms of schizophrenia in vivo. Thus, NMDAR antagonists are used for modeling schizophrenia (Javitt 2010). It has been shown that NMDAR antagonists induce a decrease in PV expression in GABAergic interneurons in rodents and nonhuman primates (Cochran et al. 2002, 2003; Keilhoff et al. 2004; Rujescu et al. 2006; Morrow et al. 2007). Indeed, PV interneurons are highly sensitive to NMDAR antagonists (Jones and Buhl 1993), which suggests that NMDARs are implicated in the control of basal synaptic activation in PV interneurons (Goldberg et al. 2003). Specifically, NMDAR subunits NR2A are expressed at higher levels in PV interneurons than in pyramidal neurons (Kinney et al. 2006) and NR2A antagonist NVP-AAM077 reduced GAD67 expression (Kinney et al. 2006), which supports the role of NMDAR subunit NR2A in reduction of GAD67 levels. Furthermore, NMDAR antagonists increase reactive oxygen species (ROS) in vitro (Xia et al. 2002) and in vivo (Zuo et al. 2007) and, thus, induce an imbalance of redox status. Oxidative stress is implicated in the pathogenesis of schizophrenia through, among others things, a decrease in glutathione (GSH) levels (Do et al. 2009). GSH, an important radical scavenger, is crucial for NMDAR activation, a redox-sensitive process (Lipton et al. 2002). We present hereafter a review of in vitro and in vivo studies that demonstrated the crucial role of oxidative stress on GAD67 expression and on PV interneurons via NMDAR hypofunction and its relevance to schizophrenia.

4.1 In Vitro Studies

CNS oxygen toxicity has been associated with generation of ROS (Li et al. 2008), which attacks enzymes like GAD67. It has been demonstrated that primary rat hippocampus neurons, exposed to prolonged hyperbaric oxygen treatment (HBO), show a decrease in GAD67 content, GAD activity, and intracellular GABA content (Li et al. 2008). As HBO exposure increases oxygen-free radicals and, then, induces oxidative stress, it has been suggested that the decrease in GAD67 expression is provoked by the increase of oxidative stress (Li et al. 2008). In fact, it has been reported that oxygen-free radicals decrease GAD67 activity by disrupting their hydrosulfide groups (−SH), which are essential for GAD67 activity (Satyanaran et al. 1985).

In primary cortical neuronal cultures, NMDAR antagonists induced a reversible decrease in GAD67 and PV levels in PV interneurons (Kinney et al. 2006), while ketamine, a NMDAR antagonist, increased superoxide production and NADPH oxidase subunit NOX2 expression in PV interneurons (Behrens et al. 2007). Furthermore, the superoxide production and the loss of PV and GAD67 immunoreactivity were prevented by treatment with apocynin, an inhibitor of NADPH oxidase activity (Behrens et al. 2007), thereby confirming the pivotal role of oxidative stress between "PCP-like" antagonism of NMDA receptors and down-modulation of GAD67 levels.

While most of these results involve posttranscriptional functional modifications, transcriptional regulation of GAD67 also raises interesting questions. The notion of transcriptional repression in the CNS in situations of oxidative stress, if confirmed, would stand in sharp contrast to what happens in the systemic compartment where oxidative stress upregulates GAD67 in an NFkB-dependent fashion (Choi et al. 2002). Among the signaling network associated with GAD67 downregulation, Daxx is well placed to achieve transcriptional repression. One other potential mechanism, given the robust evidence of epigenetic modulation of GAD67 transcription (Kundakovic et al. 2009), would be the redox modulation of DNA methylation of histone deacetylation.

4.2 In Vivo Studies

Many studies have demonstrated that hyperoxia, in vivo, decreases GAD activity (Tunnicliff et al. 1973; Davis et al. 2001; Segerbo 1979; Hori 1982). A rise-and-fall dynamic pattern of GAD activity has been reported exposing rats to hyperbaric oxygen treatment (HBO) (Li et al. 2008). Indeed, in the hippocampus, GAD content increased gradually in the first 15 min after exposure to HBO, but decreased from 20 min onward after exposure, which correlated with the development of convulsions in rats. Furthermore, this effect on GAD content came from changes in GAD67 expression, because GAD65 remained unchanged (Li et al. 2008).

Furthermore, it has been reported, in vivo, a decrease in PV and GAD67 immunoreactivity, following treatment with ketamine, in PV interneurons from mouse prefrontal cortex and an increase in superoxide production. Pretreatment of animals with apocynin, an inhibitor of NADPH oxidase (NOX) activity, prevented the ketamine-induced effects (Behrens et al. 2007). The effects were specific for the PV-interneuronal population, because other interneurons expressing the calcium-binding proteins calbindin (CB) and calretinin (CR) were unchanged by ketamine (Behrens et al. 2008). In Nox2-deficient ($gp91^{phox-/-}$) mice, ketamine did not induce an increase of superoxide production nor a loss of phenotype of PV interneurons, which suggests that the decrease in PV and GAD67 levels in PV interneurons is dependent on NOX and, thus, on oxidative stress (Behrens et al. 2008).

A decrease in GSH (glutathione) levels, which is associated with an increase of oxidative stress (Do et al. 2000), during development leads to a hypofunction of NMDARs in adulthood (Gysin et al. 2007; Tosic et al. 2006), and GABAergic neurons are highly sensitive to oxidative stress (Lipton et al. 2002; Kohr et al. 1994; Volterra et al. 1994; Mustafa et al. 2007). Catalytic (GCLC) and modifier (GCLM)

subunits of the glutamate cysteine ligase (GCL), the rate-limiting enzyme of GSH synthesis, have been associated with schizophrenia (Gysin et al. 2007; Tosic et al. 2006). It has been reported that the ventral hippocampus is vulnerable to redox dysregulation in GCLM knockout mice, which exhibit brain GSH deficits (Steullet et al. 2010), whereas no effect was observed in dorsal hippocampus. Thus, PV interneurons, but not CB or CR interneurons, were reduced in the ventral hippocampus of GCLM knockout mice, which suggests that oxidative stress-induced effects are specific to PV interneurons. The ventral hippocampus could be more vulnerable to oxidative stress because of its higher catecholamine concentration (Oleskevich et al. 1989; Gasbarri et al. 1997; Bjarkam et al. 2003), in line with the fact that reactive oxygen species (ROS) can be formed from auto-oxidation and catabolism of catecholamines (Cadet and Brannock 1998).

Thus, a redox dysregulation, through a decrease in GSH levels and/or an increase in ROS production, leads to NADPH oxidase (NOX) activation, which triggers NMDAR antagonism; for instance, the N2RA subunit of NMDAR, which is sensitive to oxidative status, maintains the function of PV interneurons (Kinney et al. 2006). As a further consequence, NMDAR hypofunction would then induce a decrease in GABAergic markers, namely, GAD67 and PV (Fig. 2) (Do et al. 2009) and downstream disruption of PV interneuron functions as well as their EEG/cognitive correlates, as discussed above.

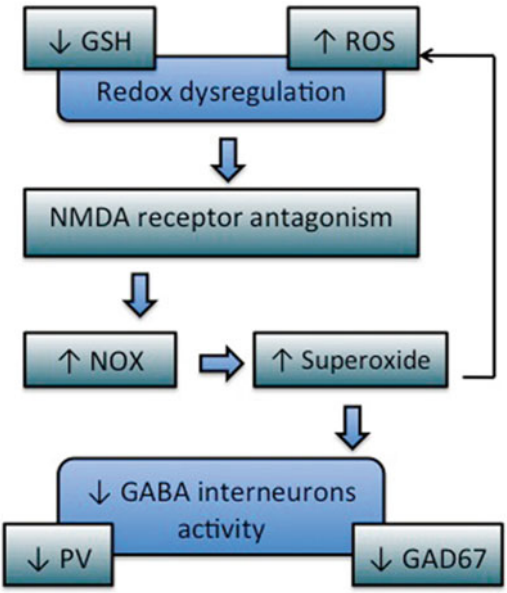

Fig. 2 Link between oxidative stress and decreased GAD67 expression. Redox regulation, induced by a decrease in glutathione (GSH) levels or an increase in reactive oxygen species (ROS) production, leads to NMDA receptor antagonism through NR2A subunit. NMDA receptor antagonism is followed by an increase in NADPH oxidase (NOX) levels, increasing superoxide production. Finally, there is a decrease in parvalbumin (PV) and the 67 kDa form of glutamic acid decarboxylase (GAD67), leading to a hypoactivity of GABA interneurons. The increase in superoxide production can also enhance ROS production through a positive feedback

5 Conclusion

GAD perturbations in severe mental disorders have been extensively replicated, making them the current neurochemical signature of these diseases. Extensive research has provided a better understanding of the upstream determinants and downstream consequences of these perturbations and suggested a prominent role of oxidative stress at the transcriptional as well as posttranscriptional level. As such, they constitute a privileged field to ascertain how oxidative status impacts the pathophysiology of psychiatric disorders.

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The Possible Role of Iron in Neurodegeneration

Jolanta Gałązka-Friedman and Andrzej Friedman

Abbreviations

AA	Atomic absorption
AD	Alzheimer disease
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
FS	Fluorescence spectroscopy
GP	Globus pallidus
Hip	Hippocampal cortex
MS	Mössbauer spectroscopy
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PSP	Supranuclear palsy
ROS	Reactive oxygen species
SN	Substantia nigra

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1 Introduction

Progressive death of nervous cells in human brain is referred to as neurodegeneration. There are several diseases affecting human brain, which are known as neurodegenerative diseases. The best known are Parkinson's disease (PD) and other atypical parkinsonisms and Alzheimer's disease (AD). AD is the most common dementia in human population. The prevalence of dementia in general population is assessed as 6.3 % and sharply raises with the age of the population studied reaching more than 40 % for the population older than 94 years (Ott et al. 1995). PD is less common with the prevalence being 0.2 % of the general population and rising up to 2 % for the population older than 65 years (Tanner and Goldman 1996). Another neurodegeneration studied by us is progressive supranuclear palsy (PSP), which belongs to a wider group of atypical parkinsonisms. The common feature of these disorders is related to formation of pathological misfolded proteins, which cannot be metabolized within nervous cells. In AD, this protein is beta-amyloid, in PD it is alpha-synuclein, and in PSP it is the protein tau. The inability to get rid of those misfolded proteins leads to formation of pathological aggregates. It is believed that the aggregates of misfolded proteins are the cause of the death of nervous cells. It is interesting to note that these diseases have predilection for specific brain areas. In AD, the pathological process starts in hippocampal cortex (Hip), while the motor symptoms of PD are related to the atrophy of the dopaminergic cells of substantia nigra (SN). In PSP, the neurodegeneration is present in basal ganglia (among others – globus pallidus – GP), substantia nigra, and cortex. It is important to notice that in all these structures, iron is present in high concentrations. Iron may play a role in initiation of neurodegeneration by triggering oxidative stress via Fenton reaction (see below).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

In this reaction, divalent iron promotes generation of free radical $OH^{\bullet}$. In normal situation, the radicals are scavenged and cannot destroy the tissue. However, when there is an increase in the production of free radicals or if the scavenging system is not working correctly, oxidative stress may occur. An excess of divalent iron may be the starting point for the oxidative stress. We decided, therefore, to study the possible role of iron in neurodegeneration related to those three human brain diseases using various methods. It is important, however, to remember that the oxidative stress represents only one possible mechanism leading to the nervous cells death and other pathways of destruction, e.g., inflammatory process, environmental toxicity, etc., may be involved in various proportions in each particular case (Dauer and Przedborski 2003).

In this chapter, we will present the results of our research lasting over 20 years being a collaboration of Warsaw University of Technology, Medical University of Warsaw, Racah Institute of Physics at the Hebrew University in Jerusalem, Mayo Clinic in Jacksonville, Florida, Warsaw University, and Polish Academy of Sciences.

2 Materials

Our studies were performed on human brain samples of SN, GP, and hippocampal cortex obtained at autopsies of patients who died with clinical diagnosis of PD, AD, or PSP and of the patients who died without any symptom of neurodegenerative disorder. This last group served as control. The clinical diagnosis in each case was confirmed by histopathological microscopic study. All samples were taken within 48 h after the death and kept in –80° C until assayed. Autopsies and histopathological diagnoses were made either in Warsaw or in Mayo Clinic, Jacksonville, Florida.

3 Methods

The samples were studied with the use of Mössbauer spectroscopy (MS), electron microscopy (EM), atomic absorption (AA), enzyme-linked immunosorbent assay (ELISA), and fluorescence spectroscopy (FS).

MS was used for the determination of the total iron concentration in the sample and assessment of divalent and trivalent iron and also for determination of iron binding compound. EM served for the assessment of the size of the iron cores of ferritin in the brain structures studied. ELISA gave us the information about the structure of ferritin in the samples with the concentration of H and L ferritin chains. In this study, specific monoclonal antibodies against human L and H ferritin were used (generous gift of Prof. Arosio from University of Brescia, Italy). AA was applied to study the concentration of non-ferritin labile iron and FS measured reactive oxygen species (ROS) activity in the samples. For the determination of the labile iron concentrations, all SN tissues that had been stored at −80 centigrade were thawed for 30 min on ice and then incubated in 1 mL of phosphate-buffered saline pH 7.2 (PBS) for 60 min on ice. After equilibration, 880-mL samples were removed and centrifuged at 6,900 rpm for 20 min at 4 centigrade on a centrifugal filter device (10,000 molecular weight cut-off) (Amicon Ultra-4; Millipore Corp, Bedford, Massachusetts), to remove all particles bigger than 10 kDa. Therefore, ferritin and transferrin, the main proteins involved in the storage and trafficking of iron, could not be present in the filtrate (Wypijewska et al. 2010).

4 Iron and Ferritin in Human Brain

In adult human body, there is about 4 g of iron. Iron plays an important role in the functioning of our organism. Two thirds of the total amount of iron is located in hemoglobin. The remaining iron is present in liver, spleen, heart, and brain. Iron in liver and spleen is distributed homogenously, which is not the case of the human brain. Distribution of brain iron is very heterogeneous. It was demonstrated already

long time ago that the highest concentration of iron in human adult brain is in the globus pallidus and substantia nigra (Hallgren and Sourander 1958). It is also high in hippocampal cortex. It is important to note, as mentioned above, that these structures are involved in three human neurodegenerative diseases: Parkinson's disease, Alzheimer's disease, and PSP. Concentration of iron in some brain structures is even higher than in human liver.

4.1 Concentration of Iron in Normal Human Brain Samples

Concentration of the total iron present in human control brain samples assessed by MS is shown in Table 1. The results of these measurements were published previously (Galazka-Friedman et al. 1996; Wypijewska et al. 2010; Galazka-Friedman et al. 2011).

Similar results were obtained by Hallgren and Sourander (1958) with the use of colorimetry (for GP, concentration of iron was 213 ± 5 ng/mg and for SN 185 ± 9 ng/mg). Much smaller value of the uncertainties is the result of much bigger number of investigated samples (over 50).

We also attempted to assess the concentration of divalent iron in SN. As the Mössbauer spectra did not show any presence of divalent iron, we assessed by computer fitting how much divalent iron could escape the detection. According to the computer simulation, the concentration of divalent iron in SN cannot exceed 5 % of the total iron (Galazka-Friedman et al. 1996).

4.2 Ferritin in Human Control Brain Samples

Hallgren and Sourander (1958) and Diezl (1955) with the use of the colorimetry were not able to identify "the forms in which this non-haemin iron exists." They cited in this paper a work of who "maintains that at least a part of it is present as ferritin."

Mössbauer studies (Galazka-Friedman et al. 1996) showed that majority of iron in human brain is bound to ferritin or hemosiderin. Analysis of Mössbauer spectra of substantia nigra demonstrated that at least 85 % of iron in SN is ferritin-like iron.

Ferritin is the most important iron storage protein. Ferritin is composed of protein shell (with diameter about 12 nm) and a cavity where iron is stored in a safe form. The diameter of this cavity is about 7 nm. It may be filled by iron in different amounts. Our own measurements with the use of electron microscopy showed that

Table 1 Concentration of total iron in human control tissue (ng/mg wet tissue)

Tissue	No. of samples	Concentration of iron
GP	12	183 ± 22
SN	29	177 ± 14
Hip	10	45 ± 10

Table 2 Concentration of H and L chains of ferritin in human control tissue (ng/mg wet tissue)

Tissue	H ferritin	L ferritin	H/L ratio
GP	335 ± 21	70.9 ± 6.2	5.1 ± 0.4
SN	375 ± 38	98 ± 12	4.3 ± 0.3
Hip	101 ± 9	9 ± 2	14.5 ± 1.8

The H/L ratios were calculated separately for each sample and the mean values calculated from all the individual measurements are given in the table

iron cores of brain ferritin are much smaller than iron cores of liver ferritin (3.5 nm vs. 6.5 nm) (Galazka-Friedman et al. 1998).

In investigated brain structures, the presence of hemosiderin was also observed. The diameter of iron core of brain hemosiderin is smaller than iron core of hemosiderin present in human spleen (2.0 nm vs. 5.7 nm) (Galazka-Friedman et al. 1998).

Protein shell of ferritin is composed of 24 subunits also referred as chains. There are two types of ferritin chains: L (light) chains and H (heavy) chains, whose functions are different. L chains are involved in building of iron cores of ferritin, and H chains are involved in trafficking of iron to and out of the ferritin shell (Friedman et al. 2011). The proportion of the L and H chains is different in different organs and structures of human body. In Table 2, concentrations of L and H chains in different brain structure are presented. The ratio of H/L is also given. Its value is calculated as an arithmetic mean of the ratio H/L obtained for each samples (Koziorowski et al. 2007; Galazka-Friedman et al. 2012).

5 Iron and Ferritin in Control and Pathological Brain Tissue (PD, PSP and AD)

Investigating the possible role of iron in neurodegeneration, we performed comparative studies of brain structures involved in three different human brain diseases: Parkinson's disease, where the structure studied was substantia nigra; progressive supranuclear palsy, in which globus pallidus and substantia nigra were targeted; and Alzheimer's disease, in which we assessed hippocampal cortex.

In these three diseases, we compared the concentrations of iron in pathological and control tissue. For PD, we also compared the concentration of labile, non-ferritin iron, and the activity of reactive oxygen species in disease and control.

Concerning PD and AD, we were also able to assess the structure of ferritin – the concentrations of H and L ferritin chains.

5.1 Parkinson's Disease

Table 3 shows the results for PD (Galazka-Friedman et al. 2004; Koziorowski et al. 2007; Wypijewska et al. 2010).

Table 3 Iron and ferritin studies – comparison of PD and control

SN	PD	Control	PD/control ratio
Total iron concentration	177 ± 18 ng/mg	177 ± 14 ng/mg	1.00 ± 0.13
Labile iron concentration	90 ± 10 ng/g	37 ± 2 ng/g	2.4 ± 0.3
H ferritin concentration	534 ± 71 ng/mg	375 ± 38 ng/mg	1.42 ± 0.23
L ferritin concentration	52 ± 8 ng/mg	98 ± 12 ng/mg	0.53 ± 0.10
H/L ratio	11.1 ± 1.4	4.3 ± 0.3	2.6 ± 0.4

The concentration of the total iron was determined with the use of Mössbauer spectroscopy, and labile iron was measured by atomic absorption. The results of Mössbauer spectroscopy have shown that in Parkinson's disease, there is no increase of the total concentration of iron in substantia nigra compared to control. These results represent an important argument in long lasting discussion in literature. Some laboratories using different methods presented studies showing important increase of the concentration of iron in parkinsonian SN, while other studies did not. Possible causes of these controversies as well as the history of the research related to iron in PD were presented by us in other reviews (Galazka-Friedman and Friedman 1997; Friedman et al. 2009; Friedman and Galazka-Friedman 2012, 2012). We also did not find any amount of divalent iron in parkinsonian SN, similarly to the measurements of control brains.

It seems that the main cause of the large discrepancy of the results in the literature may be related to artifacts due to the way of preparation and storage of samples. In some studies, the samples were stored in formalin for a long time before assessment. It is known that samples stored in formalin lose iron (Bauminger et al. 1994). Our results were obtained from samples which were fresh frozen and were not pretreated with formalin. Also the preparation of samples for measurements may cause artifact. The best example of this is the experimental study, which used pretreatment with hydrochloric acid and pepsin before the assessment of the concentration of iron with the use of spectrophotometry (Sofic et al. 1988). It is important to stress that Mössbauer spectroscopy is performed without any pretreatment of the samples studied.

Although we did not find any increase of the total concentration of iron in parkinsonian SN, we did find an increase of the labile, non-ferritin iron. These measurements were made with atomic absorption on samples void of molecules bigger than 10 kDa. The concentration of labile iron represents only about 1‰ of the total iron. This labile iron may trigger the Fenton reaction. In fact, our fluorescence spectroscopy studies demonstrated an increase of ROS activity in parkinsonian SN compared to control (Wypijewska et al. 2010).

Our ELISA study demonstrated a change in the structure of ferritin in PD with a decrease of L and an increase of H chains in pathological tissue affecting the H/L ratio by a factor of 2.6. It is important to note that a decrease of the concentration of L ferritin was found already in SN obtained from human brains showing Lewy

bodies in this structure without clinical symptoms of PD (Koziorowski et al. 2007). As these brains represent the preclinical phase of PD, it is justified to suppose that the decrease of L ferritin precedes the parkinsonian neurodegeneration.

5.2 *Progressive Supranuclear Palsy*

The results obtained for PSP are shown in Table 4.

The concentrations of iron were assessed with the use of Mössbauer spectroscopy (Galazka-Friedman et al. 2009). Our results show that in PSP, there is an increase in the concentration of iron both in GP and SN compared to control. We do not have any data concerning the labile iron and the structure of ferritin in PSP.

5.3 *Alzheimer Disease*

The results obtained for AD are shown in Table 5.

The concentration of iron in control tissue was made with the use of Mössbauer spectroscopy, while in the pathological tissue with atomic absorption (Galazka-Friedman et al. 2011). The increase of the iron concentration in pathological tissue is not significant as it is within the error limits, which is related to a wide distribution of the results between the samples. Certainly, measurements of a much larger number of samples are needed to elucidate this observation. The ELISA study has shown an important increase of both H and L chains of ferritin in AD. Apparently in AD, contrary to PD, there is an overproduction of molecules and no change in the structure of ferritin in the pathological tissue compared to control.

Table 4 Iron studies – comparison of PSP and control

Tissue	Disease (PSP) or control (C)	Concentration of iron (ng/mg)	PSP/control ratio
GP	PSP	257 ± 19	1.40 ± 0.20
	C	183 ± 22	
SN	PSP	301 ± 26	1.60 ± 0.23
	C	188 ± 22	

Table 5 Iron and ferritin studies – comparison of AD and control

	AD	Control	AD/control ratio
Total iron concentration (ng/mg)	66 ± 13	45 ± 10	1.47 ± 0.44
H ferritin (ng/mg)	397 ± 29	101 ± 9	3.93 ± 0.45
L ferritin (ng/mg)	29 ± 5	9 ± 2	3.22 ± 0.90

6 Conclusions

Mechanisms of neurodegeneration in these three diseases may be different. In PD, the process may start with a decrease of the L ferritin, which is detected already in the preclinical phase of the disease. This change of the structure of ferritin, which makes efflux of iron from the protein shell easier, may cause an increase of the labile iron, which can trigger oxidative stress via Fenton reaction.

In PSP, lack of information about labile iron and ferritin structure makes any hypothesis related to the role of iron in the process impossible.

In AD, the increase of both ferritins without significant change in iron concentration suggests different mechanism of neurodegeneration, possibly related to inflammatory process.

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Oxidative Stress and Polyunsaturated Lipid Peroxidation Products in the CNS: Focus on Retinal Bisretinoids and DHA-Derived Carboxyethylpyrroles as Potential Inducers of Vision-Threatening Pathology

Jerzy Z. Nowak

Abbreviations

A2E	*N*-Retinylidene-*N*-retinylethanolamine
AA ARA	Arachidonic acid
AGE	Advanced glycation end product
ALA	α-Linolenic acid
AMD	Age-related macular degeneration
AT-RAL	All-*trans* retinal
AT-RvD	Aspirin-triggered resolvin D
CAP	Carboxyalkylpyrrole
CEP	Carboxyethylpyrrole
CEP-HSA	Carboxyethylpyrrole-modified human serum albumin
CEP-MSA	Carboxyethylpyrrole-modified mouse serum albumin
CHP	Carboxyheptylpyrrole
CNS	Central nervous system
CNV	Choroidal neovascularization
CPP	Carboxypropylpyrrole
DGLA	Dihomo-γ-linoleic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EP	Ethylpyrrole
EPA	Eicosapentaenoic acid
ETA	Eicosatetraenoic acid
GLA	γ-Linoleic acid
HHE	4-Hydroxyhexenal

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HNE	4-Hydroxy-2-nonenal
HODA	9-Hydroxy-12-oxydec-10-enoic acid
HOHA	4-Hydroxy-7-oxyhept-5-enoic acid
HOOA	5-Hydroxy-8-oxyoct-6-enoic acid
LA	Linoleic acid
MDA	Malondialdehyde
NPD1	Neuroprotectin D1
POS	Photoreceptor outer segment
PP	Pentylpyrrole
PUFAs	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RvD	Resolvin D
RvE	Resolvin E
SOD	Superoxide dismutase
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
VEGF	Vascular endothelial growth factor

1 Introduction

Oxidative stress is a common phenomenon occurring in living systems. Although endowed with pathological potential, oxidative stress in fact originates from physiological reactions taking place practically inside each cell of an organism. And this is because of the fact that the production of so-called free radicals, especially reactive oxygen species (ROS), accompanies, for example, oxidative phosphorylation-derived ATP-based energy metabolism which takes place in the mitochondria of a cell. ATP – originating from the mitochondrial electron transport chain – is absolutely essential for life. However, during energy transduction, some electrons "leak" to oxygen, forming the oxygen free radical superoxide, which has been implicated in the aging process (the concept known as the free radical theory of aging (Harman 1956; Fossel 2003; Terman et al. 2006)), as well as in the pathophysiology of a variety of diseases (Dröge 2002; Valko et al. 2007; Alfadda and Sallam 2012).

Under physiological conditions, free radicals (both ROS and reactive nitrogen species – RNS) are rapidly neutralized by effective enzymatic and nonenzymatic defense mechanisms, contributing to the maintenance of a proper balance – "steady-state" level – between their rates of production and their rates of removal/inactivation. However, an excess of free radicals, resulting from either their overproduction or insufficient activity of antioxidant defense systems, will disturb physiological equilibrium between pro- and antioxidant systems, leading eventually to a sequence of usually nonenzymatic spontaneous reactions with possible injurious consequences, a process embraced by the term oxidative stress.

Oxidative stress and polyunsaturated fatty acids (PUFAs) are closely related, as PUFAs, occurring abundantly in the central nervous system (CNS) and possessing many "fragile" double bonds between carbon atoms (C=C), easily undergo nonenzymatic oxidation and fragmentation during lipid peroxidation and form numerous toxic products. In other words, PUFAs, especially long-, or – as some people say – very long-chain fatty acids, are targets of oxidative stress-mediated lipid peroxidation. Lipid peroxidation thus contributes to a chain reaction implicated in the generation of a variety of very reactive highly cytotoxic molecules.

In this article we will focus on oxidative stress and two families of compounds strictly related to oxidative stress as possible causes of pathologies affecting the CNS and especially the retina. These compounds include docosahexaenoic acid (DHA)-derived oxidative protein modifications represented by carboxyethylpyrroles and lipofuscin-stored bisretinoids, whose photoexcitation/photooxidation can lead to the formation of singlet oxygen and other reactive oxygen species. The retina, being an extracranial extension of the brain, is an integral part of the CNS. The retina, similar to the brain, is a tissue very rich in long-chain (LC) PUFAs, particularly in DHA, and, unlike the brain (yet in agreement with its physiology), is subjected to high level expose to light, which can produce photochemical damage. Due to its structural and functional features, the retina seems to be particularly predisposed to ROS generation. Therefore, the retina may be an excellent model tissue with which to show what may happen in the CNS tissue under condition when oxidative stress and polyunsaturated fatty acids peroxidation meet together.

2 Free Radicals, PUFAs, and Oxidative Stress: Short Introduction

2.1 Free Radicals

Free radicals are molecules that possess one or more unpaired electrons in their outermost orbits which results in an extremely unstable configuration; they may also contain a complete set of electrons – yet occurring in an unstable state. Free radicals quickly react with other molecules or radicals to achieve the stable configuration (at a lower energy state), thereby becoming less reactive. There is a wide variety of free radicals, including reactive nitrogen species (RNS) and oxygen-derived free radicals, known as reactive oxygen species (ROS). The former group is represented by nitric oxide radical ($NO^{\bullet}$), nitrosonium cation (NO^{+}), nitroxyl anion (NO^{-}) or extremely reactive peroxinitrite ($ONOO^{-}$); the latter group includes oxygen ions and peroxides. Superoxide anion ($O_2^{-\bullet}$), also named superoxide radical anion or hyperoxide, is considered the "primary" ROS which in biological tissues can be converted (also with the aid of superoxide dismutase (SOD)) into non-radical species: hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). In the presence of some transition metals, e.g., Fe^{2+}, hydrogen peroxide can easily undergo decomposition by the Fenton and Haber–Weiss reactions to hydroxyl radical ($^{\bullet}OH$) – one of

the most toxic molecules, characterized by $T_{1/2}$ in the range of nanoseconds; such a short biological half-life favors the oxidation of molecules just in their place of ˙OH generation. Hydroxyl radical, via reactions with alkanes (and their derivatives) or fatty acids, forms a new less active radical and a molecule of water. On the other hand, hydrogen peroxide, being a substrate for catalase or glutathione peroxidase, may be converted to water (Dröge 2002; Valko et al. 2007).

Free radicals occur widely in nature as they are formed throughout the whole life as by-products during various biochemical reactions taking place in different cellular compartments of a variety of cells (Valko et al. 2007; Novo and Parola 2008; Alfadda and Sallam 2012). For example, hydrogen peroxide is produced under various conditions, even in normoxia, reaching constant cellular concentration between 10^{-9} and 10^{-7} M. Being products of normal cellular metabolism, free radicals may play a dual role as both deleterious and beneficial species. If endogenous antioxidative defense systems are effective (as, e.g., highly active SOD, a superoxide scavenging enzyme which neutralizes $O_2^{-\bullet}$, or sufficiently active macular pigments in the retina), then the presence and actions of free radicals do not result in oxidative stress and overt pathology. If such systems fail – as likely takes place in a senescent organism or diseased tissues/organs – prooxidative activity predominates. Consequently, an excess of ROS (resulting either from mitochondrial electron transport chain, or excessive stimulation of NAD(P)H, or other mechanism), giving rise to oxidative stress, may lead to damage to cell structures and the development of pathological states requiring medical intervention. As far as beneficial activity of free radicals is concerned, their deployment by the immune system, and specifically macrophages and neutrophils generating ROS to kill invading microorganisms, may be a good example.

Concerning cell compartment where free radicals are formed, it is generally assumed that oxygen-derived reactive species produced in mitochondria may contribute to "physiological" aging process (Wallace et al. 1998), whereas ROS of non-mitochondrial origin, produced, for example, in the endoplasmic reticulum, cell membrane, or peroxisomes, may play a role in age-dependent diseases (Del Rio 2011; Bhandary et al. 2012). A mixture of ROS originating from both sources may play in concert as well (Wallace et al. 1998; Alfadda and Sallam 2012).

2.2 Polyunsaturated Fatty Acids

Fatty acids (FAs) are present in the most diverse forms of life and perform important functions as lipid components in the structure of the plasmatic/cellular membranes, being responsible for, e.g., membrane fluidity, and in metabolic/signaling processes – they are important sources of energy and precursors of signaling molecules (including proinflammatory, anti-inflammatory, vasoactive, and many other mediators). The vast family of fatty acids comprise saturated and unsaturated compounds; the former lipids have no double bonds between carbon atoms (C=C) in a hydrocarbon (acyl or polyene) chain, and the latter compounds possessing one C=C double bond refer to monounsaturated fatty acids; those possessing two or more double bonds refer to polyunsaturated fatty acids (PUFAs). At both ends of each fatty acid,

there is reactive group: carboxyl group and methyl group, forming, respectively, carboxyl end and methyl end. Depending on the position of the first C=C double bond (counting from the carbon of the methyl end, designated as the first or omega carbon) polyunsaturated fatty acids divide into two families/series: omega-3 (ω3, n-3) and omega-6 (ω6, n-6) PUFAs. The simplest fatty acids in each family/series are α-linolenic acid (ALA; 18:3-ω3 or 18:3n-3) for omega-3 and linoleic acid (LA; 18:2-ω6 or 18:2n-6) for omega-6; both undergo metabolism consisting of elongation of the acyl chain and insertion of additional C=C double bonds, as depicted below.

Omega-3 family/series
α-Linolenic acid (ALA; 18:3-ω3) → octadecatetraenoic acid (18:4ω3) → eicosatetraenoic acid (ETA; 20:4-ω3) → eicosapentaenoic acid (EPA; 20:5ω-3) → docosapentaenoic acid (DPA; 22:5-ω3) → 24:5-ω3 → 24:6-ω3 → [β-oxidation] → docosahexaenoic acid (DHA; 22:6-ω3)

Omega-6 family/series
Linoleic acid (LA; 18:2-ω6) → γ-linoleic acid (GLA; 18:3-ω6) → dihomo-γ-linoleic acid (DGLA; 20:3-ω6) → arachidonic acid (AA or ARA; 20:4-ω6) → docosatetraenoic acid (DTA; 22:4-ω6) → 24:4-ω6 → 24:5-ω6 → [β-oxidation] → docosapentaenoic acid (DPA; 22:5-ω6)

The metabolic reactions within the ω3 and ω6 PUFA families take place in the cell endoplasmic reticulum, with the exception of the last reaction – β-oxidation which takes place in peroxisomes, thus requiring translocation of adequate substrates (i.e., 24:6-ω3 and 24:5-ω6) into this cell compartment.

Polyunsaturated fatty acid residues of membrane phospholipids are very sensitive to oxidation and the action of ROS (Novo and Parola 2008). For example, hydroxyl radical ($^{\cdot}OH$) can easily abstract or using a more colloquial expression "steal" an electron from such unsaturated fatty acids (marked as LH from lipid-H) to give rise to a carbon-centered lipid radical ($L^{\cdot}$; fatty acid radical). Lipid radical ($L^{\cdot}$) can further interact with molecular oxygen (O_2) to produce lipid peroxyl radical ($LOO^{\cdot}$; fatty acid peroxyl radical). Being an unstable species, lipid peroxyl radical interacts with another free fatty acid (LH) to produce a different fatty acid radical ($L^{\cdot}$) and lipid peroxide (LOOH). The formed $L^{\cdot}$ reacts again with molecular oxygen to produce lipid peroxyl radical ($LOO^{\cdot}$), etc., creating the cycle that continues, as the new fatty acid radical ($L^{\cdot}$) reacts in the same way. The described process can be schematically depicted as follows:

$$\text{Lipid-H (LH)} + {}^{\cdot}OH \rightarrow L^{\cdot} \text{ (lipid/fatty acid radical)}$$
$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \text{ (lipid/fatty acid peroxyl radical)}$$
$$LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH \text{ (lipid peroxide)}$$
$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$

The whole process is known under the term "lipid peroxidation"; being initiated by the reaction of $^{\cdot}OH$ with unsaturated lipid (initiation step) and then entering the cycle (propagation step), the lipid peroxidation leads eventually to cell damage. The harmful process can however be terminated when two radicals react and produce a non-radical species. Another possibility to speed up termination is to catch free radicals, which can be achieved by molecules with antioxidant activity, e.g.,

vitamin E, antioxidant enzymes (SOD, catalase, and peroxidase), or xanthophyll-type macular pigments (Valko et al. 2007; Novo and Parola 2008).

Peroxidation of highly unsaturated lipids, i.e., fatty acids that possess more than three C=C double bonds (e.g., ω6 arachidonic acid, ω3 eicosapentaenoic acid, and particularly ω3 docosahexaenoic acid), leads to complex mixtures of products, including malondialdehyde (MDA), acrolein, 4-hydroxy-2-nonenal (HNE), 4-hydroxyhexenal (HHE), as well as a number of hydroxy-ω-oxoalkenoic acids. The latter compounds, together with their derivatives of the carboxyalkylpyrrole type, are produced in the DHA-rich brain and retina, where they may contribute to, respectively, some degenerative brain disorders and vision-threatening macular pathologies.

It should be emphasized that in the central nervous system (CNS), including the retina – the DHA-richest CNS structure (Wang and Anderson 1992; Tanito et al. 2009) – DHA, which is the most unsaturated fatty acid, plays many important physiological roles which, ironically, are related just to its structural unsaturation. Structural unsaturation (six C=C double bonds) predisposes DHA for peroxidation with resultant harmful compounds.

3 The Retina: An Integral Part of the CNS and a Suitable Model Tissue for Brain Research

The retina, together with the optic nerve (which consists of axons of retinal ganglion cells) is an extension of the brain. As such, it may be considered an integral part of the CNS (Nowak 1988; Marc 2011). The retina is a thin sheet of nervous tissue (about 90 μm in the rabbit and 300 μm in the cow) situated at the back of the eye. The complex retinal processing of visual stimuli, including the detection of brightness, contrast, color, and movement, is accomplished by the interaction of retinal neurons, i.e., photoreceptors (rods and cones), bipolar cells, horizontal cells, amacrine cells, interplexiform cells, and ganglion cells (whose axons form the optic nerve). In addition to these neuronal cells, glial cells, known as Müller cells, are also present in the retina. Morphologically, the retina is characterized by distinct laminar organization of its cells and processes, with two layers of synaptic connections: the outer and inner plexiform layers. Although both electrical and chemical synaptic junctions occur in the retina, the latter predominate, and the "interplay" between retinal neurons appears to be mostly chemically mediated. Since the vertebrate/mammalian retina is derived embryologically from the brain, it would not be unreasonable to assume that substances which are proposed as chemical messengers in the brain have similar functions in the retina – what in fact appeared to be the case. Similarly, long-chain PUFAs, which are abundantly present in the brain, also occur in high amounts in the retina, with DHA showing distinctly high levels in this ocular structure (Wang and Anderson 1992).

Light-sensitive photoreceptor cells, i.e., rods and cones, occur in different proportions among vertebrates: rods dominate, or occur even exclusively, in nocturnal animals, and cones are present in retinas of daytime active species, whereas species active at night and daytime have both types of photoreceptors. Rods contain rhodopsin as a visual pigment and are very sensitive to light, being responsible for twilight and night vision; however they do not recognize colors. In contrast, cones are generally

less sensitive to light, possess at least three visual pigments, and are responsible for color and high-acuity vision. The human retina possesses both rods and cones – the latter cell type being densely packed in a small central region of the structure named the macula, or yellow macula; "yellow" refers to the color which is related to the presence in the macula of xanthophylls, known as macular pigments (MacLeish and Makino 2011; Kijlstra et al. 2012).

Depending on environmental lighting conditions, each day the human retina absorbs approximately 10^{12} to 10^{15} photons. Although such photons are informative in terms of vision, they can cause irreparable damage to the retina: brief exposure to bright light can produce immediate thermal injury, whereas exposure to light for an extended period of time may lead to photochemical damage, including RPE monolayer disruption (Besharse and Bok 2011; Hunter et al. 2012). Ambient radiation from the sun or from artificial light sources contains varying amounts of UV irradiation from A to C range (220–400 nm) and visible light (400–700 nm). The shorter the wavelength, the greater the energy and therefore the greater the potential for photochemical cell/tissue damage. However, although the longer wavelengths are less energetic, they can penetrate the eye more deeply (Roberts 2001). The blue region (400–500 nm) of the visible spectrum is of particular importance since it has a relatively high energy and can easily penetrate ocular tissues, including the neural retina with photoreceptors (Algvere et al. 2006).

The retina is a tissue with high metabolism and the highest oxygen consumption per unit weight of all human tissues (Yu and Cringle 2005). Oxygen and nutrients are supplied by two independent circulatory systems: the retina vessels and the choroid. The retina system, whose vessels penetrate as far as the outer plexiform layer, supplies oxygen and nutrients to the inner two-thirds of the retina. The outer third part of the retina physiologically remains completely avascular – yet it receives necessary nutrients and oxygen via the choriocapillaris of the choroidal system. High blood perfusion rate of the choroidal system delivers enough oxygen and nutrients for living and functioning of the RPE and photoreceptors (in fact, the choroidal blood flow far supersedes that required to nourish the neural retina) (Yu and Cringle 2005). Thus, the high oxygen tension in this tissue, together with high levels of light exposure and high levels of various lipid compounds, with DHA accounting for more than 80 % of PUFAs in photoreceptor disk membranes, promotes the production of free radicals leading to oxidative stress and makes the retina particularly susceptible to damage by ROS and by lipid-derived oxidative protein modifications (Wang et al. 1992; Cai et al. 2000; Roberts 2001; Anderson et al. 2010; Serhan and Petasis 2011; Cai and McGinnis 2012; Jarret and Boulton 2012; Klettner 2012; Salomon et al. 2011).

Thus, it is not surprising that the macula (and to a lesser extent the rest of the retina) is abundantly equipped with macular pigments (i.e., lutein, zeaxanthin, and lutein metabolite – meso-zeaxanthin), because, due to their specific physicochemical properties, they play an important protective role of a filter for harmful shortwave light and of a scavenger of free radicals (Wiktorowska-Owczarek and Nowak 2006; Kijlstra et al. 2012; Ozawa et al. 2012). In other words, macular pigments, together with other antioxidant defense systems, protect the central retina against possible deleterious effects of blue and near-UV light entering the eye, bisretinoids which are formed as by-products during the retinoid/visual cycle in the RPE–photoreceptor complex, and lipid (especially DHA) peroxidation-derived harmful products (Nowak 2013).

4 Age-Related Macular Degeneration: The Retina Disease with a Strong Background of Oxidative Stress and Lipid Peroxidation

As stated in the preceding section, the retina, due to its structural and physiological features, is particularly predisposed to produce ROS, whose excess can generate oxidative stress, and to lipid peroxidation and fragmentation to cytotoxic products (Jarret and Boulton 2012; Klettner 2012; Salomon et al. 2011). All these phenomena, together with natural aging process, are implicated in the pathogenesis of age-dependent disease named age-related macular degeneration (AMD) – one of the most common irreversible causes of severe loss of vision, including legal blindness (Hageman et al. 2001; Nowak 2006, 2012; Bhutto and Lutty 2012). The disease develops slowly and insidiously, with clinically meaningful symptoms seen in the elderly (60+ years) population. Clinically, AMD is divided into two forms: atrophic (dry form) and exudative (wet or neovascular form). Despite intensive basic and clinical research, its pathogenesis remains unclear, likely due to the multifactorial and age-dependent character. Pathological changes take place in the macular region of the retina comprising the choriocapillaris, Bruch's membrane, retinal pigment epithelial (RPE) cells, and photoreceptors. RPE undergo degeneration in the first place, followed by photoreceptors. Clinical features common for the two types of AMD (dry and wet form) include the presence of drusen (also recently identified pseudodrusen) as well as hypo- and/or hyperpigmentation of the RPE. More than 80 % of all people with intermediate and advanced AMD have the dry form, yet this form may progress to the wet form (15–20 %), which leads to significantly more vision loss (Nowak 2006; Anderson et al. 2010).

The pathophysiology of AMD is complex, and in addition to genetic predispositions and environmental determinants, at least four specific processes contribute to the development of the pathology (Fig. 1):

- Lipofuscinogenesis – generation of lipofuscin in the form of aggregates which are stored in lysosomal compartment of RPE; linkage to oxidative stress and lipid peroxidation, which especially in predisposed individuals may act as a "vicious circle" contributing to the progression of the disease.
- Drusogenesis – formation of extracellular deposits of insoluble material in the form of drusen localized between RPE and Bruch's membrane; pseudodrusen are located between photoreceptors and RPE monolayer. Taking the RPE monolayer as a reference structure, it can be said that drusen are situated beneath RPE, whereas pseudodrusen are above RPE. Small and non-numerous drusen (so-called hard drusen) may occur in healthy eyes, yet their numerous population and especially their bigger size or confluent character are the hallmark of AMD.
- Chronic inflammation – named parainflammation, as there are no signs of typical inflammation process.
- Choroidal neovascularization (CNV) – choriocapillaris-derived pathological new vessels are formed, which break through the Bruch's membrane and direct toward the RPE–photoreceptor complex; the process contributes to the wet, neovascular (exudative), form of AMD.

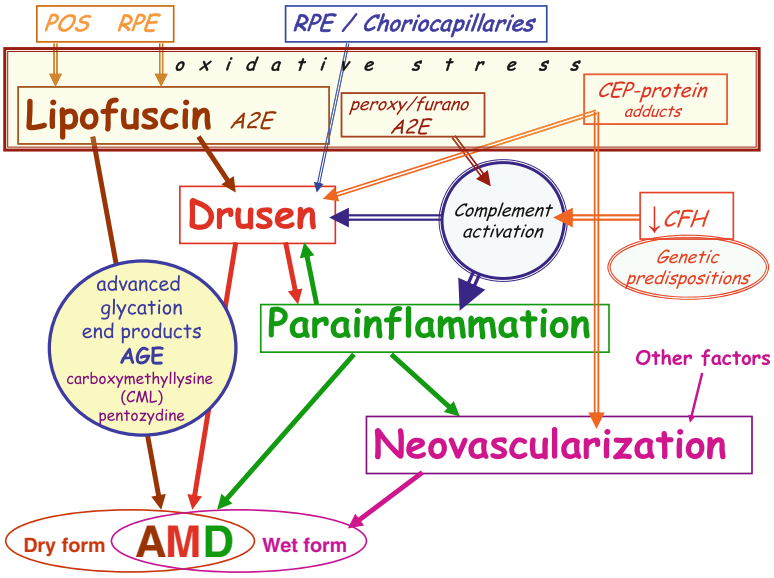

Fig. 1 Four principal processes in the pathogenesis of AMD: lipofuscin formation, drusen formation, local "atypical" inflammation (parainflammation), and neovascularization. Explanations in the text. Abbreviations: *POS* photoreceptor outer segment, *RPE* retinal pigment epithelium, *A2E* *N*-retinylidene-*N*-retinylethanolamine, *CEP* carboxyethylpyrrole, *CFH* complement factor H

It is generally accepted that the impairment of RPE cell function is an early and crucial event in the molecular pathways leading to photoreceptor degeneration and clinically relevant AMD (Nowak 2006; Thurman et al. 2009; Krohne et al. 2010; Kinnunen et al. 2012; Mettu et al. 2012). Such view has its rationale in the fact that RPE serves a variety of metabolic and supportive functions that are of vital importance for retinal photoreceptors, including maintenance of the blood–retina barrier, participation in the visual cycle (uptake, processing, transport, release of vitamin A derivatives), and phagocytic uptake and degradation of constantly shed apical photoreceptor outer segments (POS). One of driving forces of the RPE dysfunction is an age-dependent phagocytic and metabolic insufficiency of postmitotic RPE cells (lysosomal failure hypothesis). This leads to progressive accumulation of lipofuscin (or "age pigment") granules/aggregates composed mostly of lipids (≈50 %) and proteins (≈44 %) of phagosomal, lysosomal, and photoreceptor origin, modified to varying degree by oxidative processes as a result of both exposure to visible and UVA light and high oxygen levels in the eye (Sparrow and Boulton 2005).

Although lipofuscin accumulates in RPE of healthy eyes – what can be imaged ophthalmoscopically as the retina fundus autofluorescence (the natural fluorescence of retina) (Sparrow and Boulton 2005; Sparrow et al. 2012) – an excess of accumulating age pigment, as a highly reactive material, may lead to harmful consequences. And this is because of the presence of bisretinoid compounds which, upon illumination, are capable of taking up oxygen, the process increasing with age, and producing reactive oxygen species, including singlet oxygen (Gaillard et al. 1995;

Rozanowska et al. 1995, 2004). Photooxidation of bisretinoids results in the formation of epoxides, furanoid moieties, and endoperoxides (Kim et al. 2007). It has been demonstrated that photoexposure of RPE containing bisretinoid A2E led to cell damage and death (Schutt et al. 2000; Sparrow et al. 2000). In addition to bisretinoid fluorophores, lipofuscin constituents include also DHA-derived carboxyethylpyrrole (CEP) modifications of proteins known as CEP-protein adducts (Salomon et al. 2011). Due to its specific, abovementioned ingredients (bisretinoids in particular) that are absent from non-retinal lipofuscins, the term "retinal lipofuscin" or "RPE lipofuscin" is widely used to stress peculiarity of the age pigment accumulated in the RPE cells.

5 Bisretinoid Fluorophores of the Retinal Lipofuscin

5.1 Formation of Bisretinoids

At least 25 bisretinoid fluorophores originating in photoreceptor cells and resulting from reactions of all-*trans*-retinal (AT-RAL) have been identified until now (Murdaugh et al. 2011; Sparrow et al. 2012). Well-characterized members of such bisretinoid family include: A2-PE, A2E, A2-DHP-PE, A2-GPE, and AT-RAL-dimer. A2-PE refers to the compound possessing two AT-RAL molecules, i.e., A2, conjugated with phosphatidylethanolamine (PE); A2E consists of two AT-RAL-derived arms connected through a pyridinium ring (in other words, hydrolysis of the phosphate ester of A2-PE yields A2E); A2-DHP-PE (A2-dihydropyridine-PE) and A2-GPE (A2-glycerophosphoethanolamine). It is noted that the bisretinoid-starting compound, i.e., AT-RAL, is a physiological molecule that is formed in the visual cycle via conformational change – photoconversion – of photon absorbing of visual pigment cofactor, i.e., 11-*cis*-retinal [11-*cis*-RAL] (Fig. 2). A proposed synthesis of some bisretinoids is depicted below (Sparrow et al. 2012):

11-*cis*-Retinal + *hv* → **all-*trans*-retinal** (AT-RAL) + phosphatidylethanolamine (PE)
→ *N*-retinylidene-PE Schiff base (NRPE) → // [1,6] H-shift → tautomer x // + AT-RAL
→ bisretinoid phosphatidyl-dihydropyridinium (dihydropyridinium-A2-PE)
→ //automatic loss of 1 H^+//→A2-dihydropyridine-PE (A2-DHP-PE)
→ //automatic loss of 2 H^+// → phosphatidyl-pyridynium bisretinoid (A2-PE)
→ // phosphate hydrolysis // → **A2E** or A2-GPE

Another Pathway
11-*cis*-Retinal + *hv* → **all-*trans*-retinal** (AT-RAL) + phosphatidylethanolamine (PE)
→ *N*-retinylidene-PE Schiff base (NRPE) → // [1,6] H-shift → tautomer x // + AT-RAL
→ all-*trans*-retinal-dimer + PE → all-*trans*-retinal-dimer-PE

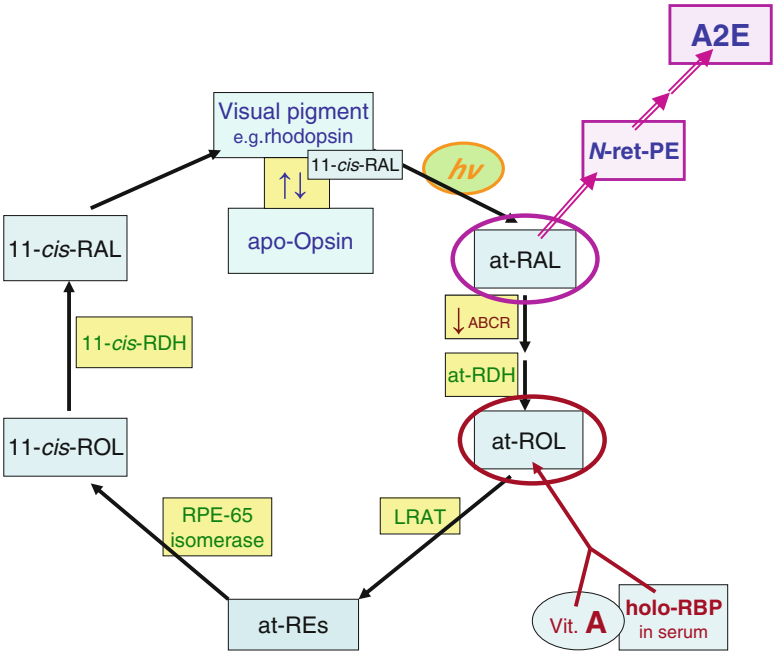

Fig. 2 Retinoid cycle of the visual cascade and generation of bisretinoid A2E. Explanations in the text. Abbreviations: *11-cis-RAL* 11-*cis*-retinal, *hv* photon of light absorbed by visual pigment, *at-TAL* all-*trans*-retinal, *ABCR* ATP-binding cassette transporter, *at-RDH* all-*trans* retinal dehydrogenase, *at-ROL* all-trans-retinol, *LRAT* lecithin/retinol acyl transferase, *RBP* retinal binding protein, *at-REs* all-trans-retinal esters, *N-ret-PE* *N*-retinylidene-phosphatidylethanolamine, other explanations as in Fig. 1

5.2 *Biological Activity of Bisretinoids*

A2E (*N*-retinylidene-*N*-retinylethanolamine) – the first RPE lipofuscin constituent to be described – was thoroughly analyzed for its biological activity in general and for its harmful effects in particular. Keeping in mind that A2E, a lipofuscin constituent, is formed practically in each human retina as a by-product of the visual cycle, the experimental findings are really impressive and simultaneously worrying. For example, A2E accumulates in mitochondrial membranes of cultured RPE cells becoming a proapoptotic molecule acting via a mitochondrial pathway. A possible explanation of this observation is that upon reaching a critical intralysosomal concentration, A2E is released from the lysosome and then specifically targets the outer mitochondrial membrane, thereby initiating apoptosis of the RPE cell (Schutt et al. 2007). Furthermore, A2E absorbs visible blue light and in consequence is able to generate singlet oxygen, superoxide anion, hydrogen peroxide, or lipid hydroperoxides – all of which may act as an injury stimulus to RPE cells (Beatty et al. 2000; Zhou et al. 2006; Wu et al. 2010). Other bisretinoids are also toxic. Interestingly, unconjugated all-*trans*-retinal-dimer was shown to be even more potent generator

of singlet oxygen than A2E, and photooxidized A2E derivatives (resulting from spontaneous photooxidation at C=C double bonds), especially furano- and peroxy-photoproducts, appeared to be capable of effectively activating complement system (Jang et al. 2005; Sparrow 2010), which is an effector mechanism of the innate immune system and plays a major role in shaping the adaptive immune response (Trouw and Daha 2011). Thus, the photoreceptor-derived lipofuscin-stored bisretinoids predispose or sensitize the macula to pathological reactions, as generation of the low-grade complement activation may lead to chronic inflammatory processes in the form of so-called parainflammation (Xu et al. 2009; Anderson et al. 2010; Khandhadia et al. 2012). In fact, all stages of AMD of both dry and wet form are associated with inflammatory cells, notably macrophages, neutrophils, or dendritic cells (Hageman et al. 2001; Xu et al. 2009; Anderson et al. 2010; Issa et al. 2011). In addition, bisretinoid photodegradation may also lead to generation of highly cytotoxic dicarbonyl molecules responsible for advanced glycation end product (AGE) modification of proteins, which are present in drusen (Wu et al. 2010) and which may contribute to age-related pathologies not only in the retina but also in other parts of the CNS (notably the brain), as well as in the peripheral tissues (Uchiki et al. 2012). Taken collectively, the mentioned mechanisms may all contribute to the development of pathological states in various body organs, including ocular pathology such as AMD (Fig. 3).

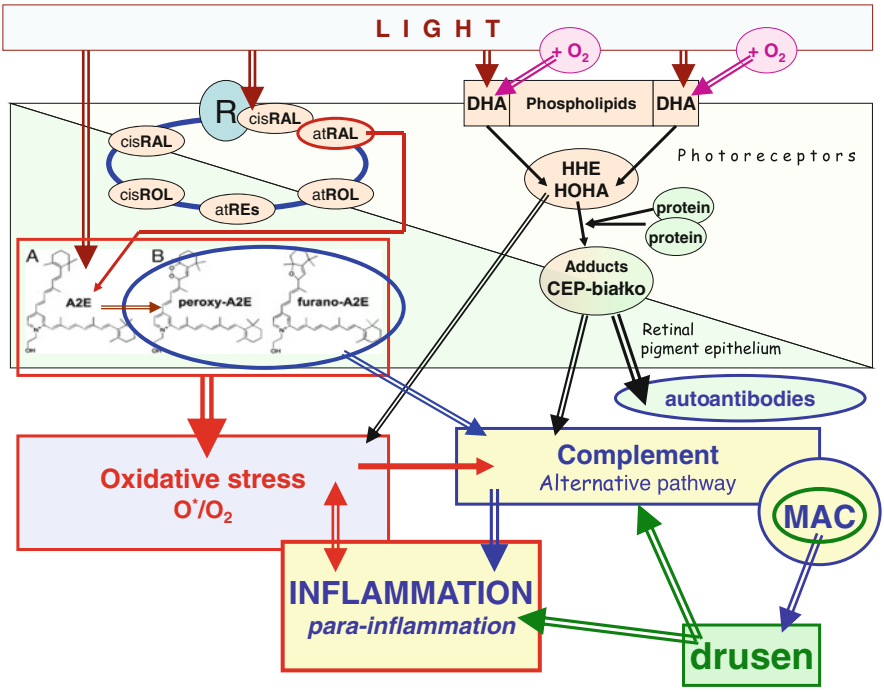

Fig. 3 Molecular and cellular processes occurring at early stages of AMD pathogenesis. Explanations in the text. Abbreviations: *HHE* 4-hydroxyhexenal, *HOHA* 4-hydroxy-7-oxyhept-5-enoic acid, *MAC* membrane attack complex; other explanations as in Fig. 2

6 PUFA-Derived Carboxyalkylpyrroles

Carboxyalkylpyrrole (CAP)-protein adducts derive from PUFAs subjected to peroxidation. Upon oxidation, PUFAs firstly undergo fragmentation to smaller molecules, such as 4-hydroxynonenal (HNE), 4-hydroxyhexenal (HHE), malondialdehyde (MDA), or an array of hydroxy-ω-oxoalkenoic acids, including HODA, HOOA, and HOHA. The generated PUFA truncated molecules can next form covalent adduction with proteins resulting in alkyl- or carboxyalkylpyrrole modifications (Lu et al. 2009). Alkylpyrrole modifications include such adducts as pentylpyrrole (PP)-protein or ethylpyrrole (EP)-protein, whereas carboxyalkylpyrrole (CAP) modifications are: CHP-protein, CPP-protein, and CEP-protein (Table 1).

The idea that CAP modifications of proteins may contribute to the development of some CNS diseases has its roots in observations made at the end of the last century (Wang et al. 1992; Alvarez et al. 1994; Sayre et al. 1996; Kaur et al. 1997). It has been shown that a γ-hydroxyalkenal product of phospholipid oxidation, i.e., HNE, can form covalent adducts that incorporate the ε-amino group of protein lysyl residues in pentylpyrrole (PP) modifications that accumulate in neurons of patients with Alzheimer's disease and in the blood of individuals with atherosclerosis. Other studies have shown the in vivo formation of carboxyheptylpyrrole (CHP)-, carboxypropylpyrrole (CPP)-, and carboxyethylpyrrole (CEP)-protein modifications from oxidized PUFA (linolenic, arachidonic, and docosahexaenoic acid, respectively)-containing phospholipids. Based on these observations, it was suggested that the formation of carboxyalkylpyrrole (CAP)-protein modifications should be greater in tissues containing high levels of respective PUFAs.

As mentioned, the retina is the DHA-richest tissue in human body. The distribution of this fatty acid is however uneven throughout the tissue, showing highest concentrations in membranes of the photoreceptor outer segments and the RPE (Wang and Anderson 1992; Alvarez et al. 1994). Thus, high amounts of DHA, together with lipofuscin-stored bisretinoids and high supply of oxygen, makes this retina region (which is responsible for capturing photons, being often endangered from intensive light irradiation) particularly suitable for generation of ROS and oxidation-forced DHA metabolites, both endowed with pathogenic potential capable of inducing oxidative stress, complement activation (with its terminal product responsible for cell lysis, i.e., membrane attack complex (MAC)), and then

Table 1 Polyunsaturated fatty acids (PUFA) peroxidation leads to generation of carboxyalkylpyrrole-protein adducts

PUFA	Hydroxy-ω-oxoalkenoic acid	Adduct carboxyalkylpyrrole-protein
LA, ALA	→ HODA + protein	→ CHP-protein
GLA, ARA, EPA	→ HOOA + protein	→ CPP-protein
DHA	→ HOHA + protein	→ CEP-protein

LA linoleic acid, *ALA* α-linolenic acid, *GLA* gamma-linolenic acid, *ARA* arachidonic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *HODA* 9-hydroxy-12-oxodec-10-enoic acid, *HOOA* 5-hydroxy-8-oxooct-6-enoic acid, *HOHA (E)*-4-hydroxy-7-oxohept-5-enoic acid, *CHP* 2-(ω-carboxyheptyl)pyrrole, *CPP* 2-(ω-carboxypropyl)pyrrole, *CEP* 2(ω-carboxyethyl)pyrrole

inflammation – three interrelated phenomena that work as self-driving mechanism with damaging effects on cells and tissues (Fig. 3).

Coming back to DHA peroxidation-driven changes, it is interesting to mention that the formation of 4-hydroxy-7-oxyhept-5-enoic acid (HOHA) and then carboxyethylpyrrole (CEP)-protein adducts can take place in many tissues, including melanoma, aging vasculature or healing wounds, as well as autistic brain (Evans et al. 2008; Lu et al. 2009; West et al. 2010); however, their generation in the retina, just due to high levels of DHA, is exceptionally prominent.

7 CEP-Protein Adducts in AMD Patients

Using rabbit polyclonal anti-CEP antibody, the presence of intense CEP immunoreactivity was found in the photoreceptor outer segments (POS) and RPE, and lighter immunoreactivity, in the inner plexiform layer of mouse retina. Similar findings were obtained in studies on whole human retina and samples of drusen-containing RPE/Bruch's membrane/choroid tissue, yet consistently more CEP immunoreactivity was present in the AMD tissue than in the normal (healthy) retina (Crabb et al. 2002; Gu et al. 2003a, b).

Since CEP-protein adducts were shown to be formed more abundantly in ocular tissues (drusen, Bruch's membrane) from AMD patients than from normal human donors, it has been hypothesized that they may be involved in the pathogenesis of this ocular disease (Crabb et al. 2002; Hollyfield et al. 2003) (see Fig. 3).

CEP immunoreactivity was detected not only in human retina, but also in human plasma, with values being again significantly higher in the plasma of AMD subjects than in the plasma samples of both younger and older healthy subjects (Gu et al. 2003a). Interestingly, the plasma CEP immunoreactivity positively correlated with CEP autoantibody titer (Gu et al. 2003a), indicating that CEP behaves as an antigen which generates production of specific anti-CEP antibodies. The immune-mediated events related to immunogenic CEP-protein adducts, which in AMD patients are probably generated through many decades, may contribute as one of many molecular links to the development of AMD pathology (Fig. 3).

Recent experiments carried out on mice immunized with CEP-modified mouse serum albumin (CEP-MSA) and Freund's adjuvant (in an attempt to raise the level of sensitivity to endogenously generated CEP) have shown that the retinas of such animals produced changes similar to those seen in retinas of AMD-suffering peoples (Hollyfield et al. 2008, 2010). The observed changes included: accumulation of drusen below the RPE monolayer, swollen Bruch's membrane, fixation of complement-C3d product in Bruch's membrane, lesions in the RPE cells, and decreased electrophysiological responses to light. In addition, in mice with laser-induced rupture of Bruch's membrane, subretinal injection of CEP-MSA significantly augmented choroidal neovascularization (CNV), the effect being similar to that produced by injections of vascular endothelial growth factor (VEGF), a major proangiogenic factor (Ebrahem et al. 2006). The in vivo angiogenic properties of

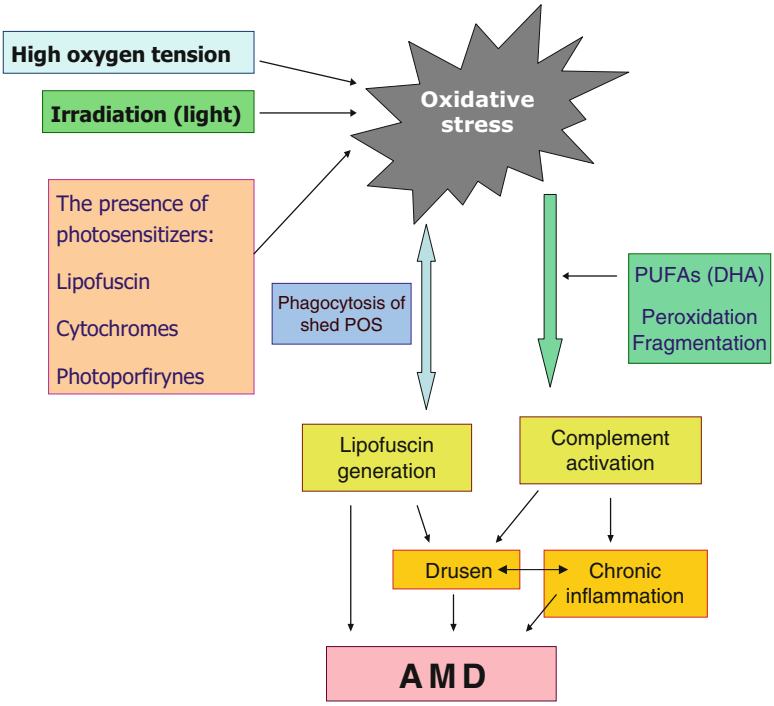

Fig. 4 Formation and characteristics of carboxyethylpyrrole (CEP)-protein adduct

the "human" adduct CEP-HSA were demonstrated in two widely used angiogenesis model systems: the chick chorioallantoic membrane and rat corneal micropocket assay. The results showed CEP-HSA to be highly potent (active at low picomolar amounts) inducer of neovascularization that utilized VEGF-independent pathways (Ebrahem et al. 2006). In conclusion, CEP-MSA has the potential, in mice, to produce a full spectrum AMD, including both dry (degenerative–atrophic) and wet (neovascular) form, raising a possibility that the human analog (CEP-HSA), formed endogenously from oxidation of DHA-containing lipids, will display similar profile of activity in humans (Fig. 4), being able to produce pro-AMD changes, together with CNV at least in some patients (Fig. 1).

8 CEP as an Inducer of Angiogenesis

The ability of CEP-protein adducts to induce angiogenesis is not restricted to ocular neovascularization, but seems to be a phenomenon of general importance. Experiments carried out on different model systems, such as tumor (melanoma) implantation and growth, hind limb ischemia model (ligature of femoral artery),

wound healing model, as well as tube formation assay and Matrigel plug assay, clearly demonstrated the angiogenic potential of CEP-protein adducts, both in a positive (physiologic or therapeutic) and negative (pathologic) sense, similar to angiogenic profile of VEGF. The mechanisms underlying VEGF- and CEP-driven angiogenesis are different – VEGF realizes its action via specific VEGF receptor-mediated signaling pathway, whereas CEP (and also CPP)-induced angiogenesis involves activation of toll-like receptor type 2 (TLR2), but not TLR4 or scavenger receptors on endothelial cells (West et al. 2010). These observations may have important practical consequences, also in ophthalmology (AMD), since CNV occurring in wet form AMD is currently treated with anti-VEGF drugs: pegaptanib (aptamer), ranibizumab and bevacizumab (monoclonal antibodies), or aflibercept (soluble decoy receptor). Yet, CNV resistant to anti-VEGF therapy is not unusual in AMD patients, indicating in such cases the role of VEGF-independent mechanism(s). Therefore, it is not unlikely that in such VEGF-independent CNV in AMD patients, CEP oxidative protein modifications and TLR2-directed signaling pathway may operate – a suggestion that is possible (based on animals' data), yet requiring experimental support for its validity in humans.

9 Concluding Remarks and Important Questions

Numerous experimental and clinical data gathered over many decades indicate that oxidative stress may contribute to various human pathologies, including CNS malfunctions and diseases, especially those with degenerative and/or inflammatory background (Cai and McGinnis 2012). In addition to oxidative stress, other molecular risk factors, e.g., lipofuscin-stored photoreactive bisretinoids and PUFA-derived metabolites and protein modifications, may also be of importance, as already demonstrated in some human diseases (Salomon et al. 2011; Jarret and Boulton 2012; Sparrow et al. 2012). The mentioned phenomena usually play in concert to induce various pathological situations, including ocular disease – AMD (Fig. 5).

However, although both lipofuscin and oxidation fragments of long-chain PUFAs can be formed in different tissues even in physiology (without obvious pathological symptoms), their pathogenic potential reveals when they are formed in excess or in the presence of some additional factors favoring or strengthening pro-pathogenic mechanisms. Substantial amounts of such potentially pathogenic compounds depend on cell/tissue levels of respective substrates, which should assure an intense formation of given products. The CNS tissue offers and under specific conditions creates a suitable milieu for generation of both oxidative stress and the end products of lipid oxidation.

The retina lipofuscin varies from lipofuscin formed and accumulated in neurons, cardiomyocytes, or skeletal muscle cells, and the difference lies in that the RPE age pigment contains an array of retinoids originating from the visual/retinoid cycle, which are absent from other age pigments. Retinoids, being highly reactive molecules, can spontaneously fuse together to form various bisretinoids (e.g., A2E)

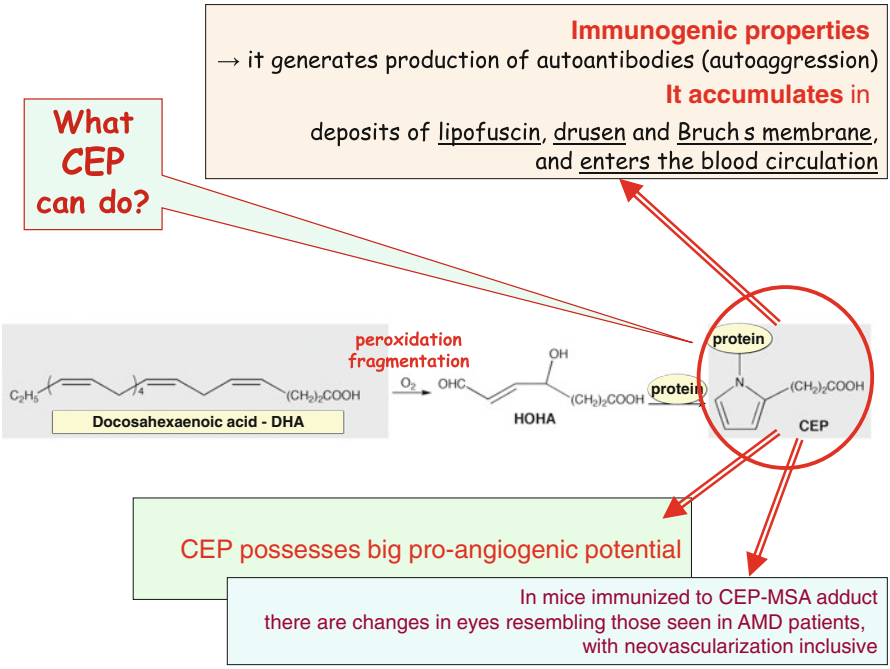

Fig. 5 Key role of oxidative stress in AMD pathogenesis

endowed with photocytotoxic potential. Concerning PUFA-derived oxidative protein modifications, some tissues due to distinctly high levels of a given fatty acid substrate will generate more oxidatively truncated potentially harmful compounds than other tissues. In this respect, the retina is an exception among different CNS structures in that its photoreceptors contain comparatively huge amounts of DHA. This, together with high oxygen supply and high levels of light exposure, results in its natural predisposition to ROS generation and PUFAs peroxidation. For these reasons, the retina can be considered an excellent CNS model tissue with which to show what may happen in the CNS under stressful conditions.

In this article a focus has been made on vision-threatening pathology such as age-related macular degeneration (AMD), which, in its molecular background, has many similarities to age-dependent brain malfunctions or disorders. One of such common features may be generation of PUFA-derived oxidative protein modifications, with CEP-protein adducts being the best example. In AMD patients, the serum levels of CEP-protein adducts and anti-CEP-autoantibodies were so pronounced that they were proposed to serve an early biomarker of this retina disease (Crabb et al. 2002; Hollyfield et al. 2003; Lu et al. 2009; Salomon et al. 2011). Perhaps such biomarkers will help to substantiate early diagnosis of developing AMD, since at early stages of the disease clinical symptoms are either absent or unclear or non-unequivocal. However, due to a multifactorial and complex nature of AMD (with genetic, immunological, and environmental determinants), there is still

an open question whether CEP-protein adducts, as well as cytotoxic bisretinoids, are really the primary cause of the disease. Current views seem to favor the concept implicating their role in the pathogenesis in AMD, but direct evidence supporting their role as major causative factors is lacking, and one cannot exclude a possibility that these molecules simply accompany the disease – evidently the problem awaits elucidation (Bhutto and Lutty 2012; Jarret and Boulton 2012; Mettu et al. 2012; Plafker et al. 2012; Sparrow et al. 2012).

There are several unanswered issues/questions concerning oxidative stress and lipid peroxidation-driven compounds. *Firstly*, it is not clear whether under in vivo conditions oxidative stress results from decreased activity of endogenous antioxidative defense systems or is simply a manifestation of accelerated aging process. *Secondly*, potentially harmful bisretinoids are formed in photoreceptors as by-products in the visual cycle and are stored in lipofuscin granules accumulating in lysosomal compartment of RPE cells. As lipofuscin (age pigment) and its formation, i.e., lipofuscinogenesis, age-dependently occur in each retina (including healthy eyes), should they be considered a strictly pathological process? *Thirdly*, long-chain PUFAs, particularly DHA in the brain and retina, are physiologically indispensable constituents of all plasma membranes in living organisms; however, based on experimental findings discussed in this survey, one could formulate a conclusion: the more unsaturated the fatty acid(s), the more problems in terms of possible pathology, of which the retinal disease – AMD – may be a good example. Of many PUFAs, DHA is the most complex compound not only in its chemical structure but also, or first of all, in its biological activity. DHA is a multifunctional molecule, and this aspect deserves short comment.

DHA can be readily oxidized, but simultaneously DHA is a substrate for neuroprotectin D1 and an array of anti-inflammatory proresolving mediators.

In this review, long-chain PUFAs were presented as substrates for generation of oxidative products such as hydroxy-ω-oxoalkenoic acids (HOAAs) and carboxyalkylpyrroles (CAPs), the latter being endowed with mainly pathogenic potential (though its role as an angiogenic factor in wound healing and tissue recovery should be considered a beneficial activity (West et al. 2010)). Concerning DHA, the respective compounds are HOHA and CEP, the latter being likely involved in the pathogenesis of several disorders both in the periphery, e.g., artheriosclerosis (Kaur et al. 1997), and in the CNS, e.g., Alzheimer's disease, autism, and AMD (Salomon et al. 2011). Connecting CEP-protein adducts and AMD, a logical relationship can be suggested: the more CEP-protein adducts generated in the photoreceptor-RPE complex, the faster AMD progression and the worse clinical prognosis.

Yet, the retinal DHA obviously has two opposite faces: a negative face was discussed above. A positive face is connected, firstly, to its role as a plasma/cell membrane constituent and, secondly, to the fact that just this fatty acid is a substrate for neuroprotectin D1 (NPD1) and an array of anti-inflammatory proresolving mediators (Bazan 2006; Serhan and Petasis 2011; Shinohara et al. 2012). The former links DHA mainly to endowing brain/photoreceptor membrane domains with physical properties that positively contribute to functional modulation (e.g., optimization of membrane fluidity or maintenance of retinal integrity), while the latter refers to

DHA-derived products such as resolvins (RvD1–RvD4, AT-RvD1–AT-RvD4; resolvins can also originate from EPA forming E-series resolvins, RvE1 and RvE2), maresins, and already mentioned NPD1. Resolvins and maresins are endogenous regulators of inflammatory process which physiologically extinguish or resolve acute phase of inflammation – hence their name proresolving mediators. NPD1 was originally discovered as a neuroprotective lipid mediator acting against harmful factors/situations such as H_2O_2/TNFα oxidative stress-triggered apoptotic RPE DNA damage (Mukherjee et al. 2004). The biosynthetic pathway for NPD1 is as follows:

DHA → [15-lipoxygenase + O_2] → 17*S*-HpDHA → [–H_2O] → 16*S*,17*S*-epoxide intermediate → [hydrolase + H_2O] → 10R,17S-dihydro-docosa-4Z,7Z,11E,13E, 15Z,19Z-hexaenoic acid (neuroprotectin D1/protectin D1, depending on the site of formation)

NPD1 displays an array of biological effects. In addition to the effect mentioned above, it appeared also to upregulate antiapoptotic proteins (e.g., Bcl-2) and to decrease proapoptotic Bax and Bad expression (Mukherjee et al. 2004). Furthermore, DHA-derived NPD1 appeared to counteract proinflammatory cell-damaging events triggered by multiple factors not only in the diseased retina but also in the Alzheimer brain (Mukherjee et al. 2007; Bazan 2009; Lukiw 2009; Bazan et al. 2010).

The retina molecular picture of pathological consequences of oxidative stress and DHA peroxidation product activity – does it predict the situation suitable for brain mechanisms?

Although the present chapter focused on oxidative stress-driven retinal pathology such as AMD, the first part of the paper title announces a wider perspective embracing not only the particular light capturing and processing CNS structure, i.e., the retina, but also other CNS structures, specifically the brain. In fact, practically everything that was written on harmful effects of oxidative stress and polyunsaturated lipid, especially DHA peroxidation products on retinal physiology, may equally well have an impact on brain tissue and some neuropsychiatric disorders (Dröge 2002; Valko et al. 2007; Lukiw 2009; Hroudova and Fisar 2011; Pandya et al. 2012). The mentioned bisretinoids, such as A2E, seem to be the retina-specific photocytotoxic chemicals, which are formed locally and are active locally, having probably no chance to enter the circulation and affect distant CNS structures. Nevertheless, the dissection of a molecular background of a highly complex AMD pathology provides valuable guidelines on biochemical reactions that may occur in the brain under condition of oxidative stress and resultant cellular pathogenic events.

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Therapeutic Aspects

Antioxidant Interventions in Neuropsychiatric Disorders

Anilkumar Pillai and Jeffrey K. Yao

Abbreviations

$^1O_2$	Singlet oxygen
AA	Arachidonic acid
BDNF	Brain-derived neurotrophic factor
BPRS	Brief psychiatric rating scale
CAD	Coronary artery disease
CAT	Catalase
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ethyl-EPA	Ethyl-eicosapentaenoic acid
GPx	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
IL-1	Interleukin-1
IL-6	Interleukin-6

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LTB_4	Leukotriene B_4
MADRS	Montgomery–Asberg Depression Rating Scale
MDD	Major depressive disorder
NAC	N-Acetylcysteine
NGF	Nerve growth factor
NO^-	Nitric oxide
NO^-_2	Nitrate
NO^-_3	Nitrite
NO•	Nitric oxide
O_2^-	Superoxide anion
OH•	Hydroxyl
PANSS	Positive and Negative Syndrome Scale
PGE_2	Prostaglandin E_2
PUFAs	Polyunsaturated fatty acids
RBC	Red blood cell
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAS	Total antioxidant status
TD	Tardive dyskinesia
TNF-α	Tumor necrosis factor-α

1 Introduction

Oxidative stress is defined as higher cellular levels of reactive oxygen species (ROS) than the cellular antioxidant defense. Brain consumes approximately 20 % of the total amount of oxygen in the body. But the enhanced metabolic rate in the brain leads to the generation of excessive levels of ROS. Mostly, oxidative stress-mediated damage of the brain occurs due to higher lipid peroxidation in the cerebrospinal fluid and plasma along with reduced membrane polyunsaturated fatty acids (PUFAs) in the brain and red blood cell (RBC) membranes (Mahadik et al. 2001). Free radicals are produced through a variety of physiological and pathological processes (Fig. 1). The radicals generated from molecular oxygen are generally known as ROS, which include superoxide anion (O_2^-), hydroxyl (OH•), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and nitric oxide (NO•).

Oxidative stress occurs when the production of ROS exceeds the natural antioxidant defense mechanisms, causing damage to macromolecules such as DNA, proteins, and lipids. Cells are protected by antioxidant defense mechanisms that remove these free radicals to prevent oxidative damage. The antioxidant system comprises of different types of functional components such as enzymatic and nonenzymatic antioxidants (Fig. 1). The enzymatic antioxidants comprise of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase, and glutathione S transferase. The nonenzymatic antioxidants include reduced glutathione, vitamin C, vitamin E (α-tocopherol), uric acid, carotenoids, flavonoids, ubiquinol, etc.

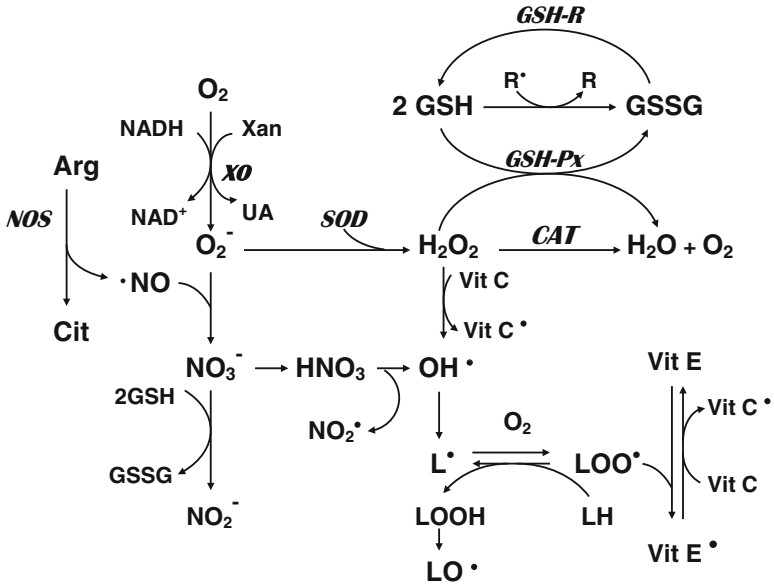

Fig. 1 Possible mechanisms involving production and removal of oxygen and nitrogen free radicals in mammalian cells (reprinted with permission from Yao and Keshavan 2011). Molecular oxygen can be converted to superoxide radicals (O_2^{-}•) in the presence of xanthine oxidase (XO). Subsequently, superoxide dismutase (SOD) catalyzes the conversion of superoxide radicals to hydrogen peroxide (H_2O_2). Catalase (CAT) and glutathione peroxidase (GSH-Px) convert hydrogen peroxide to water. Glutathione (GSH) is utilized by GSH-Px to yield the oxidized form of glutathione (GSSG), which is converted back to GSH by glutathione reductase (GR). Hydrogen peroxide is susceptible to autoxidation to form hydroxyl radicals (OH•), particularly in the presence of metal catalysts such as iron. In addition, nitric oxide (NO), which is the product of a five-electron oxidation of the amino acid L-arginine, can also produce hydroxyl radicals as well as nitrogen dioxide radical. On the other hand, **α**-tocopherol (vitamin E) has the ability to inhibit lipid peroxidation as a chain-breaking antioxidant. Vitamin E radicals can be recycled back to their native form by ascorbic acid (vitamin C)

2 Oxidative Stress in Schizophrenia

Studies suggest that genetic factors, neuronal maldevelopment, impaired neurotransmission, viral infections, environmental factors, and stressors are the main triggers of schizophrenia (Kendler 2003; Jakob and Beckmann 1986; Thome et al. 1998; Carlsson et al. 1999; Kornhuber and Weller 1994; Pearce 2001). Evidence also indicates that mitochondrial pathology and oxidative stress may be the most critical components in the pathophysiology of schizophrenia (Goff et al. 1995; Whatley et al. 1998; Ben-Shachar and Laifenfeld 2004; Bubber et al. 2004; Yao and Keshavan 2011). Lipid peroxidation products, a marker for oxidative stress-mediated damage, were found to be increased in the cerebrospinal fluid and plasma (Mahadik et al. 2001). It has been also observed that oxidative damage leads to

reduced membrane PUFAs in the brain and RBC membranes (Mahadik et al. 2001). Moreover, levels of nitric oxide and superoxides (NO^- and O_2^-) as determined indirectly as nitrate (NO^-_2) and nitrite (NO^-_3) were higher in serum (Taneli et al. 2004), RBC (Herken et al. 2001), and postmortem brain (Yao et al. 2004) samples from schizophrenia subjects.

A significant reduction in plasma total antioxidant status (TAS) has been found in patients with chronic schizophrenia (Yao et al. 1998a) as well as first-episode drug-naive patients with schizophrenia (Li et al. 2011). Individual plasma antioxidants, albumin, bilirubin (Yao et al. 2000), and uric acid (Yao et al. 1998c) were also found lower in schizophrenia subjects. Moreover, decreases in plasma levels of total and reduced GSH, along with altered antioxidant enzyme activities, have been reported in drug-naive first-episode patients with schizophrenia when compared with healthy control subjects (Raffa et al. 2011). Suboticanec et al. (1990) have demonstrated that both plasma and urinary vitamin C levels were lower in chronic schizophrenia subjects, relative to normal controls, even after controlling for diet. McCreadie et al. (1995) found lower ratios of vitamin E to cholesterol in schizophrenic patients compared with normal control subjects. Later, Brown et al. (1998) also reported decreased lipid-corrected vitamin E levels in schizophrenic patients with tardive dyskinesia, relative to healthy controls, but not in patients without dyskinesia. Decreased levels of GSH, ascorbic acid, and plasma vitamin E levels were also found in erythrocytes from schizophrenic patients compared with healthy subjects (Surapaneni 2007).

Increased SOD activities have been reported in RBC of schizophrenic patients (Abdalla et al. 1986; Reddy et al. 1991; Yao et al. 1998b). A recent study did not find any change in plasma SOD activity in drug-naive first-episode schizophrenic patients compared to control subjects (Raffa et al. 2011). However, a meta-analysis showed that SOD activity was significantly decreased in the disorganized type of schizophrenia patients versus healthy controls (Zhang et al. 2010). A significant increase in GPx activity but decrease in CAT activity was found in plasma samples from drug-naive first-episode schizophrenic patients compared to control subjects (Raffa et al. 2011). Moreover, GPx activity was found to be lower in neuroleptic-treated chronic schizophrenia patients (Stoklasova et al. 1986), in drug-free female schizophrenia patients (Abdalla et al. 1986), and in neuroleptic-naive psychotic children (Golse et al. 1977). Schizophrenia patients had significantly lower RBC GPx activity than controls (Othmen et al. 2008). Zhang et al. (1998) have reported higher plasma GPx activities in long-term neuroleptic-free as well as neuroleptic-naïve schizophrenic patients, while Yao et al. (1999) did not find any significant difference between chronic schizophrenic patients and normal subjects. Decrease in CAT activity was also observed in clinically stable patients with schizophrenia and their unaffected siblings (Othmen et al. 2008). However, CAT activity was found unchanged in erythrocytes and plasma of drug-free schizophrenic patients (Yao et al. 1998b; Yao et al. 1999). A recent meta-analysis reported no significant difference in CAT activity between schizophrenia and control subjects (Zhang et al. 2010).

Inflammatory responses induced by proinflammatory T cells provide a source of free radicals that leads to damage of proteins, lipids, and nucleic acids in neuronal cells. Increased cytokine (IL-1β, IL-6, TNF-α) levels are known to generate ROS in

the cells. A microarray gene analysis of T cells from schizophrenia patients showed prominent transcript alterations in cell cycle machinery, intracellular signaling, metabolism, and oxidative stress, suggesting that altered T cell response might induce oxidative stress in schizophrenia (Craddock et al. 2007).

3 Oxidative Stress in Bipolar Disorder

Bipolar disorder is a major mood disorder affecting an estimated 1–3 % of the population (Belmaker 2004; Kupfer 2005; Merikangas et al. 2007). Oxidative stress has also been implicated in the pathophysiology of bipolar disorder. Several studies have reported that bipolar disorder patients have significant alterations in antioxidant enzymes, lipid peroxidation, and nitric oxide levels; however, the results are conflicting. A meta-analysis by Andreazza et al. (2008) found that bipolar disorder patients have increased lipid peroxidation and increased NO levels but failed to find significant changes in GPx activity in bipolar disorder (Andreazza et al. 2009). An earlier study has found lower levels of SOD and catalase in bipolar disorder patients (Ranjekar et al. 2003). However, the above data was not in agreement with the findings by Kuloglu et al. (2002), where an increase in SOD levels with no changes in GPx was found in bipolar patients. Serum levels of NO and SOD were found significantly higher in bipolar disorder patients, with a correlation between the number of the manic episodes and NO levels (Savas et al. 2006). A recent review by Marazziti et al. (2012) indicated that mitochondrial dysfunction could contribute to cell metabolism errors and apoptosis in disorders such as schizophrenia and bipolar disorder.

4 Oxidative Stress in Major Depression

Major depression is characterized by significantly lower plasma levels of a number of key antioxidants, such as vitamin E, zinc, and coenzyme Q10, as well as lower glutathione peroxidase activity (Maes et al. 2011). A significant association has been found between depression and polymorphisms in genes involved in oxidative pathways such as manganese superoxide dismutase and catalase (Maes et al. 2011). Galecki et al. (2009) showed increases in CAT activity levels during acute episodes of depression, whereas Kodydkova et al. (2009) demonstrated decreases in GPx activity from female patients with depression. Such reduced levels of GPx were further shown in postmortem prefrontal cortex samples from patients with major depression and schizophrenia (Gawryluk et al. 2011).

In addition, accumulating evidence exists that demonstrates the presence of membrane fatty acid defects in patients with major depression (Hibbeln and Salem 1995; Peet et al. 1998; Edwards et al. 1998). Specifically, an increased ratio of 5,8,11,14-eicosatetraenoic acid (arachidonic acid, AA) to 5,8,11,14,17-eicosapentaenoic acid (EPA) and decreased levels of ω-3 fatty acids have been observed in the serum and RBC lipids of depressive patients. Furthermore, the AA/EPA ratio in serum and RBC

membrane phospholipids was correlated positively with the severity of illness (Maes et al. 1996; Seko et al. 1997). The above findings are consistent with the epidemiological studies demonstrating an association between decreased ω-3 fatty acid consumption and increased rates of depression (Hibbeln and Salem 1995). Patients with major depression may have an abnormal intake of ω-3 fatty acids (Edwards et al. 1998; Hibbeln 1998).

Both ω-6 and ω-3 PUFAs are involved the regulation of inflammatory response system. The ω-6 PUFAs, particularly AA, have the proinflammatory features, since AA is the precursor of proinflammatory eicosanoids, prostaglandin E_2 (PGE_2), and leukotriene B_4 (LTB_4) and the increase production of interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and IL-6 (Soyland et al. 1994; Tashiro et al. 1998). On the other hand, the ω-3 PUFAs, EPA, and docosahexaenoic acid (DHA) suppress the production of AA-derived eicosanoids, thus having anti-inflammatory and immunosuppressive effects (Calder 1998). Several groups have reported that ω-3 PUFA-enriched diets (e.g., fish oil) can lead to partial replacement of AA by EPA in inflammatory cell membranes and significantly reduce the ex vivo production of proinflammatory cytokines (Soyland et al. 1994; Calder 1998; James et al. 2000). Therefore, an imbalance of ω-6/ω-3 PUFAs may result in an increased production of proinflammatory cytokines. Smith (1991) proposed that abnormal fatty acid composition might be related to the inflammatory response system underlying pathophysiology of major depression. Further, Maes et al. (2000) have substantiated the role of ω-3 PUFAs in predicting the response of proinflammatory cytokines to psychological stress.

5 Antioxidant Supplementation as Adjunctive Therapy

In the above sections, we have discussed the role of free radicals and antioxidant enzymes in the pathophysiology of schizophrenia, bipolar disorder, and major depression. Accumulating evidence from clinical, preclinical, and epidemiological studies suggests that many of the antioxidant compounds possess neuroprotective and anti-inflammatory properties and could be considered as important adjunctive therapy for schizophrenia (Pillai 2008). We discuss below a few important antioxidants for their therapeutic potential in the above neuropsychiatric disorders.

5.1 Antioxidant Interventions in Schizophrenia

The section below will discuss the recent findings in studies using antioxidants as adjunctive therapeutics in schizophrenia. The major compounds explored in the treatment of schizophrenia for their antioxidant potential are vitamins, N-acetylcysteine (NAC), and ω-3 fatty acids.

5.1.1 Vitamins

Vitamin C and vitamin E are the well-studied essential nutrients that function as the major chain-breaking antioxidants. They are the first line of defense against lipid peroxidation and protecting cell membranes from free radical damage in the human body. It has been shown that the oral supplementation of vitamin C with atypical antipsychotic reverses ascorbic acid levels, reduces oxidative stress, and improves BPRS score in schizophrenic patients (Dakhale et al. 2005). Arvindakshan et al. (2003) also reported reduction in BPRS and PANSS and increase in Henrich's quality of life score after supplementation with ω-3 fatty acids, vitamin E, and vitamin C. It has been suggested that a combination of a hydrophobic agent such as vitamin E, to protect membranes, and a hydrophilic agent such as vitamin C in intracellular protection provides complete antioxidant defense (Mahadik et al. 2001). A number of studies have used vitamin E as a supplement in chronic schizophrenic patients with TD (reviewed by Yao and Keshavan 2011). Supranormal doses of vitamin E have been safely and effectively used to reduce the severity of TD. Several studies, albeit with relatively small sample sizes, have reported decreases in the severity of dyskinesia by vitamin E treatment (Peet et al. 1993; Elkashef and Wyatt 1999; Adler et al. 1993) though there are some contradictory findings (Corrigan et al. 1993; Shriqui et al. 1992).

5.2 N-Acetylcysteine (NAC)

NAC is the precursor of glutathione, which is known to restore the primary endogenous antioxidant GSH and maintain the oxidative balance in the cell. In addition, NAC has been also shown to scavenge oxidants directly, particularly the reduction of the hydroxyl radical and hypochlorous acid (Aruoma et al. 1989). A number of studies have tested the efficacy of NAC as an adjunctive therapy in schizophrenia (Berk et al. 2008a; Bulut et al. 2009; Dodd et al. 2008; Dean et al. 2011). The above studies have suggested that NAC seems to be a safe, effective, tolerable, and affordable adjunctive antioxidant molecule for the treatment of schizophrenia.

5.2.1 Omega-3 Fatty Acid

Membrane deficits have been well documented in subjects with schizophrenia (Mahadik and Yao 2006; Yao and Keshavan 2011). Therefore, boosting the lower levels of membrane phospholipid EPUFAs, predominantly AA (20:4n-6, ω6-EPUFA) and DHA (22:6n-3, ω3-EPUFA), by dietary supplementation is an attractive approach to protect the membrane from cellular damage in schizophrenia. A recent study has shown that high intake of fish, ω-3 or ω-6 PUFA has a lower rate of schizophrenic symptoms in women (Hedelin et al. 2010).

Long-chain ω-3 fatty acids have been shown to reduce the risk of progression to psychotic disorder, particularly in the early stages of illness, and may propose a safe and efficacious adjunctive strategy to prevent from psychiatric condition (Amminger et al. 2010). Thus, ω-3 fatty acids provide numerous health benefits to a variety of psychiatric symptoms (Perica and Delas 2011). Taken together, the above findings suggest the therapeutic potential of ω-3 fatty acid in the treatment of schizophrenia.

In addition to the above compounds, a number of other antioxidants such as glutathione (Berk et al. 2008b), rutin (Bishnoi et al. 2007), Ginkgo biloba (Singh et al. 2010), melatonin (Ortiz et al. 2008; Maldonado et al. 2009), hydroxytyrosol (Young et al. 2007), caffeic acid phenethyl ester (Ozyurt et al. 2007), resveratrol and quercetin (Dietrich-Muszalska and Olas 2009), and lycopene (Rao and Rao 2004) have also been suggested as alternative treatments in schizophrenia (reviewed by Bošković et al. 2011).

5.3 Antioxidant Interventions in Bipolar Disorder

As discussed above, alterations in lipid peroxidation and antioxidant enzymes have been found in subjects with bipolar disorder. In an effort to find the therapeutic potential of antioxidants in bipolar disorder, NAC has been extensively used as adjunctive therapy in bipolar disorder. A recent systematic review of clinical trials showed that adjunct treatment of NAC with standard pharmacotherapies for bipolar disorder shows positive evidence with large effect sizes (Sarris et al. 2011). NAC as an add-on treatment was found to be beneficial in few individuals in relationship to mood and functional outcomes (Magalhães et al. 2011). It has been suggested that long-chain ω-3 fatty acid supplementation has therapeutic potential to improve the disease condition of both major depression and bipolar disorder (McNamara 2013). Increase in brain-derived neurotrophic factor (BDNF) expression following ω-3 fatty acids has been suggested as a possible mechanism that may mediate at least in part the enhancing effects of ω-3 fatty acids in bipolar disorder (Balanzá-Martínez et al. 2011). Frangou et al. (2006) reported a significant improvement in depressive symptoms with ethyl-EPA (ethyl-eicosapentaenoic acid) treatment compared with placebo in subjects with bipolar disorder. In addition, an open-label study with supplementation of 1.5–2 g/day of the ω-3 fatty acids for up to 6 months showed significant improvement in depressive symptoms in bipolar disorder subjects (Osher et al. 2005). Significant changes in mania and depression were reported in an open-label study supplemented with 360 mg of EPA per day and 1,560 mg of DHA (docosahexaenoic acid) per day for 6 weeks in juvenile bipolar disorder subjects (Clayton et al. 2009). Thus, ω-3 fatty acids' intervention represents a promising therapeutic strategy for bipolar disorder.

5.4 Antioxidant Interventions in Major Depression

5.4.1 Omega-3 Fatty Acids

Increased ratio of ω-6/ω-3 PUFAs may contribute to an increased incidence of coronary artery disease (CAD) (Smith 1991; Linscheer and Vergroesen 1988). Moreover, it is now recognized that MDD is robustly associated with an increased risk of CAD. Thus, the increased ratio of ω-6/ω-3 PUFAs may be responsible for the association between MDD and CAD (Maes et al. 1996; Linscheer and Vergroesen 1988). The administration of ω-3 PUFAs has a demonstrated efficacy in reducing cardiac events and triglycerides with minimal side effects (O'Keefe and Harris 2000). The beneficial effect of dietary and supplemental ω-3 fatty acids on CAD was further supported by a recent meta-analysis of 11 randomized controlled trials of both EPA + DHA and alpha-linolenic acid (Bucher et al. 2002). Several potential mechanisms including hypotriglyceridemic, antithrombogenic, antiarrhythmic, and antiatherogenic properties might be responsible for the protective effect of ω-3 fatty acids on CAD (Connor 2000).

In addition, there have been promising results for the use of low-dose ethyl-EPA in treatment-resistant unipolar depression (Peet and Horrobin 2002; Emsley et al. 2003). The effect appears to be specific to EPA, and not DHA (Marangell et al. 2003; Ross et al. 2007; Martins 2009). There also appear to be dose-specific effects; high-dose EPA may not be effective (EPA 6 g/day) (Post et al. 2003). Of particular note is the onset of response with EPA. Peet and Horrobin (2002) found significant reduction in severity of depressed mood as early as 2 weeks and maximally at 4 weeks. Emsley et al. (2003) found significant treatment response at 4 weeks. Thus, response to EPA occurs relatively rapidly. This may be important in managing patients who are not responding to conventional treatments and remain at risk for complications of depression, such as suicide. In the above placebo-controlled trials, there were no dropouts due to EPA-related side effects. An additional advantage is that EPA is not known to alter levels of psychotropic drugs used in treatment of depression. Epidemiological data suggests that there is an inverse relation between risk of depression and postpartum depression and fish consumption (Hibbeln and Salem 1995).

Recently, a meta-analysis study has shown that supplements containing EPA ≥ 60 % of total EPA + DHA, in a dose range of 200–2,200 mg/d of EPA in excess of DHA, were effective against primary depression (Sublette et al. 2011), which is in accordance with an early meta-regression analysis from those double-blind placebo-controlled clinical trials by Ross et al. (2007). On the other hand, another recent systematic review and meta-analyses by Bloch and Hannestad (2011) indicated only a small, nonsignificant, benefit of ω-3 fatty acids treatment in major depression. However, Martins et al. (2012) questioned the validity of their conclusions on the basis of inclusion/exclusion criteria, study subgroup selection, strategy for selecting outcome measures, standard mean difference estimates, and choice of effect modifiers.

5.4.2 Zinc Supplement

Zinc is an essential metal, which plays an important role in improving depressive symptoms (Maes et al. 2011). Zinc has been found to have antidepressive effects by normalizing antioxidant concentrations (Maes et al. 2011). People with depression have significantly lower serum zinc levels than controls (Maes et al. 1994; McLoughlin and Hodge 1990. The transport of zinc to the brain occurs by crossing the blood–brain and blood–cerebrospinal fluid barriers, concentrating in areas such as the hippocampus, amygdala, and neocortex (Frederickson et al. 2000; Takeda and Tamano 2009). Zinc plays an essential role in adult hippocampal neurogenesis and synaptogenesis (Szewczyk et al. 2011). Chronic zinc treatment in high doses is required to increase BDNF mRNA and protein levels in the frontal cortex, while the hippocampus BDNF expression increased with lower, more acute doses of zinc (Cichy et al. 2009; Franco et al. 2008; Nowak et al. 2004; Sowa-Kucma et al. 2008). Earlier studies found that zinc can also regulate nerve growth factor (NGF) directly via the modulation of the zinc binding site (Szewczyk et al. 2011). The induction of NGF by zinc might serve to support neuron survival (Chen and Liao 2003; Mocchegiani et al. 2005).

5.4.3 N-Acetylcysteine (NAC)

In addition to schizophrenia and bipolar disorder, low levels of glutathione (GSH) were also found in postmortem prefrontal cortex from patients with depression (Gawryluk et al. 2011). As described above, the use of NAC in restoring GSH levels has been well established (Dodd et al. 2008). Previously, Berk et al. (2008a) have shown that NAC treatment caused a significant improvement on the Montgomery–Asberg Depression Rating Scale (MADRS) and most secondary scales at end point. A recent open-label study by this same research group also found a robust decrease in depression scores with NAC treatment in 149 individuals with moderate depression for 2 months (Berk et al. 2011).

6 Conclusion

Given the complex pathophysiology of the neuropsychiatric disorders, it is difficult to suggest that a single mechanism could explain the diversity of impairments found in these disorders. As discussed above, a large body of studies provides compelling evidence to show that oxidative stress plays an important role in the pathophysiology of schizophrenia, bipolar disorder, and major depression. However, the biochemical mechanisms underlying these psychiatric disorders remain unclear. A number of studies have suggested that important relationships exist between redox signaling molecules and neuroplasticity-related molecules. For example, neurotrophic factors such as BDNF are known to rescue cerebellar granule neurons

from oxidative stress-mediated cellular damage (Skaper et al. 1998). In addition, both peripheral and brain levels of neurotrophins are lower in subjects with schizophrenia or mood disorder (Pillai 2008). It would be important to determine whether increases in oxidative stress lead to reductions in neurotrophin levels in these psychiatric disorders. As oxidative stress is known to disturb the neuroplasticity, attempts to normalize such impairments are of great therapeutic value in psychiatry research. A few such studies using antioxidants as adjunctive therapy have shown promising leads in the treatment of schizophrenia and mood disorder. However, additional studies using large number of subjects are required to identify further viable therapeutic strategies to restore the oxidative stress-induced cellular, molecular, and behavioral deficits. Such studies will provide exciting opportunity for the treatment and long-term management of neuropsychiatric disorders.

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Antioxidant Plant Polyphenols and Cognitive Disorders

Dariusz Nowak

Abbreviations

AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BMI	Body mass index
CSF	Cerebrospinal fluid
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
FFQ	Food frequency questionnaire
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HIF1α	Hypoxia inducible factor 1α
LRP1	Low-density lipoprotein receptor-related protein1
MAP kinase	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
MDA	Malondialdehyde
MMSE	Mini–mental state examination

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NADPH	Nicotinamide adenine dinucleotide phosphate
PI3 kinase	Phosphoinositide 3-kinase
PKC	Protein kinase C
PS-1	Presenilin-1
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SOD	Superoxide dismutase

1 Introduction

Numerous clinical and experimental data prove the role of oxidative stress in the development and progression of variety of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease, and other causes of cognitive disorders and dementia. Although, currently there is no efficient method to stop progression or cure neurodegenerative diseases, diet rich in plant polyphenols seems to decrease the risk of dementia and to slow down the rate of age-related decline in cognitive performance. There are thousands of polyphenols found in nature, and majority of them occur in flowering plants, their vegetative organs, flowers, and fruits. A group of polyphenols, called flavonoids, is found in significant levels in fruits and vegetables. On the basis of the oxidation state of the central pyran ring, flavonoids could be divided into 6 subclasses: flavonols (e.g., quercetin), flavones (e.g., apigenin), flavanones (e.g., hesperidin), isoflavones (e.g., genistein), anthocyanidins (e.g., cyanidin, pelargonin, malvidin), and flavanols (e.g., catechin, epicatechin, epigallocatechin). Phenolic acids (e.g., gallic acid, hydroxycinnamic acids) and stilbenes (e.g., resveratrol) belong to the nonflavonoid group of plant phenolic compounds. Almost all phenolic acids are present in plants as esters of glucose, tartaric acids, and quinic acid. Chlorogenic acid present in coffee and tea is an ester of caffeic acid and quinic acid. Due to presence of catechol ring, conjugated double bonds, and numerous hydroxyl substitutions in the backbone structure, plant polyphenols have distinct antioxidant activity. Therefore, researches initially focused on the investigation of this activity and its significance for human health in experimental and clinical studies. However, recent data revealed that plant polyphenols and their in vivo metabolites can evoke more complicated and specific effects than direct scavenging of reactive oxygen species (ROS).

They can influence cell functions including neurons in the brain via modulation of kinase-dependent intracellular signals transduction pathways. Moreover, polyphenols in transgenic animal models of Alzheimer's disease (AD)-like pathology decreased amyloid deposition in the brain and improved animals' behavior performance. These are in accordance with the results of observational (cross-sectional, longitudinal) and interventional studies (randomized, double-blind, placebo-controlled) in older humans showing positive effect of increased polyphenols dietary intake on cognitive performance.

2 Plant Polyphenols

2.1 Antioxidant Properties of Plant Polyphenols

Antioxidant activity was the earliest discovered characteristic of plant polyphenols. This topic was extensively studied in in vitro and in vivo models using selected biomolecules sensitive to oxidative damage, subcellular fractions, a variety of isolated cell suspensions and cell cultures, and laboratory animals as well as in clinical trials. These studies were executed with chosen purified polyphenols and polyphenols extract from various fruits, flowers, leaves, seeds, and other parts of plants. Consequently, hundreds of original papers describing antioxidant effects of plant polyphenols on prevention of cancer and cardiovascular and neurodegenerative diseases have been published so far. Since these issues have recently been largely reviewed and discussed elsewhere (Williams and Spencer 2012; Ebrahimi and Schluesener 2012; Vauzour 2012; Hu 2011; Choi et al. 2012; Bubols et al. 2013), this section will describe plant polyphenols antioxidant activity in a concise way.

Plant polyphenols can act as antioxidant by direct reaction with ROS and indirectly by stimulating natural processes that enhance cellular resistance to oxidative stress. Due to various substitutions in the backbone structure (especially the presence of hydroxyl groups), polyphenols can react and inactivate numerous free radicals and oxidants including superoxide, hydroxyl, peroxyl, lipid free- and carbon-centered radicals as well as singlet oxygen, nitric oxide, and peroxynitrite.

Polyphenols can also act as chelators of transition metal ions Fe^{2+}, Fe^{3+}, and Cu^{2+} that are involved in the conversion of hydrogen peroxide into hydroxyl radicals and stimulation of lipid peroxidation. This activity is attributed to the presence of o-diphenolic groups in the 3O, 4O-dihydroxy positions in the B ring and the keto structure 4-keto, 3-hydroxy or 4-keto, and 5-hydroxy in the C ring of flavonoid backbone (Thompson et al. 1976; Rice-Evans et al. 1996; van Acker et al. 1996).

On the other hand, the antioxidant activity of selected plant polyphenols and their metabolites expressed as the ability to reduce Fe^{3+} ions (FRAP) in vitro was positively associated with the presence of a catechol structure in the compound molecule. In addition, an aliphatic substitute at a catechol ring and a double bond in an aliphatic substitute conjugated with an aromatic ring of catechol contributed to almost 40 % of the variance in the FRAP of compounds with catechol in the backbone structure (deGraft-Johnson et al. 2007). Indirect antioxidant activity of polyphenols involves stimulation of synthesis of various enzymes that increase cellular resistance to ROS. For instance, intraperitoneal injection of green tea polyphenols (epigallocatechin gallate) increased the activity of two important antioxidant enzymes, catalase and superoxide dismutase, in mouse striatum (Levites et al. 2001). Insufficient supply of blood to the brain leading to local nervous tissue hypoxia is one of the causes of cognitive impairment and dementia. Some polyphenols (catechins, epigallocatechin gallate, resveratrol) present in red wine and green tea can increase synthesis of hypoxia-inducible factor 1α subunit (HIF1α) that normally rises under hypoxic conditions and activates genes encoding proteins involved in cell survival, angiogenesis, glycolysis, and iron metabolism. Thus, these polyphenols as

factors influencing activity of HIF1α and stimulating increased activity of antioxidant enzymes would express neuroprotective activity. Consequently, polyphenols revealed a significant protective effect against neurotoxicity and neurodegeneration induced by variety of factors (e.g., homocysteine, glutamate, kainic acid, *N*-methyl-D-asparate, glucose oxidase, bacterial endotoxin, transient global cerebral ischemia) under conditions of in vitro and in vivo models.

2.2 *Anti-Inflammatory and Other Biological Activities of Plant Polyphenols*

Since ROS belong to mediators of inflammation, any polyphenolic compound with distinct antioxidant activity will also have some anti-inflammatory properties. However, detailed molecular studies revealed that more specific mechanisms contribute to inhibitory effect of polyphenols on inflammatory processes including neuroinflammation. This issue was also extensively reviewed elsewhere (Williams and Spencer 2012; Ebrahimi and Schluesener 2012; Vauzour 2012). Therefore, only the main known mechanisms leading to anti-inflammatory effect of polyphenols in cerebral tissue will be listed below.

Polyphenols (especially flavonoids) can affect neuronal and glial functions via binding to various receptors including adenosine, nicotinic, estrogen, testosterone, or δ-opioid receptors.

Flavonoid-induced receptors stimulation can cause changes in the activation state of the mitogen-activated protein (MAP) kinase (e.g., naringenin), the phosphoinositide 3- (PI3) kinase (e.g., curcumin), the nuclear factor-κB (e.g., resveratrol, epigallocatechin gallate), and protein kinase C (PKC) pathways (e.g., resveratrol). These signal transduction pathways are involved in cell differentiation and apoptosis, cell survival (apoptosis inhibition), inflammatory response and also learning and memory, and reduction of amyloid plaque formation, respectively. Polyphenols can also inhibit activation of the glial cells (resident macrophages of the brain) and thus decreasing their ability to produce pro-inflammatory cytokines (TNF-α, IL-1β) and release superoxide radicals, hydrogen peroxide, and nitric oxide.

Polyphenol-induced improvement of cerebral blood flow via stimulation of nitric oxide production in the endothelium and inhibition of platelet aggregation may be an additional important mechanism of neuroprotection. All these effects will lead to suppression of oxidative stress and inflammatory response in the cerebral tissue and reduced risk of damage to neurons and their degeneration.

2.3 *Dietary Intake and Absorption of Polyphenols*

Since beneficial effect of fruits and vegetables consumption on the risk of chronic degenerative processes is attributed to various plant polyphenols which possess variety of biological activities, it is necessary to know which food products contain

the highest content of these compounds and how they are absorbed from the gut into the blood stream. Dietary intake of plant polyphenols depends on the types and amount of plant foods consumed and was estimated to be between 150 mg and 1 g (Aura 2008). Currently, there are hundreds and thousands of plant food products with different phenolic composition available for consumption on the market. Therefore, this subsection will focus only on those polyphenolics or plant products containing them that were tested in animal models of AD-like pathology and clinical trials involving humans with various levels of cognitive impairment and dementia (described in further subsections). Table 1 lists these polyphenolics and their nutritional sources as well as their main metabolites in the human body.

Table 1 Phenolic compounds, their nutritional sources, and main metabolites detected in human body that were tested in animals models of AD-like pathology and clinical trials involving humans with various levels of cognitive impairment and dementia

Phenolic compound	Nutritional source	Main metabolite in human[a]
Catechin and epicatechin	Green and black tea, red wine, chocolate, grape seed	3-(3-hydroxyphenyl) propionic acid,
Ellagic acid and ellagitannins	Pomegranate, raspberry, strawberry, nuts	Urolithins A and B
Gallic acid	Red wine, black tea, pomegranate, blackberry	4-O-methylgallic acid
Caffeic acid	Grapes, apples, blueberries, broccoli, coffee, red wine,	Acids: 3- and 4-coumaric, 3-(3,4dihydroxyphenyl)propionic, 3-(3-hydroxyphenyl)propionic, 3-hydroxybenzoic, 3-hydroxyhippuric, hippuric
Tannic acid	Tea, grapes,	4-O-methylgallic acid, pyrogallol, resorcinol[b]
Chlorogenic acid	Blueberries, coffee, tea, sunflower seeds	Acids: hippuric, ferulic, caffeic
Quercetin	Apples, onions, tea, blackcurrant	Acids: 2-(3,4-dihydroxyphenyl)acetic, 2-(3-hydroxyphenyl)acetic, 2-(3-methoxy-4-hydroxyphenyl) acetic
Epigallocatechin gallate	Green tea	5-(3′,4′-dihydroxyphenyl)-c-valerolactone, 5-(3′,5′-dihydroxyphenyl)-c-valerolactone, 5-(3′,4′,5′-trihydroxyphenyl)-c-valerolactone
Hesperidin	Orange juice	Eriodictyol, homoeriodictyol[b]
Proanthocyanidins (oligomers of catechin or epicatechin)	Apples, grapes, wine, tea, chocolate, cranberry	Acids: 3-(3-hydroxyphenyl) propionic, 2-(4-hydroxyphenyl)acetic, 3-(4-hydroxyphenyl)propionic, 3-(3-hydroxyphenyl)propionic, 3-(3-hydroxyphenyl)acetic, 3-phenylpropionic
Anthocyanins[c]	Red fruits, red berries, red wine	Acids: 3,4-dihydroxybenzoic, 3-methoxy-4 hydroxybenzoic, 4-hydroxybenzoic acid, 3,4-dimethoxybenzoic[d]

Based on Aura (2008), D'Archivio et al. (2007), Olthof et al. (2003), Stalmach et al. (2009), Shahrzad and Bitsch (1998), Nakamura et al. (2003), and Jin et al. (2010)

[a]Results obtained from analysis of human plasma, urine, feces, and experiments with human fecal microbiota

[b]Results obtained from experiments on rats

[c]Glycosides of anthocyanidins

[d]Depending on specific anthocyanin

Majority of dietary polyphenols exists as esters, glycosides or polymers that cannot be absorbed in the native form (D'Archivio et al. 2007) They must undergo the reaction of hydrolysis catalyzed by intestinal enzymes (e.g., β-glucosidase, lactase-phlorizin hydrolase) or enzymes produced by colonic microbiota. However, there are exceptions to this rule: quercetin glucosilation facilitates its absorption. Thus, quercetin glucosides absorption is more efficient than that of the aglycone itself and its subsequent hydrolysis catalyzed by cytosolic β-glucosidase takes place in enterocytes.

Those polyphenols that are not absorbed in the small intestine reach the colon where they could be transformed into less complex compounds by gut microflora before transportation into the blood. Thus, the combination of consumed polyphenols may differ to great extent from that absorbed and reaching tissues with circulating blood (Aura 2008). Further metabolism in enterocytes and liver (reactions of methylation, sulfation and glucuronidation) can also contribute to this difference and may affect the bioavailability of dietary phenolics (Aura 2008; Silberberg et al. 2006). These are supported by clinical experiments with dietary supplementation with chlorogenic acid. In subjects with intact colon, about half of ingested chlorogenic acid was metabolized to hippuric acid; however, in ileostomy subjects (without colon due to a total colectomy in the course of ulcerative colitis or polyposis coli), only traces of hippuric acid were detected in the urine (Olthof et al. 2003). There is a great interindividual variability in polyphenol bioconversion by colonic microbiota in healthy subjects. For instance, bioconversion of polyphenols from black tea and a mixture of red wine and grape juice differed in metabolite pattern, kinetics, and concentrations of specific products (Gross et al. 2010).

Shifts in the composition of resident bacteria can occur in response to numerous factors including changes of the diet, diarrhea, or antibiotic treatment. Thus, even the same subject can present altered polyphenols bioconversion in the colon at different time points. Therefore, it is very difficult to detect all the metabolites and evaluate their biological activity as well as to transfer results of in vitro experiments and on animal models into human physiology and medicine. In addition, these observations suggest different healthful effects obtained with the same polyphenols supplementation between various subjects.

2.4 Polyphenols Penetration to Brain Tissue

To answer the question as to why plant polyphenols can protect the brain from peroxidative damage and other neurodegenerative processes, it is necessary to know whether and how they can cross the blood–brain barrier and what determines their concentration in the brain structures. The knowledge about concentrations of specific polyphenols in the central nervous system would be helpful for the statement whether observed in vitro protective effects of polyphenols could be relevant to clinical practice. Although this problem is not completely solved, the results obtained so far seem interesting and promising for future clinical trials on efficacy

of plant polyphenols in protection from neurodegenerative disorders and decline of cognitive performance.

Single oral administration of $_{14}$C-labeled grape polyphenols preparation in rats resulted in the appearance of radioactivity in cerebral interstitial fluid sampled with microdialysis probes from hippocampus and also in brain slices obtained at 24-h post-dose (Janle et al. 2010). Maximal signals for labeled catechin/epicatechin (monomers, oligomers) peonidins, cyanidins, and cyanidin glycosides occurred in cerebral fluid sampled between 0.75 and 1.5 h after grape polyphenols ingestion. However, these signals were lower than those in corresponding samples of blood. The amount of residual label in the brain did not exceeded 1.7 % of the dose, and the radioactivity was spread homogenously over all the brain structures (Janle et al. 2010).

It is interesting that repeated oral administration of plant polyphenols in rats can increase their brain bioavailability. Single administration of increasing doses of grape seed polyphenolic extract (from 50 to 150 mg/kg body weight) resulted in the dose-dependent increase of plasma circulating gallic acid, catechin, epicatechin, and some of their metabolites; however, they were not detectable in the brain tissue (Ferruzzi et al. 2009). Repeated daily ingestion of grape seed polyphenolic extract for 10 days increased both plasma and brain bioavailability of gallic acid, catechin, and epicatechin with brain concentrations of these two latter compounds reaching about 570 and 290 pg/g of tissue (Ferruzzi et al. 2009). Thus regular prolonged ingestion of plant polyphenols may enhance their deposition in the brain structures and subsequently increase protective activity.

Dietary supplementation with blueberry extract (20 g of extract per kg of diet) for 8–10 weeks also resulted in the appearance of several blueberry anthocyanins (e.g., cyanidin-3-O-b-galactoside, peonidin-3-O-b-arabinoside, malvidin-3-O-b-galactoside) in the brain (cerebellum, cortex, hippocampus, striatum) of rats (Andres-Lacueva et al. 2005). The brain cortex showed the greatest number of detected compounds ($n=8$). Negative readings were obtained for brain samples from control animals without supplementation (Andres-Lacueva et al. 2005). Similarly, HPLC analysis revealed several anthocyanins (total concentration of 0.25 nmol/g of tissue) in the brain of rats fed with blackberry anthocyanin-enriched diet (15 g blackberry extract per kg of diet) for 15 days (Talavéra et al. 2005).

On the other hand, it should be pointed out that green tea polyphenols were able to reduce the elevated blood–brain barrier permeability in the rat model of experimental cerebral ischemia induced by middle cerebral artery occlusion (Zhang et al. 2010). Reduced expression of caveolin-1 and phosphorylated extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) is the suggested mechanism by which these polyphenols can improve the function of blood–brain barrier (Zhang et al. 2010).

Putting together these results, it is clearly visible that diet supplementation with grape, blueberry, or blackberry extracts results in deposition of corresponding polyphenols and their metabolites in the brain structures in animals. Therefore, dietary polyphenolics are bioavailable in the brain and can exert protective effect directly in this organ. Although there is no data on phenolic levels in the human brain, one may assume that a diet rich in fruit and vegetables will result in increased cerebral deposition of theses beneficial compounds.

3 Oxidative Stress, Development, and Progression of Cognitive Disorders

3.1 Why Is Brain Susceptible to Oxidative Stress?

All cells, tissues, and organs of human body have a set of enzymes, numerous low-molecular-weight compounds, and metal chelating/sequestrating agents that compose the system of antioxidant defense. In general, the individual links of this system are similar throughout the human body; however, their expression and activity differ between organs. Therefore, under the same conditions of generation of ROS, the capacity of the antioxidant defense in a given organ could be overloaded with free radicals and lead to oxidative damage, but in another one it would not. When two organs have a similar capacity of antioxidant defense but they differ in intensity of generation of ROS, tissues exposed to higher ROS activity will be at a higher risk of peroxidative damage. This situation is complicated by two other factors. Firstly, when a given tissue contains a lot of biomolecules that can rapidly react with ROS with further generation of secondary radicals and cytotoxic products, this will result in a higher cellular destruction. Secondly, tissues can contain high amounts of transition metals (e.g., iron, copper) that are normally "silent" (sequestrated). When these metals are released, they can catalyze conversion of hydrogen peroxide (H_2O_2) into more toxic hydroxyl radicals and induce larger tissue damage. Moreover, high concentration of vitamin C that can maintain oxidation state of these metal cations optimal for hydroxyl radicals generation will augment cell injury (McGrath et al. 2001; Nowak et al. 1991). All these four factors that predispose to peroxidative damage to tissues occur in the brain.

There is a low activity of catalase in the brain. Other key enzymes of antioxidant defense such as superoxide dismutase and glutathione peroxidase reach moderate activities in comparison to those noted in other organs (Lau et al. 2005). Superoxide dismutase catalyzes dismutation of superoxide into oxygen and H_2O_2. H_2O_2 can be subsequently decomposed into water and oxygen by catalase. Glutathione peroxidase uses glutathione as an electron donor and is active with H_2O_2 and also (some isoenzymes) with organic hydroperoxide substrates. Thus, lower activity of these enzymes can increase the possibility of conversion of superoxide and H_2O_2 into highly reactive hydroxyl radicals with subsequent damage to brain biomolecules.

Formation of superoxide radical (and then H_2O_2) in normal tissues is an ongoing process in the respiratory chain electron transport that is localized at the mitochondrial inner membrane.

Electrons given by NADH and succinate pass through the electron transport chain to oxygen which is reduced to water. At a rough estimate, 3–5 % of consumed oxygen undergoes incomplete reduction due to direct leakage of electrons from respiratory chain to oxygen with subsequent formation of superoxide radical. The rate of mitochondrial oxygen consumption determines the rate of superoxide and H_2O_2 formation. The brain constitutes about 2 % of the body mass; however, it utilizes around 20 % of the total oxygen consumption. Thus, the rate of brain mitochondrial oxygen

consumption per g of tissue is very high in comparison to other organs in human body and determines high secondary stream of oxidants capable of damaging brain structures. Additionally, the reaction of oxygen and ROS with brain neurotransmitters can turn on various mechanisms leading to the oxidation of lipids, proteins, and DNA (Pattison et al. 2002).

The brain contains large amounts of lipids including polyunsaturated fatty acids (PUFAs). They are present in neuronal as well as in mitochondrial membranes and are highly susceptible to lipid peroxidation. ROS are the most important initiators of PUFAs peroxidation process that can easily evolve into the propagation phase characterized by excessive formation of fatty and peroxyl-fatty acid radicals with subsequent formation of 4-hydroxy-nonenal and malondialdehyde which in turn are toxic for various cells including neurons (Cheng et al. 2011; Bai and Mei 2011).

Since the brain is not homogeneous in its lipid composition including PUFAs (O'Brien and Sampson 1965), one may assume that some brain regions would be more susceptible to peroxidative attacks. There are relatively high concentrations of iron and ascorbic acid in the brain. Because of the fact that they stimulate lipid and catecholamines oxidation (with subsequent radicals formation) under experimental conditions (Fan et al. 2010; Hašková et al. 2011; Hasegawa et al. 2009), one may assume that they can additionally enhance the risk of ROS-induced damage to brain biomolecules.

Taking into consideration the fact that in the majority of regions of the brain, neuronal cells are post-mitotic and are not able to wash out themselves from accumulated oxidative damage, it can explain the high brain susceptibility to oxidative stress.

3.2 Association Between Persistent Oxidative Stress and Cognitive Dysfunction

Numerous pathogenetic mechanisms leading to the development of neurodegenerative diseases characterized by progressive decline of cognitive performance involve damage to neurons caused by the oxidative stress (Albarracin et al. 2012).

Without the decision whether oxidative stress is the primary or secondary mechanism in the progression of these diseases, one may assume that subjects with a different degree of cognition impairment may present features of systemic or local (in the cerebral tissues) oxidative stress. Therefore, in the last few years, numerous studies comparing biomarkers of oxidative stress in specimens of the brain tissue, cerebrospinal fluid, blood, and urine obtained from subjects with and without cognitive dysfunction or dementia have been executed (Tables 2 and 3).

In general, these studies involved patients with mild cognitive impairment (MCI) that were compared with two additional references group: patients with AD and age-matched controls with normal cognition. The chosen biomarkers reflected intensity of lipid peroxidation (malondialdehyde, hydroxynonenal, F2-isoprostanes), status of low-molecular-weight antioxidants (glutathione, vitamin C), activity of enzymes involved in antioxidant defense (superoxide dismutase, glutathione peroxidase, glutathione

reductase, glutathione-S-transferase) and ROS generation (NADPH oxidase), degree of peroxidative damage to DNA (8-hydroxy-2-deoxyguanosine) and proteins (proteins carbonyls), as well as the iron and cooper metabolism (transferrin saturation, ferritin, iron, and copper concentration).

Patients with mild cognitive impairment (MCI) revealed increased concentration of markers of lipid peroxidation in body fluids (Table 2). They had an increased plasma concentration of malondialdehyde (MDA) (Padurariu et al. 2010; Torres et al. 2011; Umur et al. 2011), as well as the plasma, urinary, and cerebrospinal fluid (CSF) levels of isoprostane 8,12-iso-iPF(2alpha)-VI (Praticò et al. 2002). In addition, in the group of clinically normal subjects, the concentration of F2-isoprostanes in CSF raised over the adult human life span, and the subgroup with biomarker signature of AD (CSF amyloid (A) β(42) and tau) had the highest levels of this biomarker (Montine et al. 2011). Plasma levels of MDA were negatively correlated with cognitive performance expressed with Mini–mental state examination (MMSE) score in MCI patients (Torres et al. 2011). Similar correlation between circulating MDA and MMSE score was observed in the group of elderly residents from nursing homes (Umur et al. 2011).

However, other researches that used F2-isoprostanes to evaluate intensity of lipid peroxidation reported completely opposite results. Plasma and urinary levels of F2-isoprostanes did not discriminate subjects with MCI and AD patients from individuals with no cognitive impairment (Mufson and Leurgans 2010). Moreover, in one prospective study, the concentration of F2-isoprostanes in plasma did not correlate with changes in cognitive function in non-demented older adults over the 8 years of follow-up (Fiocco et al. 2011). Thus, the usefulness of F2-isoprostanes monitoring for prediction of the risk of cognitive decline remains open to question. Perhaps, the measurement of more specific isoprostane 8,12-iso-iPF(2alpha)-VI (Praticò et al. 2002) would give promising results.

Low-molecular-weight antioxidant (total glutathione, GSH, GSH/GSSG ratio) and its metabolite cysteinylglycine were reported to be decreased in plasma of MCI patients (Bermejo et al. 2008; Hernanz et al. 2007).

Both total glutathione and cysteinylglycine positively correlated with total score of cognitive performance in these subjects (Hernanz et al. 2007). Although AD patients presented a low content of GSH in brain tissues as evaluated using noninvasive magnetic resonance spectroscopy, this was not observed in the MCI group (Mandal et al. 2012). Activities of glutathione reductase (involved in the maintenance of high tissue GSH level) and glutathione peroxidase (that uses GSH for tissue protection against oxidants) were suppressed in plasma and erythrocytes of individuals with MCI [(Padurariu et al. 2010; Torres et al. 2011; Umur et al. 2011). These were accompanied by the decrease of serum superoxide dismutase activity (Padurariu et al. 2010). In respect to transition metals predisposing to enhanced peroxidative reactions, patients with cognitive dysfunction had elevated concentration of free iron, ferritin, and transferrin saturation in serum (Umur et al. 2011). Moreover, the increased ratio of copper to iron reflected the increased risk of dementia development in the group of MCI individuals during the 5-year observation (Mueller et al. 2012). Probably, as a result of the decrease in the antioxidant defense and the

Table 2 Results of selected studies on markers of oxidative stress in patients with various impairment of cognitive performance

Studied group	Main results	References
Patients with mild cognitive impairment vs. AD vs. matched controls	Plasma – increased protein carbonyls, decreased GSH, and GSH/GSSG ratio in patients with MCI and AD subjects compared to controls	Bermejo et al. (2008)
Patients with mild cognitive impairment vs. AD vs. matched controls	Plasma – decreased total glutathione and its metabolite cysteinylglycine in AD and MCI patients vs. control. Positive correlations between total score of cognitive performance and glutathione and cysteinylglycine levels in MCI and AD patients	Hernanz et al. (2007)
MCI patients vs. AD patients vs. matched healthy controls	Serum – similarly decreased SOD and GPX activity and increased MDA in MCI and AD patients vs. controls	Padurariu et al. (2010)
MCI patients (mild probable AD) vs. matched healthy controls	Plasma – increased MDA, erythrocytes – decreased glutathione reductase in MCI patients. MMSE score was negatively associated with MDA levels	Torres et al. (2011)
Clinically normal individuals	CSF – significant increase of F2-isoprostanes over the adult human life span. Increased F2-isoprostanes in the subjects with the biomarker signature of AD (CSF amyloid (A) β(42) and tau)	Montine et al. (2011)
Subjects with no cognitive impairment vs. MCI vs. AD	Plasma and urine – F2-isoprostane levels did not differ between these three clinical groups	Mufson and Leurgans (2010)
Subjects with no cognitive impairment vs. MCI vs. AD	GSH content in brain regions using noninvasive magnetic resonance spectroscopy – decreased GSH in AD patients in comparison to controls. MCI did not differ from controls	Mandal et al. (2012)
MCI vs. healthy controls	CSF, urine, plasma – elevated isoprostane 8,12-iso-iPF(2alpha)-VI in MCI patients	Praticò et al. (2002)
Non-demented older adults	No association between plasma F2-isoprostanes and change in cognitive function over 8 years. F2-isoprostanes are not a valuable biomarker in predicting cognitive decline in non-demented older adults	Fiocco et al. (2011)
Amnestic MCI vs. preclinical AD	Brain – postmortem-obtained inferior parietal lobule samples had more protein carbonyls in MCI subjects (despite equal levels of neuropathology)	Aluise et al. (2011)
Non-demented Puerto Rican adults	Urine – higher 8-hydroxy-2-deoxyguanosine concentration was significantly associated with lower global cognitive scores	Gao et al. (2010)
Elderly residents from nursing homes with and without cognitive dysfunction	Serum – increased iron, transferrin saturation, ferritin, and MDA, decreased GPX activity in subjects with cognitive dysfunction. Negative correlation between MMSE score and serum MDA	Umur et al. (2011)
MCI vs. early stage senile dementia vs. subjects with normal cognition	Serum – increase in the ratio of copper to non-heme iron predicted which subjects with MCI would progress to dementia over the 5 years follow-up	Mueller et al. (2012)

AD Alzheimer's disease, *MCI* mild cognitive impairment, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *SOD* superoxide dismutase, *GPX* glutathione peroxidase, *MDA* malondialdehyde, *CSF* cerebrospinal fluid, *MMSE* mini–mental state examination

rise of circulating free transition metals (iron, copper), MCI subjects had increased concentration of protein carbonyls in plasma (Bermejo et al. 2008) and in postmortem-obtained brain specimens (Aluise et al. 2011). Consistently, higher urinary levels of 8-hydroxy-2-deoxyguanosine (marker of DNA oxidation) were associated with lower cognitive performance (e.g., global cognitive score, scores for word list learning, recognition) in a large group of non-demented adults (Gao et al. 2010).

In conclusion, the results mentioned above clearly show that oxidative stress resulting from decreased antioxidant defense and probably from increased deposition of transition metals (Fe, Cu) occurs in subjects with mild cognitive impairment. This oxidant–antioxidant imbalance was strong enough to induce detectable products of peroxidative damage to lipids, proteins, and DNA in circulating blood, cerebrospinal fluid, urine, and brain tissue. Thus, one may assume that oxidative stress may be an important event in the early steps of cognitive disorders.

3.3 Clinical Evidences of Oxidative Stress in Patients with Dementia

Table 3 summarizes results of selected studies on intensity of oxidative stress in patients with dementia and its association with cognitive performance scores. These studies mostly involved AD patients and compared them to matched healthy controls as well as to patients with vascular dementia. AD patients had increased plasma concentrations of both lipid peroxidation products, malondialdehyde and 4-hydroxynonenal (McGrath et al. 2001; Gustaw-Rothenberg et al. 2010), and decreased levels of vitamin C (McGrath et al. 2001). There was a significant increase in 4-hydroxy-2-nonenal bound to transmembrane low-density lipoprotein receptor-related protein1 (LRP1) in samples of hippocampus-obtained postmortem from AD patients (Owen et al. 2010). LRP1 is responsible for the efflux of amyloid-β peptide (the main component of senile plaques in the gray matter of the brain, one of the characteristic features of AD) from the brain to the blood across the blood–brain barrier. Thus oxidative damage to LPR1 can impair transport of amyloid-β peptide with subsequent enhancement of its accumulation in the brain.

In other studies, specimens of brain cortex obtained from AD patients revealed suppression of antioxidant defense (decreased concentration of glutathione and activities of superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase an enzyme detoxifying peroxidized lipids and other xenobiotics) (Ansari and Scheff 2010) and increased activity of NADPH oxidase (Ansari and Scheff 2011) that generates superoxide radicals by transferring electrons from NADPH to O_2. These changes in the brain cortex favoring free radical-mediated processes as well as the plasma levels of 4-hydroxy-2-nonenal were negatively correlated with the scores of cognitive performance (McGrath et al. 2001; Ansari and Scheff 2010, 2011).

Apart from AD patients, decreased circulating total and reduced glutathione was noted in Lewy body dementia and in demented individuals in the course of Parkinson's disease (Gironi et al. 2011). In accordance with the observed

Table 3 Results of selected studies on markers of oxidative stress in patients with dementia

Clinical setting	Main results	References
AD vs. matched healthy controls	Plasma – elevated 4-hydroxy-nonenal, normal MDA, decreased vitamin C. Inversely relation of 4-hydroxy-noneal levels with MMSE score	McGrath et al. (2001)
Vascular dementia patients vs. nonvascular dementia vs. healthy controls	Urine – increased concentration of 8-hydroxyl-deoxyguanosine in vascular dementia patients in comparison to both reference groups	Shi et al. (2012)
Vascular dementia patients vs. AD vs. matched healthy controls	Plasma – increased MDA in both dementia groups vs. controls. Higher MDA in vascular dementia patients than in AD patients	Gustaw-Rothenberg et al. (2010)
AD vs. dementia with Lewy bodies vs. Parkinson's disease with dementia vs. MCI vs. healthy controls	Serum – decreased total and reduced glutathione in groups with dementia	Gironi et al. (2011)
AD (mild/moderate, late stage) vs. MCI patients vs. subjects with no cognitive impairment	Frontal cortex – decreased glutathione, glutathione peroxidase, glutathione-S-transferase, and superoxide dismutase in mitochondrial and synaptosomal fractions of AD patients. Levels of oxidative markers correlated with MMSE scores	Ansari and Scheff (2010)
AD vs. MCI vs. matched healthy controls	Plasma – elevated carbonyl proteins in AD and MCI in comparison to healthy controls	Greilberger et al. (2010)
AD different stages (preclinical, MCI, early, mild to moderate) vs. subjects with no cognitive impairment	Postmortem samples of frontal and temporal cortex – elevated NADPH oxidase activity in AD patients. Negative correlation between NADPH oxidase activity and cognitive performance	Ansari and Scheff (2011)
AD patients vs. age-matched controls	Postmortem samples of hippocampus – significant increase in the levels of the lipid peroxidation product 4-hydroxy-2-nonenal bound to transmembrane LRP1 in AD patients in comparison to controls	Owen et al. (2010)

AD Alzheimer's disease, *MCI* mild cognitive impairment, *MMSE* mini–mental state examination, *MDA* malondialdehyde, *NADPH* nicotinamide adenine dinucleotide phosphate, *LRP1* low-density lipoprotein receptor-related protein1

suppression of antioxidant defense, AD patients had increased concentrations of carbonyl proteins in plasma (Greilberger et al. 2010). However, patients with vascular dementia in comparison to AD subjects revealed higher levels of plasma malondialdehyde (Gustaw-Rothenberg et al. 2010) and increased urinary concentration of 8-hydroxydeoxyguanosine (Shi et al. 2012).

Taking the above into consideration, one may conclude that oxidative stress can occur in different forms of dementia regardless of the specific etiology and pathogenetic mechanism (neurodegenerative disorders, vascular dementia). An overlap between various mechanisms leading to oxidative stress in different forms of dementia and high brain susceptibility to oxidative damage could be the possible explanation of these findings. Moreover, these suggest that pharmacological or dietary interventions that will augment antioxidant defense or suppress ROS generation could be effective in prevention of cognitive disorders.

4 Dietary Plant Polyphenols in Prevention of Cognitive Disorders

4.1 Results of Selected Experiments on Laboratory Animals

In order to find neuroprotective activity of plant polyphenols, several experiments employing animal models of neurodegenerative diseases and dementia have been executed in the last few years. In the majority of them, products rich in plant polyphenols or placebo (added to drinking water or standard chow) were applied to transgenic mice bearing mutations causing AD-like pathology. At the end of treatment, cognitive performance of animals was estimated with battery of various tests (e.g., water maze test, step-down test, step-through test, open field test), and then animals were sacrificed, and samples of brain were subjected to microscopic and molecular analysis.

Tg2576 mice overexpress a mutant form of amyloid precursor protein (APP), and they are a model of early onset familial AD (formation of amyloid plaques in the cerebral cortex and progressive cognitive deficits). Seven-month consumption of wine (Cabernet Sauvignon delivered in drinking water equivalent to 6 % ethanol, about 7 % of the total energy consumption was derived from wine) reduced AD-type neuropathology (lower content of amyloidogenic Aβ1-40 and Aβ1-42 peptides in the neocortex and hippocampus) and attenuated spatial memory decline in comparison to animals drinking 6 % ethanol or water alone (Wang et al. 2006). Since no differences were noted between ethanol and water groups, the authors suggested that phenolics present in this wine (gallic acid, caffeic acid and its derivatives, catechin, gallotannins) can stimulate the nonamyloidogenic processing of amyloid precursor thus preventing formation of Aβ peptides (Wang et al. 2006).

In another study employing the same AD-like pathology, murine model pomegranate juice (rich in ellagic acid, gallic acid, tannins, and anthocyanins) inhibited accumulation of soluble Aβ42 and amyloid deposition in the hippocampus as compared to control mice treated with sugar water (Hartman et al. 2006). Moreover, this 6.5-month dietary supplementation (equivalent to daily polyphenol consumption 0.3–0.6 mg) improved animals' behavior performance including learning ability and visual acuity.

Similarly, 5-month administration of grape seed polyphenolic extract 200 mg/kg/day, delivered in the drinking water, containing mostly catechin and epicatechin monomers and oligomers attenuated AD-type cognitive deterioration and reduced accumulation of soluble high-molecular-weight oligomeric Aβ peptides in the brains of Tg2576 mice (Wang et al. 2008). Moreover, under in vitro conditions, this grape seed extract inhibited oligomerization of synthetic Aβ1-42 and Aβ1-40 peptides (Wang et al. 2008).

Microtubule-associated protein tau is a phosphoprotein that regulates microtubule stability and polymerization. Hyperphosphorylation of tau protein that causes its function disturbance and aggregation with subsequent formation of neuropil threads and neurofibrillary tangles in the brain is observed in numerous neurodegenerative disorders including AD. The same grape seed polyphenolic extract given in the dose of 200 mg/kg/day for 2 months inhibited accumulation of sarkosyl-insoluble tau in the brain of TMHT mice, a model of age-dependent development of tau pathology (Wang et al. 2010). Analysis of pulverized brain tissue lysates with ELISA-based multiplex cell signaling assays showed important suppression of ERK 1/2 kinase activity. Since ERK 1/2 is involved in hyperphosphorylation of tau protein, this may be the mechanism of protective effect of grape seed polyphenols (Wang et al. 2010).

Several experiments were devoted to prove neuroprotective and neuromodulating activity of green tea polyphenols. Catechins are the main bioactive constituents of green tea leaves and account for about 30 % of their dry weight. Catechin, epicatechin, gallocatechin, epigallocatechin, catechin gallate, epicatechin gallate, gallocatechin gallate, and epigallocatechin gallate are included in the green tea extract. Green tea polyphenols protected AD-like mice from decline in learning ability and memory induced by intraperitoneal injection of D-galactose and intracerebroventricular injection of Aβ25-35 peptide (Lü et al. 2006). Restraint stress (6-h inhibition of movement per day with the tube fit closely to the body of the animal for 3 weeks) caused significant cognitive impairment in rats with subsequent decrease of total antioxidant activity and increase of malondialdehyde in brain tissue. Simultaneous addition of green tea extract to feedstuff resulted in the improvement of cognitive performance and normalization of oxidative stress biomarkers (Chen et al. 2009). Green tea polyphenols given in the intragastric dose from 5 to 20 mg/kg for 7 days revealed also antidepressant effect in adult mice (Zhu et al. 2012). Addition of green tea extract to drinking water (final concentration 0.5 %) for 8 weeks significantly improved learning and memory as well as suppressed the acetylcholinesterase activity in cerebrum of older rats (Kaur et al. 2008). This suggests usefulness of green tea polyphenols in prevention of age-related deficits of learning and memory.

Presenilin-1 (PS-1) overexpression is associated with AD by favoring the formation of Aβ peptide with subsequent generation of ROS and amyloid deposition. Feeding normal and apolipoprotein E-deficient mice (ApoE−/−) with the diet without folate and vitamin E and supplemented with iron as a prooxidant resulted in PS-1 overexpression in frontal cortex (Chan and Shea 2006). Addition of apple juice concentrate to drinking water (final concentration 0.5 %) prevented this increase in

both animal groups. Apple derived antioxidants and S-adenosylmethionine present in the juice were believed to be involved in this protection; however, the latter via its effect on normalization of DNA methylation seems to be more important.

Six-month administration of tannic acid (daily dose 30 mg/kg) in PSAPP mice that overproduce human Aβ1-40 and Aβ1-42 peptides decreased formation of amyloid deposits in brain parenchyma in comparison to control animals (Mori et al. 2012). This treatment also reduced the behavioral impairment including hyperactivity, decreased object recognition, and defective spatial reference memory. Molecular analysis of brain parenchyma and supplemental in vitro experiments proved inhibition of β-secretase activity as possible mechanism of protective effect of tannic acid against AD-like pathology (Mori et al. 2012).

SAMP8 mice develop early abnormalities of learning and memory due to overproduction of β-amyloid peptide. Therefore, these animals are a useful model of senescence acceleration and geriatric disorders with increased oxidative stress and neuronal deficit. Addition of oligonol (product of polyphenols oligomerization containing catechin-type monomers and oligomers of proanthocyanidins) to standard diet in the daily dose of 60 mg/kg body weight prolonged life span, improved locomotive activity, and suppressed the inflammatory response in SAMP8 mice infected with mouse hepatitis virus and pinworm (Tomobe et al. 2007).

Taking into consideration the results of abovementioned studies, it seems that plant polyphenols may protect organisms from neurodegenerative diseases and resulting dementia. The mechanisms of this protective action seem more complicated than direct unspecific antioxidant activity (however, this does not exclude the latter) and involve molecular interactions with peptides and reactions leading to amyloid formation and interference with intracellular pathways of signal transduction.

4.2 Epidemiological Data: Results of Cross-Sectional and Longitudinal Studies

Numerous in vitro and in vivo studies on experimental animals proved neuroprotective action of plant polyphenols. This was inspiration for planning and execution of epidemiologic observations on association between dietary ingestion of plant polyphenols/antioxidants, the cognitive performance, and risk of developing neurodegenerative diseases. Table 4 summarizes most important cross-sectional and longitudinal studies on the topics that were published in the last dozen years or so. They involved middle-aged and elderly subjects free of dementia at baseline, and the number of studied groups ranged from around 1,500 to 5,500 subjects, and the duration of follow-up in the case of longitudinal studies ranged from 6 to about 32 years.

Analysis of cross-sectional data obtained from 2031 older subjects revealed association between habitual consumption during the previous year of chocolate, wine, and tea (food products rich in variety of flavonoids) as estimated with validated food frequency questionnaire and performance on battery of 6 cognitive tests. Consumers of chocolate, wine, or tea had significantly higher mean test scores and lower prevalence

Table 4 Cross-sectional studies on relation between dietary intake of antioxidants, vitamins, food products rich in plant polyphenols, and cognitive performance

Aim of the study	Study description	Main results	References
Relation between cognitive tests performance and consumption during the previous year of chocolate, wine, and tea in elderly subjects	Cross-sectional, 2031 subjects (age 70–74 year), 6 cognitive tests, FFQ, multivariate models with adjustments for various sociodemographic variables	Consumers of chocolate, wine, or tea had significantly higher mean test scores and lower prevalence of poor cognitive performance than non-consumers	Nurk et al. (2009)
Relation between cognitive function and dietary intake during the previous year of β-carotene and vitamins C and E in elderly subjects	Cross-sectional, 5,182 subjects (aged 55–95 years), 30-point MMSE, FFQ, multivariate models with adjustments for various clinical and sociodemographic variables	Lower intake of β-carotene was associated with impaired cognitive function. Vitamins C and E intake was without significance	Jama et al. (1996)
Relationship between intake during the previous year of different plant foods and cognitive performance in elderly individuals	Cross-sectional, 2,031 subjects (aged 70–74 years), 6 cognitive tests, FFQ, multivariate models with adjustments for various clinical and sociodemographic variables	Intake of many plant foods (especially carrots, cruciferous vegetables, citrus fruits, high-fiber bread) is associated with better cognitive performance	Nurk et al. (2010)
Association between dietary flavonoid intake and cognitive decline in older persons over 10-year period	Cross-sectional at baseline, longitudinal with 10-year follow-up. 1,640 individuals without dementia (aged ≥65 years), 5 cognitive tests repeated 4 times (including baseline assessment), FFQ at baseline, analyses were adjusted for sociodemographic variables	Higher flavonoid intake was associated with better cognitive performance at baseline. Low flavonoid consumers had higher decline in cognitive performance over 10 years follow-up	Letenneur et al. (2007)
Association between midlife level of total and class-specific polyphenol intake and cognitive performance assessed 13 years later	Longitudinal with 13-year follow-up, 2,574 subjects, six 24-h dietary records for calculation of polyphenol intake at baseline. Four neuropsychological tests performed at the end of study. Analyses were adjusted for sociodemographic variables	High polyphenol intake (including catechins, theaflavins, flavonols, and hydroxybenzoic acids) was associated with better language and verbal memory. Intake of catechins, flavonols, proanthocyanidins was negatively associated with executive functioning	Kesse-Guyot et al. (2012)
Association between the French National Nutrition and Health Program Guideline Score (PNNS-GS) assessed at baseline and cognitive performance evaluated 13 years later	Longitudinal with 13-year follow-up, 2,135 subjects (French cohort) (age at cognitive evaluation 65.5 ±4.6 years) PNNS-GS estimated at baseline, four neuropsychological tests performed at the end of study. Analyses were adjusted for sociodemographic variables	Strong compliance with nutritional recommendations was related to better verbal memory	Kesse-Guyot et al. (2011)

(continued)

Table 4 (continued)

Aim of the study	Study description	Main results	References
Consumption of fruit and vegetable juices (rich in polyphenols) and the risk of incident probable AD in dementia-free Japanese Americans at baseline	Longitudinal with 8-year follow-up, 1,589 subjects. FFQ at baseline. Cognition, dementia, AD criteria evaluated at baseline and at every 2 years. Analyses were adjusted for sociodemographic variables and dietary intake of vitamins C, E, and β-carotene	63 incident cases of probable AD. Significantly lower hazard ratio for probable AD in subjects drank juices at least 3 times per week than in those who drank less often than once per week	Dai et al. (2006)
Does high dietary intake of antioxidants decrease the risk of Parkinson's disease?	Cross-sectional, 5,434 subjects (aged 55–95 years) without dementia, FFQ, all analyses were adjusted for sociodemographic variables	High intake of vitamin E (but not vitamin C, flavonoids, β-carotene) may protect from Parkinson's disease	de Rijk et al. (1997)
Relationship between dietary intake during the previous year of antioxidants (vitamins C and E, flavonoids, β-carotene) and the risk of AD in adults free of dementia at baseline	Longitudinal with 6-year follow-up, 5,395 subjects (aged ≥ 55 years), FFQ, 3-stage protocol for case-finding and diagnosis of dementia and AD performed at baseline, during and at the end of follow-up. All analyses were adjusted for clinical and sociodemographic variables	197 subjects developed dementia (146 had AD); high intake of vitamins C and E was associated with lower risk of AD. Flavonoids and β-carotene protected only in the subgroup of current cigarette smokers	Engelhart et al. (2002)
Association of midlife dietary intake of antioxidants to late-life dementia and its subtypes in dementia-free Japanese Americans at baseline	Longitudinal with 32-year follow-up, 2,459 men (aged 45–68 years) included in the study between 1965 and 1968, 24-h dietary recall and dementia assessment at baseline and several times during the follow-up. Analyses were adjusted for clinical and sociodemographic variables	235 incident cases of dementia (102 AD, 44 vascular dementia). No association between intake of β-carotene, flavonoids, vitamins E and C and the risk of dementia or its subtypes	Laurin et al. (2004)
Relationship between dietary intake during the previous year of antioxidants (vitamins C and E, flavonoids, β-carotene) and the long-term risk of AD in adults free of dementia at baseline	Longitudinal with 10-year follow-up, 5,395 subjects (aged ≥ 55 years), FFQ, 3-stage protocol for case-finding and diagnosis of dementia and AD performed at baseline, during, and at the end of follow-up. All analyses were adjusted for clinical and sociodemographic variables	465 incident cases of dementia (365 AD). Higher intake of vitamin E was associated with lower risk of dementia and AD. Vitamin C, β-carotene, and flavonoids were not protective	Devore et al. (2010)

FFQ food frequency questionnaire, *MMSE* mini–mental state examination, *AD* Alzheimer's disease

of poor cognitive performance than non-consumers. The strongest risk-reducing effect of poor cognitive performance was related to wine then to chocolate consumption (Nurk et al. 2009). Ingestion of tea had a weak effect and significantly reduced the risk of poor cognitive performance only in 2 out of 6 tests. These effects were dose dependent (mean test scores raised with increased intake of this food products); however, a plateau was observed for daily consumption of 75–100 ml of wine, 10 g of chocolate, and 200 ml of tea during the previous year (Nurk et al. 2009).

Two other cross-sectional studies had similar aim and design (Jama et al. 1996; Nurk et al. 2010). They reported association between cognitive function and dietary intake during the previous year of β-carotene and vitamins C and E (Jama et al. 1996) and different plant foods (Nurk et al. 2010) in elderly subjects. Lower dietary intake of β-carotene was associated with impaired cognitive function, while consumption of vitamins C and E was without significance (Jama et al. 1996). On the other hand, subjects with intakes of >10th percentile of fruits, vegetables, grain products, and mushrooms performed significantly better in cognitive tests than those with very low or no intake (Nurk et al. 2010). Combined intake of fruits and vegetables had strongest influence on mean test scores with dose-dependent relation up to daily consumption of 500 g. Analysis of individual plant foods revealed a positive effect of consumption of carrots, cruciferous vegetables, citrus fruits, and high-fiber bread on cognitive performance in elderly subjects (Nurk et al. 2010).

The first 3 longitudinal studies reported in Table 4 were devoted to investigate possible relations between dietary intake of flavonoids (Letenneur et al. 2007; Kesse-Guyot et al. 2012), adherence to rules of good nutrition, and cognitive performance assessed several years later (Kesse-Guyot et al. 2011). Higher dietary ingestion of flavonoids was associated with better cognitive performance in a group of 1,640 older adults free from dementia at baseline (Letenneur et al. 2007). Analysis over the 10-year follow-up revealed higher decline in MMSE score in the subjects with the lowest flavonoid intake (Letenneur et al. 2007).

Similar results were obtained in the study analyzing the effect of total and class-specific polyphenol intake on cognitive performance assessed 13 years later. High total polyphenol intake as well as ingestion of catechins, theaflavins, flavonols, and hydroxybenzoic acids was associated with better language and verbal memory. On the other hand, it should be pointed out that scores on executive functioning were negatively associated with intake of dihydrochalcones, catechins, proanthocyanidins, and flavonols (Kesse-Guyot et al. 2012). The last study from this group analyzed the association between adherence to rules of the French National Nutrition and Health Program and the cognitive performance evaluated 13 years later in the cohort of middle-aged adults (Kesse-Guyot et al. 2011). This program had a set of 9 priority objectives focusing on nutrition and physical activity. Those related to nutrition listed below are: increase fruit and vegetable consumption, reduce dietary fat intake (reduce consumption of saturated fats by 25 %; reduce total fat intake to less than 35 % of total dietary intake), increase consumption of carbohydrates (increase carbohydrate consumption to more than 50 % of total dietary intake through 25 % reduction in simple sugars; 50 % increase in fiber and increased consumption of complex carbohydrates starches), increase consumption of calcium

(reduce vitamin D deficiency by 25 %; reduce by 25 % the number of people with calcium intake below recommended levels), and reduce alcohol intake (reduce calorie intake from alcohol consumption in the general population to no more than two drinks per day). To evaluate the adherence to these rules, the special index score (French National Nutrition and Health Program Guideline Score) was constructed (Estaquio et al. 2008). There was a positive association between this index score and verbal memory and executive functioning (Kesse-Guyot et al. 2011). This suggests that strong compliance with nutritional recommendations in midlife could positively affect cognitive performance in the late life and thus prevent the development of dementia.

Five other studies (one cross-sectional and 4 longitudinal) analyzed the association between dietary intake of antioxidants including consumption of polyphenol-rich vegetable, fruit juices, the risk of neurodegenerative diseases (Parkinson's disease, AD), and the development of late-life dementia (Dai et al. 2006; de Rijk et al. 1997; Engelhart et al. 2002; Laurin et al. 2004). In a studied group of 5,434 subjects without dementia (in which 31 had diagnosed Parkinson's disease), high dietary intake during the previous year of vitamin E estimated with food frequency questionnaire was associated with the lower risk of Parkinson's disease (de Rijk et al. 1997). However, ingestion of other antioxidant plant compounds such us vitamin C, flavonoids, and β-carotene had no protective effect.

One hundred forty-six persons out of the group of 5,395 subjects free of dementia at baseline developed AD during the 6 years of prospective observation. In this group high intake of vitamin C and E was associated with the lower risk of AD; however, consumption of flavonoids and β-carotene had no protective effect (Engelhart et al. 2002). On the other hand, when the subgroup of current cigarette smokers ($n = 1{,}257$) was analyzed separately, the significant protective effect of all compounds was noted (Engelhart et al. 2002). The same group of subjects analyzed again after 10-year follow-up revealed protective effect of higher consumption of vitamin E but not vitamin C, flavonoids, and β-carotene (Devore et al. 2010).

In another study counting 1,589 Japanese Americans observed for 8 years, fruit and vegetable juice consumption at least 3 times per week decreased the risk of AD. Dietary intake of vitamins E, C, and β-carotene as well as tea consumption was not protective (Dai et al. 2006). Similarly, almost 32-year prospective observation of large group of men revealed no association between the midlife dietary intake of vitamins E, C, β-carotene, and flavonoids and the risk of late-life dementia and its subtypes (AD, vascular dementia, combination of AD, and cerebrovascular disease) (Laurin et al. 2004).

Age can induce changes in taste. In older subjects the loss of identification of sour and bitter taste in some regions of the tongue has been described (Nordin et al. 2007). Moreover, the sensitivity to sweetening agents is also decreased (Easterby-Smith et al. 1994; Kennedy et al. 2010). These may influence the food preference and in consequence the dietary intake of flavonoids and other antioxidants in elderly people. Therefore, estimation of dietary intake of plant antioxidants at midlife cannot precisely reflect consumption of these compounds in older people. Similarly, data from food frequency questionnaire (cross-sectional studies) obtained from

elderly subjects can be only partially compatible with their consumption at midlife. Moreover, decline of cognitive performance and dementia can alter dietary habits and subsequently intake of plant polyphenols and other antioxidants. Consequently, it cannot be excluded that lower intake of plant polyphenols and antioxidant vitamins reported in older subjects with worse cognitive performance (cross-sectional studies) was just the secondary effect of these disturbances on food preference.

Nevertheless, all studies except for one (Table 4) in general revealed a positive effect of dietary intake of antioxidant vitamins or plant polyphenols on cognitive performance. Their results need confirmation in further more extensive studies. Since data obtained from food frequency questionnaires have some limitations (especially for the group of elderly subjects), inclusion of monitoring of main phenolic metabolites or total phenolic concentration in plasma or urine in the prospective study protocols could be helpful to overcome interpretation troubles of obtained results.

Another interesting question is that intake of some polyphenols (catechins, flavonols) was simultaneously associated with better language and verbal memory but worse executive functioning (Kesse-Guyot et al. 2012). This suggests at least bidirectional action (beneficial or rather unfavorable) of some plant compounds on cognitive function in humans and requires further studies especially in respect to ingested dose and plasma levels of these compounds.

4.3 Interventional Studies: Results of Randomized, Double-Blind, Placebo-Controlled Clinical Trials

Epidemiologic data on positive association between antioxidant vitamins and plant polyphenols dietary intake and cognitive performance in elderly subjects as well as results of experimental studies on relationship between oxidative stress and cognitive decline inspired scientists to perform interventional studies on effects of diet supplementation with antioxidants and polyphenols on cognitive performance and markers of oxidative stress in humans.

Some promising reports (with randomized, double-blind, placebo-controlled design) on these topics have been published during the last several years (Table 5). Because an increased fruits and vegetables consumption involves increased vitamins, microelements, and numerous polyphenols ingestion, the intervention in the majority of these trials consisted in supplementation with mixture of variety of plant compounds (sometimes not precisely defined chemically) with direct and indirect antioxidant and anti-inflammatory properties.

Part of these studies focused on possible suppression of circulating markers of oxidative stress in AD patients and healthy elderly subjects after oral supplementation with cocktails of plant polyphenols or antioxidant vitamins with or without simultaneous monitoring of cognitive function. Since oxidative stress is involved in the development of AD and subsequent dementia, one may assume that inhibition of oxidative stress may protect from further progression of the disease.

Table 5 Interventional studies (randomized, double-blind, placebo-controlled) on plant polyphenols and antioxidants effect on cognition in various clinical settings

Clinical setting	Intervention	Treatment duration and patients number	Outcome	References
Total plasma homocysteine level in patients with AD	Antioxidant drink, 200 ml/day (polyphenols from apple, lemon, green tea extracts, vitamins B and C)	8 months, 48 patients with AD, 52 controls	Attenuation of the increase in homocysteine level along with time in AD patients	Morillas-Ruiz et al. (2010)
Cognitive performance in healthy elderly women	One capsule (set of antioxidant vitamins, magnesium, selenium) per day	6 months, 220 women (aged 60–91 years)	No effect on cognitive performance in female seniors	Wolters et al. (2005)
Memory function in community -dwelling seniors without dementia	Complex antioxidant blend (34 components, e.g., grape seed extract, ginkgo biloba, gotu kola)	4 months, 86 subjects (aged 50–75 years)	Significant memory improvement	Summers et al. (2010)
Oxidative stress markers in patients with AD	Cholinesterase inhibitor (donepezil 5 mg/day) plus mixture of antioxidants vs. donepezil plus placebo	6 months, 52 patients with AD	Decrease of circulating homocysteine and hydroperoxides	Cornelli (2010)
Cognitive function and circulating isoprostanes and amyloid β in AD patients	Curcumin in a daily dose 0, 1, or 4 g followed by monitoring of curcumin metabolites in plasma	6 months, 34 patients with AD, age ≥ 50 years	No effect on circulating isoprostanes and amyloid β. Inconclusive for cognitive function	Baum et al. (2008)
Cognitive function and circulating lipid peroxidation products in older adults	Flavonoid antioxidant Pycnogenol 150 mg per day (100 mg with breakfast, 50 mg with evening meal)	3 months, 101 healthy adults (age 60–85 years) free of any medications	Significant improvement of spatial working memory. Tendency to decrease circulating F2-isoprostanes	Ryan et al. (2008)

Dementia in Down syndrome subjects over age 40 years	Daily oral antioxidant supplementation (900 IU of α-tocopherol, 200 mg of ascorbic acid and 600 mg of α-lipoic acid)	2 years, 53 individuals with Down syndrome and dementia	Lack of improvement in cognitive performance and inhibition of cognitive decline	Lott et al. (2011)
Memory and cognitive performance in community-dwelling adults without dementia	Vitamins and antioxidants (folic acid, B12, vitamin E, *S*-adenosylmethionine, *N*-acetylcysteine, acetyl-L-carnitine)	3 months, 115 adults (aged 22–73 years)	Significant improvement of memory and cognitive performance	Chan et al. (2010)
Cognitive function in older adults with stable state of health	Dried powdered rosemary in single 458 ml drink of tomato juice (doses 0 mg placebo, 750, 1,500, 3,000, and 6,000 mg)	Cognitive function tested after 1, 2.5, 4, and 6 h after single dose of rosemary (7 day washout between doses), 28 adults (mean age 75 years)	Dose-specific effect on speed memory; at 750 mg significant improvement, at 6,000 mg significant impairment versus placebo	Pengelly et al. (2012)
Memory function in older adults with MCI	Concord grape juice (daily dose 6–9 ml/kg body weight)	12 weeks, 12 adults (78 ± 5 years) with memory decline but not dementia	Significant improvement in verbal learning	Krikorian et al. (2010a)
Memory function in older adults with MCI	Wild blueberry juice (daily dose 6–9 ml/kg body weight)	12 weeks, 16 older adults with memory decline but not dementia	Significant improvement of memory functions	Krikorian et al. (2010b)
Neurocognitive function in older adults with mild age-related memory decline	Concord grape juice (daily dose 6.3–7.8 mL/kg of body weight)	16 weeks, 21 adults (76.9 ± 6.1 years)	Reduced interference during recognition memory	Krikorian et al. (2012)
Neuropsychological functioning of cognitively intact older adults	Cranberry juice (900 g/day of 27 % vol. juice)	6 weeks, 50 community-dwelling adults (age ≥60 years)	No significant effect	Crews et al. (2005)

AD Alzheimer's disease, *MCI* mild cognitive impairment

Concentration of circulating homocysteine that can exert direct neurotoxic effect and induce endothelial dysfunction via promoting oxidative stress is elevated in AD patients. Increased homocysteine levels (even within the normal range) were reported to strongly promote the cognitive decline in AD patients (Oulhaj et al. 2010) and in healthy elderly subjects (McCaddon et al. 2001). Since polyphenols can normalize plasma homocysteine levels in humans, one double-blind placebo-controlled study on the effect of brisk consumption of 200 ml drink rich in antioxidant polyphenols from apple, lemon concentrate juice, apple, green tea extracts, and vitamins B and C on total plasma homocysteine levels in AD patients (initial and moderate phase) has been done (Table 5). Eight-month consumption of this drink significantly attenuated the rise of homocysteine level along with time in AD patients especially in those with moderate phase (Morillas-Ruiz et al. 2010). However, in any studied group (AD patients and age-, sex-, BMI-matched controls), the homocysteine levels after intervention were not lower than at the baseline (Morillas-Ruiz et al. 2010). Therefore, the effect of this treatment was rather weak because it did not decrease (normalized) but only inhibited the rise of total plasma homocysteine levels in AD patients. Since no monitoring of cognitive performance was done, the clinical outcome of this polyphenolic cocktail is not known.

In another placebo-controlled study, 6-month multivitamin supplementation (including 150 mg vitamin C, 36 mg vitamin E, 50 mg magnesium, and 60 μg selenium per day) lowered total plasma homocysteine levels in the group of 220 healthy elderly women without dementia (Wolters et al. 2005). On the other hand, this intervention did not change the cognitive performance in vitamin group comparing to the placebo receivers. Perhaps, the period of lowered homocysteine levels was too short to reveal any positive effect on cognitive performance in this group of healthy women. It should be pointed out that observational studies proving relationship between increased circulating homocysteine and the rate of decline of cognitive performance lasted at least a few years (Oulhaj et al. 2010; McCaddon et al. 2001).

Four-month administration of complex antioxidant blend (34 components including antioxidant vitamins, microelements, ginseng, grape seed extract, gotu kola, ginkgo biloba) reduced circulating homocysteine along with improvement of memory function in community-dwelling seniors without dementia at baseline (Summers et al. 2010).

Mixture of various direct and indirect antioxidants (carnosine, coenzyme Q 10, vitamin E, vitamin C, β-carotene, selenium, L-cysteine, ginkgo biloba, vitamins B1, B2, B3, B6, B9, B12) was also tested in combination with donepezil (cholinesterase inhibitor) in patients with AD. After six-month treatment, significant attenuation of some markers of oxidative stress (hydroperoxides) and circulating homocysteine in comparison to AD patients treated with donepezil plus placebo was noted (Cornelli 2010).

Curcumin (a polyphenolic molecule) is an effective scavenger of reactive oxygen and nitrogen species in vitro and was effective in animal models of AD reducing brain amyloid, plaques, and markers of oxidative stress. However, curcumin given in a maximal oral daily dose of 4 g for 6 months had no significant effect on circulating isoprostanes and serum concentrations of amyloid β (AD biomarker) in patients presenting with progressive decline in memory and cognitive function for half of the

year (probable or possible AD) (Baum et al. 2008). Although MMSE scores were noted for all participants at baseline and at the end of treatment, the study was inconclusive for any effect of curcumin on cognitive performance since there was no decline in cognitive function in the group treated with placebo over the study period (Baum et al. 2008). Pycnogenol (the trade name for a specific blend of procyanidins extracted from the bark of French maritime pine with strong antioxidant properties, Horphag Research, Geneva, Switzerland) given in the daily oral dose 150 mg for 3 months significantly improved spatial working memory while had no distinct effect on plasma concentration of lipid peroxidation products in healthy older adults (Ryan et al. 2008).

Effect of fruit juice consumption (Concord grape juice, wild blueberry juice, cranberry juice) on cognitive function in older subjects with and without MCI is the main feature in the second group of these interventional studies (Table 5). Moreover, this group involves also trials with dried rosemary leaf powder and combination of vitamins with antioxidants on neurocognitive performance in older adults and subjects with Down syndrome and dementia (Lott et al. 2011; Chan et al. 2010; Pengelly et al. 2012). Consumption of Concord grape juice or wild blueberry juice in a daily dose 6–9 ml/kg body weight for 12 weeks revealed some neurocognitive effects in older subjects with early memory decline but not dementia (Krikorian et al. 2010b). This consisted of significant improvement in a measure of verbal learning and nonsignificant enhancement of verbal and spatial recall (Krikorian et al. 2010a). Results of this study were confirmed recently with the same daily dose of Concord grape juice consumed for 16 weeks. Active treatment group revealed reduced interference during recognition memory versus placebo group (Krikorian et al. 2012). Moreover, functional magnetic resonance imaging revealed increased activity in the right anterior and posterior regions of the brain cortex during performance of memory tasks after supplementation with the concord grape juice (Krikorian et al. 2012), which may suggest greater hemodynamic response and neuronal activity. On the other hand, cranberry juice consumed for 6 weeks had no significant effect on neuropsychological functioning (assessed by a battery of tests, e.g., selective reminding test, Wechsler Memory Scale III Faces I and Faces II subtests) of community-dwelling, cognitively intact older adults (Crews et al. 2005). Similar results were found in individuals with Down syndrome and dementia. Although 2 years supplementation with antioxidants (α-tocopherol, ascorbic acid, and α-lipoic acid) was safe, well tolerated, and increased about 2 times the plasma levels of α-tocopherol in these subjects, no improvement in cognitive performance and inhibition of cognitive decline was noted compared to the placebo group (Lott et al. 2011).

In another study composition of vitamins and antioxidants (folic acid, B12, Vitamin E, *S*-adenosylmethionine, *N*-acetylcysteine, and acetyl-L-carnitine) improved memory and cognitive performance in adults without dementia (Chan et al. 2010). However, performance declined to baseline following withdrawal of this treatment (3 month washout) and again statistically improved when subjects involved in the study resumed taking this supplementation for three additional months (Chan et al. 2010).

The study on short-term effect of various single doses of dried rosemary leaf powder (R. officinalis L. from 750 mg to 6,000 mg) on cognitive performance

revealed a very interesting dose-dependent effect (Pengelly et al. 2012). Lower doses (750 mg, almost equal to normal culinary consumption) improved the speed memory in older adults. The highest dose of 6,000 mg had opposite effect – significantly impaired the speed memory as estimated with Cognitive Drug Research computerized system at 1 to 6 h of post-ingestion. Similarly, biphasic dose–response curve was noted for self-reported alertness with computerized questionnaire scale (Bond–Lader Visual Analogue Scales of Mood and Alertness) (Pengelly et al. 2012).

Although the majority of these trials revealed positive effects of dietary interventions on circulating markers of oxidative stress and cognitive performance, they should be recognized as pilot studies that can be used for future planning of more extensive trials. As one can see from Table 5, these studies have some limitations. Low number of studied patients/volunteers and relatively short duration of dietary supplementation seem to be the most important factors suggesting caution for application of these results into clinical practice. The majority of these studies (9 of 13) involved older adults in a stable state of the health with or without mild impairment of cognitive performance. For that reason, their results could be rather applicable for prevention than for treatment of cognitive disorders.

Dietary supplementation included consumption of various juices, combination of direct and indirect antioxidants (vitamins, plant phenolics, microelements), plant extracts, and just dried leaves. Thus, in some cases the observed improvement of cognitive performance or suppression of circulating markers of oxidative stress could be the result of simultaneous action of several dozen of bioactive compounds including polyphenols. In addition, dietary polyphenols before absorption into the blood could be transformed into less complex compounds by gut microflora (Aura 2008). Therefore, the composition of polyphenols supplement may differ significantly from that absorbed and reaching brain tissues with circulating blood. Taking these into consideration, it is necessary to determine active substance or substances responsible for improvement of cognitive performance in order to construct the most effective supplement. It is interesting to investigate whether supplementation-induced improvement of cognitive performance is stable or transient. What dose of polyphenols is necessary for the maintenance of the positive effect? Only one study of those listed in Table 5 tried to answer these questions. In this study improved memory and cognitive performance after 3-month supplementation with vitamins and antioxidants declined to baseline already after 3-month washout period. Moreover, they raised again significantly after consecutive 3-month supplementation (Chan et al. 2010). This indicates that the effect of supplementation is reversible, and its maintenance needs continuous addition of these antioxidants into the diet.

Another question is the optimal daily dose of dietary supplements that can improve or inhibit decline in cognitive performance in elderly subjects. As was shown in the study with short-term effect of rosemary on speed memory, the supplement can improve or damage cognitive performance depending on the amount of ingested active substance. These questions should be solved in future trials. To do this, more extensive studies with a large number of patients with different stages of cognitive impairment, various doses of supplements, and longer duration of dietary intervention are necessary.

5 Future Directions

Some important questions necessary to solve were already listed at the end of the last subchapter. However, having established set of plant polyphenols (or their metabolites) with anti-neurodegenerative activity, we should ask the following questions: is it necessary to use them for dietary supplementation? What about addition to the diet of food products with high content of these compounds?

Polyphenols are almost ubiquitous in plant foods. Therefore, it seems that their content in the average diet would be sufficient to cause neuroprotection and inhibit age-related cognitive decline under condition of better absorption from the gastrointestinal tract into the blood. Dietary polyphenols are poorly absorbed in the small intestine, and the majority of them can reach the colon where they are exposed to action of variety of enzymes synthesized by the colon microbiota. These enzymes can hydrolyze glycosides, glucuronides, sulfates, amides, esters, and lactones. Apart from reactions of reduction, decarboxylation, demethylation, and dehydroxylation, they can also break down the polyphenolic backbone structure, thus producing numerous efficiently absorbed low-molecular-weight metabolites. Therefore, it seems that stimulation of intestinal growth of bacterial species that effectively process dietary polyphenols into easily absorbed compounds with neuroprotective activity would be another approach. This could be obtained with the usage of appropriate probiotics and/or prebiotics and would not require additional dietary supplementation with plant polyphenols.

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Hyperbaric Oxygen Treatment in Autism Spectrum Disorders

Daniel A. Rossignol

Abbreviations

ASD	Autism spectrum disorder
ATEC	Autism Treatment Evaluation Checklist
ATM	Atmosphere
ATP	Adenosine triphosphate
CARS	Childhood Autism Rating Scale
CSF	Cerebrospinal fluid
fMRI	Functional magnetic resonance imaging
HBOT	Hyperbaric oxygen treatment
IHA	International Hyperbarics Association
PET	Positron emission tomography
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SPECT	Single-photon emission computed tomography
SRS	Social Responsiveness Scale
TBI	Traumatic brain injury

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1 Introduction

Hyperbaric oxygen treatment (HBOT) involves inhaling up to 100 % oxygen at a pressure greater than one atmosphere (atm) in a pressurized chamber (Feldmeier 2003). The use of HBOT in adults and children has been shown to be safe at pressures of 2.0 atm for 2 h per day (Ashamalla et al. 1996; Perrins and James 2005). Many of the clinical indications for HBOT are at higher pressures (over 2.0 atm) including treatment of decompression sickness, healing of problem wounds, arterial gas embolism, and carbon monoxide poisoning (Leach et al. 1998). However, interest has increased recently in using high levels of oxygen in a way resembling a drug to improve brain function (Harch et al. 2011). For example, in healthy young adults, the use of supplementary oxygen when compared with room air significantly enhanced memory (Moss and Scholey 1996), cognitive performance, word recall, and reaction time for 24 h (Scholey et al. 1999) as well as attention and picture recognition (Moss et al. 1998) in several double-blind studies. Some investigators have also reported that HBOT may possess neuroprotective effects (Henninger et al. 2006; Huang and Obenaus 2011).

HBOT has been used to treat certain neurological disorders, some of which are considered incurable. Many of these disorders have been treated with lower HBOT parameters (e.g., 1.3–1.5 atm and oxygen at 24–100 %) than traditional indications. For example, one investigator reported significant improvements in a 15-year-old child who had fetal alcohol syndrome using HBOT at 1.5 atm/100 % oxygen for 73 sessions (Stoller 2005). Several recent studies have reported a beneficial effect from HBOT in traumatic brain injury (TBI) in both animal models (Harch et al. 2007; Henninger et al. 2006; Palzur et al. 2008; Vlodavsky et al. 2006; Wang et al. 2010) and humans (Eovaldi and Zanetti 2010; Golden et al. 2002, 2006; Harch et al. 2009, 2011; Hardy et al. 2007; Lv et al. 2011; Rockswold et al. 2010; Sahni et al. 2011; Shi et al. 2003, 2006; Stoller 2011; Wright et al. 2009), including two recent controlled studies (Rockswold et al. 2010; Sahni et al. 2011). Larger multicenter trials are ongoing in attempt to confirm these findings (Helms et al. 2011). In a recent study of 16 individuals who had TBI, individuals exhibited significant improvements with the use of HBOT at 1.5 atm/100 % oxygen (40 hourly treatments over 30 days) in their neurological exam, IQ, memory, posttraumatic stress symptoms, depression, anxiety, and quality of life. They also displayed objective improvements in brain perfusion measured by pre- and post-HBOT single-photon emission computed tomography (SPECT) scans (Harch et al. 2011). Other studies have also reported significant improvements in cerebral perfusion in TBI or chronic brain injury as measured by pre- and post-HBOT SPECT scans (Golden et al. 2002; Harch et al. 2009; Shi et al. 2003).

Starting around 2005, some investigators speculated that HBOT may be useful to treat children with autism spectrum disorders (ASD) (Buckley 2005; Harch and Small 2005; Rossignol 2007; Rossignol and Small 2006; Stoller and Small 2006). Several abnormalities reported in individuals with ASD suggest that HBOT may be beneficial. For example, multiple studies have reported relative cerebral

hypoperfusion in individuals with ASD compared to controls as measured by positron emission tomography (PET), SPECT, or functional magnetic resonance imaging (fMRI). This hypoperfusion correlates with certain autistic behaviors such as repetitive behaviors (Starkstein et al. 2000), desire for sameness (Ohnishi et al. 2000), impairments in processing facial expressions and emotions (Critchley et al. 2000), and decreased language development (Wilcox et al. 2002). Furthermore, lower perfusion to the brain has been significantly correlated with more severe autistic behaviors (Gendry Meresse et al. 2005) and increasing age in children with ASD (Wilcox et al. 2002). Improvements in cerebral perfusion have been documented with the use of HBOT at 1.3–1.5 atm (Golden et al. 2002; Harch et al. 2009, 2011; Heuser et al. 2002; Shi et al. 2003).

Furthermore, data has accumulated that some individuals with ASD have evidence of neuroinflammation or gastrointestinal inflammation. One recent review article reported that 416 publications implicated inflammation or immune abnormalities in ASD, including 65 publications of neuroinflammation and 31 publications of gastrointestinal inflammation (Rossignol and Frye 2011b). Since hypoxia is involved in inflammation (Cramer et al. 2003), treatment with HBOT may help lower inflammation (Rossignol 2007). In fact, HBOT has been shown to possess potent anti-inflammatory properties in both animal (Akin et al. 2002; Luongo et al. 1998; Sumen et al. 2001) and human studies (Abbot et al. 1994; Granowitz et al. 2002; Lavy et al. 1994; Nelson et al. 1990; Takeshima et al. 1999). HBOT has also been reported to decrease the production of proinflammatory cytokines (including TNF-alpha, interferon-gamma, IL-1, and IL-6) in both animal (Inamoto et al. 1991; Yang et al. 2006) and human studies (Granowitz et al. 2002; Weisz et al. 1997) as well as increase IL-10 levels (Buras et al. 2006). The effect of HBOT on inflammation may be mediated through a pressure-related effect and not necessarily by the oxygen delivered, as one human study reported a reduction in interferon-gamma production by lymphocytes with HBOT at 2.0 atm/10.5 % oxygen but an increase in interferon-gamma with 100 % oxygen at 1.0 atm (Granowitz et al. 2002).

Some individuals with ASD also have evidence of mitochondrial dysfunction (Chauhan et al. 2011; Frye and Rossignol 2011; Rossignol and Frye 2011a). A recent review article reported that 145 publications implicated mitochondrial dysfunction in ASD (Rossignol and Frye 2011b). Although treatments for mitochondrial dysfunction remain relatively limited (Rossignol and Frye 2011a), interest has increased recently in using HBOT as a potential treatment. Since hypoxia is known to impair mitochondrial function (Magalhaes et al. 2005) and because only approximately 0.3 % of inhaled oxygen is ultimately delivered to mitochondria (Lane 2002), increasing oxygen delivery to dysfunctional mitochondria through HBOT may aid in improving mitochondrial function (Daugherty et al. 2004; Dave et al. 2003).

Both animal and human studies have examined the effects of HBOT on mitochondrial function. In a mouse model with an intrinsic impairment of mitochondrial complex IV activity, HBOT at 2.0 atm significantly improved mitochondrial dysfunction and delayed the onset of motor neuron disease when compared with mice not treated with HBOT (Dave et al. 2003). In other animal studies, HBOT increased the amount of work performed by mitochondria (Boveris and Chance 1973),

improved mitochondrial function after brain injury (Daugherty et al. 2004), and prevented mitochondrial deterioration (Gosalvez et al. 1973) when compared with room air pressure and 100 % oxygen levels. HBOT has also been reported to increase sperm motility by augmenting mitochondrial oxidative phosphorylation in fructolysis-inhibited sperm cells (Bar-Sagie et al. 1981). HBOT prevented apoptosis and improved neurological recovery after cerebral ischemia by opening mitochondrial adenosine triphosphate (ATP)-sensitive potassium channels (Lou et al. 2006). In another animal model, hypoxia and ischemia led to diminished ATP and phosphocreatine production; the addition of HBOT restored these levels to near normal and increased energy utilization when compared with room air oxygen and pressure levels (Calvert and Zhang 2007). Furthermore, HBOT was recently shown to activate mitochondrial DNA transcription and replication, in part, through the increased production of reactive oxygen species (ROS) and thus increase mitochondrial biogenesis in the rat hippocampus. In this study, older mitochondria were removed (through autophagy) and were replaced with more healthy mitochondria (biogenesis) (Gutsaeva et al. 2006). In another study of rats with normal mitochondrial function, HBOT increased the production of ATP compared to a control group (Kurt et al. 2008). Finally, in a recent controlled study of 69 patients with severe TBI, HBOT at 1.5 atm/100 % oxygen significantly increased brain oxygen levels, increased cerebral blood flow, and decreased cerebrospinal fluid (CSF) lactate levels. In this study, HBOT also improved brain metabolism and mitochondrial function compared with both room air treatment and 100 % oxygen given at normobaric pressure (Rockswold et al. 2010).

It should be noted that, theoretically, HBOT might increase oxidative stress through the augmented production of ROS from the high concentration of oxygen (Alleva et al. 2005). This may occur because increased oxygen delivery to mitochondria can increase ROS production. However, increasing oxygen levels in the blood reduces blood flow by autoregulation (Jacobson et al. 1963). This autoregulation may help to limit the potential increase in oxygen delivery to mitochondria by HBOT and therefore curb increases in ROS and oxidative stress. Furthermore, HBOT has also been shown to upregulate the production of antioxidant enzymes such as superoxide dismutase (Gregorevic et al. 2001; Ozden et al. 2004), glutathione peroxidase (Gulec et al. 2004), catalase (Nie et al. 2006), paraoxonase (Sharifi et al. 2004), and heme-oxygenase 1 (Rothfuss et al. 2001; Speit et al. 2000). This increase in antioxidant enzyme levels has been termed “conditioning” and can protect against damage caused by ROS (Rossignol 2007; Rothfuss and Speit 2002).

Since some children with ASD have evidence of elevated oxidative stress (Chauhan and Chauhan 2006; Rossignol and Frye 2011b), some investigators have been concerned that HBOT could increase oxidative stress in these children (Rossignol et al. 2007). An increase in oxidative stress appears to be less of a concern at HBOT pressures under 2.0 atm (Wada et al. 2001). However, at pressures above 2.0 atm, the production of ROS may increase exponentially and overwhelm the protective effect afforded by conditioning and therefore lead to increased oxidative stress (Wada et al. 2001). Therefore, keeping HBOT treatment protocols to 2.0 atm or less appears prudent in children with ASD as higher pressures might be

detrimental. However, as previously discussed, a slight increase in ROS produced by HBOT may be beneficial as these ROS appear to play a role in augmenting mitochondrial biogenesis (Gutsaeva et al. 2006).

2 The Effect of HBOT on Biomarkers of Inflammation and Oxidative Stress in ASD

Three studies have examined the effects of HBOT on biomarkers of inflammation and oxidative stress in children with ASD (Audhya 2007; Bent et al. 2012; Rossignol et al. 2007). In addition, two of these studies (Bent et al. 2012; Rossignol et al. 2007) reported behavioral outcomes (discussed shortly). In the first study (unpublished), HBOT was administered at 1.3 atm to 48 children with ASD, and superoxide dismutase (SOD), catalase, and glutathione peroxidase levels were measured before starting HBOT and after 1 day and 32 days of HBOT (Audhya 2007). After 1 day, mean SOD increased by 4.5-fold and after 32 days was 4.7-fold higher than before beginning HBOT. Mean catalase increased by 1.9-fold after 1 day and after 32 days was 90 % of the initial level before beginning HBOT. Finally, mean glutathione peroxidase increased by 1.4-fold after 1 day and after 32 days was 1.2-fold higher than before beginning HBOT. The effects of HBOT on these antioxidant enzymes may be an example of conditioning as previously discussed.

In another prospective study, HBOT was administered at 1.3 atm/24 % oxygen (12 children) and 1.5 atm/100 % oxygen (6 children), and biomarkers were measured before HBOT was initiated and after 40 HBOT sessions (Rossignol et al. 2007). C-reactive protein (a general marker of inflammation) dropped in both the 1.3 atm ($p=0.123$) and 1.5 atm ($p=0.084$) groups. This drop was significant when all study participants were examined ($p=0.021$). This finding is notable because some of the children in this study did not initially have elevated C-reactive protein, and children with the highest C-reactive protein levels had the largest decrease. Plasma-oxidized glutathione levels did not significantly change at 1.3 atm ($p=0.557$) or 1.5 atm ($p=0.583$). Since oxidized glutathione is exported from cells when intracellular levels exceed the redox capacity (Dickinson and Forman 2002), this finding suggests that intracellular oxidative stress did not significantly worsen. Strengths of this study included the prospective nature and the use of objective measurements (oxidative stress and inflammatory markers). Limitations of this study included the open-label nature, the lack of a control group, and a relatively small sample size.

Finally, in another prospective study, plasma cytokine levels, including some associated with inflammation, were measured before and after 80 HBOT sessions at 1.5 atm/100 % oxygen over a 20-week period in 10 children with ASD (Bent et al. 2012). This study reported no significant changes in cytokines during the study. However, a significant limitation of this study was that none of the children had abnormal cytokine levels at the beginning of the study, making it unlikely that a significant change could be observed. Another limitation was the lack of cytokine measurements in the cerebrospinal fluid or brain, especially since cytokine abnormalities

have been reported in these areas in some children with ASD (Ashwood and Van de Water 2004; Chez et al. 2007; Vargas et al. 2005). Further studies of HBOT in children with ASD who have abnormal cytokines and markers of inflammation are warranted to investigate these findings in more depth.

3 The Effects of HBOT on Cerebral Perfusion in ASD

Several studies have examined changes in cerebral perfusion with HBOT in children with ASD. One case report noted improvements in cerebral blood flow in a child with ASD as measured on pre- and post-HBOT SPECT scans. In this case, HBOT was administered at 1.3 atm/24 % oxygen for 1 h per day for 10 consecutive days (Heuser et al. 2002). In another report, two children with ASD had improvements in cerebral perfusion on pre- and post-HBOT SPECT scans. In these two cases, HBOT was administered at 1.3 atm/24 % oxygen for 40–80 treatments (Rossignol 2008). Kinaci et al. reported on the effects of HBOT at 1.5 atm/100 % oxygen for 50 sessions at 60 min per day in 108 children with ASD (Kinaci et al. 2009). This study also reported behavioral effects of HBOT (discussed shortly). All 108 patients had normal MRI scans, and all had decreased temporal lobe perfusion as measured by SPECT scans, while 88 % had decreased frontal lobe perfusion, and 61 % had decreased perfusion to other areas of the brain. Comparing pre- and post-HBOT SPECT scans, 82.4 % of the patients had an improvement in temporal lobe perfusion, 85.3 % improved in frontal lobe perfusion, and 75.8 % had improvements in perfusion to other brain areas.

4 The Effects of HBOT on Behavioral Measurements in ASD

4.1 Studies Lacking a Control Group

Several case studies have reported improvements in several behavioral domains using HBOT in individuals with ASD. The first published report of the use of HBOT in an individual with ASD was in 1994 (Anonymous 1994). In this report, a 3-year-old child with ASD was treated with HBOT, and improvements were reported in mood and social interaction. The number of treatments and other HBOT parameters were not reported. Heuser et al. (2002) reported a "striking improvement" in behavior, memory, social interaction, verbalizations, and cognitive functioning in a 4-year-old boy with ASD after using HBOT at 1.3 atm/24 % oxygen for 10 consecutive days. Burke (2007) noted improvements in two children with ASD using HBOT at 1.3 atm/28 % oxygen, including improvements in communication, aggressiveness, and social interaction. Another report noted improvements in one child with ASD in handwriting after 40 treatments with HBOT at 1.3 atm/24 % oxygen, and a second child had improvements in chronic diarrhea, distended abdomen, and

eczema with HBOT at 1.5 atm/100 % oxygen for 40 treatments (Rossignol 2008). One investigator reported improvements in language, social interaction, and overall cognition in a 3-year-old boy with ASD using HBOT at 1.3 atm/24 % oxygen for 40 treatments (Van Dyke 2009). This child also had chronic diarrhea and had his first normal bowel movement of his life during HBOT. In another report, 23 patients with ASD had various improvements in social interaction, language, and repetitive behaviors with HBOT at 1.5 atm (Harch and Small 2005). One larger, prospective study (unpublished) of 20 children with ASD used 20 sessions of HBOT at 1.5 atm/100 % oxygen and reported various improvements in communication, social interaction, and stereotypical behaviors (Markley 2007). Some investigators have also reported improvements using HBOT in children with concomitant mitochondrial disease and ASD (Van Dyke 2009). Limitations of these reports include the lack of a control group, the open-label nature, the small number of participants, and the retrospective nature. However, it should be noted that for a new treatment to be recognized, it is common for case reports to first be published to introduce and confirm a new treatment before sufficient interest is generated to commit resources to completing larger high-quality, stronger studies.

The first published case series to examine the effects of HBOT in children with ASD administered HBOT at 1.3 atm/28 % oxygen (1 h treatments for 40 treatments) to 6 children (Rossignol and Rossignol 2006). Improvements were reported on the Autism Treatment Evaluation Checklist (ATEC), the Childhood Autism Rating Scale (CARS), and the Social Responsiveness Scale (SRS). No adverse effects were noted. More significant improvements were observed in children under age 5 compared to those older. Limitations of this study included the retrospective nature, the small number of participants, the use of parent-rated scales, and the lack of a control group.

A follow-up prospective study examined the effects of HBOT in 18 children with ASD (Rossignol et al. 2007). Twelve children were treated at 1.3 atm/24 % oxygen, and 6 were treated at 1.5 atm/100 % oxygen. Hyperbaric sessions were 45 min in duration for 40 total sessions. As previously noted, markers of oxidative stress and inflammation were measured. Pre- and post-HBOT parent-rated SRS and ATEC indicated significant improvements in each group, including motivation, speech, and cognitive awareness ($p < 0.05$ for each). No major adverse events were observed. Strengths of this study included the prospective nature and the use of objective measurements (oxidative stress and inflammatory markers). Limitations of this study included the open-label nature, the lack of a control group, the use of parent-rated scales, and a relatively small sample size.

One small, prospective case series of three children with ASD used a multiple baseline design, and the authors reported no apparent improvements (compared to baseline) with HBOT at 1.3 atm/88 % oxygen for 27–40 treatments of 60 min each. However, one child had an increase in spontaneous communication, and another child had a decrease in problem behaviors with HBOT and an immediate increase in problem behaviors when HBOT was stopped (Lerman et al. 2009). Strengths of this study included the prospective nature, the multiple baseline design (including a baseline prior to initiating HBOT), as well as evaluations by therapists and videotaping. Limitations included a small sample size, the open-label nature, and the lack of control children.

Another prospective study from Thailand reported the effects of HBOT at 1.3 atm/100 % oxygen for 10 sessions (one session per week) in 7 children with ASD (Chungpaibulpatana et al. 2008). Significant improvements ($p<0.001$ for each) were observed in social interaction, fine motor and eye-hand coordination, language, gross motor skills, and self-help scores. There were no adverse effects except for transient tinnitus in one child which resolved within one week. Strengths of this study included the prospective nature and the objective measurements of self-help and motor skills by therapists. Limitations of this study included the lack of a control group, a small sample size, and the open-label nature.

A large, retrospective study from Turkey used HBOT at 1.5 atm/100 % oxygen for 50 sessions at 60 min per day and reported pre- and post-HBOT clinical scores measured on the ATEC (Kinaci et al. 2009). As previously noted, improvements were observed on pre- and post-HBOT SPECT scans. As rated by clinicians/therapists for 54 children with ASD, improvements were observed in speech/language/communication in 79 %, sociability in 85.5 %, sensory/cognitive awareness in 87 %, and health/physical/behavior in 75.2 %. Strengths of this study included objective measurements (SPECT imaging), evaluations by clinicians, and a larger sample size than other studies. Limitations included the retrospective nature, the lack of a control group, and the open-label nature.

One prospective study used a multiple baseline design and examined the effects of HBOT at 1.3 atm/24 % oxygen for 40 treatments in 16 children with autism treated over an average of 56 days (Jepson et al. 2010). The mean frequency of treatments was 4.78 sessions per week with a range of 2.46–7.0 sessions. No consistent positive or negative effects were observed. Strengths of this study included the multiple baseline design (including a baseline prior to initiating HBOT), as well as evaluations by therapists and videotaping. Limitations included the open-label nature, a small sample size, lack of control children, and the use of an observational technique which may not have been sufficient to measure changes in certain areas, such as attention and memory.

Finally, a more recent prospective study in 10 children with autism measured the effects of HBOT at 1.5 atm/100 % oxygen for 1 h per day, 5 days per week for 80 treatments (completed over 20 weeks, with a 4-week break between the 40th and 41st dive) on several behavioral scales as rated by parents and clinicians (Bent et al. 2012). As previously noted, cytokine markers were measured before and after HBOT. Significant improvements were observed as measured by parent-rated ABC in irritability, lethargy, hyperactivity, and overall scores ($p=0.02$ or less for each). On the parent-rated PDD-BI, significant improvements were observed in sensory problems, specific fears, and aggressiveness ($p=0.006$ or less for each). Parents reported improvements in eye contact, imitation, language, tantrums, gastrointestinal problems, and eczema. A significant improvement of two points ("much improved") was observed on the clinician-rate CGI-I scale in all 10 children. Several nonserious adverse events were reported, including ear discomfort (4 children), ear infections (2 children), and for 1 child each: hyperactivity, increased vocal sensitivity, increased sensory needs, insomnia, fatigue, dehydration, irritability, mouthing of objects, and a seizure. Strengths of this study included the prospective nature,

evaluations by clinicians, and objective measurements (cytokine levels). Limitations of this study included the open-label nature, the lack of a control group, and a small sample size.

4.2 Studies with a Control Group

The first study to use a control group investigated the effects of HBOT at 1.3 atm/24 % oxygen for 40 treatments, using a concentrated protocol of two treatments per day, 5 days per week, over 4 weeks in 62 children with autism (Rossignol et al. 2009). Compared to the group of 29 children receiving slightly pressurized room air (1.03 atm and 21 % oxygen), significant improvements were found in the group of 33 treated children on the clinician-rated CGI scale and the parent-rated CGI and ATEC scales. Significant improvements were reported in overall functioning, receptive language, social interaction, eye contact, and sensory/cognitive awareness in the treatment group compared to the control group. Six centers were used in the study, and study findings did not significantly differ across centers. Side effects were minimal. Strengths of this study included evaluation by blinded clinicians and parents (only the HBOT technician was aware of group assignment), an assessment of blinding (which was adequate), an intention-to-treat analysis (children who did not finish all treatments were still included in the analysis), the prospective nature, the use of a control group, and the use of 6 centers (which may have minimized potential biases associated with a single site study). Limitations included the lack of measurements of the long-term effects of HBOT beyond the study period.

In a smaller controlled study, 34 children with autism were treated with HBOT at 1.3 atm/24 % oxygen for 80 sessions (18 children) or "placebo" (16 children), and no obvious changes were reported (Granpeesheh et al. 2010). The number of treatments per week was less concentrated than the previous study (6–10 sessions per week, with 80 sessions completed within 15 weeks). Twelve participants withdrew from the study (it was not noted if these participants were in the treatment group or the control group); the scores from these children were not included in the final analyses. No significant adverse events were reported. Strengths included the prospective nature, the use of a control group, and evaluation by blinded therapists. Limitations of this study included a high dropout rate (12 out of 46 initial patients enrolled or 26 %), a smaller sample size, and no assessment of blinding efficacy as described in other HBOT studies (Clarke 2009; Clarke et al. 2008).

5 Conclusions

HBOT has been reported to improve cerebral hypoperfusion, reduce inflammation, and improve mitochondrial dysfunction, all of which have been observed in some individuals with ASD. HBOT at the pressures commonly used in ASD (up to 1.5 atm/100 %

oxygen) was reported to decrease markers of inflammation and did not worsen oxidative stress markers. HBOT also improved cerebral perfusion in some children with ASD. Most studies of HBOT in children with ASD reported improvements in several behavioral domains, although many of these studies were not controlled. Although the two studies employing a control group appear to have conflicting results, this may have been due to different sample sizes and frequencies of HBOT sessions. The study using a greater number of treatments per week reported the most favorable findings. Similar findings have been reported in studies of TBI where a greater intensity of treatments leads to the largest benefits (e.g., 40 treatments within a one month period) (Harch et al. 2011). Additional studies would be helpful in determining the number and frequency of treatments required to obtain optimal benefits as well as the pressure and oxygen levels required.

Competing Interests The author treats individuals with HBOT in his clinical practice and derives revenue from this. He has previously received research funding from the International Hyperbarics Association (IHA) for two studies of hyperbaric treatment in children with autism (Rossignol et al. 2007, 2009) and is a medical advisor (unpaid) for IHA.

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Effects of Lithium on Oxidative Stress

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Abbreviations

ATPase	Adenosine triphosphatase
CAT	Catalase
CNS	Central nervous system
GPx	Glutathione peroxidase
GSK-3	Glycogen synthase kinase 3
GST	Glutathione S-transferase
NAD+	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced forms of NAD + and NADP +
NOS	Nitric oxide synthase
PLC	Phospholipase C
PI3K	Phosphatidylinositol 3-kinase
SOD	Superoxide dismutase
TAS	Total antioxidant status
TBARs	Thiobarbituric acid reactive substances

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1 Introduction

Lithium salts are one of the oldest psychotropic drugs. Highly valued in psychopharmacotherapy, they are used primarily in treatment and prophylaxis of bipolar disorder. Lithium is also used in schizoaffective disorder – bipolar type, cyclothymia and augmentation of antidepressant treatment. Lithium long-term therapy decreases total risk of suicide as well as suicidal tendencies, which is highlighted in all international guidelines (Jefferson and Greist 2006; Young and Hammond 2007).

2 Mechanism of Action of Lithium in the Central Nervous System

The mechanism of action of lithium in the human central nervous system (CNS) is unknown. It may lead to a sequence of molecular events that start in the synaptic fissure, and their final effect is their impact on gene expression and neuronal plasticity (Lenox and Frazer 2001).

Lithium influences synaptic transmission and membrane properties, neurotransmitter and intracellular systems. It shows neuroprotective activity and inflict changes in gene expression (Chang et al. 1999; Coyle and Manji 2002; Devaki et al. 2006; Gill et al. 2005; Jope 1999; King and Jope 2005; Lai et al. 2006; Layden et al. 2000; Lenox and Wang 2003; Li and El- Mallakh 2000; Nunes et al. 2007; Pardo et al. 2003; Philips et al. 2008; Rosack 2002; Sassi et al. 2002; Schlecker 2006; Shin et al. 2007). These mechanisms seem to be important for therapeutic effects of lithium ion activity. The efficacy is usually observed a few days after the onset of the treatment and the recurrence of symptoms is not present immediately after the drug withdrawal. It suggests that lithium acts on genetic-molecular level (Brandish et al. 2005; Lai et al. 2006; McColl et al. 2008).

Lithium action in CNS overlaps resulting in molecular and functional net, with phosphatidylinositol system and glycogen synthase kinase (GSK) in its centre.

Apart from these effects, it also affects lengthening of biological cycle (McColl et al. 2008), antidepressant action of lithium achieved by its influence on neuronal nitric oxide synthase (NOS) (Ghasemi et al. 2008), protection against apoptosis by increasing calcium ion concentration mediated through phospholipase C (PLC) activation and phosphatidylinositol 3-kinase (PI3K) (Kang et al. 2003), lithium modifying function of cell skeletons, and action of lithium on oxidative stress.

3 Effects of Lithium on Oxidative Stress

Several studies (carried out in cell lines and laboratory animals) indicate that lithium may influence oxidative stress. However, most of the data of these studies are ambiguous.

Shao et al. carried out experiments on glutamate-induced toxicity in cultures of rat cortical cells and showed that chronic use of lithium compounds protects from peroxidation of membrane lipid and protein as well as DNA fragmentation and cell death. A few years later, the same researchers suggested that lithium activates mRNA of glutathione S-transferase (GST) isoenzymes (Shao et al. 2005). Similar results were obtained by Lai et al. in human SH-SY5Y neuroblastoma and glioma (SVG and U87) cells. They found that lithium (1 mM) protects against oxidative stress-mediated cell death in neuroblastoma cells that were cultured in lithium ion medium for seven days before stressor action. This activity was thought to be independent from GSK-3 inhibition by lithium. Allagui et al. also demonstrated protective activity of lithium (at a concentration of 0.5 mM and 6-month incubation) on SH-SY5Y cell line, as decreased level of lipid peroxidation and increased cell survival induced by oxidative stress compared to control was observed (Allagui et al. 2009). However, a reverse effect was observed at lithium concentration of 6–8 mM, which after 4 days, caused pro-oxidative activity and an increase in cell death.

Kiełczykowska et al. conducted an 8-week experiment on rats and did not find any effect of lithium (at wide range of concentrations) on oxidative stress parameters in peripheral blood (Kiełczykowska et al. 2007). Lipid peroxidation also was not changed in the tissues, except in brain, where statistically significant decrease in oxidative stress parameter was seen. However, in all tissues, a decrease in superoxide dismutase (SOD) activity was observed.

Almost 20 years ago, Song et al. published results from an interesting study on antioxidative enzyme activity in two different rat populations treated with lithium chloride (Song et al. 1994). Changes in catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were different in normal laboratory rats than in animal model of depression. Frey et al. also showed that in some brain structures (prefrontal cortex, hippocampus), lithium may prevent and decrease amphetamine-induced lipid peroxidation in animal model of mania (Frey et al. 2006). However, it does not change oxidation reduction reactions under physiological conditions. Castro et al. and Jornada et al. also found a lithium-induced decrease in oxidative stress parameters in rat brains in animal model of mania (Castro et al. 2009; Jornada et al. 2011). Bhalla and Dhawan showed that lithium decreases lipid peroxidation and normalizes level of antioxidative enzymes in different areas of animal brain exposed to aluminium pro-oxidative activity (Bhalla and Dhawan 2009). Lithium supplement improved histoarchitectonics of the investigated regions.

Vasconcellos et al. investigated different brain structures in rats exposed to long-term stress and found that although lithium has antioxidative properties (increased the total antioxidant reactivity in the hippocampus), they are not sufficient to prevent damage caused by oxidative stress in vivo (Vasconcellos et al. 2006). It has also not been found that lithium changed parameters of lipid peroxidation or antioxidant defence (SOD and GPx activity) in erythrocytes and brain tissue in rats, which received intraperitoneally lithium carbonate for seven days (Abdalla and Bechera 1994). Similarly, Nciri et al. showed no changes in oxidative status in rat kidney tissue and liver (Nciri et al. 2012). Despite antiapoptotic activity, lithium did not change the effects of oxidative stress in mouse models of neurodegenerative

diseases (Shin et al. 2007). Few studies indicate pro-oxidative properties of lithium. Chadha et al. found increased lipid peroxidation as well as significant changes in activity of antioxidative enzymes in liver after exposure of lithium for several months (Chadha et al. 2008). Investigations carried out by (Oktem et al. 2005; Nciri et al. 2008) also indicated lithium-induced nephrotoxicity by increased oxidative stress (Oktem et al. 2005; Nciri et al. 2008). According to Engin et al. and Malhotra et al., lithium-induced lipid peroxidation lead to structural and functional damage in erythrocytes (decreased antioxidative defence potential secondary to sub- or clinical hypothyroidism caused by lithium therapy) (Engin et al. 2005; Malhotra and Dhawan 2008). Similar phenomenon was described by Sahin et al. 2006.

There are very few human studies on the effects of lithium on parameters of oxidative stress. They were carried out on patients with bipolar disorder. Machado-Vieira et al. showed that lithium inhibited lipid peroxidation and normalized ratio of SOD to CAT during mania (Machado-Vieira et al. 2007). Similar results were obtained by Aliyazicioglu et al. who also reported an increase in total antioxidative potential in patients with bipolar disorder (Aliyazicioglu et al. 2007).

In a recent publication, Banerjee et al. determined plasma lipid peroxidation and sodium/potassium adenosine triphosphatase (ATPase) activity in the erythrocytes from peripheral blood in patients treated with and without lithium and healthy volunteers (Banerjee et al. 2012). Increased level of lipid peroxidation was found in all patients compared to control but also a significantly higher lipid peroxidation was observed in patients not treated compared with patients given lithium. The value of lipid peroxidation was negatively correlated with sodium/potassium ATPase activity.

Effects of lithium activity on oxidative stress parameters depend on many factors. One of them, beyond doubt, is lithium concentration and possibly also time of its treatment. Initial balance between pro- and antioxidative balance as well as target tissue may also play an important role.

In our in vitro studies, no statistically significant changes in concentration of lipid peroxidation markers (thiobarbituric acid reactive substances – TBARS) and plasma total antioxidant status (TAS) were found in healthy volunteers given lithium. Similarly, lithium had no effect on parameters of oxidative stress in SH-SY5Y neuroblastoma cells. Lack of effect was observed not only under normal conditions but also under induced oxidative stress conditions. However, increased lipid peroxidation was found in human plasma incubated with a combination of lithium and haloperidol despite the fact that these drugs were used in therapeutically relevant concentrations and no increase in lipid peroxidation in plasma incubated separately for each drug was detected. Caution is advised for the use of haloperidol in combination with lithium (Gawlik and Rabe-Jabłońska 2014). These findings were in accordance with numerous well-documented clinical trials and confirmed neurotoxicity of this combination (Cipriani et al. 2006; Goldman 1996).

Chan et al. compared activity of haloperidol combined with lithium/valproate to zotepine combined with the same drugs (Chan et al. 2010). Patients with mania given haloperidol manifested extrapyramidal symptoms significantly more often, and level of uric acid in their blood serum was higher than in the patients treated with zotepine.

In the 1970s and 1980s, it was hypothesized that processes involved in increasing oxidative stress in the CNS and peripheral tissues are responsible for toxicity of haloperidol combined with lithium. Savas and Glibert, in investigations on rat tissues, found increased peroxidation in cerebral cortex and kidney homogenates induced by both lithium carbonate and haloperidol (Sawas and Gilbert 1985). Combination of these drugs caused significant increase in levels of oxidative stress compared with the drugs given separately. A few years earlier, Guynn and Faillace carried out experiments on effects of lithium, haloperidol and combination of these drugs on metabolic processes in rat brains and found no significant differences between them (Guynn and Faillace 1979). However, one of the differences was decreased ratio of [NAD+]/[NADH][H+] and [NADP+]/[NADPH], types of nucleotides which have an important role in cellular respiration processes, induced by combination of lithium and haloperidol. It may suggest respiratory chain defect in cells and change in redox potential balance induced by combination of lithium and haloperidol.

4 Conclusion

Most data on lithium antioxidative activity come from experiments carried out in animal, which shows disturbed pro- and antioxidant balance. Very few human studies were carried out on patients with initially increased level of oxidative stress. It seems that many factors are responsible for effects of lithium activity on oxidative stress parameters. One of them is lithium concentration and also time of its exposure in the system/organism. Initial state of pro- and antioxidant balance as well as type of target cells may also play an important role.

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Cryostimulation as Adjunct Treatment in Psychiatric Disorders

Elżbieta Miller

Abbreviations

ACTH	Adrenocorticotropic hormone
BDI	Beck Depression Inventory
BDNF	Brain derived neurotrophic factor
CAT	Catalase
CRH	Corticotropin-releasing hormone
DHEA	Dehydroepiandrosterone
EDSS	Expanded Disability Status Scale
GPx	Glutathione peroxidase
HARS	Hamilton anxiety rating scale
HDRS	Hamilton depression rating scale
HPA	Hypothalamic–pituitary–adrenal axis
IGF	Insulin-like growth factor
IL	Interleukin
MRI	Magnetic resonance image
MS	Multiple sclerosis
POMC	Pro-opiomelanocortin
PPMS	Primary-progressive multiple sclerosis
PRMS	Progressive-relapsing multiple sclerosis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis

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SOD	Superoxide dismutase
SPMS	Secondary-progressive multiple sclerosis
TAS	Total antioxidative status
TNF	Tumor necrosis factor
UA	Uric acid
WBCT	Whole-body cryotherapy

1 Introduction

Currently, the use of medications is the standard and most common method of biological treatment in psychiatry. However, sometimes pharmacotherapy does not lead to remission, so research is being carried out into other, non-pharmacological strategies of treatment (Wayne et al. 2003; Werneke et al. 2006).

Many persons with psychiatric disorders turn to non-pharmacologic and nonconventional interventions (Olivieri et al. 2011; Cabral et al. 2011; Sánchez González et al. 2009), including cryostimulation. There is increasing scientific interest in the potential effectiveness of these interventions for the treatment of anxiety and depression, especially for mild to moderate levels of disorder severity (van der Watt et al. 2008). There is a need for developing new therapies such as cryostimulation that can be used as adjunct psychiatric therapy. The mechanisms of hypothermic protection are not entirely understood. It is known that lower temperature protects tissues against hypoxia by slowing down the rate of cellular damage due to formation of free radicals, chemical metabolites, and tissue edema (Gordon 2001; Miller et al. 2010b). According to increasing evidence, hypothermia can significantly improve outcomes of diseases with oxidative stress background such as neonatal hypoxic–ischemic encephalopathy (Perrone et al 2010; Fatemi et al. 2009), brain injury (Varon et al. 2011), multiple sclerosis (Miller et al. 2010a), depression (Miller et al. 2011b), and cerebral ischemia (Varon et al. 2011). Treatment with the total immersion of the body at extremely low temperatures was first introduced in Japan towards the end of the 1970s by Toshiro Yamauchi (Yamauchi 1989) who constructed the first cryogenic chamber and successfully used cryotherapy to treat rheumatism (Lange et al. 2008). At present, cryostimulation is recommended not only for inflammatory diseases of the locomotor system, degenerative joint and spine diseases, or soft tissue rheumatic diseases but also for psychiatric disorders especially for anxiety-depressive disorders (Rymaszewska et al. 2000).

2 Non-pharmacological Treatments in Psychiatry

Currently, a variety of supplementary therapies for treatment psychiatric disorders are used worldwide. Complementary medicines are either used as an alternative or in addition to conventional medicine (Cabral et al. 2011; Lavretsky 2009; Olivieri et al. 2011). Their use by those with chronic disorders such as cancers, with their

associated physical and psychological problems, is well documented (Ernst and Cassileth 1998; Schraub 2000). In psychiatric patients, estimates of their use range from 8 to 57 %, with the most frequent use being in depression and anxiety. Acupuncture, aromatherapy, enzyme therapy, homeopathy, hypnotherapy, massage, reflexology, relaxation techniques, and spiritual healing were frequently used forms of treatment (Cabral et al. 2011; Edzard 2001; Shapiro et al. 2007). Phototherapy is one of the most popular and effective methods that was used in treating the seasonally occurring mood disorder occurring in bipolar disorders at the beginning of the 1980s. Electroconvulsive therapy is also still applied therapy in psychiatric disorders (Sánchez González et al. 2009). Other modern biological treatment methods such as cryostimulation are constantly being developed. Methods involving neurostimulation include repetitive transcranial magnetic stimulation (Rau et al. 2007; Lavretsky 2009), magnetic seizure therapy, vagus nerve stimulation (Rush et al. 2000), deep brain stimulation, and also transcranial direct current stimulation (Grunhaus et al. 2000). These methods may be effective in treating depression and have minimal side effects.

A population-based study from the USA found that 9 % of respondents had anxiety attacks, 57 % of whom used complementary medicines, and that 7 % of respondents reported severe depression, with 54 % of these using complementary medicines (Kessler et al. 2001). Another survey from the USA reported mental disorders in 14 % of respondents, 21 % of whom used complementary medicines. People with mental health problems may take complementary medicines to treat anxiety and depression or to counter side effects of conventional treatments, for example, late occurring dyskinesia and weight gain (Unger et al. 1992).

Some patients with chronic anxiety and depression use complementary medicine which seems to be more holistic treatment with no side effects especially when ineffectiveness of conventional treatment became evident (van der Watt et al. 2008; Berchtold et al. 2010).

3 Hypothermia as a Neuroprotective Therapy

Nowadays, hypothermia seems to be the most promising neuroprotective therapy that has been implemented to clinical practice (Shintani et al. 2011; Ceulemans et al. 2010; Dietrich and Bramlett 2010; Fatemi et al. 2009).

The first clinical studies of brain cooling in the 1960s showed decrease of O_2 consumption, CO_2 production, and other indicators of metabolism (Adelson 2009: Dietrich and Bramlett 2010). Even small fluctuations in the temperature of the brain alter hemodynamic, calcium-dependent intercellular signaling, excitotoxicity, inflammation and edema, apoptosis, as well as molecular makers (Adelson et al. 2005; Ceulemans et al. 2010; Kuo et al. 2011). Excitotoxicity is one of the most important processes in the brain. Many studies reported that extracellular levels of the excitatory amino acid glutamate and other neurotransmitters after brain injury were reduced following mild posttraumatic hypothermia. Additionally,

hypothermia inhibits generation of oxygen free radicals involved in the secondary damage, associated with reperfusion (Christian et al. 2008; Perrone et al. 2010; Adelson 2009).

Mild to moderate hypothermia was also reported to reduce abnormal blood–brain barrier permeability after both ischemic and traumatic insults (Katz et al. 2004; Ji et al. 2007). Another mechanism of hypothermic cytoprotection is reducing inflammatory processes including trauma-induced increases in the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF α) (Ceulemans et al. 2010; Jorgensen 2008). Current research lead to use the temperature modifications to discover the critical ligand/receptor and cellular signaling specific responsible for anti-inflammatory effect (Bayir et al. 2009), although the precise way of neuroprotection by hypothermia is not known (Gordon et al. 2003).

Apoptotic cell death also participates in the vulnerability of different cell types after neurotrauma. Several clinical studies suggest that posttraumatic hypothermia can significantly reduce levels of caspase 3, an important initiator of apoptotic cell death, and reduce cytochrome C release from dysfunctional mitochondria (Hasegawa et al. 2009; Bayir et al. 2009). Hypothermia may affect signaling cascades associated with hippocampal-dependent learning and memory. It may represent a molecular mechanism by means of which hypothermia improves psychological aspects of neurotrauma (Dietrich and Bramlett 2010). Additionally, hypothermia decreases the global cerebral metabolic rate for glucose and oxygen but maintains a slightly better energy level by reducing ATP breakdown (Bayir et al. 2009).

4 Physiological Effect of Cryotherapy

Cryostimulation is an exposure of whole human body to extremely low temperatures (⁻130 °C) in a cryogenic chamber. The cryogenic chamber (usually liquid nitrogen is a coolant) is consists of two rooms: the vestibule, with the temperature of (⁻60 °C), and the main chamber, with temperature (⁻130 °C). Sessions in the cryochamber last 3 min (Bauer and Skrzek 1999; Zagrobelny et al. 1993). During cryostimulation, the subjects wear bathing suits, surgical masks, caps, gloves, socks, and shoes. Cryostimulation sessions are usually applied every day. The participants are entered to the main chamber in groups of five or four persons. In the cryogenic chamber, subjects are instructed to keep walking in a circle, moving slowly, one behind another, without verbal contact. Just before each session of cryostimulation, systolic and diastolic blood pressures are measured, because arterial hypertension is one of the main contraindication to this therapy (Gregorowicz and Zagrobelny 2007; Lubkowska et al. 2010).

The maintenance of a constant body temperature in human body during cold stress occurs through changes in the endocrine, circulatory, neuromuscular, and immunological systems (Kellogg 2006). The major effector of human

thermoregulation is cutaneous circulation. In the dermis, there are tenfold more cold receptors than heat receptors. Experimental evidence indicates that the early phase of vasoconstriction due to cooling is mainly dependent on neural regulation and that late-phase vasoconstriction relies mainly on nonneural mechanisms (Hampl et al. 2006).

Shivering is a form of thermogenesis, which consumes large amounts of energy but is not effective during severe cold (Silva 2006) such as cryostimulation. It is the earliest and most primitive response to increase heat production. Humans have evolved a more efficient and long-lasting form of non-shivering facultative thermogenesis that uses pure metabolic mechanisms to generate heat. The non-shivering thermogenesis has two categories: obligatory and facultative (Kellogg 2006). The facultative thermogenesis is regulated mainly by catecholamines released from adrenals and the sympathetic nervous system (Hampl et al. 2006). The most important endocrine factors modulating obligatory thermogenesis are thyroid hormones, which increase metabolic rate and thermogenesis. Obligatory thermogenesis proceeds continuously in all organs and tissues of the body (Silva 2006).

Currently cryostimulation is one of the most promising adjunct therapies in psychiatric disorders especially in anxiety-depressive disorders. Cryogenic temperatures induce vasoconstriction followed by vasodilation after 4 min which is connected with increasing blood flow seen as skin hyperemia and return to normal skin temperatures (after about 14 min). Vasodilation appears about 4 min after whole-body cryotherapy (WBCT) and achieves fourfold higher value than before cryostimulation and can last a few hours (Bauer and Skrzek 1999) increasing blood flow and stimulating elimination of metabolic products. There are a variety of individual responses to cold due to such factors as body size, fitness level, amount of subcutaneous fat, and sex (Gordon 2001). Cooling the skin below 20 °C causes a marked reduction in the production of acetylcholine and in the rate of conduction along cooling nerves, which varies according to the size of fibers, thus producing asynchrony of impulses (Woźniak et al. 2007). Females have a reduced cold temperature tolerance compared to men because of their lower aerobic capacity (Gordon 2010).

Cryostimulation treatment resulted in decreased levels of testosterone and estradiol in football players, although there were no changes in the concentration of luteinizing hormone and dehydroepiandrosterone (DHEA-S) (Korzonek-Szlacheta et al. 2007). After cryostimulation, decreased hemoglobin and iron in erythrocytes were observed (Banfi et al. 2009a) which probably cause decreased testosterone. Smolander et al. (2009) reported that ten sessions of WBCT in healthy females did not lead to disorders related to altered secretions of growth hormone, prolactin, thyrotropin, or thyroid hormone. The mechanisms of action of hypothermic protection are not entirely understood. It seems that lower temperature protects tissues against hypoxia by slowing the rate of cellular damage due to formation of free radicals, chemical metabolites, and tissue edema. In addition to protection from ischemic damage, hypothermia has been show to ameliorate the toxicity of various drugs and environmental toxicants as well as to protect against other disorders such as hemorrhage, hypergravity, and hypoglycemia (Gordon 2001).

5 Cryostimulation and Oxidative Stress

Acute cold temperature represents an obvious stress, which could lead to some adaptive mechanisms, which increase in body resistance against cold. It has been suspected that an adaptation to cold stimuli and the improvement in the body hardening could be related to an increase in the protection against oxidative stress by significant augmentation of antioxidant levels (Fig. 1) (Miller et al. 2011a). Since oxidative stress is very important factor of many psychiatric disorders, it is important to find therapy that could improve the protection of the body against oxidative stress and could have some practical applications in the development of therapies for a large numbers of individuals. Siems et al. (1999) reported a higher enzymatic protection (i.e., in the increased activity of red blood cell enzymes) for those who regularly practice winter swimming activities or after heavy endurance physical exercise in comparison with control. Recent data suggest that cold stress increases antioxidant defenses in human body (Miller et al. 2010a, b; Siems and Brenke 1992). Activation of the antioxidant system can be an adaptive defensive mechanism to cope with increased oxidative stress especially in immunoactive disorders.

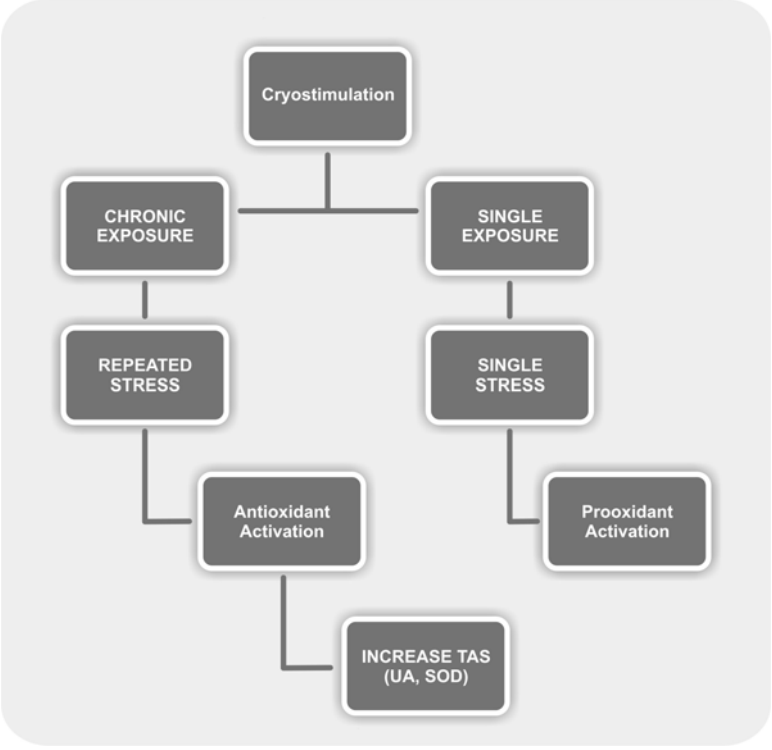

Fig. 1 The influence of single and chronic exposures of whole-body cryostimulation on oxidative stress in human. *ROS* reactive oxidative species, *TAS* total antioxidative status, *UA* uric acid, *SOD* superoxide dismutase

Cold exposure elicits substantial alterations both in metabolic and physiological aspects. Firstly, free radical formation is increased during cold stress (Armario et al. 2008). Further, cold stress activates stress responses and induces shivering and muscle movement to maintain body temperature, and this action increases production of reactive oxygen species (ROS) (Fig. 1). The ratio of oxidized glutathione to glutathione is increased after short-term whole-body cold exposure in human (Teramoto and Ouchi 1999). However, winter swimmers have a higher concentration of glutathione and greater activities of glutathione peroxidase and catalase than do healthy controls (Siems et al. 1999). These findings indicate that glutathione metabolism and function might be impaired during acute cold-water immersion but can be preserved during chronic or repeat immersion.

Thermoregulation induced by low temperatures is associated with an increase in lipid metabolism (Westerlund et al. 2003). The human body uses energy derived mainly from the conversions of carbohydrates and lipids (Vallerand and Jacobs 1989). The release of norepinephrine from the terminal endings of sympathetic neurons during non-shivering thermogenesis leads to the mobilization of fatty acids from intracellular stores of triglycerides and their oxidation in the mitochondria (Florez-Duquet and McDonald 1998).

In the course of normal human activity – energy production, detoxification of pollutants, and immunologic defense mechanisms – free radicals are produced. Dietary antioxidants (such as proanthocyanidins found in blueberries and bioflavonoids found in citrus fruits) as well as the human antioxidant enzymes and nonenzymatic provide critical protection against free radical formation and reduce damage induced by their action.

In depression, oxidative stress is increased (Kodydkvo et al. 2009; Cumurcu et al. 2009). There is a need for developing new therapies such as cryostimulation which can be used as adjuvant antioxidative therapy. After 10 sessions of cryostimulation, total antioxidative status (TAS) level in plasma was distinctly higher ($p<0.001$) (Miller et al. 2011a). These observations that presented the suppression of oxidative stress by cryostimulation are consistent with other reports (Duqué et al. 2005; Siems et al. 1999; Miller et al. 2010a, b). Woźniak et al. (2007) showed that cryostimulation induces an increase in the activity of superoxide dismutase (SOD) by 36 % ($P<0.001$) and glutathione peroxidase (GPx) by 68 % ($P<0.01$) in the human erythrocytes.

Siems et al. (1999) reported a higher enzymatic protection (i.e., in the increased activity of red blood cells, catalase (CAT), GPx, SOD) for those who regularly practice winter swimming activities or after heavy endurance physical exercise in comparison with control. This activation can be viewed as an adaptive defensive mechanism to cope with increased oxidative stress.

Cryostimulation stimulates the antioxidative response of organism via augmentation of SOD activities ($p<0.001$) and increase of uric acid (UA) level ($p<0.001$) compared to non-WBCT subjects. In humans, over half the antioxidant capacity of blood plasma comes from UA. UA can scavenge superoxide, the hydroxyl radical, and singlet oxygen and may assist in the removal of superoxide by preventing the degradation of SOD, the enzyme that is responsible for clearing superoxide from the cell (Miller et al. 2011c). Uric acid like ascorbic acid is a strong reducing agent

and a potent antioxidant responsible for TAS level in plasma (Kutzing and Firestein 2008; Miller et al. 2011c).

Longitudinal measurement of uric acid level in plasma after ten sessions of cryostimulation showed an increase of uric acid concentration for 3 months after therapy. Therefore, cryostimulation could be a therapy elevating uric acid concentration in plasma. It is very important because low level of uric acid is suggested as characteristic of depression and may be normalized after antidepressant pharmacologic treatment (Wen et al. 2011).

Taking into account the above data, it has been suspected that an adaptation to cold stimuli and the increase in body resistance could be related to an increase in the protection against oxidative stress (Duqué et al. 2005).

6 Cryostimulation in Psychiatric Disorders

Most pharmacological treatments of anxiety and other mental disorders rely on the hypothesis that there are underlying neurochemical or neurophysiological abnormalities that can be corrected with pharmacological treatment (Werneke et al. 2006; Unger et al. 1992). However, there may also be a component of some mental disorders that responds to the environmental factors that occurs with some forms of temperatures stimulus, such as cryotherapy.

Recent data point at a positive role of cryostimulation in affective and anxiety disorders, particularly in depression. The study of Rymaszewska et al. (2008) on 26 patients with affective and anxiety disorders reported significant reduction of 13 from 14 Hamilton Anxiety Rating Scale (HARS) items after 15 exposures of WBCT. Only gastrointestinal symptoms did not improve significantly. Concerning the Hamilton Depression Rating Scale (HDRS) items, it was the reduction in most of the items at the level of 0.001 except guilt feelings, early waking, psychomotor retardation, and hypochondrias on the level below 0.01 (gastrointestinal symptoms and body mass did not change within 3 weeks). So, after 15 (2–3 min) exposures of WBCT (1 exposure per day), a decrease of at least 50 % from the baseline HDRS-17 scores in 34.6 % of the study group and 2.9 % of the control group and a decrease of at least 50 % from the baseline HARS score in 46.2 % of the study group and in none of the control group were noted.

The next study of Rymaszewska et al. (2003) reported that the HDRS sum score for each patient ($n=33$) after ten exposures of WBCT was lower than that of the baseline and reached statistical significance. Recent results (Miller et al. 2011b) demonstrate that ten exposures of cryostimulation significantly increased not only TAS level in 15 patients with mild to moderate depression (13–18 BDI) but also reduced 19 from 21 items in Beck Depression Inventory (BDI) self-report rating scale (17 items: $p<0.001$).

Cryostimulation is a relatively new therapeutic method of physical medicine with a history of about 20 years. Thus, research works on mechanisms of therapeutic action of cryogenic temperatures are still carried on.

6.1 Cryostimulation and Aerobic Training

Persons with severe psychiatric disabilities in addition to their mental illness also frequently suffer the adverse effects of poor physical fitness, including weight problems, sleeplessness, poor cardiovascular fitness, fatigue syndrome, and low self-assessment. These conditions represent serious barriers to the treatment and rehabilitation (Tkachuk and Garry 1999).

Cryostimulation is often connected with exercise, especially aerobic training to increase acceleration of body temperature to normal value.

Voluntary physical activity affects brain plasticity by facilitating neurodegenerative, neuroadaptive, and neuroprotective processes (Daley 2002; Greenwood and Fleshner 2011; Lafenetre et al. 2011; Hortobgyi and Maffiuletti 2011).

At least some of the processes are mediated by neurotrophic factors (Dishman et al. 2006). Motor skill training and regular exercise concomitant with cryostimulation enhance executive functions of cognition and some types of learning. Chronic physical activity increases the expression of brain-derived neurotrophic factor (BDNF) (Heyman et al. 2011; Ding et al. 2011). In vitro and vivo studies showed that chronic exercises can increase the expression of genes that encode several brain neurotrophins such as BDNF, nerve growth factor, and galanin. BDNF supports the survival and growth of many neuronal subtypes, including glutamatergic neurons, and emerged as a key mediator of synaptic efficacy, neuronal connectivity, and use-dependent plasticity (Berchtold et al. 2010). IGF-1 levels increase in both the periphery and brain after exercise, and at least part of the increase in the brain reflects increased transport from the periphery across the blood–brain barrier (Cotman and Berchtold 2002).

Chronic training may also have neurodegenerative and neuroprotective effects on the brain by stimulating the growth and development of new cells and protecting against ischemic damage in the hippocampal formation and neurotoxic damage in the neostriatum (Helmich et al. 2010; Pajonk et al. 2010; Sacerdote et al. 2000). The neural consequence of chronic cold stress and aerobic training is that it may contribute to these stress protective effects including alterations in serotonergic 5-hydroxytryptamine and splenic norepinephrine systems (Dishman et al. 2006).

6.2 Endorphin Hypothesis of Cryostimulation

Low temperature activates not only thermoregulation system but also hormonal response, which changes cellular metabolism and the concentrations of epinephrine, norepinephrine, adrenocorticotropic hormone (ACTH), cortisone, pro-opiomelanocortin (POMC), and β-endorphins in blood plasma as well as testosterone levels (Campeau et al 2004; Belda et al. 2008).

POMC is the source of several important biologically active substances, such as ACTH in the anterior pituitary gland and melanocyte-stimulating hormone (α-MSH) and β-endorphin. α-MSH has a role in the regulation of appetite and sexual behavior. One neurobiological hypothesis of depression is based on dysregulation of the hypothalamic–pituitary–adrenal axis (Stranahan et al. 2008). The brain's

opioid peptide systems are known to play an important role in motivation, emotion, attachment behavior, response to stress and pain, and the control of food intake (Droste et al. 2003; Sharp et al. 1998).

The physical and psychological stress such as cryostimulation coordinates the adaptive responses of the organism to stressors (Teramoto and Ouchi 1999). Activation of the stress system leads to behavioral and peripheral changes that improve the ability of the organism to adjust homeostasis. The main components of the stress system are the corticotropin-releasing hormone (CRH) and autonomic systems with their peripheral effectors and the hypothalamic–pituitary–adrenal axis (HPA) (Mellon and Bayer 1998; Sacerdote et al. 2000).

On the basis of available data, it seems that during the period of hypothermic stress, the brain releases a number of chemical mediators, including opioid peptides such as β-endorphin, which stimulate an inhibitory effect on the immune system (Carr et al. 1996; Guan et al. 1995; Shavit et al. 1986).

The activation of the HPA starts the production of adrenocorticotropin from the pituitary that in turn causes the release of glucocorticoids that could suppress the immune system (Freier and Fucks 1993; Van Den Eede and Moorkens 2008). Circulating ACTH is the key regulator of glucocorticoid secretion by the adrenal cortex. Other hormones or cytokines, either originating from the adrenal medulla or coming from the systemic circulation, as well as neuronal information from the autonomic innervation of the adrenal cortex may also participate in the regulation of cortisol secretion. Glucocorticoids play main regulatory role in the activity of the HPA axis and in the termination of the stress response by acting at extra-hypothalamic centers, the hypothalamus and the pituitary gland. The inflammatory cytokines TNF α, IL-1β and IL-6 can cause stimulation of the HPA axis alone, or in synergy with each other. It is unclear how repeated cryostimulation influences the level of pro-inflammatory and anti-inflammatory mechanisms. There is a report of increased anti-inflammatory cytokine IL-10 and decreased pro-inflammatory IL-2 and IL-8 after five systemic cryostimulation sessions (Banfi et al. 2009b). On the other hand, another report shows increased levels of IL-6 in response to ten cryostimulation sessions (Lubkowska et al. 2009). Some authors report that cryostimulation leads to an increase in plasma ACTH and cortisol, epinephrine, and norepinephrine (Zagrobelny et al. 1993), while others have not observed any stimulation of traditional stress hormones (Leppäluoto et al. 2008) (Fig. 2).

Further studies are required to explain the mechanisms of multisystem cold stress reaction in humans and determine the possible role of cryostimulation in the treatment mental disorders.

6.3 Cryostimulation in Depressive Multiple Sclerosis Patients

Multiple sclerosis (MS) is a complex disease with several pathophysiological processes: inflammation, demyelination, oxidative stress, axonal damage, and repair mechanisms that participate in this disorder (Peterson and Fujinami 2007).

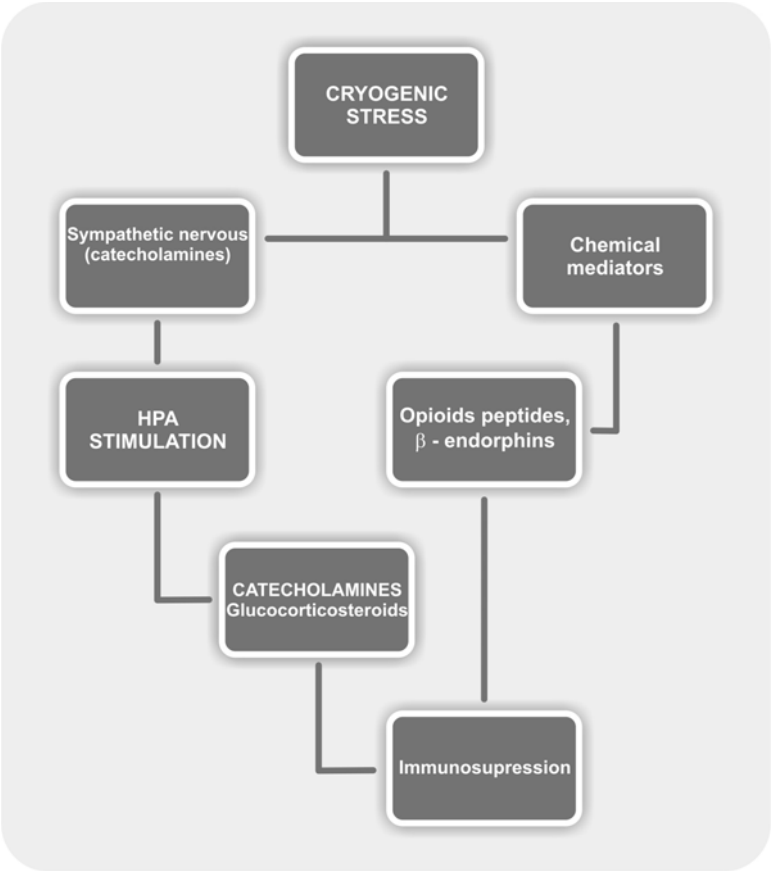

Fig. 2 The hypothesis of immunosuppression by cryogenic stress. *HPA* hypothalamus–pituitary–adrenal axis

These processes are not uniformly represented in patient populations but can selectively predominate in individual patients (Bielekova and Martin 2004). Therefore, there is a need for developing new antioxidative pathways such as cryostimulation especially in progressive phase of MS that are more process specific and can be used in specific patient subpopulations (Miller et al. 2011b, c). There are three main types of MS: relapsing-remitting (RRMS), secondary progressive (SPMS) and primary-progressive (PPMS) with progressive-relapsing (PRMS) recently distinguished as an additional subtype (Miller et al 2011c). SPMS patients have irreversible disability with a wide range of ameliorative symptoms. MS is variable in onset and progression. First, the most common symptoms are impaired vision due to optic neuritis (inflammation of the optic nerve) and deficits in sensation (or over-sensation as burning or prickling). In the mature form of MS appear other symptoms including paresis and paralysis, ataxia, fatigue, spasticity, and incontinence. Cognitive impairment (difficulties with memory, concentration, and other mental skills), depression,

and fatigue also occur frequently. Initially, more than 80 % of individuals with MS have a RRMS disease course with defined clinical exacerbations of neurologic symptoms, followed by complete or incomplete remission (Miller 2011). RRMS is dominated by multifocal inflammation, edema, and the physiologic actions of cytokines (Miller 2011; Racke 2009). After 10–20 years, about half of those with RRMS gradually accumulate irreversible neurological deficits in the absence of clinical relapses or new white matter lesions by magnetic resonance image (MRI). This stage is known as SPMS characterized by progression of clinical symptoms (Tullman et al. 2004 ; Liguori et al. 2000). Disability levels in SPMS patients often worsen despite a stable MRI T(2) lesion burden. Oxidative stress and brain atrophy in the absence of measurable inflammation are possible explanations for this phenomenon (Koch et al. 2007). It is difficult to predict the clinical course of this disease. Progression of disability seems to be increased in patients with higher number of relapses during the first and second year of the disease (Lublin and Reingold 1996; Bashir and Whitaker 1999). A higher incidence of depressive symptoms and major depressive disorder in patients with MS is well documented and reported in both large community surveys and studies of persons with MS. Depressive symptoms in MS patients are associated with reduced quality of their lives (Sollom and Kneebone 2007).

Accumulating data indicate that oxidative stress (OS) plays a crucial role in the pathogenesis of MS and depression (Miller et al. 2011b; Gilgun-Sherki et al. 2004). Reactive oxygen and nitrogen species (ROS/RNS), leading to oxidative stress, generated in excess primarily by macrophages, have been implicated as mediators of demyelination and axonal damage in MS (Gonsette 2008). Excessive release of ROS causes damage to main cellular structures and components such as lipids, proteins, and nucleic acids (e.g., RNA, DNA) and promotes transendothelial leukocyte migration as well as contributes to oligodendrocyte damage and axonal degeneration. Additionally, weakened cellular antioxidant defense systems in CNS in MS and its vulnerability to ROS effects may ameliorate damage (Miller 2011). Therefore, treatment with antioxidants might theoretically prevent propagation of tissue damage and improve both survival and neurological outcome. Cryostimulation in MS patients with neurological deficits has increased not only muscle strength, decreased spasticity, and reduced disability in EDSS (Expanded Disability Status Scale),but also higher level of antioxidative status has been observed (Miller et al. 2010b). During hypothermia, reduced demand for oxygen slows the rate of lipid peroxidation and protects ischemic cell membranes by stabilizing potassium efflux. Recent clinical studies showed that the level of TAS was distinctly reduced ($p < 0.0003$) in depressive MS patients in comparison with MS patients without depression (Miller et al. 2011b). Treatment with cryostimulation caused significant increase of TAS level in plasma of depressive MS patients compared to untreated patients and reached the values of healthy controls. It is unclear exactly how exactly cryostimulation might reduce depression in non-MS populations; however, several theories have been proposed to suggest a possible role for cryogenic treatment for mood and anxiety disorders including regulation of the hypothalamic–pituitary–adrenal axis (HPA), increased

β-endorphin levels, normalization of hippocampal brain-derived neurotrophic factor (BDNF), regulation of monoamines, and improved perceptions of self-efficacy. The HPA, BDNF, and serotonin have all been implicated in MS pathology (Miller et al. 2011a). If cryostimulation like exercise affects HPA function, BDNF concentration, or serotonin concentration in persons with MS, this provides a possible explanation for the decreased incidence of depression observed in persons with MS who regularly participate in physical activity. Alternatively, depression etiology in MS may have a psychological rather than neurobiological explanation. Clinically significant depression can affect up to 50 % of patients with multiple sclerosis over the course of their lifetime (Feinstein 2011). Therefore, the etiology and the influence of cryostimulation on depression are areas that warrant further investigation. Cryostimulation could be an effective aid to psychopharmaceutical treatment of MS patients. Results (Miller et al. 2010a) demonstrate that ten exposures of cryostimulation significantly increased the level of TAS ($p<0.002$) in MS patients. It seems that the lower level of TAS observed in plasma of MS patients is dependent on the low concentrations of endogenous antioxidants, mainly uric acid. The results suggest that cryotherapy may play an important role by suppressing oxidative stress and ROS production especially in MS patients with depression.

7 Conclusions

It seems that cryostimulation may be used as adjuvant therapy in the treatment of psychiatric diseases with oxidative stress background since it improves the antioxidant capacity of organism.

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Index

A
Aetiology, 45
Age-related macular degeneration (AMD), 486–488, 492–497
Alzheimer's disease (AD), 78, 152–155, 220, 234, 237, 253, 334, 404, 417–418, 423, 470–475, 491, 496, 522, 525, 529, 530, 532–536, 540, 541, 544–545
Amyotrophic lateral sclerosis (ALS), 10, 16, 78, 152, 157–159, 253, 418, 423
Antioxidant properties antidepressants, 137
Antioxidants, 9–12, 29–30, 48–53, 91, 137, 224, 225, 249–252, 271–272, 399, 400, 406, 422–424, 505–515, 521–547
Anxiety, 100, 121–128, 225, 234–236, 315, 343, 394, 400, 445, 554, 576, 577, 582
Anxiety disorders, 121–128, 134, 144, 234, 334, 582, 586–587
Attention deficit hyperactivity disorder (ADHD), 103, 143–148
Autism, 89–107, 234, 237–238, 334, 355–367, 373–388, 393–402, 406–409, 417–430, 553–562
Autoimmune system, 133, 137–138

B
Behavior, 45, 90–93, 95, 99, 100, 102–104, 123–125, 128, 224, 235, 237, 252, 279, 280, 310, 334, 359, 361, 366, 383, 393, 396, 406, 408, 417, 421, 425, 426, 522, 534, 558, 560, 583–584
Bipolar disorder (BD), 73–83, 100, 144, 147, 171–189, 233–236, 270, 276–278, 288, 307, 309, 317, 334, 396, 437, 439–443, 455–456, 509, 510, 512, 514, 568, 570, 577
Bisphenol A (BPA), 97–98, 101–103
Blood platelet, 52, 56, 307, 317, 327–344

C
Calcium metabolism, 79–81
Calcium signaling, 74, 81, 365–367, 376, 454
Carboxyalkylpyrrole (CAP), 484, 491–492, 496
Carboxyethylpyrrole (CEP), 479–497
Central nervous system (CNS), 9, 17, 46, 47, 52, 60, 74, 77, 78, 94, 100, 125, 138, 156, 158–160, 181, 198, 213, 215, 221, 233, 234, 247, 249, 250, 252–253, 255, 259, 270, 271, 285, 302, 304, 307, 309, 317, 329, 333, 334, 359, 380, 382, 401, 423, 452–454, 459, 460, 479–497, 526, 568, 571, 586
Cognitive impairment, 61, 103, 125, 154–155, 256–257, 259, 279, 523, 525, 529, 530, 532, 535, 546, 585–586
Cortex, 55, 57, 61, 75, 77–79, 82, 93, 95, 97, 100, 101, 124, 133, 137, 153, 155–158, 172, 174, 178, 180, 185, 189, 199, 202, 203, 206, 235, 236, 251, 256, 258, 273, 276–279, 281, 283–285, 287, 302, 304, 307–309, 311–314, 317, 360, 362, 365, 366, 382–385, 399, 439, 443, 452, 454–458, 460, 470–473, 509, 514, 527, 532, 534, 535, 545, 571, 584
Cryostimulation, 575–587

A. Dietrich-Muszalska et al. (eds.), *Studies on Psychiatric Disorders*,
Oxidative Stress in Applied Basic Research and Clinical Practice,
DOI 10.1007/978-1-4939-0440-2

D
Dementia, 126, 153, 234–235, 252–253, 418, 470, 522, 523, 525, 529, 530, 532–534, 536, 539–541, 544, 545
Depression, 74, 80, 100, 122, 134, 137, 138, 144, 212, 225, 233–236, 253–254, 310, 315, 334–336, 343, 426, 440, 443, 509, 510, 512–514, 569, 576, 577, 581–583, 585–587
Dopamine (DA), 46, 47, 55–57, 59, 77, 100, 125, 145, 147, 156–157, 198–201, 273, 278–283, 285, 301–304, 306, 310, 313–315, 335, 336, 429
D2 receptor (D2R), 199, 203–206, 281, 283–285, 313

E
Effects of lithium on oxidative stress, 567–571
Electron transport chain (ETC), 3, 46, 47, 74, 75, 77–79, 81, 89, 96, 97, 158, 160, 173, 188, 189, 221, 232, 233, 235, 236, 305, 356–358, 361–364, 366–367, 382, 399, 480, 482, 528
Environment, 45, 47, 59, 89–107, 126, 144, 153, 154, 156, 216, 232, 233, 272, 280, 286, 310, 333, 341, 342, 356, 359, 364, 365, 376, 378–379, 381, 383–384, 386, 395, 397, 398, 404–405, 417–419, 422, 423, 427–430, 470, 485, 486, 495–496, 507, 579, 582
Epigenetic, 47, 52–53, 82, 153, 232, 376–378, 385, 386, 397–398, 417, 418, 427–429, 456, 460
Etiopathophysiology, 74

F
Ferritin, 14, 471–476, 530
Flavonoids, 13, 15, 189, 506, 522–524, 536, 539, 540, 581
Free radicals (FR), 2, 4, 6, 11–14, 17–22, 28, 30, 46, 47, 52–56, 59, 75, 76, 81, 91–94, 96–97, 122, 123, 127, 128, 134, 144, 155, 158, 172, 173, 188, 189, 215–216, 237, 246–247, 250, 253, 269–271, 301, 303, 305, 312, 334, 337, 340, 356, 357, 362, 364–366, 377–382, 386, 398–400, 418, 420, 459, 470, 480–485, 506–508, 510, 511, 523, 528, 532, 576–579, 581

G
Genetic susceptibility, 91, 95–96, 126, 395, 400, 430
Glutamate (Glu), 56, 57, 132, 133, 138, 158–159, 172–175, 179, 212–214, 220, 221, 223, 254, 274, 303, 304, 309, 310, 452
Glutamic acid decarboxylase (GAD), 206, 287, 452–454, 456–462
GSH deficit in schizophrenia and in animal models, 278–280
GSH functions, 47, 56, 125, 270–274

H
Hyperbaric oxygen treatment, 459, 460, 553–562
Hypochlorite, 2, 5, 7–9, 20, 26, 29

I
Inflammation, 47, 59, 96, 97, 101, 137–138, 159, 160, 232, 234, 245–260, 333–334, 338, 359, 381, 405, 423, 436, 445, 447, 457, 486, 487, 491–492, 497, 524, 555, 557–559, 561–562, 577, 584–586
Initial balance between pro-and antioxidative balance, 570
Iron (Fe), 5, 10, 14, 47, 52, 93, 98, 122, 156, 201, 251–252, 286, 339, 356, 364–365, 385, 404, 469–476, 507, 523–524, 528–532, 535, 579

K
Kynurenic acid (KA), 60, 220, 248–249

L
Lithium concentration, 569–571

M
Major depression (MD), 80, 100, 234, 236, 315, 334, 335, 343, 437, 439–443, 445–446, 509–510, 512–514, 586
Metals, 4, 5, 9, 13–15, 19, 47, 93, 97–99, 102–103, 107, 122, 152, 200, 249–251, 254, 296, 336, 359, 364, 374, 386, 398, 403, 404, 429, 481, 507, 514, 523, 528, 530, 532
Minicolumn, 383, 384

Mitochondria, 3, 13, 14, 46, 47, 60, 74, 81, 96–97, 136, 147, 152, 154, 158, 160, 173, 179, 186, 187, 199, 213, 232, 253, 278, 301, 329, 356, 378, 399, 439, 480, 507, 528, 555, 578
Mitochondrial DNA (mtDNA), 76–78, 155, 158, 160, 173, 187, 233–236, 301, 357–359, 361–364, 556
Mitochondrial dysfunction (MD), 47, 56–58, 74–79, 81, 82, 96–97, 107, 158, 173, 174, 178–180, 185–189, 231–238, 355–367, 399, 509, 555, 561
Multiple sclerosis (MS), 78, 152, 159–160, 575, 584–587

N
N-acetylcysteine in therapy of schizophrenia, 288
NADPH oxidase (Nox), 4, 15, 16, 27, 46, 125, 153, 173, 216, 219, 222, 223, 225, 312, 460, 461, 529–530, 532
Neurodegeneration, 9, 45, 46, 59, 60, 123, 125, 144, 151–160, 198, 199, 206, 212, 224, 225, 234, 247, 249–251, 255, 256, 269, 271, 281, 301, 330, 358, 423, 430, 469–476, 522–524, 526–527, 529, 534–536, 540, 547, 569–570, 583
Neuroprotection, 188, 224, 249, 271, 278, 279, 305, 306, 496, 497, 510, 523–524, 534–537, 547, 554, 568, 577–578, 583
Nitrative/oxidative stress, 48–53, 201–202, 215, 219, 301, 342
Nitric oxide (NO), 6, 7, 10, 15–17, 26, 47, 52, 75, 79, 92, 94, 145, 152, 156, 173, 179, 215, 219, 223, 248, 251, 258, 271, 299–318, 337, 338, 362, 381, 398, 423, 506–509, 523, 524
N-methyl-D-aspartate receptor (NMDA), 47, 57, 59–60, 179, 188, 212–214, 216–220, 222–225, 273–274, 283–288, 302–305, 310–315, 380, 452, 454–457, 460, 461
Nox. *See* NADPH oxidase (Nox)

O
Obsessive-compulsive disorders (OCD), 122, 131–138, 235–237, 334
Oxidation, 22, 24–25, 75–77, 385, 404
Oxidative, 3, 45, 74, 92, 123, 135, 144, 152, 173, 212, 232, 246, 269, 301, 328, 356, 380, 404, 418, 437, 452, 506, 523, 556, 569, 580
Oxidative damage, 14, 17, 18, 20, 24, 28, 29, 46–48, 52, 53, 59, 74–79, 81, 82, 91–95, 107, 122–125, 127, 147, 152–155, 157, 160, 173, 221, 246, 251, 269, 278, 313, 329, 337, 339, 340, 365, 437, 439, 506–508, 523, 526, 528–530, 532, 534
Oxidative/nitrative stress, 48–53, 201–202
Oxidative status, 46, 123, 124, 135, 138, 143–148, 200–201, 204–206, 215–220, 428, 452, 461, 462, 569
Oxidative stress, 3, 8, 9, 11–13, 17, 43–61, 73–83, 89–107, 121–128, 131–138, 151–160, 171–189, 197–207, 211–226, 245–260, 335, 393–409, 417–430, 435–447, 451–462, 479–497, 567–571

P
Parkinson's disease (PD), 78, 152, 156–157, 199–201, 234, 404–406, 417–418, 423, 470, 522, 532, 540, 742–475
Parvalbumin (PV), 58, 206, 225, 287, 451–462
Peroxynitrite ($ONOO^-$), 2, 5–7, 9, 10, 26, 52, 75, 91, 92, 94, 158, 179, 188, 201, 223, 251, 252, 270, 302, 303, 305–307, 315–317, 337, 338, 340–342, 382, 399, 523
Pesticides, 101, 103, 106–107, 398
Phthalates, 97–98, 101, 105–106
Plant polyphenols, 51, 446, 521–547
Plasticity, 60, 97, 154, 172, 206, 212–214, 233, 234, 283, 286, 302, 312, 357, 358, 384–387, 454, 568, 583
Polyunsaturated fatty acids peroxidation, 481
Post-traumatic stress disorder (PTSD), 122, 126–128
Psychiatric diseases, 127, 255, 306, 315, 329, 336, 337, 341, 458, 587
Psychiatric disorders, 56–58, 122, 126, 132, 134, 138, 144, 146–148, 206–207, 225, 226, 231–238, 249, 252, 255, 260, 269–288, 310, 312, 315, 327–344, 435–447, 452, 462, 514, 515, 575–587
PV. *See* Parvalbumin (PV)

Q
Quinone, 11, 157, 200, 270, 273, 281, 404, 405

R
Reactive nitrogen species (RNS), 2, 7, 17, 18, 30, 46, 47, 59, 74, 75, 91, 92, 97, 125, 212, 249, 301–307, 316, 317, 321, 337–342, 480, 481, 586

Reactive oxygen species (ROS), 2–9, 12–13, 46, 74–75, 82, 90, 91, 144, 152, 172, 178–179, 200, 212, 216, 233, 246, 256, 271, 378, 379, 399–400, 403–404, 406, 408, 419, 437, 459, 461, 471, 473, 480, 481, 487, 506, 522, 556, 581
Redox, 12, 17, 30, 46–48, 53, 56–58, 82, 94, 123, 125, 172, 188, 198, 217, 218, 252, 258, 269, 342, 373–388, 429, 461
Retina, 481, 482, 484–497

S
Schizophrenia (SCZ), 43–61, 77, 78, 82, 99, 103, 198, 201, 205, 206, 232, 235–236, 245–260, 276–278, 280–283, 285–288, 299–318, 334, 337, 427, 437–440, 445–446, 455–459, 507–511, 514

T
Telomerase, 436, 437, 443, 445–446
Telomere length, 435–447
Thalidomide, 99–100
Time of its treatment, 570
Tryptophan (Trp), 7, 12, 25, 26, 60, 247, 248, 252, 253, 255, 258, 306, 340

V
Valproic acid (VPA), 100–101, 385, 396, 397, 399–402, 406–409, 426