Dopamine and Glutamate in Psychiatric Disorders

EDITED BY

Werner J. Schmidt
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HUMANA PRESS

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Humana Press Totowa, New Jersey

999 Riverview Drive, Suite 208
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humanapress.com

Production Editors: Mark J. Breaugh and Amy Thau

Cover design by Patricia F. Cleary

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ANSI Z39.48-1984 (American National Standards Institute) Permanence of Paper for Printed Library Materials.

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

eISBN:978-1-59259-852-6

Library of Congress Cataloging-in-Publication Data

Dopamine and glutamate in psychiatric disorders / edited by Werner J.Schmidt and Maarten E.A. Reith.

p. cm. Includes bibliographical references and index.

ISBN 978-1-58829-325-1 (alk. paper)

1. Neuropsychiatry. 2. Dopamine--Pathophysiology. 3.Glutamate--Pathophysiology. 4. Neurotransmitter receptors. I. Schmidt,Werner J. II. Reith, Maarten E.A.

RC347.D674 2005

616.8--dc22

2005001495

PREFACE

Among the medical disciplines, psychiatry has for a long time held a special position separate from natural sciences. This may be rooted in the old philosophical problem of the mind–body dichotomy. Accordingly, psychiatry, with its focus on the mind, developed separately from natural sciences, which were concerned with the body. Thus, psychiatry laid out its own hypotheses, constructs, and methods. The substrate of the mind is formed by neuronal networks, and neurobiology as a natural science discipline developed on its own, focusing primarily on neuronal mechanisms, from computationally integrated networks all the way down to electrical, cellular, and molecular processes underlying neuronal communication. In the last decades, psychiatry has moved from psychoanalytical to biological approaches. Biological psychiatry has completely changed the treatment of psychoses, allowing outpatient treatment of psychotics who previously would have been locked up inside psychiatric institutions; more recently, neurotic symptomology is also being treated more and more by chemical approaches. In the meantime, neurobiology has been revolutionized by new techniques, among which the development of molecular biological tools is of primary importance. Now psychiatry and neurobiology are approaching each other, and our knowledge about the neurobiological basis of mental functions is increasing rapidly. *Dopamine and Glutamate in Psychiatric Disorders* is dedicated to fostering interactions between the two disciplines.

One could highlight two approaches to understanding psychiatric diseases within the realm of neurobiological and natural sciences. Psychiatric diseases can be regarded from a molecular genetic point of view, i.e., to be genetically caused by, or at least be susceptible to, a predisposition, with proteins being the end product of the genetic machinery. This view equates a psychiatric disease to a proteinopathy. In this sense Parkinson's disease can be regarded as a synucleinopathy, Alzheimer's disease as a tauopathy, and so forth. A book could easily be filled summarizing this type of knowledge. Another approach is to first study the biological properties and functions of proteins we know play an important role in mental processes. Thus, dopamine and glutamate receptors can be singled out as crucial targets for endogenous transmitters known to play a role in pychoses or other complex psychiatric diseases. The molecular biology of such receptors, their subtypes and subunits could also easily fill a book. *Dopamine and Glutamate in Psychiatric Disorders* wishes to focus on the combination of these approaches. We plan to address the basic molecular mechanisms, but psychiatric diseases will be primarily regarded as "synaptic or extrasynaptic diseases," taking into account changes in dopamine and glutamate neurotransmission that can occur by communication through synaptic connections between neurons as well as by longer-range action through the extracellular space, sometimes referred to as volume transmission. This approach has led to effective medications in the past, for example, antipsychotics and antidepressants. In turn, the pharmacotherapy of psychiatric diseases has significantly contributed to concepts and hypotheses about neuronal dysfunctions underlying these diseases, such as the dopamine hypothesis of schizophrenia, or the monoamine-deficiency hypothesis of depression. However, better treatments are still badly needed. For example, antipsychotics, even the newer atypicals, have undesirable side effects; antidepressants, including the newer

Prozac-type, develop their therapeutic effect too slowly and offer no therapeutic help to a large percentage of depressed patients. Drug development is still an urgent priority.

Dopamine and Glutamate in Psychiatric Disorders reviews our progress in the field of dopamine and glutamate in psychiatric diseases. It includes both basic and clinical approaches and should be of interest to both basic scientists working at the bench on dopamine or glutamate neurotransmission and clinicians treating psychiatric diseases. In addition, graduate students and advanced undergraduates seeking a comprehensive overview of the field of dopamine and glutamate in psychiatric disorders will be interested in the book.

There is a fine line between symptoms of psychosis and symptoms of mood disorder. The latter can be secondary to an underlying psychosis; conversely, psychotic symptoms such as phobia can accompany depression. To make matters more complicated, many disorders that are targets for antidepressant treatment, such as obsessive compulsive phobic states, acute panic attacks, social phobias, and bulimia, are now considered to be clinical anxiety disorders rather than manifestations of an underlying depression. *Dopamine and Glutamate in Psychiatric Disorders* addresses many of these diseases originating in the central nervous system. Stress, as it is intricately related to depression, is also covered, as well as addiction, which is considered by many to be another brain disease, if not in origin, then created by repeated drug use.

Each chapter of *Dopamine and Glutamate in Psychiatric Disorders* summarizes the prevalence and symptoms of the disease, covers involvement of dopamine and/or glutamate systems with emphasis on findings with new molecular approaches, such as transgenic knockout or knockin mice and newer analytical techniques, such as brain imaging, and describes future directions and possibilities for new therapy development.

Werner J. Schmidt, PhD
Maarten E. A. Reith, PhD

ACKNOWLEDGMENT

We thank Mrs. Daniela Binder for her excellent secretarial assistance. Without her help, the production of this book would not have been possible.

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I Dopamine

1
Dopamine Receptors

Kim A. Neve

1. INTRODUCTION

Beginning with the initial suggestion that antipsychotic neuroleptic drugs block dopamine receptors *(1)*, and continuing with the demonstration that the affinity of antipsychotic drugs for dopamine receptors is highly correlated with clinical potency *(2,3)*, and that the density of [^{3}H]neuroleptic-labeled dopamine receptors is enhanced in postmortem brain tissue of schizophrenics *(4)*, the study of dopamine receptors has been inextricably linked with hypotheses for the mechanism of action of antipsychotic drugs and the etiology of schizophrenia. As described in other chapters in this volume, the role of dopamine in numerous other neuropsychiatric disorders, such as parkinsonism, attention deficit hyperactivity disorder, and addiction, has made consideration of the properties of dopamine receptor subtypes important for attempts to provide improved pharmacological treatments for these disorders. This chapter summarizes the molecular cloning of the five mammalian dopamine receptor subtypes, and reviews their structural, pharmacological, signaling, and regulatory properties.

2. DOPAMINE RECEPTOR SUBTYPES

2.1. Classification Into D1 and D2 Receptor Subfamilies

Although the existence of a receptor for dopamine was suggested indirectly by the effect of blockade of those receptors on dopamine turnover *(1)*, more direct evidence for such a receptor came in 1972 with the identification of dopamine-stimulated adenylate cyclase activity and cyclic adenosine monophosphate (AMP) accumulation first in retina *(5)*, and subsequently in rat neostriatum *(6)* and other basal forebrain nuclei including the nucleus accumbens and olfactory tubercle *(7)*. Importantly, the dopamine-stimulated adenylate cyclase was inhibited by antipsychotic drugs such as chlorpromazine, haloperidol, and fluphenazine much more potently than by drugs without antipsychotic or extrapyramidal actions such as imipramine and promethazine *(6–9)*. Dopamine receptors were first identified by radioligand binding in 1975 using both [^{3}H]dopamine and [^{3}H]haloperidol to label the receptors *(10–12)*, followed shortly by the synthesis and characterization of [^{3}H]spiperone *(13–15)*, still perhaps the most commonly used radioligand for D2-like dopamine receptors because of its high affinity and selectivity for the receptors.

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

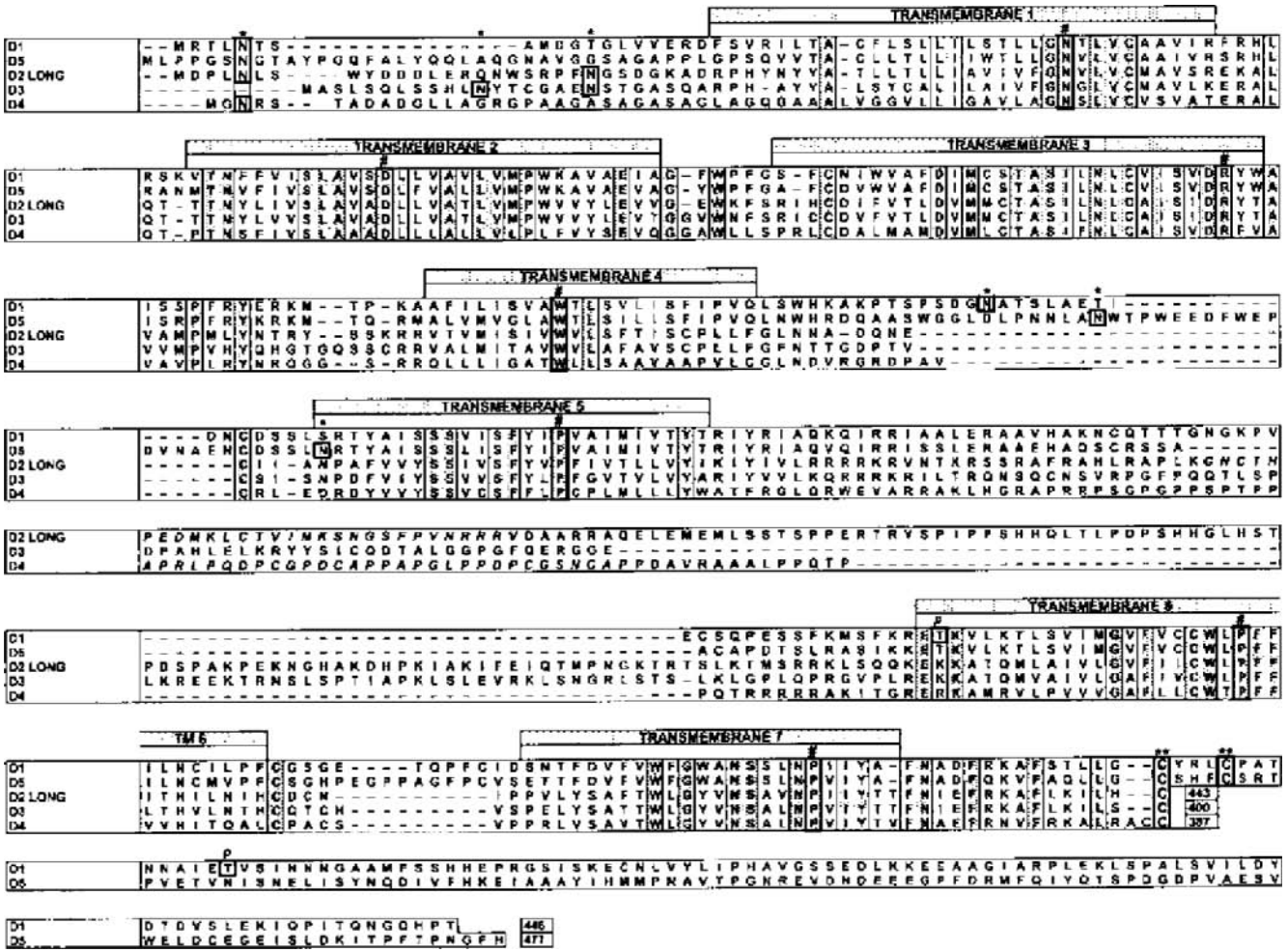
TRANSMEMBRANE 1
TRANSMEMBRANE 2
TRANSMEMBRANE 3
TRANSMEMBRANE 4
TRANSMEMBRANE 5
TRANSMEMBRANE 6
TM 6
TRANSMEMBRANE 7
D1
D5
D2 LONG
D3
D4

Fig. 1.

Two seminal papers in 1978 and 1979 summarized several lines of evidence that are inconsistent with the notion of a single type of dopamine receptor *(16,17)*. For example, the pharmacological profiles of dopamine-stimulated adenylate cyclase and the dopamine receptor identified by radioligand binding studies differ in key ways; in particular, domperidone and substituted benzamide derivatives, such as metoclopramide and sulpiride, that are potent inhibitors of radioligand binding are weak antagonists of dopamine-stimulated adenylate cyclase *(18–20)*, and butyrophenone antipsychotic drugs, such as spiperone and haloperidol, are also less potent inhibitors of enzyme activity than would be predicted based on their binding affinity *(21)*. Furthermore, dopamine-stimulated adenylate cyclase was shown to be physically distinct from the receptor predominantly labeled by most of the dopamine receptor radioligands in use at that time. Thus, dopamine does not stimulate adenylate cyclase activity in the anterior pituitary *(16)*, a tissue with abundant binding of several dopamine receptor ligands *(15,21)*, and axon terminal-sparing lesions of the cell bodies in the neostriatum (kainic acid) and substantia nigra (6-hydroxydopamine) selectively abolish or spare, respectively, dopamine-stimulated adenylate cyclase *(22–24)*. Data such as these led to the proposal that dopamine receptors belong to two subtypes, with the D1 subtype being coupled to adenylate cyclase and having low affinity for dopamine, ergots, such as bromocriptine, and substituted benzamine antagonists, and the D2 subtype being unassociated with adenylate cyclase, having high affinity for dopamine, substituted benzamide derivates, and butyrophenone antipsychotic drugs, and serving as the autoreceptor that regulates dopamine release *(17)*. This classification is still valid, with the major modifications to it being the recognition that, rather than being uncoupled from adenylate cyclase, D2 receptors are coupled to inhibition of adenylate cyclase *(25)*, and the fulfillment of the prediction that subcategories of D1 and D2 receptors would be discerned *(17)*; that is, D1 (henceforth referred to as D1-like) and D2 (D2-like) receptors are subfamilies, rather than subtypes.

2.2. Molecular Cloning of Dopamine Receptor Subtypes

The molecular cloning of a rat D2 receptor cDNA, reported in December of 1988 *(26)*, was the first step in the cloning of five dopamine receptor subtypes, all of which were discovered by 1991. As this work has been reviewed in detail elsewhere *(27)*, in this chapter I will summarize the cloning of the human receptors (Fig. 1). The cloning of the rat cDNA was rapidly followed by isolation of cDNA encoding the human D2 receptor, with four reports appearing in 1989 *(28–31)*. The first unanticipated result of the cloning of the dopamine receptors was the observation by all four of these reports that the D2 receptor gene product is alternatively spliced to produce long ($D2_L$; gene accession no. NM_000795) and short ($D2_S$; NM_016574) variants, 443 and 414 amino acids long, respectively. The variants differ by the presence or absence of an alternatively spliced

Fig. 1. Amino acid sequence-alignment of the human dopamine receptors. Positions that are conserved among all five subtypes are shaded. Residues that are marked with a dark border and a symbol above the alignment include the most highly conserved residue in each transmembrane domain (#), predicted sites of *N*-linked glycosylation (*), predicted sites of palmitoylation (**), and experimentally determined sites of phosphorylation (*p*). The alternatively spliced insert in $D2_L$ and the tandem repeat in the D4.2 variant are in italicized font.

exon encoding 29 amino acids in the third cytoplasmic loop of the receptor. $D2_L$ and $D2_S$ have essentially the same pharmacological profile, which corresponds to that of the pharmacologically defined D2-like receptor.

The following year saw the molecular cloning of DNA encoding the human D1 dopamine receptor (NM_000794), a 446 amino acid protein with a pharmacological profile corresponding to that of the pharmacologically defined D1-like receptor *(32–34)*. The same year brought a second major unanticipated result, the cloning of cDNA encoding a rat D3 receptor *(35)*, followed closely by the cloning of the human D3 receptor (NM_000796), a 400 amino acid protein with a pharmacological profile that, although similar to that of the D2 receptor, is distinct in ways that were not predicted by previous pharmacological studies of native dopamine receptors *(36,37)*. The distribution of D3 receptor mRNA also differs from that of the D2 receptor and D2-like receptor binding, being absent from the anterior pituitary and overall much less abundant than D2 receptor mRNA in brain, low in the dorsal neostriatum where the D2 receptor is most abundant, and highest in ventral forebrain nuclei such as the nucleus accumbens and the olfactory tubercle *(38)*.

The human D4 (gene accession no. NM_000797; ref. *39*) and D5 dopamine receptors (gene accession no. NM_000798; refs. *40,41*) were cloned in 1991. The D4 receptor is structurally and pharmacologically related to the D2 receptor, but has a unique distribution in brain, being relatively most abundant outside of the basal ganglia in retina, amygdala, cerebral cortex, hypothalamus, and hippocampus *(42)*. Although there are numerous allelic variants of the D4 receptor that differ in length (*see* Subheading 7.2.), the two-repeat version D4.2 is 387 amino acids long (Fig. 1). The 477 amino acid D5 receptor is very closely related to the D1 receptor, but its cognate mRNA is both much less abundant and more widely distributed, including in brain regions that do not have a substantial dopaminergic innervation *(38)*.

The criteria that are used to group the dopamine receptors into D1-like (D1, D5) and D2-like (D2, D3, D4) subfamilies include primary and secondary structure, organization of the genes, pharmacological profiles, and signaling properties. The D1 and D5 receptors have over 60% amino acid similarity, and each has only approx 30% similarity to the D2 receptor, whereas D2 and D3 are greater than 50% homologous, and the D4 receptor has approx 40% amino acid identity with the D2 or D3 receptors. The D1-like receptors have in common a relatively short third cytoplasmic loop and a long C-terminus, whereas the D2-like receptors have a long third cytoplasmic loop and a short C-terminus (Fig. 1). The D1-like receptor genes are intronless within their coding regions; in contrast, the D2-like receptor coding regions are interrupted by numerous introns of variable length, with a conserved intron/exon organization *(27)*. The D2-like receptors share high affinity for a number of D2 antagonists such as the prototypical D2 radioligand [^{3}H]spiperone, whereas the D1-like receptors are pharmacologically indistinguishable, particularly regarding antagonist affinity, and have high affinity for the prototypical D1 antagonist [^{3}H]SCH 23390 (Table 1).

3. STRUCTURAL FEATURES OF DOPAMINE RECEPTORS

3.1. Shared Structural Features

The dopamine receptors are all family A G protein-coupled receptors (GPCRs). Many dopamine receptor models are based primarily on the homology of the receptors

Table 1
Dopamine Receptor Affinity for Antagonists

	Affinity (K_i, n*M*)					
	Receptor subtype					
Drug	D2	D3	D4	D1	D5	References
A-69024	1320	—	—	19	—	*88,332*
Aripiprazole[a]	0.5	9.1	260	410	1200	*111*
(+)-Butaclamol	0.8	4.8	51	3	6	*333*
BW 737C89	54	—	—	0.3	—	*89,332*
Chlorpromazine	4	3	13	44	83	*333,334*
Clozapine	145	238	29	124	343	*333*
Domperidone	0.7	12	90	~10,000	—	*333,335,336*
Eticlopride	0.1	0.25	27	>10,000	>10,000	*333*
Flupentixol, *cis*	1.2	2.0	—	2.9	5.2	*40,333,334*
Fluperlapine	316	255	76	57	328	*333,334*
Fluphenazine	0.9	0.5	28	10	8	*333,334*
Haloperidol	2	10	4.2	124	87	*333*
L741,626	4	63	320	790	630	*337*
Metoclopramide	64	16	—	>10,000	—	*338*
NNC 756	782	—	—	0.34	0.6	*332,334*
Olanzapine	24	36	23	68	74	*333*
Perphenazine	0.6	0.6	40	30	—	*333*
Pimozide	3	4	30	>10,000	—	*333*
Quetiapine	470	506	1705	1900	1513	*333*
Raclopride	2.1	3.4	1990	>50,000	—	*333*
Remoxipride	344	1700	2600	>10,000	—	*333*
Risperidone	5	7	11	540	560	*333*
SCH23390	480	1450	2910	0.3	0.3	*333*
SCH39166	>1000	—	—	0.2	0.4	*87,339*
SDZ PSD 958	63		810	0.16	0.18	*90*
Spiperone	0.08	0.4	0.1	420	3550	*333*
Sulpiride	35	60	52	>10,000	>10,000	*35,39,333*
Thioridazine	7	4	14	100	300	*333*
YM-09151-2 (nemonapride)	0.05	0.13	0.32	2600	—	*333*

Affinity values are shown for dopamine receptor antagonists. Data were obtained from the cited papers except for ref. *333* which indicates that the data were obtained from the NIMH Psychoactive Drug Screening Program K_i database, and were obtained by averaging all the affinity values for each drug that were obtained using clone (i.e., heterologously expressed) receptors.

(http://kidb.bioc.cwru.edu/pdsp.php).

[a]Aripiprazole is a low-efficacy partial agonist.

to rhodopsin, the only member of this family for which the crystal structure has been determined *(43)*. Other data that contribute to receptor models are obtained from affinity-labeling studies, in which a chemically reactive moiety is attached to a receptor ligand, and by site-directed mutagenesis, in which the effect of mutations on ligand binding to and activation of receptors is determined, or in which the mutations are designed to create

a "binding" site for multivalent ions, cross-linking reagents, or specific side chain-reactive affinity reagents. The data that contributed to our dopamine receptor models have been reviewed in detail elsewhere *(44–46)*; in this chapter I will provide an overview of receptor domains thought to be important for ligand binding and signaling. Although there is no case in which the role of a particular residue has been confirmed in all dopamine receptor subtypes, the extensive conservation of GPCR structure and function makes it possible to generalize certain findings from one dopamine receptor subtype, or from other biogenic amine receptors, to all dopamine receptors. In referring to specific residues I will use the index of Ballesteros and Weinstein *(47)*, in which a residue is referred to by the transmembrane segment (TM) in which it resides and according to its position relative to the most highly conserved residue in that TM. Thus, the most highly conserved residue in TM1 of GPCRs is Asn1.50. In the D2 receptor this residue is Asn52$^{1.50}$, and Gly51$^{1.49}$ and Leu54$^{1.52}$ are immediately to the N terminal side and two residues to the C terminal side of Asn52$^{1.50}$, respectively. In Fig. 1, the most highly conserved residue in each TM is designated by this symbol: #.

The family A GPCRs have in common a relatively short N-terminal extracellular domain and seven α-helical membrane-spanning segments. In rhodopsin, the cytoplasmic extension of TM7 is an α helix (helix 8) extending parallel to the plane of the membrane, a structural feature thought to be shared by most family A GPCRs *(44)*. Broadly speaking, the intracellular loops and C-terminal tail form the contact surfaces for G proteins and other receptor-interacting proteins, whereas the binding of small molecule neurotransmitters such as the biogenic amines involves residues in the outer third of the TMs (i.e., toward the extracellular face of the membrane). With the possible exception of the second extracellular loop between TM4 and TM5 *(45)*, the extracellular regions are not thought to participate directly in ligand binding or receptor signaling. The primary binding pocket for dopamine consists chiefly of residues in TM3, TM5, and TM6 that are conserved among catecholamine receptors. These residues include Asp$^{3.32}$ (Asp103 in D1 and Asp114 in D2), which participates in an electrostatic interaction with the protonated amine of the ligand, Ser$^{5.42}$, Ser$^{5.43}$, and Ser$^{5.46}$, which interact with the catecholamine hydroxyl groups, and a cluster of aromatic residues in TM6 (Trp$^{6.48}$, Phe$^{6.51}$, Phe$^{6.52}$) that have been demonstrated to contribute to ligand binding to and activation of many biogenic amine receptors *(45,46)*. Other residues that contribute to the primary binding pocket formed by TM3, TM5, and TM6 are often adjacent to or one helix turn away from primary contact residues such as Asp$^{3.32}$ (residues 3.29, 3.33, and 3.36), Ser$^{5.46}$ (residue Phe$^{5.47}$), and Phe$^{6.51}$/Phe$^{6.52}$ (residue 6.56).

To use receptor models as tools to aid in the development of subtype-selective drugs, it is important to know not only binding pocket residues that are shared among the subtypes, but also residues that differ and could account for pharmacological differences between subtypes. Some of the primary pocket residues listed above differ between D1-like and D2-like receptors, or among the D2-like receptors *(46)*. Also important for subtype-selective binding is the ancillary binding pocket *(46,48)*, formed by residues in TM2, TM3, and TM7 on the extracellular side of the primary binding pocket. Elegant work by Javitch and colleagues determined that selectivity between D2 and D4 receptors is owing in large part to a cluster of nonpolar residues in this region that they refer to as a divergent aromatic microdomain, because the nonconserved D2 and D4 residues

often differ with respect to the presence or absence of an aromatic side chain *(49)*. The value of this work is demonstrated by the development of the highly selective D4 receptor antagonist FAUC 213, designed to exploit differences between the D2 and D4 receptor in this microdomain *(50)*. In addition to residues that line the binding pocket in the TMs, it has been proposed that the second extracellular loop, which reaches into the binding crevice and contacts retinal in rhodopsin *(43)*, also forms part of the binding pocket in dopamine receptors and other GPCRs and could contribute to pharmacological specificity *(44,45)*.

The structural basis for receptor activation may not be fully understood until a GPCR is crystalized in an active conformation, but studies of rhodopsin and other GPCRs have yielded a basic model of receptor activation that is probably valid for the dopamine receptors. In this model, a number of interhelical bonds constrain the receptor in an inactive conformation. Of particular importance is $Arg^{3.50}$, which forms an ion pair with $Glu^{6.30}$ and hydrogen bonds with residue 6.34. Activation of the receptor can result from disruption of interhelical bonds by agonist binding, by mutation of a residue participating in an interhelical bond, or by nonspecific disruption of helix packing *(51–54)*. Releasing the interhelical constraints allows the movement of TM6 so as to increase the distance between TM3 and TM6 at the cytoplasmic face of the membrane, also exposing residue 6.34 to the solvent *(46,55,56)*. Alterations in the relative positions of TM3, TM5, and TM6 presumably expose domains of the receptor cytoplasmic loops that bind to and activate heterotrimeric G proteins. These domains have not been mapped in detail for all of the dopamine receptors subtypes, although work with dopamine and other biogenic amine receptors has implicated the second cytoplasmic loop, the membrane-proximal portions of the third cytoplasmic loop, and the membrane-proximal region of the cytoplasmic tail in G protein selection and activation *(57–61)*.

3.2. *Posttranslational Modifications*

3.2.1. Glycosylation

All dopamine receptor subtypes have one or more potential sites of *N*-linked glycosylation in the amino-terminal region (Fig. 1). The D1-like receptors have additional consensus sequences in the second extracellular loop (one for D1 and two for D5), whereas the D3 receptor has potential sites in both the first and second extracellular loops. Endogenous D1-like receptors (probably mostly D1) are heavily glycosylated, and partial deglycosylation has little or no effect on [^{3}H]SCH 23390 binding to the receptors *(62)*. Studies of recombinant D1 receptor show that it is probably glycosylated at both potential sites, although preventing glycosylation does not alter receptor trafficking to the membrane *(63)*. The D5 receptor is glycosylated on the amino-terminal site and the first site (N-W-T) in the second extracellular loop, but perhaps not on the second site (N-R-T) in that loop. Prevention of glycosylation, by either mutation or treatment with tunicamycin, prevents trafficking of the D5 receptor to the plasma membrane. Interestingly, although glycosylation *per se* is not required for ligand binding because enzymatic deglycosylation does not affect binding levels, inhibition of glycosylation during receptor biosynthesis prevents the acquisition of ligand binding *(63)*. Endogenous and recombinant D2 receptors are also heavily glycosylated *(64,65)*, but enzymatic deglycosylation does not greatly affect ligand binding *(66)* or coupling of the receptors to G proteins *(67)*.

3.2.2. Palmitoylation

The D1-like receptors have two potential sites of palmitoylation in the cytoplasmic tail, whereas the D2-like receptors terminate in a cysteine that is thought to be palmitoylated; attachment of the palmitoylated cysteine to the membrane could create a fourth cytoplasmic loop out of helix 8. Many GPCRs are palmitoylated either constitutively or dynamically (e.g., agonist-stimulated palmitate turnover), and palmitoylation may be involved in receptor processing and targeting to the membrane, in coupling to G proteins and signaling, and in desensitization, sequestration, and internalization *(68)*. Although incorporation of [^{3}H]palmitic acid into the D1 receptor expressed in Sf9 cells has been reported to be enhanced by dopamine *(69)*, in other work by this group the D1 receptor was found to be constitutively palmitoylated, on both Cys347 and Cys351 *(70)*. Preventing palmitoylation by mutation of these residues does not hinder receptor expression, activation of G proteins, or dopamine-induced uncoupling from G proteins (desensitization) *(71)*. In contrast, another report described loss of desensitization (as measured by diminished stimulation of adenylate cyclase) after mutation of Cys351, and speculated that the mutant receptor was constitutively desensitized *(72)*. The $D2_L$ receptor is also constitutively palmitoylated in Sf9 cells *(73)*.

3.2.3. Phosphorylation

Phosphorylation by GPCR kinases (GRKs), second messenger-dependent kinases, such as protein kinase A (PKA) and protein kinase C (PKC), and other kinases, is a general mechanism for regulating the signaling and trafficking of GPCRs. All of the dopamine receptor subtypes have multiple potential sites of phosphorylation by these kinases in the cytoplasmic loops and tail. Agonist-dependent phosphorylation of the D1 receptor *(69,74)* is catalyzed by GRKs *(75)* and by PKA *(76)*. Mutagenesis studies in which potential phosphorylation sites are mutated to alanine suggest that Thr360 in the cytoplasmic tail is a site of phosphorylation by GRK2 *(77)*, whereas Thr268, at the junction of the third cytoplasmic loop and TM 6 (Fig. 1), is a site of phosphorylation by PKA *(76)*. The D2 receptor is phosphorylated both constitutively *(73)* and in an agonist-stimulated manner *(78)*, whereas agonist treatment causes little phosphorylation of the D3 receptor *(78)*. Agonist-dependent phosphorylation of the D2 receptor is enhanced by overexpression of GRK2 *(78,79)*.

4. PHARMACOLOGICAL PROFILES OF DOPAMINE RECEPTOR SUBTYPES

4.1. Differentiation Between D1-Like and D2-Like Receptors

At the time of the division of dopamine receptors into what were then considered D1 and D2 subtypes, characterization of D2-like receptors and their contribution to dopamine-dependent behaviors was aided by the development of butyrophenone radioligands that labeled D2-like receptors with high affinity and selectivity, of highly selective substituted benzamide antagonists, and D2-like receptor-selective agonists, such as bromocriptine. The addition of equally selective but more efficacious agonists, such as quinpirole *(80),* and substituted benzamide radioligands such as [^{3}H]YM-09151-2 ([^{3}H]nemonapride) *(81)*, facilitated what was already an explosion of research on the behavioral and biochemical properties of D2-like receptors. Progress on D1-like receptors was hindered by the lack of D1-selective agonists and antagonists until the identification

of selective benzazepine ligands, such as the antagonist SCH23390 *(82,83)*, also extremely useful as a [^{3}H]-labeled radioligand *(84)*, and the partial agonist SKF38393 *(85)*. Numerous D1-like receptor-selective agonists that vary in efficacy and D1/D2 selectivity have subsequently been developed *(86)*, as well as several D1-like receptor-selective antagonists (Table 1) including SCH39166 *(87)* and the non-benzazepines A-69024 *(88)*, BW 737C89 *(89)*, and SDZ PSD 958 *(90)*. Because different D1-like receptor antagonists may have different behavioral properties *(91)*, this research area is weakened by the shortage of structurally diverse and commercially available antagonists to supplement SCH23390, by far the most commonly used D1-like receptor antagonist.

The D1-like D1 and D5 receptors have similar affinities for most antagonists (Table 1) as would be predicted from their extensive homology in the TMs (Fig. 1). Although the D5 receptor has higher affinity for most agonists than does the D1 receptor *(40)*, this is because of the higher constitutive activity of the D5 receptor *(92)*, and probably does not reflect differences in the binding pockets of the two receptor subtypes that can be exploited to develop selective antagonists. Both because homology among the D2-like receptors is lower and because D2-like subtype-selective drugs held the promise of significant improvements in the treatment of schizophrenia and other disorders, many agonists and antagonists that differentiate among the D2-like receptors have been developed.

4.2. Differentiation Among D2-Like Receptors

The development of D3 receptor-selective ligands is challenging because of the close homology of D2 and D3 receptors. Thus, there are very few amino acid positions exposed to the binding pocket where there are nonconservative substitutions between the subtypes *(46)*. The affinity of the human D3 receptor for most D2-like receptor antagonists does not differ greatly from that of the D2 receptor; most of the antagonists have similar or modestly lower affinity for the D3 receptor *(37,93,94)*. In membrane-binding assays, the D3 receptor has higher affinity than the D2 receptor for D2-like receptor agonists, such as dopamine, quinpirole, and 7-OH-DPAT *(35,37,95)*, but much of the apparent selectivity of D2-like agonists for the D3 receptor is related to the unusual guanosine triphosphate(GTP)-resistant nature of the agonist binding to the D3 receptor, so that careful control of assay conditions is required to ensure that only the D3 receptor is being labeled, and not the D2 receptor in an agonist high-affinity conformation *(96–98)*. Nevertheless, because of the possible therapeutic benefits of selective blockade or stimulation of the D3 receptor *(99,100)*, considerable effort has been invested in the development of D3 receptor selective agonists and antagonists. A number of antagonists have been developed that have 100-fold or greater selectivity for the D3 receptor over the D2 receptor, including PD 5849, the benzopyranopyrrole S33084, the arylpiperazines NGD 2849 and NGD 2904, *N*-[4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butyl]-7-methoxy-2-benzofurancarboxamide (compound 41), and the tetrahydroisoquinoline SB-277011 (Table 2). As mentioned above, determination of the D3 receptor selectivity of agonists is complicated by differences in the coupling of D2 and D3 receptors to G proteins, but among the agonists thought to be at least modestly selective for the D3 receptor are 7-OH-DPAT, pramipexole (SND 919), quinerolane, PD128,907, and FAUC 725 *(35,101–104)*.

The initial pharmacological characterization of the D4 receptor demonstrated that the subtype has a pharmacological profile that, although clearly "D2-like," differs significantly

Table 2
D2-Like Receptor Affinity for Antagonists Differentiating Among Subtypes

	Affinity (K_i, n*M*)			
	Receptor subtype			
Drug	D2	D3	D4	Reference
D3-selective				
GR 103,691	24	0.4	81	*340*
GR 218,231	63	1.0	10000	*337*
Nafadotride	3	0.3	1780	*341*
NGB 2849	262	0.9	>5000	*342*
NGB 2904	217	1.4	>5000	*342*
PD 58491	2400	20	>3000	*343*
S 14297	300	13	1380	*344*
S33084[a]	32	0.3	2000	*337*
SB-277011	1000	10	—	*345*
U99194A	2280	223	>10000	*346*
Compound 41	373	720	0.13	*112*
D4-selective				
CI-1030	413	679	4.3	*347*
CP-293,019	>3000	—	3.4	*348*
FAUC 213	3400	5300	2.2	*50*
L-745,870 (CPPMA)	960	2300	0.43	*110*
L-750,667[a]	>1700	>4500	0.5	*110*
NGD 94-1[a]	2230	>10000	4	*349*
PB12[a]	1900	—	0.04	*350*
PD 89211	>5000	>3000	3.6	*351*
PD 172938	5882	2700	8	*352*
RBI-257	568	145	0.33	*353*
U-101387	5000	>2500	10	*354*

[a]Has been used as a radioligand.

from that of the D2 receptor *(39)*. Although D2 and D4 receptors have similar affinities for some D2 receptor antagonists, such as spiperone and YM-09151-2, and the D4 receptor has slightly higher affinity than the D2 receptor for clozapine, the D2 receptor has higher affinity than the D4 receptor for most D2 receptor antagonists, with marked D2 selectivity observed for raclopride, fluphenazine, and (+)-butaclamol (Table 1). These data tend to support a conclusion also suggested by the lower homology between the D2 and D4 receptors (compared to that between D2 and D3)—the binding pockets of the D2 and D4 receptors are sufficiently different that the development of highly selective drugs is straightforward. Among the D4 receptor-selective antagonists that have more than 1000-fold lower affinity for the D2 receptor are L-745,870 and its 4-iodo analog L-750,667, PB12, RBI-257, CP-293,019, PD 89211, and FAUC 213 (Table 2; *see* also ref. *42*). With the exception of PD 168077, an agonist with reasonable efficacy and greater than 300-fold selectivity for D4 over D2 and D3 receptors *(105)*, and FAUC 312 *(106)*, reports of high-efficacy D4 receptor-selective agonists have appeared only in

meeting proceedings. Interestingly, several of the antagonists listed above are partial agonists under some conditions *(107)*, and a number of other D4 receptor-selective partial agonists have also been developed *(108,109)*.

D2 receptor-selective drugs, i.e., drugs that bind with higher affinity to the D2 receptor than to the D3 or D4 receptors, would also be useful tools. As shown in Table 1, three compounds with moderate selectivity for the D2 receptor are L741,626 *(110)*, domperidone *(20)*, and aripiprazole *(111)*; these could be lead compounds for the development of more D2-selective drugs. Compounds have also been described that are quite selective for both D3 and D4 over the D2 receptor *(112)* and for both D2 and D4 over the D3 receptor *(113)*.

5. DOPAMINE RECEPTOR SIGNALING

5.1. G Protein Coupling

5.1.1. D1-Like Receptors and G Proteins

All dopamine receptor subtypes are GPCRs whose signaling is at least partially mediated by interaction with and activation of heterotrimeric G proteins. As receptors that stimulate adenylate cyclase, the D1-like receptors were assumed to couple to the adenylate cyclase stimulatory G protein $G\alpha_s$. $G\alpha_{olf}$, the heterotrimeric G protein involved in olfaction, is very closely related to $G\alpha_s$ (88% amino acid homology) and also stimulates adenylate cyclase *(114)*. In the neostriatum, the brain region with the densest dopamine innervation and the highest expression of the D1 receptor, expression of $G\alpha_{olf}$ is very high whereas expression of $G\alpha_s$ is very low *(115)*. The nucleus accumbens and olfactory tubercle also express abundant $G\alpha_{olf}$ and little $G\alpha_s$ *(116)*. Unlike wildtype mice, $G\alpha_{olf}$ null mutant mice do not increase their locomotor activity in response to cocaine or a D1-selective agonist and exhibit little dopamine-stimulated adenylate cyclase or cocaine-induced c-fos expression in the neostriatum or nucleus accumbens, strongly suggesting that $G\alpha_{olf}$ mediates D1 receptor signaling to adenylate cyclase in these basal ganglia nuclei *(115,117)*. When expressed in HEK293 cells, D1 receptor stimulation of adenylate cyclase, but not D5 receptor stimulation, requires the expression of endogenous γ_7 subunit, presumably as part of the heterotrimer $G\alpha_s\beta_1\gamma_7$ *(118)*. Because γ_7 is abundantly expressed in neostriatal medium spiny neurons *(119)*, particularly in neurons that also express D1 receptor mRNA *(118)*, neostriatal D1 receptors may signal via a G protein heterotrimer that includes both $G\alpha_{olf}$ and γ_7. In other brain regions, including dopamine target areas that express D1 and/or D5 receptors such as the cerebral cortex and hippocampus, where the expression of $G\alpha_{olf}$ is much lower than that of $G\alpha_s$ *(116)*, it seems likely that $G\alpha_s$ mediates D1 and D5 receptor signaling to adenylate cyclase. Coupling of D1 receptors to $G\alpha_s$, and to other heterotrimeric G proteins such as $G\alpha_o$ and $G\alpha_q$, has also been described *(120,121)*.

5.1.2. D2-Like Receptors and G Proteins

D2-like receptor signaling is mediated primarily by activation of the pertussis toxin-sensitive G proteins $G\alpha_{i/o}$. For the D2 receptor, the possibility that the alternatively spliced insert in the third cytoplasmic loop of $D2_L$ might influence G protein interactions and result in differential G protein selection by $D2_S$ and $D2_L$ has meant that analyses of G protein selection are often carried out in the context of comparisons between the two isoforms. As reviewed in detail elsewhere *(46,122)*, there is quite a bit of disagreement in the literature concerning which G proteins interact with $D2_S$ and $D2_L$. It seems likely that

both receptor isoforms are inherently able to activate multiple $G\alpha_{i/o}$ subtypes, including $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o}$ *(123,124)*. $D2_S$ and $D2_L$ can also activate the pertussis toxin-insensitive G protein $G\alpha_z$ *(125,126)*. Recently, however, several different approaches have identified $G\alpha_o$ as the $G\alpha_{i/o}$ subtype that is most robustly activated by $D2_L$ *(127–129)* and by $D2_S$ *(130–132)* and, furthermore, the G protein subtype that is predominantly coupled to D2 receptors in the mouse brain *(133)*.

The D3 receptor is anomalous in that agonists bind to the receptor with a high affinity that is relatively insensitive to GTP. The GTP insensitivity could reflect GTP-resistant coupling to G proteins or a receptor structure that has inherently high affinity for agonists; interesting work by Leysen and colleagues expressing the D3 receptor in *Escherichia coli*, and thus in the absence of endogenous G proteins with which the receptor can interact, indicates that the latter explanation is more likely, and also suggests that G proteins bind to the D3 receptor with an affinity similar to that for the D2 receptor *(134)*. Work by several groups has identified $G\alpha_o$ as being activated by the D3 receptor and mediating D3 signaling, with some evidence for signaling via $G\alpha_z$ and $G\alpha_{q/11}$ *(126,135–137)*. The complexity of the mechanisms regulating G protein selection is indicated by the work of Zaworski et al. *(137)*, who found that the D3 receptor couples more efficiently to $G\alpha_o$ in SH-SY5Y cells than in HEK293 cells, despite the abundance of that G protein subtype in both cell lines. Zaworski et al. suggest that the additional presence in SH-SY5Y cells of effectors regulated by the D3 receptor contributes to the efficient activation of $G\alpha_o$ by the D3 receptor in those cells. This hypothesis is consistent with other work showing that receptors form complexes with effectors, and that G proteins participate in complex formation *(138)*.

The human D4 receptor is similar to D2 in that it activates multiple pertussis toxin-sensitive G proteins, including $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o}$ *(135,139)*. The rat D4 receptor has been reported to couple preferentially to $G\alpha_z$ *(126)* and to the pertussis toxin-sensitive transducin subtype, $G\alpha_{t2}$ *(140)*.

5.2. Signaling Pathways

5.2.1. D1-Like Receptor Signaling

The most thoroughly characterized signaling pathway for the D1-like receptors is $G\alpha_s$- or $G\alpha_{olf}$-mediated stimulation of adenylate cyclase, primarily adenylate cyclase type 5 *(141,142)*, which increases cyclic AMP (cAMP) accumulation, activates PKA, and increases the phosphorylation of a number of proteins involved in signal transduction and regulation of gene expression *(143,144)*. D1 receptor-stimulated gene expression is mediated by PKA-dependent phosphorylation of the cAMP response element-binding protein (CREB) *(145,146)*. D1-like receptor stimulation of PKA increases the phosphorylation of the glutamate *N*-methyl-D-aspartate NMDA receptor NR1 subunit *(147)*, thus enhancing (NMDA)-evoked currents *(148)* and activating L-type calcium curents *(149,150)*. D1 receptor stimulation also causes PKA-dependent inhibition of voltage-gated sodium channels *(151)*, and γ-aminobutyric acid $(GABA)_A$ receptor currents *(152)*. DARPP-32 (dopamine and cAMP-regulated phosphoprotein, 32 kDa) plays a central role in signaling by dopamine receptors. DARPP-32 is a neostriatum-enriched bifunctional signaling protein that inhibits protein phosphatase 1 (PP1) when phosphorylated on Thr34 by PKA and several other kinases *(153,154)*, and inhibits PKA when phosphorylated on Thr75 by cyclin-dependent kinase 5 *(155)*. Thus, in addition to

direct phosphorylation of numerous PKA substrates including those mentioned above, D1 receptor stimulation of PKA prevents PP1-catalyzed dephosphorylation of the same phosphoproteins by phosphorylating DARPP-32 on Thr34. D1 receptor stimulation simultaneously disinhibits PKA by activating protein phosphatase-2A and promoting Thr75 dephosphorylation of DARPP-32. Studies with DARPP-32 null mutant mice have demonstrated that DARPP-32 is required for acute D1 receptor-mediated responses, at both the cellular and behavioral levels *(154)*, and mice deficient in degradation of cAMP as a result of a phosphodiesterase 1B null mutation have enhanced D1 agonist-induced phosphorylation of DARPP-32 and enhanced methamphetamine-stimulated locomotor activity *(156)*.

One finding that is difficult to reconcile with a model of D1 receptor signaling that includes a central role for a cAMP/PKA/(protein phosphatase)/DARPP-32 cascade is that a null mutation of adenylate cyclase type 5 virtually abolishes D1 receptor stimulation of adenylate cyclase activity while enhancing D1 agonist-stimulated locomotor activity *(141,142)*. Although interpretation of the results is complicated because D2 receptor signaling is also disrupted in the adenylate cyclase 5 null mutant mouse, one possible explanation is that a cAMP-independent signaling pathway mediates D1 receptor locomotor activation, and perhaps other behavioral effects of D1 receptor stimulation. An alternative pathway that has been proposed for D1-like receptor signaling is phospholipase C-mediated mobilization of intracellular calcium. There are at least two distinct mechanisms by which this might occur. Bergson and colleagues demonstrated that heterologously expressed D1 and D5 dopamine receptors, when coexpressed with calcyon, stimulate the release of calcium from intracellular stores following priming of the cells with a $G\alpha_q$-coupled receptor agonist *(157)*. Endogenous D1-like receptors in neocortical or hippocampal neurons, but not neostriatal neurons, display a similar priming-dependent ability to mobilize calcium *(158)*. The second mechanism invokes a novel SCH23390-binding D1-like receptor that is linked to phospholipase C via $G\alpha_q$. The regional distribution and pharmacological profile of this novel receptor differ from both D1 and D5 receptors *(159)*. Furthermore, this $G\alpha_q$-coupled receptor does not react with a D1 receptor antibody, is not a product of the *D1DR* gene, and may be encoded by mRNA of a different size from that encoding the D1 receptor *(121,160–162)*.

5.2.2. D2-Like Receptor Signaling

The first signaling pathway identified for D2-like receptors was inhibition of cAMP accumulation *(25,163)*. In the rodent neostriatum, this response is primarily mediated by adenylate cyclase type 5; genetic ablation of this adenylate cyclase abolishes D2 receptor-mediated inhibition of adenylate cyclase and also eliminates the locomotor inhibitory effects of D2 receptor-blocking antipsychotic drugs *(141)*. The lack of responsiveness to antipsychotic drugs is a phenotype also seen in D2 receptor *(164)* and DARPP-32 *(154)* null mutant mice, suggesting that this signaling pathway contributes to D2 receptor-stimulated locomotor activity. The D2 and D4 receptors both inhibit adenylate cyclase activity in a variety of tissues and cell lines *(42,143)*. Inhibition of adenylate cyclase by the D3 receptor is weaker and often undetectable although, interestingly, the D3 receptor robustly inhibits adenylate cyclase type 5 *(165,166)*, in contrast to several other adenylate cyclase subtypes including the closely related type 6. Whereas D2 and D4 receptors markedly increase the activity of the G protein $\beta\gamma$-stimulated type 2 adenylate cyclase, the D3 receptor has little or no effect *(165,167)*.

As is typical of $G\alpha_{i/o}$-coupled receptors, D2-like receptors modulate many signaling pathways in addition to adenylate cyclase, including phospholipases, ion channels, mitogen-activated protein (MAP) kinases, and the Na^+/H^+ exchanger *(143)*. Most if these pathways are regulated by G protein βγ subunits that are liberated by receptor activation of $G\alpha_{i/o}$ proteins. One such pathway is activation of the G protein-regulated inwardly rectifying potassium (GIRK or Kir3) channel, a channel that carries one of several potassium currents modulated by dopamine in midbrain dopamine neurons *(168,169)*. All of the D2-like receptors activate GIRK *(170)*, presumably via Gβγ *(171,172)*. The D3 receptor is approximately as efficient as the $D2_L$ receptor at coupling to homomeric GIRK2 *(173)*, the GIRK subtype predominantly expressed by dopamine neurons in the rat ventral mesencephalon *(174,175)*, and regulation of GIRK channels contributes to inhibition of secretion by the D3 receptor heterologously expressed in AtT-20 mouse pituitary cells *(176)*. D2 and D4 receptors both coprecipitate with GIRK channels in a heterologous expression system, and the rat neostriatal D2 receptor coprecipitates with GIRK2, suggesting the existence of a stable complex that forms during receptor/channel biosynthesis *(138)*. Evidence that dopamine release-regulating autoreceptors are coupled to potassium channels *(177)* rather than to inhibition of adenylate cyclase *(178)*, together with the robust regulation of GIRK currents by D2 receptors in substantia nigra dopamine neurons *(179)*, suggests that D2 receptor activation of GIRK currents contributes to D2 autoreceptor inhibition of dopamine release and dopamine neuronal activity. The hyperactivity and facilitation of D1 receptor signaling observed in GIRK2 null mutant mice *(180)* is also consistent with a loss of inhibitory autoreceptor function.

MAP kinases are components of parallel protein kinase cascades that transmit signals from a variety of extracellular stimuli to the cell nucleus, thus participating in cell proliferation, differentiation, and survival *(181)*. Many GPCRs, including those coupled to $G\alpha_{i/o}$, regulate the activity of MAP kinases *(181,182)*. Activation of the D2 receptor also stimulates MAP kinases, including extracellular signal-regulated kinase (ERK) 1 and 2 *(183–189)* and stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK) *(185)*. D3 *(190)* and D4 *(189,191)* dopamine receptors also activate ERK1/2. D2-like receptors activate ERK1/2 in brain slices *(192,193)* and in the brain after administration of agonist in vivo *(194)*.

Although the pathway from D2-like receptors to activation of ERK1/2 has not been thoroughly described, and may differ depending on cell type, D2 receptor activation of ERK is frequently mediated by Gβγ *(183,187,188)*, phosphatidylinositol 3-kinase *(186,190)*, the small-molecular weight G protein Ras *(185,191)*, and the MAP kinase kinase MEK *(185,187,192,194)*. As for many other GPCRs, D2-like receptor signaling to MAP kinase pathways is in at least some cases mediated by transactivation of a receptor tyrosine kinase (RTK), thus recruiting the RTK signaling cascade in response to dopamine. Whereas the epidermal growth factor receptor has frequently been identified as an RTK that is transactivated by GPCRs *(195,196)*, transactivation of the platelet-derived growth factor receptor can be a necessary intermediate step in the activation of ERK1/2 by recombinant and endogenous D2 and D4 receptors *(189,197)*.

D2 receptor activation of ERK stimulates DNA synthesis and mitogenesis in many different cell types *(185,188,198,199)*. In postmitotic neurons, activation of MAP kinases is involved not only in cell survival and in synaptic plasticity *(200–202)*, but also in acute behavioral responses to dopamine receptor stimulation *(194)*. D2 receptor

signaling to ERK in pituitary lactotrophs may be more complicated; in both primary lactotrophs and a prolactin-secreting cell line, D2 receptors are reported to inhibit ERK1/2, leading to suppression of prolactin promoter function *(131)*. A conflicting report using a different prolactin-secreting cell line describes D2 receptor stimulation of ERK1/2 leading to inhibition of cell proliferation *(203)*.

Considerable data support D2-like receptor modulation of additional signaling pathways. D2 receptors in neostriatal large aspiny interneurons inhibit N-type Ca^{2+} channels by a membrane-delimited pathway that probably involves Gβγ, and that is postulated to mediate D2 receptor inhibition of acetylcholine release *(204)*. Voltage-dependent Ca^{2+} channels are also inhibited by D2 receptors in the anterior pituitary *(123)* and by the D3 receptor heterologously expressed in AtT-20 cells *(205)*; inhibition of Ca^{2+} channels in these cells would be expected to inhibit secretion of pituitary hormones. D2 receptors in neostriatal medium spiny neurons activate a cytosolic, Gβγ-stimulated form of phospholipase C, PLCβ1, causing calcium mobilization that activates calcium-dependent proteins, such as the protein phosphatase calcineurin *(206)*. The D2 receptor potentiates arachadonic acid release induced by calcium-mobilizing receptors in heterologous expression systems *(207,208)*, a response that is mediated by cytosolic phospholipase A_2 *(209)*. The D4 receptor also activates this pathway *(210)*. The D2 receptor stimulation of arachidonate has been reported to be insensitive to pertussis toxin and to be mediated by activation of protein kinase C *(207)*. These characteristics are shared by D2 receptor stimulation of phospholipase D, a response that may be mediated by interaction with a small–molecular weight G protein in the Rho family and activation of protein kinase Cε *(211)*. Heterologously expressed D2 *(212)*, D3 *(213,214)*, and D4 receptors *(210)* activate the Na^+/H^+ exchanger NHE1. Interestingly, this response, too, is insensitive to pertussis toxin in some cell lines *(212)*, as is the inhibition of Na^+/H^+ exchanger activity mediated by endogenous D2 receptors in primary lactotrophs *(215)*.

5.3. Modulation of Receptor Responsiveness

Altered dopamine receptor responsiveness has been implicated in the etiology, treatment, or treatment side effects of a variety of psychiatric, neurological, and endocrine disorders including schizophrenia, drug addiction, Parkinson's disease, Tourette syndrome, tardive dyskinesia, Huntington's chorea, and hyperprolactinemia, stimulating a tremendous amount of research on dopamine receptor regulation *(216)*. Although a comprehensive review of the topic is beyond the scope of this chapter, because of the importance of the phenomenon for the understanding of the role of dopamine in neuropsychiatric disorders, I will endeavor to provide a broad-brush treatment of the major characteristics of the regulation of dopamine receptor function and expression.

Most neurotransmitter receptors compensate for over- and understimulation with a reduction in responsiveness, or desensitization, and enhanced responsiveness, or supersensitivity, respectively *(217,218)*. In general, results from in vivo and in vitro studies of dopamine receptor regulation fit within this scheme. Denervation or chronic antagonism of D2-like dopamine receptors in vivo causes an increase in the density of the receptors, enhanced biochemical responsiveness, and behavioral supersensitivity to dopamine receptor agonists *(219–227)*. In vivo denervation or chronic antagonism also induces behavioral and biochemical supersensitivity of D1-like receptors and, in the case of

chronic antagonist treament, an increase in receptor number *(228–233)*, whereas the effect of denervation on D1 receptor density is more variable, with small decreases in receptor number being observed most frequently *(216)*. The lack of a consistent effect of denervation on D1 receptor density is one example of a broader mismatch between denervation-induced changes in dopamine receptor density and behavioral responsiveness. It is difficult to reconcile the unchanged or decreased density of D1 receptors and a 25–50% increase in the density of D2-like receptors with behavioral responsiveness to D1 or D2 receptor agonists that may be enhanced up to 40-fold after denervation *(234,235)*. One explanation for this discrepancy is that, rather than being a result of altered receptor density *per se*, most of the behavioral supersensitivity that is observed is due to a denervation- or antagonist-induced breakdown in the D1/D2 receptor synergism that, in the intact or untreated animal, requires stimulation of both receptor subtypes to obtain a functional response *(236)*.

Although treatment of intact rats with the dopamine precursor L-DOPA decreases D1 receptor-stimulated adenylate cyclase *(237)*, the effect of treatment with D1 receptor-selective agonists is less well understood. Chronic administration of the partial agonist SKF38393 has no effect on or increases the density of D1-like receptors in the intact animal, but decreases the density of receptors in a dopamine-depleted rat model *(238,239)*. The lack of a desensitization response in intact animals could be owing to the partial agonist nature of SKF38393; that treatment with a full D1 receptor agonist may cause behavioral tolerance *(240)* and internalization of D1 receptors *(241)* supports this hypothesis, although at least one study failed to find decreased receptor number after treatment with a full agonist *(233)*. Studies of the in vivo regulation of D2 receptors by agonists are also not in complete agreement. Two groups have described downregulation of neostriatal D2 receptors following chronic treatment with the D2 agonist quinpirole *(239,242)*, and some *(237,243)*, but not all *(244,245)*, have reported D2 receptor downregulation following repeated treatment with the partial agonist bromocriptine. Overall, the data suggest that agonist administration causes downregulation of D2 receptor expression in vivo *(216)*.

The D3 receptor represents an exception to the general model described above. Receptor expression in the basal forebrain is unaffected by D2-like receptor antagonist treatment, but is decreased by dopaminergic denervation *(246–248)*. Furthermore, chronic treatment with a D1-like agonist restores D3 receptor expression *(247,248)*, via increased striatal release of brain-derived neurotrophic factor (BDNF) *(249)*. BDNF regulates D3 receptor expression in the rat nucleus accumbens both during development and in adulthood *(249)*.

A variety of preparations have been used to demonstrate desensitization of D1 receptors in vitro *(216,250)*. Desensitization of D1-like receptors generally conforms to a model in which phosphorylation of the receptor (see subheading 3.2.3.) leads to rapid functional uncoupling of the receptor followed by β-arrestin-dependent sequestration or internalization and either dephosphorylation and resensitization or, after prolonged agonist treatment, downregulation and degradation of the receptors *(216)*. D1 receptor desensitization is mediated by both PKA and GRK2 *(75,251)*, with Thr268 in the third cytoplasmic loop being a site of phosphorylation by PKA *(76)* and Thr360 in the cytoplasmic tail a site of phosphorylation by GRK2 *(77)* (Fig. 1). The work of Jackson et al. *(252)* suggests that phosphorylation of residues distal to Thr360 in the cytoplasmic tail

also contributes to D1 receptor desensitization. The mechanisms of regulation of the D5 receptor appear to be similar to that of the D1 receptor *(253)*, except that the D5 receptor may normally exist in a partially desensitized condition as a result of the high constitutive activity of the receptor *(92)*.

Whereas downregulation of the D1 receptor is readily observed in cell lines, prolonged agonist treatment of cells expressing the D2 receptor generally does not decrease and often increases the density of receptors *(216,254)*. As for D1-like receptors, however, agonist activation of the D2 receptor leads to rapid phosphorylation of the receptor by GRK2 and/or GRK6, functional uncoupling including diminished inhibition of adenylate cyclase, sequestration of D2 receptors away from the surface of the membrane, and β-arrestin-dependent receptor internalization *(78,79,254–256)*. In contrast, the D3 receptor is only weakly phosphorylated and internalized *(78)*.

Functional desensitization of the D2 receptor, as measured by inhibition of adenylate cyclase, is typically modest and obscured by a more robust response that is a frequently described consequence of stimulation of $G\alpha_{i/o}$-coupled receptors: enhanced responsiveness of adenylate cyclase to activating stimuli, or heterologous sensitization *(257–259)*. Activation of D2 and D4 receptors, but not the D3 receptor, causes heterologous sensitization of adenylate cyclase *(213,258,260,261)*. D2 receptor mediated heterologous sensitization is detectable within minutes of stimulation by physiological concentrations of dopamine and other agonists and persists for some time after removing the agonist. In NS20Y neuroblastoma cells, D2 receptor-stimulated heterologous sensitization is mediated by $G\alpha_o$ *(127)*. As reviewed by Watts *(259)*, the pathway from $G\alpha_o$ to enhanced adenylate cyclase activity appears to involve $G\beta\gamma$ and a $G\alpha_s$-dependent facilitation of adenylate cyclase *(262,263)*. The characteristics of D2-like receptor-mediated heterologous sensitization suggest that it is likely to occur in vivo under conditions of prolonged overstimulation of the receptors, such as during cocaine binging, although whether heterologous sensitization of adenylate cyclase contributes to cocaine-induced behavioral sensitization is unknown.

6. DOPAMINE RECEPTOR PROTEIN–PROTEIN INTERACTIONS

6.1. Receptor Oligomerization

Evidence is accumulating that GPCRs exist as both homo- and hetero-oligomers of two or more individual GPCR monomers *(264,265)*. Several mechanisms of dimerization have been proposed, including domain swapping in which TM1-5, for example, from one GPCR monomer form a bundle with TM6-7 from another monomer, interreceptor disulfide bonds in the amino terminus, and interreceptor helix–helix interactions. There is also considerable disagreement on the function and regulation of receptor oligomers. There are data to support agonist-induced formation, agonist-induced dissociation, and constitutive existence of oligomers. Some of the disagreement is no doubt owing to differences in methods used, since each has weaknesses; for example, coprecipitation studies are subject to artefactual in vitro association of membrane proteins, whereas bioluminescence resonance energy transfer or fluorescence resonance enegy transfer studies may not be able to differentiate between association/dissociation of monomers and changes in receptor conformational states. Furthermore, there may be multiple mechanisms contributing to the formation of dimers and higher order multimers, and to the formation of homo- and hetero-oligomers, and it is likely that in some

cases apparent oligomerization reflects the interactions of two or more receptors with a scaffolding protein.

Considerable data indicate that at least some proportion of D1, D2, and D3 dopamine receptors exist as homo-oligomers *(69,266–273)*. Homodimerization has been proposed to alter the ligand binding characteristics of the D2 receptor *(266,268)*. Although the effect of TM6 and TM7 peptides on the presence of oligomers suggests a role for TM6-7 interhelix interactions in forming or stabilizing the D2 homo-oligomer *(266)*, recent cysteine cross-linking studies implicate the extracellular end of TM4 as the homodimer interface and also suggest that functional D2 receptors exist as constitutive dimers *(273)*. Similarly, that coexpression of nonfunctional mutant D2 receptors blocks the cell surface expression and function of the wildtype D2 receptor indicates that homodimerization is constitutive and necessary for expression of active receptor at the cell surface *(269)*. Hetero-oligomerization also occurs between the D3 receptor and its truncated splice variant D3nf *(267)*, with D3nf preventing trafficking of D3 to the cell membrane *(270)* or inhibiting ligand binding to the D3 receptor *(274)*, and between D2 and D3 receptors *(166)*. In the latter case, coexpression of D2 and D3 receptors in COS-7 cells with adenylate cyclase type 6 substantially increased the potency of 7-OH-DPAT for inhibition of cAMP accumulation, suggesting that the hetero-oligomer has increased potency for agonists and/or couples more efficiently to adenylate cyclase type 6.

Hetero-oligomers have also been described between dopamine receptors and other GPCRs, including the D2 receptor and the somatostatin receptor subtype SSTR5 *(275)*, the D2 and adenosine $A2_A$ receptors *(276)*, and the D1 and adenosine A1 receptors *(277)*, and between D1-like receptors and ion channel-coupled receptors *(278–280)*. The formation of hetero-oligomers is generally regulated by ligand binding, particularly agonists (but *see* ref. *280*), and typically serves to inhibit the function of at least one of the receptors in the complex (but *see* ref. *275*)

6.2. Receptor-Interacting Proteins

Another area of research that is rapidly expanding our view of how GPCRs function involves the identification and characterization of novel receptor-interacting proteins. GPCRs are defined by their interactions with heterotrimeric G proteins, and earlier I alluded to interactions of dopamine receptors with small-molecular weight G proteins, protein kinases, and β-arrestin, but it is now evident that many other GPCR–protein interactions regulate the trafficking and function of GPCRs *(281,282)*.

Interactions between the proximal cytoplasmic tail of the D1 receptor and the endoplasmic reticulum (ER) protein DRiP78 (*D*opamine *R*eceptor *i*nteracting *P*rotein of M_r *78*K) and γ-COP, a COPI golgi/ER-coated vesicle coatomer subunit, regulate transport of the receptor out of the ER *(283,284)*. DRiP78 binds to an FxxxFxxxF motif in the proximal C terminus that is shared by all dopamine receptor subtypes (Fig. 1) and many other GPCRs, whereas binding of γ-COP requires maintaining the hydrophobic face of the helix (helix 8) that is thought to run parallel to the membrane between the cytoplasmic end of TM7 and the palmitoylated cysteine residue (Fig. 1); thus, neither of these interactions is likely to be unique to the D1 receptor. The intermediate filament protein neurofilament-M binds to the third cytoplasmic loop of the D1 receptor. Overexpression of neurofilament-M in D1 receptor-expressing cells also causes the accumulation of D1

receptor in intracellular compartments, although it is not clear whether this is owing to reduced transport of newly synthesized receptor to the membrane or to constitutive internalization of functional membrane receptors. This interaction appears to be selective for D1-like receptors, as neurofilament-M binds weakly to the D5 receptor and not at all to D2, D3, or D4 receptors *(285)*. Association of the D1 receptor with protein phosphatase-1 may be involved in dephosphorylation and resensitization of the D1 receptor *(286)*, whereas binding of calcyon to residues 421–435 in the cytoplasmic tail of the D1 receptor promotes D1 receptor enhancement of $G\alpha_q$-coupled receptor-stimulated calcium mobilization, without altering the ability of the D1 receptor to stimulate cAMP accumulation *(157)* (*see* Subheading 5.2.1.).

D2 and D3 receptors, but not D1 or D4 receptors, bind the actin-binding protein filamin A, or ABP-280. Zhou and colleagues report that binding is to a segment in the carboxyl terminus of the third cytoplasmic loop, where both D2 and D3 receptors have a potential site of phosphorylation by PKC, and that D2 and D3 receptors expressed in cells that lack ABP-280 have diminished ability to inhibit adenylate cyclase *(287,288)*. Furthermore, PKC-catalyzed phosphorylation of the D2 receptor on Ser358 may inhibit binding of ABP-280, thus attenuating D2 receptor signaling *(287)*. In contrast, Lin et al. *(289)* report that ABP-280 binds to a segment toward the amino terminus of the third cytoplasmic loop, and that expression of ABP-280 is necessary for trafficking of D2 and D3 receptors to the cell surface *(289)*. The latter group has also described an interaction of D2 and D3 receptors with protein 4.1N and other members of the 4.1 family of cytoskeletal proteins; virtually the same binding site (amino terminus of the third cytoplasmic loop) and function (trafficking to the cell surface) has been attributed to the binding of 4.1N as to ABP-280/filamin A *(289,290)*. More recently, heart-type fatty acid binding protein (H-FABP) has been identified as a protein that binds to $D2_L$, but not $D2_S$, and thus selectively retains $D2_L$ in intracellular compartments in NG108-15 cells *(291)*.

In addition to the possible effect of ABP-280 binding on signaling to adenylate cyclase, other protein–protein interactions are likely to influence D2-like receptor signaling. The third cytoplasmic loop of the D2 receptor includes a binding site for spinophilin, a scaffolding protein that also binds and targets protein phosphatase-1 to dendritic spines *(292)*. Calmodulin binds in a calcium-dependent manner to the amino terminal end of the D2 receptor third cytoplasmic loop and inhibits D2 receptor activation of, but not binding to, $G\alpha_i$ *(293)*. Another EF-hand calcium-binding protein, neuronal calcium sensor-1 (NCS-1), binds to the proximal cytoplasmic tail of the D2 receptor to a region that overlaps the conserved DRiP78 and γ-COP binding sites identified in the D1 receptor *(294)*, although there is presumably no temporal overlap since the latter proteins bind during biosynthesis and transport, whereas NCS-1 interacts with the receptor at the cell surface. Overexpression of NCS-1 in D2 receptor-expressing cell lines attenuates agonist-induced internalization of the receptor *(294)*. NCS-1 also binds to D3 and D5 receptors, but not D1 or D4. Proteins, such as Nck, Grb2, and c-Src, that contain Src homology 3 (SH3) domains, a modular protein–protein interaction domain that is essential for the formation of functional signaling complexes, bind to the third cytoplasmic loop of the D4 receptor, which has multiple copies of the proline-rich SH3 binding motif *(295)*. Several SH3 domain-containing proteins also bind to the D3 receptor, although the site of binding has not been identified *(295,296)*. The functional role of SH3 protein binding to D2-like receptors is unknown, although mutation of the SH3 binding motifs in the D4

receptor causes constitutive internalization of the receptor *(296)*, and binding of the protein tyrosine kinase c-Src to other GPCRs has important consequences for receptor signaling and desensitization *(297,298)*. As discussed in Subheading 5.2.2., D2 and D4 receptors form stable complexes with GIRK potassium channels *(138)*. The formation of large multiprotein complexes that include GPCRs and their effectors may be a general characteristic of GPCR signaling *(299)*.

7. DOPAMINE RECEPTOR VARIANTS

There are numerous polymorphisms of the dopamine receptor genes that are in introns or otherwise outside the coding region, or that are synonymous single nucleotide polymorphisms *(27,300)*. Although such polymorphisms may affect gene transcription or message stability and translation *(301,302)*, and have been useful in exploring genetic relationships between neuropsychiatric disorders and dopamine receptors *(303,304)*, a review of this area is outside the scope of this chapter. Instead, I will provide a brief overview of structural variants that result from alternative RNA splicing (*see also* Chapter 2) or from nonsynonymous sequence polymorphisms within coding exons.

7.1. Splice Variants

The $D2_L$ and $D2_S$ splice variants of the D2 receptor, generated by alternative splicing of an 87-nucleotide exon that encodes 29 residues in the third cytoplasmic loop of $D2_L$, were the first GPCR splice variants to be identified *(28–31)*. Most tissues express both variants, with $D2_L$ being most abundant. Because of the location of the alternatively spliced insert in the third cytoplasmic loop, where a direct effect on the binding of ligands would not be expected, many comparisons of $D2_L$ and $D2_S$ have focused on identifying the G protein subtypes that are activated by each splice variant. As reviewed in detail elsewhere *(46,122)*, there is considerable evidence that $D2_L$ and $D2_S$ differ in the efficiency with which they bind to and activate different Gα subunits, but little agreement in the literature concerning the specific Gα subunits activated by each variant. Factors that could influence G protein selection to produce disparate results include the signaling pathway being examined, the relative abundance of Gα subtypes in a given tissue, the abundance of particular Gβγ subtypes, the presence of appropriate effectors *(137)*, and the choice of agonist used to activate the receptor *(128)*.

Recent studies of two independently generated lines of mice that express only $D2_S$ have provided intriguing evidence for functional differences between $D2_L$ and $D2_S$. In both lines of $D2_L$ null mutant mice, responses to D2 receptor agonists that are thought to be mediated by dopamine autoreceptors are spared or enhanced compared to wildtype mice. These autoreceptor-mediated responses include inhibition of locomotor activity by low doses of agonists, agonist inhibition of nigral cell firing, inhibition of dopamine release, and inhibition of tyrosine hydroxylase phosphorylation at Ser40 *(305–308)*. These studies show only that D2S *can* function as an autoreceptor, whereas if $D2_S$ null mutant mice are found to lack autoreceptor function that will be compelling support for the idea that $D2_S$ *normally* serves as the autoreceptor, but the latter hypothesis is supported by the observation that, in nonhuman primates, $D2_S$ is the predominant variant in dopaminergic neurons, whereas $D2_L$ is more abundant in neurons innervated by dopamine pathways *(309)*. Interestingly, $D2_L$ null mutant mice show deficits in behaviors mediated by postsynaptic D2 receptors: haloperidol-induced catalepsy and spontaneous

Table 3
DNA Sequence Polymorphisms of the Human Dopamine Receptors

Receptor	Polymorphism	Location	Reference
D2	Val96→Ala	TM2	*319*
	Pro310→Ser	IC3	*319*
	Ser311→Cys	IC3	*355*
D3	Ser9→Gly, creates *Bal*I/*Msc*I RFLP	NT	*322*
D4	Gly11→Arg	NT	*328*
	12-bp repeat in exon 1	NT	*327*
	21-bp deletion in exon 1	TM1	*329*
	13-bp deletion in exon 1	TM2	*328*
	Val194→Gly	TM5	*330*
	48 bp repeat in exon 3	IC3	*323–325*
D5	Leu88→Phe	TM2	*317*
	Ala269→Val	IC3	*316,317*
	Pro330→Gln	EC3	*316,317*
	Cys-335→stop	EC3	*316,317*
	Asn351→Asp	TM7	*316,317*
	Ser453→Cys	CT	*316,317*

or agonist-induced locomotor activity *(305,306)*. Furthermore, D2 receptor inhibition of D1 receptor-stimulated phosphorylation of DARPP-32, a response central to the postsynaptic actions of dopamine, is absent in $D2_L$ null mutant mice *(308)*. Not all nonautoreceptor-mediated responses require $D2_L$, however, because dopamine-dependent inhibition of neostriatal GABA transmission, lost in D_2-null mutant mice, is spared in the mice lacking only $D2_L$ *(310)*, as are certain quinpirole-induced stereotyped behaviors *(311)*.

Several variants of the human D3 receptor result from alternative splicing of exon 2 *(312,313)* or exon 3 *(313,314)*, as well as from cleavage of an atypical 3′ splice site, deleting a portion of exon 6 *(315)*. All are frame-shifted variants with D3 receptor sequence through the first two transmembrane segments, through the the first three transmembrane segments, or through the first five transmembrane segments, respectively; thus, none would be expected to function as a GPCR. Nevertheless, a protein-encoded by the latter variant, $D3_{nf}$, is expressed in brain *(315)*, and $D3_{nf}$ or any other truncated receptor variant could serve to regulate the expression of the full-length receptor *(267)*. No splice variants have been described for the D4 receptor. As they are encoded by genes that lack introns within the coding region, the D1 and D5 receptors also have no splice variants.

7.2. Allelic Variants

No DNA sequence polymorphisms have been identified that alter the coding sequence of the D1 receptor. The D5 receptor, however, has several nonsynonymous single nucleotide polymorphisms (SNPs) that are summarized in Table 3, including a nonsense change that would result in truncation of the protein between TM6 and TM7, the substitution of Asp for the highly conserved residue $Asn351^{7.45}$, and the substitution of Phe for

Leu88$^{2.51}$ *(316,317)*, adjacent to the highly conserved aspartic acid residue in TM2 that participates in a sodium-binding pocket in the D2 receptor *(318)*. Both of the missense changes in the transmembrane regions have modest effect on D5 receptor affinity for ligand *(317)*.

The human D2 receptor has three nonsynonymous SNPs: a substitution of Ala for Val96$^{2.66}$ and two adjacent substitutions in the third cytoplasmic loop *(319)*. Each of these substitutions has modest effects on ligand potency *(320)*. The two cytoplasmic loop substitutions also decrease the ability of the D2 receptor to inhibit cAMP accumulation *(321)*. The human D3 receptor has one nonsynonymous SNP in which a glycine residue replaces Ser9 in the amino terminus *(322)*.

The human D4 receptor has numerous allelic variants as a result of the presence of an imperfect tandem repeat of 48 nucleotides (16 amino acids) in the third cytoplasmic loop of the receptor *(323)*. At least 19 different repeat units (i.e., 19 different nucleotide sequences) encoding 10 different amino acid sequences have been identified. The order and number of copies of the repeat units can vary, so the potential number of alleles is large; 27 unique DNA sequence variants encoding 20 different amino acid sequences have been identified *(324,325)*. The functional significance of the allelic variants is still in question. When expressed in cells, differences among the allelic variants in terms of affinity for ligands, responsiveness to agonists, and coupling to G proteins are small or nonexistent *(139,261,325,326)*. As the SH3 binding region of the D4 receptor overlaps with the 48 bp repeats, so that the variants have differing numbers of SH3 binding motifs, it is possible that the variants will be found to participate in distinct SH3-dependent protein:protein interactions *(42)*. The D4 receptor also has a 12 bp sequence (Ala-Ser-Ala-Gly) in the amino terminus immediately extracellular to TM1 that is repeated perfectly in the most common variant of the receptor, but occurs only once in the rarer allele *(327)*. Two additional sequence polymorphisms consist of 21 and 13 bp deletions in TM1 and TM2, respectively (Table 3). The 21-bp deletion removes residues Ala36$^{1.34}$ to Val42$^{1.40}$ *(328)*. The 13-bp deletion interrupts TM2 at Ala99$^{2.48}$; because this frame-shifted variant has only one complete membrane-spanning domain it is predicted to be a null (nonfunctioning) allele *(329)*. Finally, one D4 variant has a Gly substitution for Val194$^{5.40}$ *(330)*. The Gly194 variant may also be a null allele, as it has reduced affinity for dopamine and a number of D2-like receptor antagonists and may be unable to inhibit cAMP accumulation *(331)*.

8. CONCLUSIONS

The aim of this chapter was to present background information on dopamine receptors that would provide a context within which the reader can evaluate the evidence for a contribution of dopamine to the neuropsychiatric disorders that are reviewed elsewhere in this volume. Because of this limited aim, and the space restrictions inherent in that aim, there are many important research areas, such as elucidating the structural basis of dopamine receptor function, characterizing dopamine receptor knockout mice, and determining the distinct functional roles of the dopamine receptor subtypes, that are described in only a narrow context or omitted entirely. In some areas where disagreement exists in the literature, it has not been feasible to give appropriate consideration to all points of view. In other research areas, work is advancing at such a pace that sections of this chapter are certain to be outdated by the time of publication. In particular, I predict

that a similar chapter written several years from now would have much more specific information about the mechanisms and function of protein–protein interactions involved in dopamine receptor function, from receptor oligomerization to interactions with G proteins to novel interactions with scaffolding and signaling proteins. Finally, and perhaps most relevant for the topic of this book, it seems likely that the insights gained from the confluence of work using increasingly selective drugs and transgenic and null mutant mice, including inducible and targeted mutations, will enhance our understanding of the behavioral roles of dopamine receptor subtypes and of how selectively manipulating the function of specific subtypes can be useful for the treatment of neuropsychiatric disorders

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2
Dopamine Receptor Alternative Splicing

Neil M. Richtand, Laurel M. Pritchard, and Lique M. Coolen

1. INTRODUCTION

The five dopamine receptor subtypes (D1–D5) are members of the superfamily of G protein-coupled receptors (*see also* Chapter 1). Dopamine receptors. have been known since 1978 to be divided between two families differing in biochemical and pharmacological properties *(1)*. Although the G protein and second messenger systems affected by dopamine receptors in vivo have not been clearly established, in vitro D1-family receptors (D1 and D5) couple to Gs stimulatory proteins, activating adenylyl cyclase, whereas D2-family receptors (D2, D3, D4) couple to Gi inhibitory proteins, inhibiting adenylyl cyclase. Dopamine receptors couple effectively to a wide range of signaling cascades in vitro, including calcium channels, phospholipase C, potassium channels, arachidonic acid release, Na^+/H^+ exchangers, Na+-H+-ATPase, and cell growth and differentiation pathways (reviewed in ref. *2*), suggesting that dopamine may mediate a complex array of neural signaling pathways in vivo. Dopamine systems are believed to exert functional effects through these second-messenger signaling pathways via modulation of the activity of more rapidly acting ionotropic glutamatergic, GABAergic, and nicotinic cholinergic neuronal systems *(3)*.

D1-family receptors are encoded by intronless genes, resulting in expression of a single D1 and a single D5 receptor protein in each tissue expressing these receptors. In contrast, D2-family receptors are encoded by intron-containing genes, as illustrated in Figs. 1 and 2, providing the opportunity for the production of different transcripts, and therefore different proteins, via alternative splicing. The genes for the D2-family receptors are transcribed in the cell nucleus into heteronuclear-RNA (hn RNA or pre-mRNA). This primary pre-mRNA transcript contains sequences for both the exons, or expressed protein sequences, and introns, or non-protein-coding intervening sequences. Alternative splicing in the cell nucleus by spliceosome complexes is the process through which the intronic, non-protein-coding mRNA sequences are removed, leading to the formation of mature, functional mRNA. During this splicing process, individual exon sequences can be included or excluded; the initiation site for protein coding may be altered; exons may be spliced together so that a portion of the protein-coding sequence is excised; exons may be spliced together in a manner in which a new open reading frame is generated, leading to formation of a different protein sequence; or, rarely, intron sequences may be retained in the mature functional mRNA *(4,5)*. In this manner, because of the large number

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

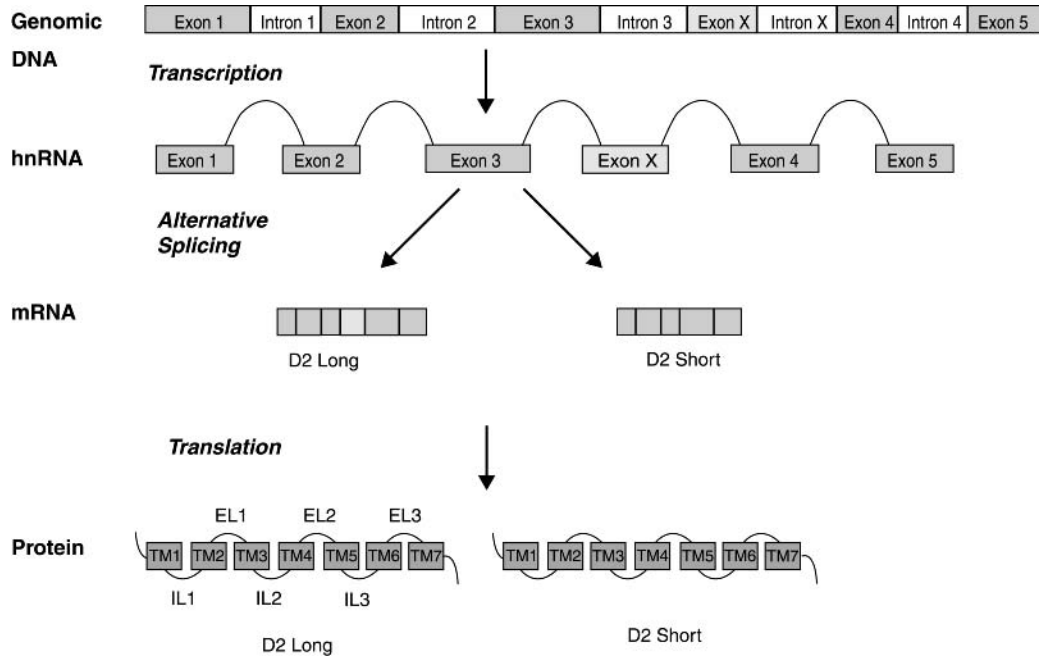

Fig. 1. D2 receptor alternative splicing.

of possible recombinations, alternative splicing provides the potential for significantly increased functional diversity in a given protein.

2. STRUCTURAL DOMAINS INVOLVED IN RECEPTOR FUNCTION

Dopamine receptors mediate ligand-induced modulation of second-messenger signaling through interactions of distinct functional domains. Ligand binding is achieved through interaction of ligand with a cleft formed by seven highly conserved primarily hydrophobic membrane-spanning domains *(6–8)*. The third intracytosolic loop, which extends between transmembrane domains five and six, is more variable, allowing greater functional diversity among receptor subtypes. Interactions between receptor and G protein occur through interaction with this third intracytosolic loop and also the region of the second transmembrane domain *(9–11)*. Dopamine receptors are believed to exist in vivo as higher ordered multimeric structures *(12–18)*. Receptor dimerization is believed to be achieved through specific intermolecular noncovalent interactions requiring at least one full transmembrane domain, in addition to intermolecular disulfide bonds *(13,18,19)*.

Alterations in primary protein sequence structure resulting from alternative splicing may lead to both predictable alterations in receptor function, based on the information described above, and may also lead to alterations in secondary and tertiary protein structure, and receptor function, which would be difficult to predict from the change in primary protein sequence. Functional consequences of receptor alternative splicing would include alterations in ligand-binding properties, signaling pathways, coupling efficiency to G proteins, receptor localization, and temporal expression of receptors. One of the major areas in which receptor function might be expanded through alternative splicing would be receptor desensitization, trafficking, and internalization, which represents an important mode of regulation of G protein-coupled receptor function.

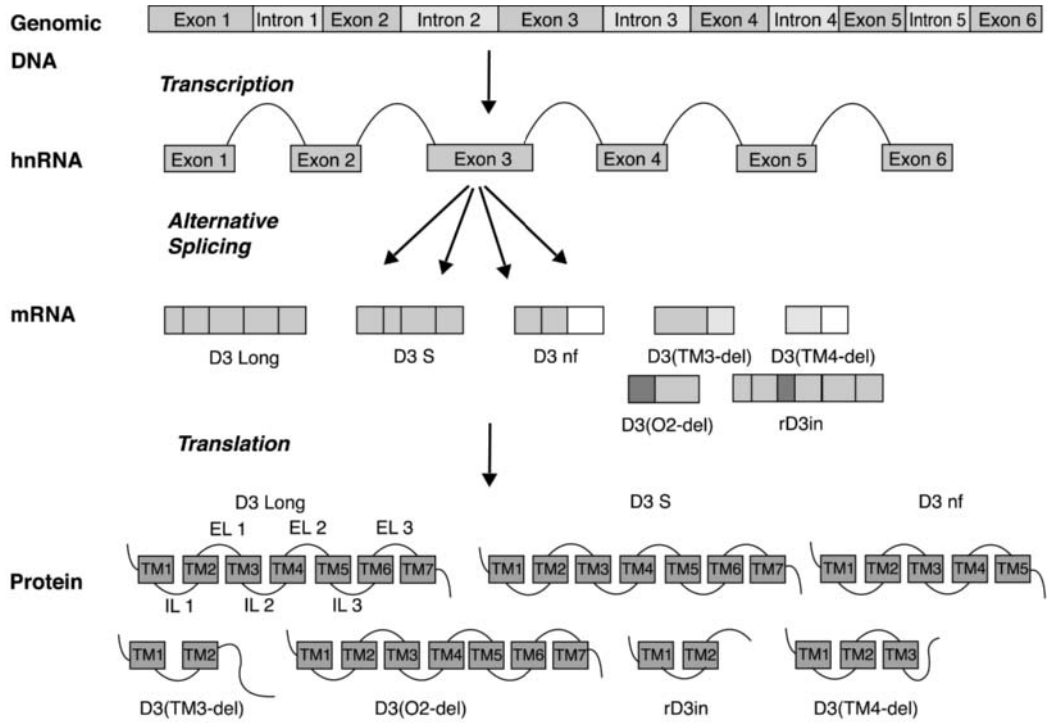

Fig. 2. D3 receptor alternative splicing.

3. REGULATION OF DOPAMINE RECEPTOR FUNCTION

G protein-coupled receptor function is regulated via receptor desensitization, a process involving receptor phosphorylation resulting in alterations in receptor internalization and receptor trafficking. Receptor desensitization, the tendency of a receptor-mediated response to diminish in the face of continued agonist stimulation, may be either homologous (agonist-specific) or heterologous (agonist-nonspecific) desensitization. G protein receptor desensitization has been most thoroughly characterized in β-adrenergic receptors, in which desensitization has been shown to involve receptor phosphorylation by a G protein-coupled receptor kinase (GRK). This results in binding of an arrestin-like protein to the receptor, uncoupling the receptor and G protein and decreasing receptor function. Arrestin binding promotes receptor internalization. Following internalization β-adrenergic receptors may be either proteolyzed in lysosomes, or recycled to the plasma membrane through a process involving dephosphorylation by a G protein-coupled receptor phosphatase *(20,21)*. Whether an analogous machinery exists for any of the dopamine receptor subtypes has not been clearly established. Of the dopamine receptors, D1 receptor desensitization has been most thoroughly characterized. D1 receptor is phosphorylated by both GRK and PKA (protein kinase A), however the relative importance of these kinases in desensitization in vivo has not been established *(22)*. The receptor is internalized into endosomal compartments following agonist activation *(23)*; however, dephosphorylation may precede internalization mediated by a phosphatase distinct from G protein-coupled receptor phosphatase *(24)*. The D2 receptor, in contrast, has been shown in a variety of settings to be resistant to agonist-induced desensitization, and in some cases

agonist treatment results in increased receptor expression *(25,26)*. D3 receptor desensitization has been far less well characterized, in part because of the difficulty in identifying second-messenger systems tightly coupled to D3 receptor stimulation. D3 receptors coupled to G protein-coupled inward rectifier potassium channels in Chinese hamster ovary cells exhibit desensitization following agonist activation *(27)*, however a more detailed understanding of an analogous desensitization process that might occur in vivo awaits further study.

4. D2 DOPAMINE RECEPTOR ALTERNATIVE SPLICING

4.1. Overview

D2 receptor mRNA and protein are expressed in most projection regions of dopamine neurons, consistent with a postsynaptic function, and are also expressed in presynaptic brain regions, suggesting the D2 receptor also functions as an autoreceptor. D2 receptor mRNA and protein are expressed in nucleus accumbens, caudate-putamen, piriform cortex, and olfactory tubercle, with lower expression detected in amygdala, hippocampus, lateral septum, hypothalamus, and other regions of limbic cortex. Abundant D2 receptor mRNA and protein expression is also observed presynaptically in dopamine cell body regions including substantia nigra, zona incerta, and ventral tegmentum *(28–42)*.

4.2. Splice Variants

The first known example of alternative splicing identified for a G protein-coupled receptor was that of D2 receptor alternative splicing *(43–46)*. The splice event results in retention or omission of a 29 amino acid sequence within the third cytoplasmic loop, resulting in formation of D2 receptors referred to as D2 (short) (lacking the 29 amino acid sequence) and D2 (long) (retaining the 29 amino acid sequence) (Fig. 1). More recently, a third alternatively spliced human receptor has also been identified, called D2 (longer), containing an additional two amino acids within the third cytoplasmic loop *(47)*. The location of the alternative splice site within the third cytoplasmic loop, the protein region believed to be integrally involved in coupling with G protein, would suggest a critical role in functional diversity between receptor isoforms, though unambiguously elucidating functional differences between D2 (short) and D2 (long) has proven difficult. The D2 (short) and D2 (long) receptor isoforms are differentially glycosylated, suggesting that differences in posttranslational modification may result in different intracellular trafficking pathways for the receptor isoforms *(48)*. Following dopamine-depleting lesions, however, expression of both D2 (short) and D2 (long) increases significantly and in the same ratio as prior to lesion in the denervated neostriatum, suggesting that splicing is regulated by tissue-specific factors *(49)*. Consistent with these observations, recent studies suggest that the D2 (short) isoform serves primarily in an autoreceptor role, whereas the D2 (long) receptor is expressed primarily at postsynaptic sites *(50)*. The two receptor isoforms also assume different subcellular localizations when transfected into NG 108-15 cells, with D2 (short) receptors localized at the plasma membrane, whereas D2 (long) receptors were localized, in this cell culture system, in the perinuclear region around the Golgi apparatus, associated with heart-type fatty-acid binding protein *(51)*. Also in keeping with a presynaptic role for the D2 (short) isoform, this isoform was found to be relatively more abundant in substantia nigra, as well as in hypothalamus *(52)*. In the same study, manipulation of sex steroid hormone levels by castration or sex steroid hormone

substitution altered the relative ratio of D2 (long)/D2 (short) expression, suggesting that sex hormones, through their hormone receptors, may play a regulatory role in D2 receptor alternative splicing *(52)*.

Of interest, D2 (long) receptors were found to be resistant to agonist-induced desensitization when expressed in culture in Sf9 cells *(25)*. Whether D2 receptor alternative splicing impacts receptor desensitization remains an area for future study.

4.3. Role in Disease

Alternative splicing of the D2 receptor has not been implicated, to date, in the pathophysiology of neuropsychiatric disease. There has been significant interest in reported associations between a *Taq*I-A1 polymorphism, which maps to a noncoding region 3′ to the *D2* receptor gene *(53)*, and several psychiatric conditions, including alcohol dependence *(54)*, pathological gambling *(55)*, obesity *(56)*, and schizoid and avoidant personality traits *(57)*. Several studies *(58,59)*, including a large, family-based sample *(60)*, have failed to confirm the reported association between this polymorphism and alcohol dependence. Further studies will be needed to elucidate the functional importance of D2 dopamine receptor splicing in these and other neuropsychiatric conditions (Table 1).

5. D3 DOPAMINE RECEPTOR ALTERNATIVE SPLICING

5.1. Overview

D3 dopamine receptor mRNA and protein are expressed primarily in olfactory tubercle, nucleus accumbens, islands of Calleja (located ventral to the ventral pallidum and nucleus accumbens), substantia nigra, ventral tegmentum, and prefrontal cortex *(41,42,61–66)*, phylogenetically ancient limbic brain regions linked to motivated and emotional behaviors. The earliest reports describing the highly restricted expression pattern of the D3 receptor suggested a role for this receptor in psychosis *(67,68)*. The cellular pattern of D3 protein expression does not overlap with expression of synaptic proteins such as synaptophysin, suggesting that receptor localization is primarily extrasynaptic *(66)*. Protein and mRNA expression are highly colocalized, suggesting receptor expression occurs primarily on perikarya, proximal dendrites, and short axons as opposed to long axon terminals from other brain regions *(61)*. D3 receptor protein has been described in tyrosine hydroxylase-positive neurons in substantia nigra and ventral tegmentum, indicative of presynaptic D3 receptors *(66)*, although the functional role of these D3 autoreceptors has not yet been elucidated *(69,70)*.

Colocalization studies of D1, D2, and D3 receptors indicate that the majority of D3 expressing neurons in islands of Calleja and nucleus accumbens shell also express D1 receptor mRNA *(71)*. In human brain, most D3-mRNA-expressing cells also express D2 mRNA *(64)*, whereas in rodent brain, in contrast, D2 and D3 receptors appear to have predominantly complementary rather than overlapping patterns of expression *(62)*.

D3 receptor function is of particular interest because evidence suggests its effects are primarily inhibitory *(72–77)*, and that loss of this inhibitory function might contribute pathologically to neuropsychiatric disease *(77–79)*.

5.2. D3 Receptor Splice Variants

The single gene coding for the D3 receptor is organized to allow for the production of different transcripts via two distinct types of alternative splicing of D3 receptor

Table 1
Functional and Physiological Correlates of Dopamine Receptor Alternative Splicing

Receptor	Isoform	Splice site location	Functional effect	Physiological effect	Reference
D2	D2S	Third cytoplasmic loop	Altered glycosylation and membrane trafficking	Autoreceptor	*43–46,50*
	D2L	Third cytoplasmic loop	Altered glycosylation and membrane trafficking	Postsynaptic signaling; resistant to agonist-induced desensitization	*25,50*
D3	D3 (short)	Third cytoplasmic loop	High-affinity dopamine binding intact	Unknown	*84*
	D3 (TM3-del)	Third transmembrane domain	No dopamine binding	Unknown	*82,86*
	D3 (TM4-del)	Fourth transmembrane domain	No dopamine binding	Unknown	*88*
	D3 (O2-del)	Second extracellular loop and fifth transmembrane domain	No dopamine binding	Unknown	*82*
	rD3in	First extracellular loop	No dopamine binding	Unknown	*89*
	D3nf	Third cytoplasmic loop	No dopamine binding; altered membrane trafficking of D3	Potential involvement in development of behavioral sensitization and psychosis	*85,90*

heteronuclear RNA (hn RNA or pre-mRNA) in the cell nucleus *(80–83)*. At least seven distinct alternative splicing variants of the D3 receptor are produced through these splicing events.

Similar to D2 splicing, a splicing event with classical donor and acceptor splice sites within the third cytoplasmic loop results in formation of either the full-length D3 receptor (called "D3"), or a shorter receptor isoform, D3S, lacking 21 amino acids *(84)* (Fig. 2). Both D3 and D3S exhibit high-affinity dopamine binding.

A distinct set of splicing events lead to formation of deletion receptor variants. These splicing events involve cleavage at an unusual, nonconsensus sequence 3′ acceptor splice site *(85)*. Five additional alternatively spliced variants have been described that do not bind dopamine, and are believed to function instead through regulation of receptor dimerization *(86)* and receptor localization *(87)*. These include D3 (TM3-del) *(82,86)*, D3 (TM4-del) *(88)*, D3 (O2-del) *(82)*, rD3in *(89)*, and D3nf *(85,90)*.

5.2.1. D3nf

D3nf is the best characterized of the non-dopamine-binding splice variants. D3nf is formed through a deletion of 98 base pairs in the third cytoplasmic loop, causing a coding frame shift resulting in creation of a novel 55 amino acid peptide and appearance of a new premature stop codon. The prematurely truncated protein thus lacks transmembrane domains 6 and 7 *(85,90)*, and does not bind dopamine *(91)*. D3nf mRNA and protein are expressed in rat, monkey, and human brain *(15,85)*. Importantly, the highly conserved nature of D3nf splicing and protein expression, from rat to human, suggests D3nf likely plays an important, although currently unknown, biological function. Evidence from at least three labs demonstrates that D3nf binds to the full-length D3 receptor subunit *(15,87,91)*. D3nf expression inhibits dopamine binding to full-length D3 receptor *(91)*, and also redirects full-length D3 receptor localization away from the plasma membrane, and instead into an intracellular compartment *(87)*. Importantly, D3 mRNA expression is decreased in cortex of schizophrenia patients *(90)*, whereas increased D3nf splicing efficiency was observed in cortex of postmortem tissue from schizophrenia patients *(92)*. These findings suggest that increased D3nf expression may contribute to functional states of altered dopaminergic activity. Collectively, these studies suggest that, in a manner analogous to dimerization playing an important role in modulation of cell signaling for the homologous insulin and gonadotropin-releasing hormone receptors *(93,94)*, dimerization of D3nf with full-length D3 receptor (or perhaps D1 or D2 receptor) could regulate dopamine signaling. A major gap in our current understanding of D3 dopamine receptor function lies in a lack of information regarding the occurrence of alterations in D3 receptor isoform expression in altered functional states.

5.2.1.1. Proposed Model of D3nf Role in Behavioral Sensitization and Psychiatric Disease

The information described above suggests a model of a mechanism underlying behavioral sensitization, an altered functional state characterized by the progressive and enduring enhancement of certain stimulant-induced behaviors that develops following repetitive stimulant drug administration (*see* ref. *95* and Chapters 15 and 16). Behavioral sensitization serves as a well-studied model of behavioral plasticity with some features in common with the development of psychosis in man *(95–98)*. Following repeated, intermittent treatment with stimulant drugs such as amphetamine (AMPH) or cocaine, behavioral responses may occur more intensely, with shorter latency, and at lower stimulant doses *(99)*. Sensitization is an enduring behavioral change, persisting for at least 1 yr in rats *(100)*. In humans, sensitized behaviors following extended repetitive stimulant drug administration may include psychotic symptoms of paranoia, ideas of reference, and auditory and visual hallucinations in otherwise healthy individuals *(101,102)*. Because pretreatment with amphetamine also facilitates the later acquisition of drug self-administration *(103)*, it has been theorized that sensitization may also underlie the development of drug craving, and thus initiate addictive behaviors seen in drug dependence *(104)*. Additionally, it has been postulated that aspects of behavioral sensitization may play a role in the development of recurrent affective disorders *(105)* and posttraumatic stress disorder *(106)*.

Behavioral sensitization could be accounted for by the increase in D3nf expression that one would predict accompanies repetitive stimulant drug administration, as illustrated

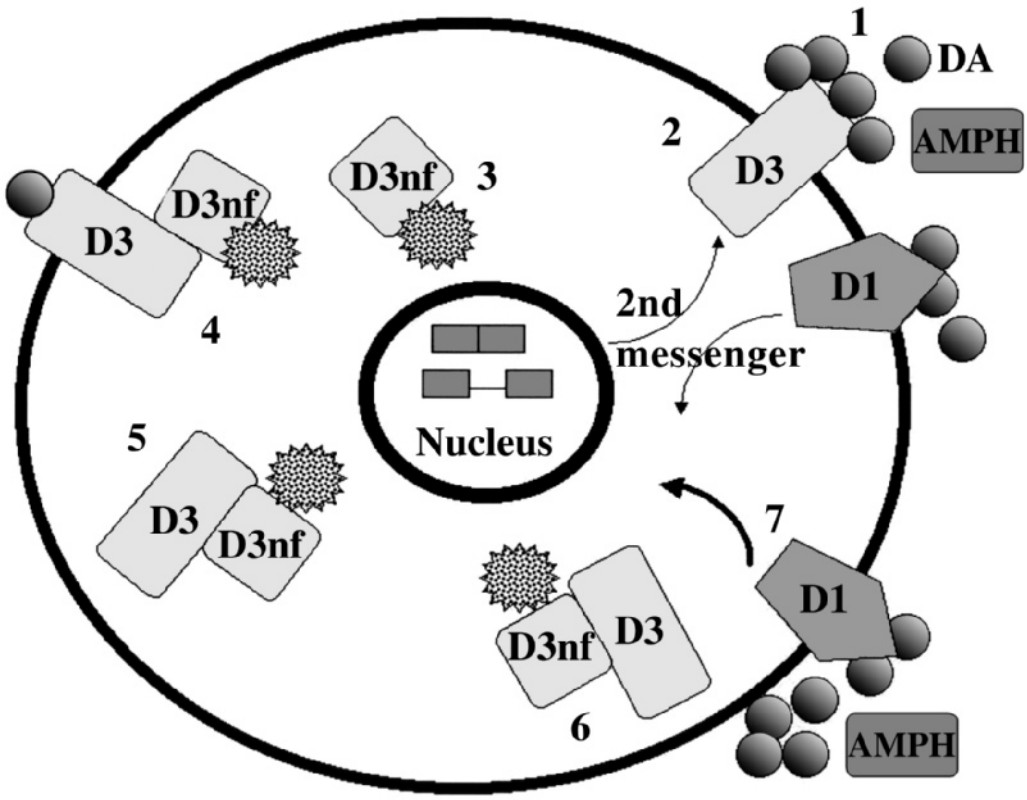

Fig. 3. D3/D3nf mechanism of behavioral sensitization to amphetamine. **(1)** Amphetamine increases extracellular dopamine, activating second-messenger systems downstream of dopamine receptor stimulation. **(2)** D3 receptor has highest dopamine affinity. D3 stimulation results in homeostatic mechanisms opposing receptor stimulation, including **(3)** increased D3nf expression. **(4)** D3nf and D3 dimerize, directing the D3/D3nf dimer **(5)** toward intracytoplasmic trafficking pools and removing D3 receptor from the synaptic membrane. **(6)** At the next amphetamine exposure D3 receptor is not available to bind dopamine. **(7)** The result is release of D3 receptor–mediated opposition to D1 receptor stimulation of adenylate cyclase activity. (Details are described in text.)

in Fig. 3. **(1)** AMPH increases extracellular dopamine, activating second-messenger systems downstream of dopamine receptor stimulation. D3 and D1 receptor stimulation interact at the second-messenger level *(71,107)*, for example through opposing effects on adenylate cyclase activity. Because D3 and D1 receptors are frequently coexpressed *(71)* this interaction is depicted in the diagram as occurring within the same cell; however, the interaction could also occur at the systems level *(108–110)*. **(2)** The D3 receptor has highest dopamine affinity, and excessive D3 stimulation results in homeostatic mechanisms opposing receptor stimulation. This homeostatic response would include **(3)** increased D3nf expression resulting from a change in D3 hnRNA alternative splicing in the cell nucleus. D3nf is a receptor splice variant whose function is thought to oppose D3 receptor stimulation. D3nf shares an NH3-terminus "dimerization domain" with the D3 receptor, resulting in **(4)** dimerization between D3nf and D3. D3nf also contains a unique protein sequence at the COOH-tail, which

may direct the D3/D3nf dimer **(5)** toward intracytoplasmic trafficking pools, thereby removing the D3 receptor from a functional position at the synaptic membrane. In this manner at the next AMPH exposure **(6)** the D3 receptor is localized in an intracytoplasmic pool, and is not available to bind dopamine at the synapse. The resulting loss of D3 receptor "brake," at both the cellular and systems level, includes for example **(7)** the loss of D3 receptor-mediated opposition to D1 receptor stimulation of adenylate cyclase activity.

5.3. Role in Disease

The model described above suggests D3 receptor alternative splicing may play a critical role in sensitization phenomenon. As sensitization may underlie the development of drug craving, and thus initiate addictive behaviors of drug dependence *(104)*, D3 receptor alternative splicing could play an important role in drug dependence, consistent with a large body of data implicating the D3 receptor in various aspects of drug-dependent behavior *(111–115)*. Through a similar mechanism, sensitization and D3 receptor alternative splicing could also play a role in psychosis *(79)*. An amino acid substitution polymorphism in the amino terminus of the D3 receptor has been reported to modulate vulnerability to schizophrenia (*see* refs. *116,117*; reviewed in ref. *118*), suggesting an interaction between D3 receptor function and other genetic and environmental factors in mediating development of a chronic psychotic illness. The D3 receptor has also been implicated in vulnerability to other neuropsychiatric disorders, including tardive dyskinesia (reviewed in ref. *119*).

6. D4 DOPAMINE RECEPTOR ALTERNATIVE SPLICING

6.1. Overview

The pattern of D4 receptor mRNA and protein expression is distinct from that of D2 and D3 receptors, with D4 receptor expressed in highest levels in limbic regions including prefrontal cortex, hippocampus, nucleus accumbens, and amygdala, and relatively lower expression in striatum *(42,120–124)*. Interest in the clinical relevance of D4 receptor function has been heightened by the observation that clozapine, an atypical antipsychotic medication with unique antipsychotic efficacy, exhibits higher affinity binding to D4 than to D2 and D3 receptors *(125)*. Additionally, elevated D4 receptor binding has been reported in the brains of schizophrenia patients *(126,127)*. Although selective D4 receptor antagonists have not demonstrated antipsychotic efficacy *(128)*, interest in the clinical relevance of D4 receptor function remains high.

6.2. D4 Receptor Splice Variants

Alternatively spliced variants analogous to those reported for D2 and D3 receptors have not been reported for the D4 receptor. Although alternative splicing is not known to contribute to variability in receptor structure, D4 sequence variability is conferred by genetic variability of D4 receptor isoforms. Specific D4 receptor isoforms have been linked, in some studies, to neuropsychiatric disease. Within the human D4 receptor third cytoplasmic loop, a 48-base pair sequence is variably repeated between 2- to 8- or 10-fold *(129,130)*. Each human therefore has two copies of the D4 gene, each gene containing 2–8 or 10 repeat units. (The sequences of the rat D4 receptor differs significantly from the human homologue in this third intracytosolic loop domain, and the rat

receptor does not have a corresponding variable repeat region.) Although it would be expected that variability within the third cytoplasmic loop protein domain thought to be integrally involved in G protein coupling would exert an important functional effect, elucidating the functional role of D4 receptor polymorphisms has been challenging. Receptor isoforms differ in sensitivity to the effect of sodium chloride on ligand binding affinity *(129)*, however overall the presence or absence of repeat sequences appears to have only minor effects on ligand binding, G protein interactions, and second-messenger signaling *(131,132)*. Further studies will be needed to clearly elucidate a functional mechanism through which D4 receptor polymorphisms play a role in neuropsychiatric disorders.

6.3. Role in Disease

The role of D4 receptor third cytoplasmic loop polymorphisms in neuropsychiatric conditions has been an area of both interest and controversy. The 7-repeat allele has been associated in some studies with the personality trait of novelty seeking *(133,134)*; however this finding has not been confirmed in other samples *(135,136)*. There has been similar interest and controversy surrounding the potential role of this polymorphism in the risk for Tourette syndrome (*see* e.g., *137,138*). To date, stronger evidence appears to support a contribution of the polymorphism within this region as one of several factors contributing to the heritable vulnerability for attention deficit hyperactivity disorder (reviewed in ref. *139*). Again, further studies will be needed to clarify the potential contribution of variability within this region of the D4 dopamine receptor in these neuropsychiatric conditions.

7. CONCLUSIONS AND FUTURE DIRECTIONS

The greatly expanded functional diversity provided by alternative splicing suggests the likelihood that dopamine receptor alternative splicing could play an important role in both the pathophysiology, as well as treatment response, of a range of neuropsychiatric conditions, including psychotic disorders, substance dependence, Parkinson's disease, Tourette syndrome, and attention deficit hyperactivity disorder. Much remains to be learned, however, regarding the functional neuroanatomy of receptor isoform expression, particularly as it relates to receptor subcellular localization and important mechanisms of receptor regulation such as receptor desensitization, trafficking, and internalization. Further studies are needed evaluating the role of receptor isoforms in neuropsychiatric disease; elucidating the effect of receptor isoform overexpression in mouse models; elaborating the effect of receptor isoform coexpression on subcellular localization and second-messenger signaling in cell culture systems. These studies will identify the cellular function of alternatively spliced isoforms, and may thereby suggest specific, previously untested interventions for neuropsychiatric conditions in which dopamine is known to play an important role, including psychosis and drug abuse.

ACKNOWLEDGMENTS

This work was supported by the Department of Veterans Affairs Medical Research Service (NMR) and the Scottish Rite Schizophrenia Fellowship Award (LMP).

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II GLUTAMATE

3

Glutamatergic Pathways

Their Relevance for Psychiatric Diseases

Yoland Smith

1. INTRODUCTION

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS). Its effects are mediated through a large variety of ionotropic and metabotropic receptors abundantly expressed along the whole extent of the neuraxis. Abnormal regulation of glutamatergic transmission is, therefore, a key factor that underlies the appearance and progression of many neurodegenerative and psychiatric diseases. Unfortunately, the success of therapeutic strategies aimed at modulating glutamatergic transmission has been variable owing to the widespread distribution of glutamate receptors throughout the brain and the importance of glutamate in normal brain functioning. Although the importance of glutamatergic transmission in the modulation of neuronal activity involved in processing limbic and cognitive information has long been established, the complexity of the neuronal pathways involved combined with the multifarious effects glutamate could mediate via pre- and postsynaptic interactions with various receptor subtypes, have led to important controversies regarding the exact role glutamate plays in psychiatric diseases. However, substantial progress has been made over the past 10 yr in dissecting out the anatomy, physiology, and pharmacology of various neuronal pathways whereby glutamate could functionally modulate integrative processing of complex cognitive information. This chapter briefly summarizes some of these observations and considers their implications in our understanding of the anatomo-patho-physiology of psychiatric diseases, particularly schizophrenia, for which various hypotheses based on abnormal glutamatergic/dopaminergic transmission have been put forward to explain the neurochemical dysfunction of this disease *(1–15)*.

This review does not intend to cover the whole literature on the potential implications of glutamatergic pathways in psychiatric diseases, but will rather focus on recent developments regarding the anatomy and the potential mechanisms whereby glutamatergic pathways may interact to modulate neuronal integration in cortical and subcortical brain regions known to be affected in psychiatric diseases (Fig. 1).

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

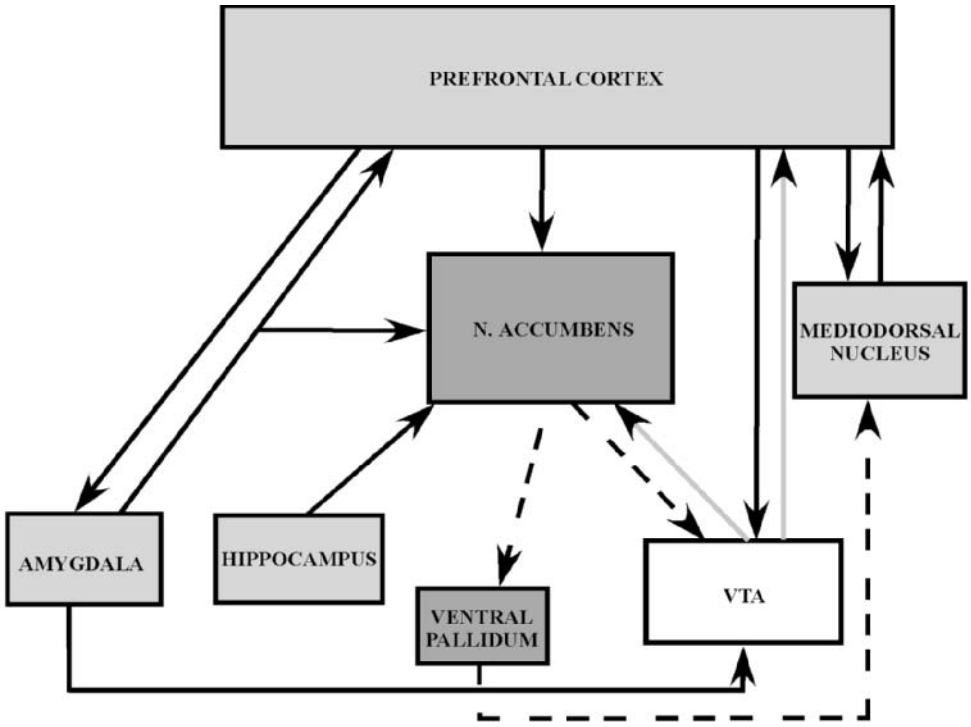

Fig. 1. Summary diagram and the main subcortical glutamatergic circuitry (black arrows) involved in the processing of limbic and cognitive information related to psychiatric diseases. These pathways play important roles in regulating dopaminergic outflow from the ventral tegmental area (light gray arrows) and γ-aminobutyric acid output from the nucleus accumbens (dashed arrows). Note that many connections have been purposefully omitted from this diagram.

2. DOPAMINERGIC/GLUTAMATERGIC HYPOTHESES OF PSYCHIATRIC DISORDERS

It has long been thought that schizophrenia and other psychiatric disorders were mediated by direct alterations of dopamine neuronal activity. This long-term belief was based on two main observations:

1. Drugs that increase dopamine levels in the brain create conditions that resemble those of schizophrenic psychosis in normals and exacerbate the psychosis problems in schizophrenic patients.
2. Drugs currently used to treat schizophrenics block dopamine receptors.

Although abnormal dopaminergic transmission remains a key component of the changes in neural activity that underlie psychiatric disorders *(14)*, it appears that the main abnormality of dopamine transmission in schizophrenics is largely mediated by changes in extrinsic regulatory influences of dopamine release either at the level of the ventral tegmental area or in the prefrontal cortex and nucleus accumbens *(1,3,5,12,14)*.

There are now various sets of data suggesting that forebrain dopamine systems may not be the primary site of neuropathology that is schizophrenia. Numerous studies have found structural and metabolic abnormalities in anterior temporal lobe and prefrontal

cortices *(8,14,16)*. Therefore, it seems that dopamine transmission is not affected in schizophrenia owing to a major defect in midbrain dopamine cell functions but rather results from an abnormal modulation by glutamatergic influences from limbic and prefrontal cortical regions (*1,3,5,12* and Chapter 7). In addition to the cerebral cortex, other forebrain structures and pathways that use glutamate as a neurotransmitter have been considered potential targets of schizophrenia and other psychiatric diseases. These include the amygdala, hippocampal formation, and mediodorsal thalamic nucleus *(6,14)*. Furthermore, the functional interactions between cortical and subcortical glutamatergic pathways at the level of the nucleus accumbens have received considerable attention over the past decades in regard to their potential involvement in neuropsychiatric diseases *(5)*. In this chapter, I will give an overview of the main features that characterize the anatomical and functional organization of these glutamatergic pathways (Fig. 1) and discuss recent findings that suggest their involvement in cognitive, emotional, and limbic-related behaviors.

3. CHANGES IN THALAMOCORTICAL AND INTRINSIC CORTICO-CORTICAL GLUTAMATERGIC CONNECTIONS IN PSYCHIATRIC DISEASES

The prefrontal cortex plays a major role in cognitive, limbic, and memory functions. Abnormalities in information processing or neurological damage to the prefrontal cortex may lead to a myriad of symptoms ranging from changes in personality traits to working memory deficits, and psychiatric diseases *(6,14)*. The anatomical organization of the prefrontal cortex in primates is very complex and comprises a multitude of functional areas characterized by differential patterns of connectivity and electrophysiological properties *(17–19)*. The activity of the prefrontal cortex is under the control of various afferent inputs that use glutamate as neurotransmitter. One of the main sources of thalamic afferents to the prefrontal cortex is the mediodorsal nucleus (MD), although projections from high-order, intralaminar and midline thalamic nuclei have also been reported *(20,21)*. The MD comprises various subdivisions and it appears that each of these subnuclei contribute to the innervation of different prefrontal cortical regions. For instance, the ventral part of the magnocellular MD (MDmc) projects to lateral regions of the ventral and medial prefrontal cortex including Walker's areas 11 and 12, whereas the dorsal part of MDmc is mainly connected with ventromedial regions of the prefrontal cortex (areas 13 and 14). In contrast, the lateral parvicellular MD (MDpc) innervates preferentially dorsolateral and dorsomedial prefrontal areas (Walker's 46, 9, and 8B); the multiform MD (MDmf) is mainly connected with area 8A, whereas area 10 has connections with the anterior part of MD. Thalamic inputs from MD are invariably confined to layer IV and adjacent deep layer III *(22)*. Interestingly, both the number of neurons and volume of MD are reduced in the brains of schizophrenic patients *(23,24)*. In line with these observations, other studies have reported fewer putative thalamic axon terminals and fewer dendritic spines on cortical pyramidal neurons in the prefrontal cortex of schizophrenics *(25,26)*. Although the exact functional implication of decreased thalamic influences on the prefrontal cortex in schizophrenia remains to be established, it has been suggested that they may lead to abnormalities in the inhibitory γ-aminobutyric acid (GABA)ergic microcircuitry of the primate prefrontal cortex *(27,28)*. However, acute lesion of the MD does not result in any significant changes in the expression of glutamic acid decarboxylase

67 (GAD67) mRNA in the prefrontal cortex of rats suggesting that the cortical abnormalities in GABAergic transmission observed in schizophrenia may be mediated by more complex changes in cortical microcircuitry than a mere decreased activity of thalamocortical glutamatergic inputs *(29)*.

The prefrontal cortex is also endowed with extensive glutamatergic corticocortical connections that may be affected in schizophrenia *(6)*. Although some of these connections involve posterior and temporal association areas, profuse horizontal axonal projections from layers II and III of dorsolateral prefrontal areas 9 and 46 to neighboring cortical areas have been described. These local projections are organized in a cluster-like manner that forms a series of elongated stripes within the same areas of the dorsolateral prefrontal cortex. Furthermore, these connections are reciprocal, suggesting that they form distinct interconnected functional modules that could play an important role in the integration and processing of prefrontal cortical information relating to working memory, one of the most fundamental cognitive process affected in schizophrenia. Although there is no direct evidence that these connections are specifically affected in psychiatric diseases, the fact that the size of layer III neuronal perikarya is reduced, combined with the evidence for a decrease in the density of dendritic spines on layer III pyramidal neurons in the prefrontal cortex of schizophrenic patients, are strong evidence in favor of abnormalities in the intrinsic glutamatergic microcircuitry in schizophrenia *(6)*.

4. GLUTAMATERGIC INPUTS TO MIDBRAIN DOPAMINERGIC NEURONS: KEY FACTORS IN CHANGES OF DOPAMINERGIC TRANSMISSION IN PSYCHIATRIC DISORDERS

Extrinsic glutamatergic inputs play a critical role in controlling the firing rate and firing pattern of midbrain dopaminergic neurons in the ventral tegmental area (VTA) *(3)*. Local application of glutamate or stimulation of glutamatergic afferents from the prefrontal cortex or the subthalamic nucleus results in an increased burst firing in midbrain dopaminergic neurons, thereby increased phasic dopamine release in the nucleus accumbens *(3,30,31)*. Midbrain dopaminergic neurons, in particular those in the VTA, receive massive glutamatergic inputs in primates *(32)*. Almost 70% of the total synaptic innervation of VTA dopaminergic neurons arises from glutamatergic boutons in monkeys *(32)*. The prefrontal cortex, subthalamic nucleus, and the brainstem pedunculopontine tegmental nucleus are likely to be the main sources of this innervation *(3,33–35)*.

The VTA is made up of largely segregated populations of dopaminergic and non-dopaminergic projection neurons that project to various cortical and subcortical brain structures, including the nucleus accumbens and the prefrontal cortex. Interestingly, glutamatergic inputs from the prefrontal cortex display a high degree of synaptic specificity in the rat VTA, targeting selectively GABA-containing mesoaccumbens neurons and dopamine-containing mesocortical cells *(36)*. These anatomical data provide a basic substrate for highly specific mechanisms through which prefrontal inputs may control the activity of ascending dopaminergic and GABAergic outflow from the VTA. It is noteworthy that the prefrontal cortex may also control the burst firing of midbrain dopaminergic neurons via its projections to the nucleus accumbens, which, in turn, sends GABAergic inputs to the VTA either directly or indirectly through disinhibition of the ventral pallidum *(3,37)*.

Other sources of glutamatergic projections that mediate changes in firing pattern of VTA neurons include the ventral hippocampus, entorhinal cortex, and amygdala, most likely via polysynaptic pathways that involve projections to the ventral striatum. However, it is important to note that the extended amygdala *(38)*, including the central nucleus and the bed nucleus of stria terminalis provides direct topographic inputs to midbrain dopaminergic neurons *(39,40)*. These represent additional routes through which glutamate could exert direct control on midbrain dopaminergic neuron activity.

5. THE NUCLEUS ACCUMBENS: A CRITICAL SITE FOR PREFRONTAL CORTICAL GLUTAMATERGIC MODULATION OF TONIC DOPAMINE RELEASE

Another way through which prefrontal glutamatergic outputs regulate subcortical dopaminergic transmission is via projections to the striatum (*see* Section 7). Corticostriatal glutamatergic afferents utilize multiple pathways to regulate striatal dopamine release and levels of extracellular dopamine *(3)*. In vitro and in vivo studies have proposed various pre- and postsynaptic mechanisms that involve both ionotropic and metabotroic glutamate receptors, as well as indirect multisynaptic pathways that could mediate these effects *(3)*. Grace and his colleagues have proposed that the glutamatergic modulation of intrastriatal dopamine release is mainly responsible for the maintenance of tonic dopamine levels in the striatum, whereas glutamatergic inputs to midbrain dopaminergic neurons regulate phasic dopamine release *(3)*. Although the prefrontal cortex is a key component for the control of intrastriatal dopamine levels, other glutamatergic inputs from the amygdala and hippocampus also appear to be involved through complex interactions functional interactions at the level of the nucleus accumbens (*see* Section 7).

6. STRESS-INDUCED DISRUPTION OF GLUTAMATERGIC TRANSMISSION FROM THE PREFRONTAL CORTEX AND ITS IMPACT FOR PSYCHIATRIC DISORDERS

Because of its functional importance in regulating dopaminergic transmission at cortical and subcortical levels, abnormal activity of prefrontal glutamatergic influences on the nucleus accumbens and the VTA may play a critical role in various psychiatric diseases *(12)*. The role of stress in the induction, maintenance, and relapse of psychiatric dysfunctions is well established and there is good evidence that changes in glutamatergic transmission in the prefrontal cortex and, possibly the hippocampus, may be responsible for the dopamine-mediated behavioral abnormalities seen in psychiatric diseases *(12)*. Stress induces two temporally different glutamate-mediated events in the prefrontal cortex. The first is an initial acute response characterized by an increase of fast glutamatergic synaptic transmission. This first event, which underlies immediate responses to stress, is likely to be induced by increased transmission of thalamocortical sensory inputs to prefrontal and limbic cortical areas *(12)*. This acute response is followed by long-lasting increases of glutamate and monoamine releases in prefrontal, limbic, and hippocampal cortices. Long-lasting changes in gene expression and protein synthesis also characterize this second event.

The prefrontal cortex also plays an important role in regulating the hypothalamo–pituitary axis (HPA) and glucocorticoid secretion during stress. It appears that the rather slow increased glutamate release in the hippocampus following stress might be mediated through HPA-regulated mechanisms, whereas the fast changes in glutamatergic transmission that

occur in the prefrontal cortex might be independent of the HPA axis and, rather, involve increased synaptic release of glutamate from intracortical or extrinsic afferents *(12)*.

7. FUNCTIONAL INTERACTIONS BETWEEN GLUTAMATERGIC INPUTS FROM THE AMYGDALA, HIPPOCAMPUS, AND PREFRONTAL CORTEX TO THE NUCLEUS ACCUMBENS

The nucleus accumbens is thought to be a key structure in the neuronal circuitry that underlies the neurobiological bases of psychiatric disorders, most particularly schizophrenia. The convergence of glutamatergic inputs from the amygdala, the prefrontal cortex, and the hippocampus, three brain regions that are affected in schizophrenic patients, combined with the dopaminergic inputs from the VTA, set the stage for multifarious and complex functional interactions that underlie the processing and integration of cognitive and limbic-related information flowing through this brain region. The anatomy and electrophysiology of these projections have been studied in great detail, which led to various hypotheses regarding the mechanisms by which these glutamatergic and dopaminergic projections interact to mediate their functional effects on behavior *(3,5)*. This section briefly summarizes some of the main anatomical features that characterize the organization and synaptic connectivity of these pathways, and discusses recent electrophysiological observations that support an important role for amygdala and hippocampal inputs to gate information flow from the prefrontal cortex to the nucleus accumbens *(5)*.

7.1. The Corticostriatal Projection

Various areas of the prefrontal and cingulate cortices provide substantial inputs to the monkey nucleus accumbens *(43–45)*. Price and his colleagues *(46)* defined the organization of prefronto-cortical projections to the striatum according to two major prefrontal networks involved in the integration and processing of functionally different information. These two networks are characterized by different corticocortical connections and distinct connections with subcortical brain regions including the thalamus, hypothalamus, and amygdala. The *"orbital network"* is thought to be involved predominantly in the processing of sensory information relating to food and feeding, whereas the *"medial network"* is more closely related to visceromotor or emotional motor functions *(45,46)*. The two pathways are tightly connected with various cortical and subcortical limbic structures including the amygdala, entorhinal cortex, and hippocampus, through which they may play important roles in controlling mood and guiding behaviors *(46)*. The two networks are differentially connected with the dorsal and ventral striatum. The ventromedial striatum, which includes the ventral putamen, medial caudate nucleus, and nucleus accumbens, receives its main input from the medial cortical network. Projections from caudomedial areas 32, 25, and 14r innervate mainly the medial edge of the caudate nucleus, the nucleus accumbens, and the ventromedial putamen, whereas projections from cortical areas 10o, 10m, and 11m remain restricted to the medial edge of the caudate nucleus *(46)*. Projections from areas 12o, 13a, and Iai terminate in the lateral accumbens and ventral putamen. On the other hand, projections from the "orbital network" are mainly directed toward the central part of the rostral striatum, which includes the central and lateral parts of the caudate nucleus and the ventromedial putamen *(46)*.

In addition to the prefrontal cortex, the nucleus accumbens also receives cortical inputs from limbic- and associative-related areas of the temporal lobe including the

entorhinal and perirhinal cortices, as well as the rostral portion of the superior temporal gyrus *(41,47–49)*. The cingulate cortex (areas 25, 24a–c, 24 a′–c′) is another major source of topographic cortical inputs to the monkey ventral striatum. The medial ventral striatum is mainly innervated by parts of the anterior cingulate cortex (areas 25, 24a,b) whereas the shell region of the accumbens receives fibers from areas 25, 24a,b and 24 a′,b′. Projections to the core of the accumbens arise primarily from areas 25, 24a,b and the medial part of area 24c, whereas the lateral part of the ventral striatum is mainly targeted by fibers coming from areas 24b,b′ and 23b and medial 24c *(43)*.

The organization of prefrontal corticostriatal projections to the core or shell of the nucleus accumbens has been studied in great detail in rodents by means of retrograde and anterograde tracing methods. The main prefrontal cortical inputs to the medial and lateral shell of the rat accumbens arise from the dorsal peduncular and infralimbic cortices, whereas the dorsal and ventral prelimbic and anterior cingulate cortices innervate preferentially the core. In addition, the lateral shell also receives strong cortical inputs from the agranular insular, perirhinal, rostral piriform, and lateral entorhinal cortices. On the other hand, additional cortical inputs to the medial shell arise from the caudal piriform cortex as well as the lateral and medial parts of the entorhinal cortex, whereas the core is preferentially targeted by inputs from the agranular insular and perirhinal cortices *(50,51)*.

Cortical inputs to the accumbens target preferentially the spines of striatal output neurons. Direct synaptic convergence of prefrontal inputs with dopaminergic terminals and hippocampal afferents have been demonstrated *(52–54)*, which provide a solid anatomical substrate for the gating properties of hippocampal projection on prefrontal cortical inputs in the rat accumbens *(5)*.

7.2. The Amygdalostrial Projection

In primates, the amygdalostriatal projection arises preferentially from various components of the basal and accessory basal nuclear complexes *(48,55–57)*. The main striatal target of amydala projections is the ventromedial striatum. Very few, if any, amygdala inputs are sent to the central striatum. The basal and accessory basal inputs innervate both the shell and core of accumbens, except for a restricted region in the dorsomedial shell that receives few basal nucleus inputs. The projection is topographically organized so that parvicellular basal inputs terminate in ventral shell and core, whereas magnocellular inputs target ventral shell and ventromedial putamen *(48)*. The intermediate division of the basal nucleus projects broadly across the whole ventromedial striatum except the dorsomedial part of the shell. The shell also receives specific inputs from the medial part of the central nucleus and periamygdaloid cortex and additional inputs from the medial nucleus *(48,57)*.

In the rat *(51,58–60)*, the amygdalostriatal projection is much more extensive than in monkeys and involves the whole extent of the ventral and dorsal striatum except for the rostrodorsolateral part of the caudate–putamen complex. This projection is highly topographic: the rostral basolateral nucleus projects preferentially to rostral and caudolateral portions of the accumbens and large portions of the dorsal striatum, whereas the caudal basolateral nucleus projects to the rostromedial caudate–putamen complex and caudomedial portion of the nucleus accumbens.

Amygdala terminals form asymmetric synapses mainly with spines and distal dendrites of projection neurons. At the light microscopic level there is a certain degree of overlap of axons from amygdala, hippocampus, prefrontal cortex, and thalamus in

nucleus accumbens *(61,62)* and some studies suggest functional convergence of these inputs onto individual neurons *(63)*. Electron microscopic studies demonstrated synaptic convergence of amygdala inputs with dopamine terminals *(53)* and hippocampal (ventral subicular) afferents onto single striatal neurons *(64)*. These convergent inputs may possibly mediate some of the complex functional interactions disclosed between these various glutamatergic afferents to control accumbens neuronal activity.

7.3. The Hippocampostriatal Projection

In monkeys, the subiculum is the main source of hippocampal inputs to the nucleus accumbens, but additional minor inputs come from parasubiculum, prosubiculum, and CA1 and CA3 regions *(48)*. These projections, which travel through the fornix and arise predominantly from the rostral hippocampus, terminate most densely in medial and ventral portions of accumbens. There is overlap of subicular and amygdala inputs to the medial division of the nucleus accumbens, suggesting potential interactions between these two pathways to modulate information processing in the primate accumbens *(48)*.

In rats and cats, the subiculum, CA1 region, and parahippocampal cortex provide massive heterogeneous projections to the ventral striatum *(65,66)*. The ventral subiculum projects mainly to the caudomedial part of the nucleus accumbens, whereas the dorsal and septal subiculum innervate preferentially its lateral and rostral components. Hippocampal inputs converge with dopaminergic, prefrontal, and amygdala afferents at the single-cell level in the rat accumbens *(33,54,67)*.

7.4. Functional Gating of Prefrontal Cortical Inputs by Hippocampal and Amygdala Afferents to the Nucleus Accumbens

Grace and his colleagues have published a series of elegant studies over the past 5 yr that provide a solid support for tight functional interactions between cortical, amygdala, and hippocampal glutamatergic inputs within the rat nucleus accumbens *(3,5)*. In vivo, accumbens neurons exhibit a bistable steady-state membrane potential alternating from a hyperpolarized nonfiring state to a depolarized state during which neurons can fire action potentials. Inputs from the hippocampal subiculum are responsible for generating the bistable state in these neurons. If fimbria/fornix is transected, none of striatal neurons exhibit the bistable membrane potential *(5,68,69)*. Prefrontal cortical stimulation induces only brief excitatory responses that, by themselves, are unlikely to result in action potentials in accumbens neurons. However, if hippocampal inputs are stimulated first, subsequent stimulation of prefrontal cortical afferents generate action potentials in accumbens neurons *(5,68)*. Activation of the subicular inputs cause the cells to shift to a depolarized state under which conditions prefrontal inputs can generate spike discharges. The hippocampal input, therefore, appears to act as a gate for prefrontal cortical influences to accumbens neurons *(5)*. Once this gate is opened, it allows prefrontal cortical inputs to get through and activates striatal neurons. This interaction is modulated by drugs that affect dopamine transmission because such compounds have an effect on the bistable state frequency of striatal neurons. For instance, systemic injection of D1 and D2 agonists decrease the frequency at which the membrane potential of striatal neurons exhibit depolarized states. Because the depolarized state is necessary for the gating of prefrontal cortical inputs by hippocampal afferents, the effects of prefrontal cortical inputs on striatal neurons are attenuated under these conditions *(5,70)*.

Amygdala inputs also appear to gate prefrontal cortical excitatory afferents to accumbens neurons. Stimulation of amygdala induces a brief depolarization of striatal neurons. If a

stimulus from amygdala is delivered before stimulation of the prefrontal cortex, there is facilitation of prefrontal cortical inputs to induce action potential in striatal neurons. This potentiation depends on the delay between the two stimuli. The amygdala has to be activated 7–30 ms before prefrontal cortical stimulation to mediate the potentiating effects. Inputs from both the amygdala and hippocampus are, therefore, capable of gating prefrontal cortical throughput to the accumbens, but in the case of amygdala, the response is brief and likely represents a phenomenon-related event *(5)*. It is important to note that there are reciprocal functional relationships between the amygdala and the prefrontal cortex; that is, prefrontal cortical stimulation influences neuronal activity in the amygdala and vice versa. Interestingly, cortical stimulation exerts inhibitory influences on the amygdala. This inhibitory effect appears to be mediated through various mechanisms that recruit amygdala GABAergic interneurons including a chloride-mediated hyperpolarization, persistent decrease in neuronal inputs resistance, and shunting of postsynaptic potentials *(71)*. Dopamine appears to be an important modulator of this functional interplay between the prefrontal cortex and amygdala *(71)*.

Although the exact functions of these gates are not clearly defined, Grace and colleagues have hypothesized that the hippocampal subiculum inputs may gate context-related events, whereas the amygdala may be involved in modulating prefrontal cortical stimuli related to emotion or affective states. Given the fact that schizophrenics show deficits in tasks that contain context-related information, one may hypothesize that a primary pathology of these brains relies upon the malfunctioning of the hippocampal gate of prefrontal cortical information at the level of the nucleus accumbens *(3,5)*.

8. CONCLUDING REMARKS

The importance of glutamatergic transmission in psychiatric diseases is now well established. Although much emphasis has been devoted to the prefrontal cortex, data presented in this review highlight the importance of other glutamatergic pathways from the amygdala and hippocampus. The complex functional interactions between these glutamatergic afferents to the nucleus accumbens, combined with the direct and indirect modulation these glutamatergic brain structures may exert on the activity of midbrain dopaminergic neurons, emphasize the importance of a tight balance of activity between glutamatergic and dopaminergic transmission to the prefrontal cortex and the nucleus accumbens for normal integration and processing of cognitive and limbic information. A shift in that balance leading to an increase release of dopamine at cortical and subcortical levels may be a critical factor that underlies the appearance, maintenance, and relapse of psychiatric diseases in humans.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health and the US Army.

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4

Glutamate Receptors

Ionotropic

Daniel T. Monaghan, Julia C. A. More, Bihua Feng, and David E. Jane

1. INTRODUCTION

L-Glutamate is the primary excitatory neurotransmitter in the vertebrate central nervous system (CNS) *(1–5)*. This conclusion, based on innumerable pharmacological, physiological, and biochemical studies, is now succinctly confirmed by the characterization of the vesicular glutamate transporters and their localization throughout the brain *(6)*. In contrast to the neuromodulatory neurotransmitters that are commonly released by brainstem nuclei projecting diffusely to large regions of the brain, and in contrast to the inhibitory, nonprojecting, local circuit neurons that use γ-aminobutyric acid (GABA) or glycine, glutamate-using pathways provide fast signaling between discrete brain regions. (For further discussion of glutamate-using pathways, *see* Chapter 3). L-Glutamate released from presynaptic nerve terminals binds to glutamate receptors on the receiving neuron. The ionotropic glutamate receptors span the plasma membrane and the binding of L-Glutamate causes a conformational change that opens a pore in the membrane formed by the receptor complex. The opened ion channel allows the influx of Na^+, and sometimes Ca^{++} ions, causing the cell to depolarize. If sufficiently depolarized, the neuron is activated. It is the fast-acting ionotropic glutamate receptors that underlie fast electrical responses in the CNS. Unexpectedly, there is also a wealth of slower-acting G protein-coupled glutamate receptors, the metabotropic glutamate receptors. The metabotropic receptors are the subject of Chapter 5 in this volume. The discovery and characterization of L-glutamate as the major CNS neurotransmitter was a major breakthrough and has opened the door to understanding many essential aspects of brain function at all levels of investigation.

L-Glutamate was first shown to be excitatory by two independent groups. In 1954 Hayashi reported in the *Keio Journal of Medicine* that L-glutamate and L-aspartate caused convulsions after intracerebral injections into dog brain *(7)*. Independently, Watkins and colleagues, in the process of screening several known brain chemicals for excitatory and inhibitory activity, found that L-glutamate directly excited spinal cord neurons *(8)*. Of the many active agents identified in this study, Watkins and colleagues

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

focused the next three decades on glutamate and aspartate (excitatory amino acids). This work characterized the excitatory action of various excitatory amino acid agonists and developed and identified antagonists that could block their action. Ultimately, these studies led to the conclusion that the excitatory actions of glutamate and aspartate were mediated by at least three distinct types of receptors, which were named for agonists by which the receptors were selectively activated: *N*-methyl-D-aspartate (NMDA), kainate, and quisqualate. The quisqualate receptor subsequently was renamed as the "AMPA" receptor (named for the selective agonist α-amino-3-hydroxy-5-methylisoxazole-4-propionate) to help avoid confusion generated by the observation that quisqualate is also a potent metabotropic glutamate receptor agonist *(3,9)*. These physiologically identified receptors differed in their physiological properties, pharmacological profiles, and anatomical distributions *(10,11)*. This receptor classification was subsequently confirmed using various radioligands (L-[^{3}H]glutamate, which bound to all three receptor types *(12)*, and the subtype-specific radioligands [^{3}H]AMPA *(13)*, [^{3}H]kainate *(14)* and D-[^{3}H]AP5 *(15)*, which bound specifically to quisqualate, kainate, and NMDA receptors, respectively). Using receptor autoradiography to map out each of these radioligand binding sites *(16–21)*, revealed discrete distributions, but collectively, glutamate receptors are found in nearly every region of the CNS. This is consistent with observations that essentially all neurons are excited by L-glutamate.

With the realization that glutamate mediates most fast synaptic transmission throughout the brain, came the pessimistic perception that the glutamate system was too widespread and of fundamental importance to be involved in subtle neurological and psychiatric disease states. Along these lines, it was felt that drugs that modulate L-glutamate receptors would be too nonspecific in their actions (e.g., general anesthetics) to be useful as therapeutic agents. Unexpectedly, however, the receptors that mediate the synaptic actions of L-glutamate were found to each be a family of receptors with discrete brain distributions and with significant differences in physiological activity and biochemical signaling. Furthermore, these glutamate receptors can have fast signaling (<10 ms), intermediate timescale signaling (100–1000 ms), and slow excitatory actions (>1 s). Consequently, there is a rich diversity of glutamate receptors and their corresponding actions. Within this diversity there is significant potential for specific receptor systems to be involved in the etiology and/or therapeutic treatment of neurological and psychiatric disorders.

Several years after the initial physiological and biochemical characterization of AMPA and kainate receptors, the distinction between these two receptors became controversial, leading to the term "non-NMDA" receptors to signify AMPA and/or kainate receptors. However, with the cloning of separate genes coding for AMPA and kainate receptors, it is clear that these two receptor families are distinct. In this chapter, we discuss the AMPA and kainate receptors together to better compare and contrast these two closely related receptors. NMDA receptors, which are functionally quite different, though closely related, will be discussed separately.

The cloning of proteins related to ionotropic glutamate receptors not only confirmed the three-receptor classification scheme initially proposed by Watkins and colleagues *(11)*, but revealed an additional subunit family termed delta (δ) *(22,23)*. These subunits have their closest homology to the kainate and AMPA subunits, but in contrast to the other glutamate ionotropic receptors, they do not form glutamate or glycine-responsive channels. Presently these receptors are considered orphan receptors. These receptors do

Table 1
Glutamate Receptor Subunits and Subunit Families[a]

Receptor	Subunit Family	Subunits
AMPA	GluR1-4	GluR1 (GluRA, α1)
		GluR2 (GluRB, α2)
		GluR3 (GluRC, α3)
		GluR4 (GluRD, α4)
Kainate	KA	KA1 (γ1)
		KA2 (γ2)
	GluR5-7	GluR5 (β1)
		GluR6 (β2)
		GluR7 (β3)
GluRδ	GluRδ	GluRδ1
		GluRδ2
NMDA	NR1	NR1a-h (ζ1)
	NR2	NR2A (ε1)
		NR2B (ε2)
		NR2C (ε3)
		NR2D (ε4)
	NR3	NR3A (χ1)
		NR3B (χ2)

[a]Alternative nomenclature is noted in parentheses.

appear to be involved in some aspect of synaptic transmission and synapse formation. In the δ-2 knockout, there is an impairment in cerebellar long-term depression (LTD) and Purkinje cell synapse formation *(24,25)*. In the lurcher mouse, the defect is a mutation in δ-2 that renders the channel constitutively active and associated with cerebellar neuronal cell loss and ataxia *(26)*. Recently, Yuzaki and colleagues have presented evidence for heteromeric complex formation between δ-2 and AMPA and kainate receptor subunits *(27)*. For an overview of glutamate receptor subunit families, *see* Table 1.

2. AMPA AND KAINATE RECEPTORS

2.1. AMPA/Kainate Receptor Function

Agonist binding to either AMPA or kainate receptors opens a channel permeable to Na^+ and K^+ ions. With the influx of Na^+ ions, the cell membrane is depolarized. In special circumstances, some receptor channels also exhibit high Ca^{2+} permeability, depending on subunit composition and posttranscriptional editing *(28)*. For both AMPA and kainate receptor ion channels, they are rapidly activated and, in the presence of L-glutamate, are rapidly inactivated owing to desensitization. It is this rapid activation/deactivation that allows fast synaptic transmission to accurately follow high-frequency CNS activity. AMPA receptors can activate in the submillisecond time-scale and then desensitize in the 1- to 10-ms range (e.g., ref. *29*). Recovery from desensitization takes longer, in the 10s of ms scale. Kainate receptors also have fast activation/deactivation kinetics; however, they can differ from AMPA responses in being slower *(30)*. On the basis of pharmacological experiments and receptor distribution studies, AMPA receptors are thought to be the primary signal for fast excitatory synaptic transmission in the vertebrate CNS. Only in recent

years have the pharmacological tools become available to study kainate receptor function. As found for AMPA receptors, kainate receptors also mediate fast synaptic transmission, but also play other roles in synaptic signaling.

In addition to ionotropic effects, each of the ionotropic glutamate receptors have been suggested to have G protein-coupled receptor-like activity. For example, some physiological responses mediated by kainate receptors have been reported to require G protein activation *(31)*. Though such signaling mechanisms are difficult to explain, recent evidence for ionotropic receptor subunit association with G protein-coupled receptors indicates that there may be many potential signals arising from glutamate ionotropic receptors *(32,33)*.

2.2. AMPA/Kainate Receptor Subunits

Molecular cloning has led to the isolation of four AMPA receptor subunits, GluR1-GluR4, and five subunits that combine to form kainate receptors. There are two types of kainate receptor subunits: GluR5-GluR7 are low-affinity kainate receptor subunits that have been shown to form functional ion channels when homomerically expressed in HEK 293 cells or Xenopus oocytes *(34,35)*. KA1 and KA2 are high-affinity kainate binding proteins that combine with GluRs 5–7 in native receptors but do not form functional homomeric channels (for reviews, *see* refs. *28,36*).

AMPA and kainate iGluR subunits are approx 900 amino acids long with a molecular weight of around 100 kDa. Hydrophobicity analysis originally suggested that each subunit contained four membrane-spanning domains but *N*-glycosylation, site-specific antibodies, and mutagenesis studies have since led to the currently accepted topology where the second proposed transmembrane domain is actually a re-entrant loop *(28,37)*. Functional non-NMDA iGlu receptors have been postulated to consist of homo- or heteromeric assemblies of either four or five subunits, although the tetrameric assembly is now the more widely accepted stoichiometry *(38,39)*.

Studies using chimeric assemblies of AMPA and kainate receptor subunits and site-directed mutagenesis demonstrated that the agonist-binding site of the receptor is formed between two segments, termed S1 and S2 *(40,41)*. S1 is a 130 amino acid section preceding the M1 transmembrane domain and S2 is made up of most of the extracellular amino acids between transmembrane domains M3 and M4 *(40)*. *See* Fig. 1 for the general structure of ionotropic glutamate receptors.

2.3. Receptor Subunit Structure

2.3.1. Ligand-Binding Domain Structure

A major advance in the study of glutamate receptors in recent years has been the development of crystal structure analysis of the ligand-binding core of the receptors. In order to attain high-resolution X-ray structures, a soluble form of the ligand-binding core of GluR2 was made by substituting the first two transmembrane regions, M1 and M3, with a peptide linker and removing the extreme amino terminal domain and M4 *(42)*. After the original description of this ligand binding core in complex with kainate *(42)*, several studies have described the high-resolution X-ray structure of the GluR2 construct in complex with agonists, antagonists and in its unbound, apo state *(43–47)*. A representation of the GluR2 ligand-binding domain crystal structure is shown in Fig. 2.

The crystal structure of the truncated GluR2 subunit in complex with kainate indicated that two domains are involved in the binding of an agonist. One domain is formed by the

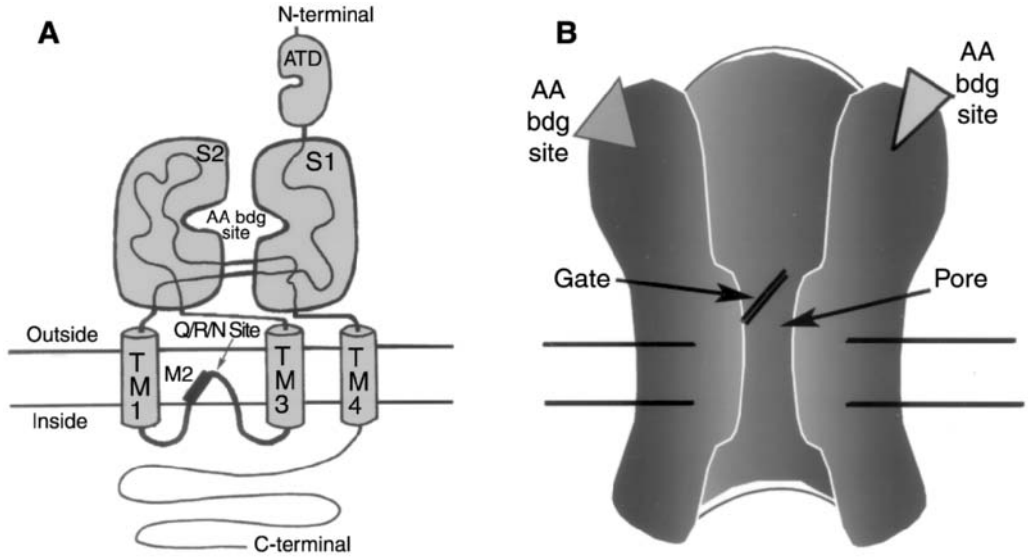

Fig. 1. Glutamate receptor subunit and complex structure. **(A)** A schematic representation of the ionotropic glutamate receptor subunit topology. The S1 and S2 domains together form the amino acid binding site (AA bdg site) for glutamate or glycine. The critical site for determining channel permeability (Q/R/N site) is shown at the center of the M2 domain. Four subunits assemble **(B)** to form a pore structure through the membrane with the M2 region of each subunit contributing to the pore constriction.

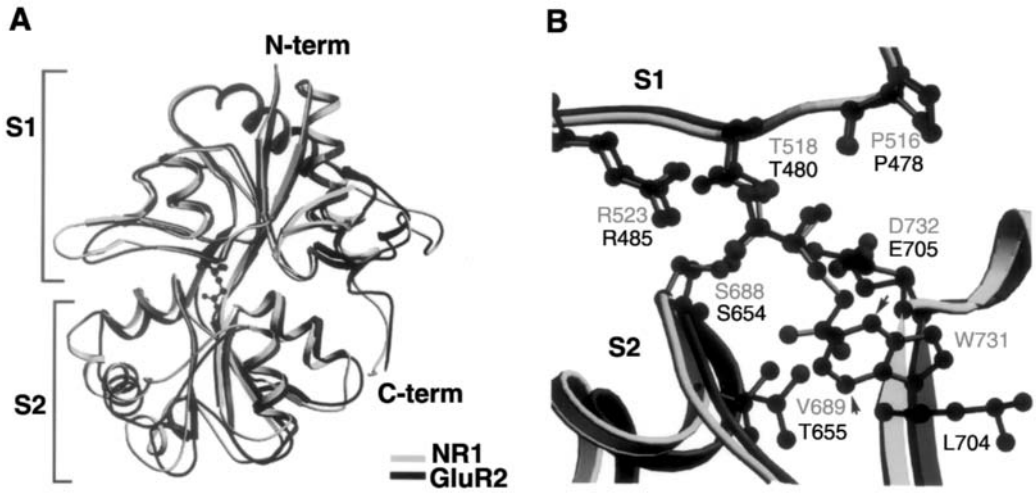

Fig. 2. Crystal structure of the S1/S2 ligand-binding domain of GluR2 and NR1. **(A)** The S1 and S2 domains of GluR2 and NR1 are superimposed. The N-terminals (N-term) and C-terminals (C-term) are shown. **(B)** Glutamate and glycine are shown docked into their respective binding sites. The peptide amino acid residues critical to ligand binding are shown. Although the α amino and carboxy groups of glutamate and glycine bind in a similar fashion, tryptophan (W) 731 of NR1 blocks the binding of the longer glutamate structure, but allows binding of glycine. Figure adapted from Furukawa and Gouaux *(47a)* and used by permission of the publishers.

Table 2
Key Residues in the Agonist-Binding Cavity of AMPA and Kainate Receptors[a]

GluR1-4	GluR5/6/7	KA1/2	Interaction with kainate
Arg485	Arg	Arg	α-carboxyl group of kainate
Thr480	Thr/Ala/Thr	Thr	protonated amino group of kainate
Glu705	Glu	Glu	protonated amino group of kainate
Ser 654	Ser/Ala/Ser	Ser	ω-carboxyl group of kainate
Thr655	Thr	Ser/Thr	ω-carboxyl group of kainate

[a]The GluR2 crystal structure was used to demonstrate the residues that interact with kainate and the residues present at equivalent positions in the other subunits are indicated *(48)*.

S1 segment and a 33 amino acid segment in the C-terminal end of S2 (which includes the flip/flop site). The second domain is made up of the 134 amino acids in the N-terminal end of the S2 segment. When kainate was docked in the receptor construct it bound between the two domains with its glutamate-like backbone forming a bridge between domains 1 and 2. The study also identified amino acids that are likely to be essential for agonist binding. Proposed interactions between kainate and amino acids in the GluR2 agonist binding pocket are summarized in Table 2. Mutagenesis studies have shown that these five residues, at equivalent positions in other glutamate receptors, are important for agonist interactions. An example of another residue that appears to be important for agonist interaction in GluR2 is Tyr450. This amino acid residue appears to prevent full closure of the binding domain by forming a wedge between the pyrrolidine ring and the isopropenyl group of kainate and domain 1. The authors proposed that this steric clash is what causes kainate to be a partial agonist, whereas AMPA and glutamate, whose structures would not clash with this residue, can allow further closure of the binding domains and therefore act as full agonists *(42)*. The degree of domain closure has also been correlated to the extent of desensitization induced by agonists *(46)*.

In Table 2 some of the key residues present in the agonist-binding core at equivalent sites in AMPA and kainate receptor subunits are compared. The five key residues believed to interact with the glutamate backbone of kainate in the GluR2 subunit are conserved in GluR1-4, GluR5, GluR7, and KA2. The differences seen in the GluR6 agonist-binding cleft may explain why various classes of compound act selectively at AMPA and GluR5 receptors but not GluR6 receptors. Another residue, Met708, of GluR2 is replaced by a serine residue in GluR5 and this amino acid switch has been proposed as an explanation for the GluR5 selectivity of ATPA and 5-iodowillardiine *(46,47)*.

Crystallographic studies have also demonstrated that the extent of cleft closure seems to correlate well with the activity of ligands at the GluR2 subunit. For example, the full agonists glutamate and AMPA induce a cleft closure of approx 20° when compared to the ligand free (apo) state whereas the partial agonist kainate leads to a cleft closure of only approx 12° *(43)*. A further study demonstrated that AMPA receptor agonists with an isoxazole ring bind in slightly different ways, depending on the substituents added to the isoxazole ring, and there was a strong correlation between the degree of domain closure and efficacy in electrophysiological studies *(44)*. Evidence from studies using antagonists in complex with the ligand-binding core of GluR2 agree with the concept that cleft closure is related to activation. Two structurally unrelated antagonists, DNQX and ATPO, have been shown to stabilize an open form of the ligand-binding core *(43,44)*.

2.3.2. Structural Basis of Desensitization

A recent study by Sun and colleagues *(49)* used structural and functional studies to develop a mechanistic scheme for the process of desensitization in AMPA and kainate receptors. The authors suggest that the four subunits of each receptor form dimers. They demonstrated that cyclothiazide can promote dimerization of the subunits and, using crystallography, showed that cyclothiazide interacts with a pocket formed at the interface of two subunits. It was proposed that after agonist binding the agonist is trapped in a cleft between two domains of the subunit, which leads to conformational strain causing the opening of the ion channel. When desensitization occurs the dimer interface changes and the domain closure no longer leads to ion channel opening. Normally, the energy barrier for activation is lower than that for desensitization. Once the receptor is desensitized, however, it is more stable than an active receptor and therefore prolonged agonist application leads to the desensitization of most of the receptor population *(49)*.

2.3.3. Ion Channel Structure

Glutamate receptor ion channels are thought to be formed as a tetramer of M2 pore-lining segments *(50)*. The M2 loop penetrates only partially into the membrane with a key amino acid residue position, termed the "Q/R" site, at the tip of the loop *(51)*. The constriction of the pore appears to be two amino acid postions C-terminal to the Q/R site. On the N-terminal side of the Q/R site, M2 forms an α helix with a dipole that has the negative end inside the membrane near the Q/R site and the positive end near the cytoplasmic surface. Near the Q/R site is a kink and C-terminal to this is an extended form of polypeptide chain (descending random coil) that returns to the cytoplasmic surface. This structure is generally similar to that found for various potassium channels. Recently, bacterial glutamate receptors have been identified that gate potassium channels and have specific structural similarities to both mammalian glutamate receptors and potassium channels *(49)*.

2.4. Multiple Isoforms of AMPA and Kainate Receptor Subunits

2.4.1. Alternative Splicing

Different forms of AMPA and kainate receptors exist owing to alternative splicing and RNA editing (Table 3). The AMPA receptor subunit GluR4 can exist in an alternative splice variant form, GluR4c, which has a short C-terminus. Of the kainate receptor subunits, both GluR5 and GluR7 exist as various splice variants. GluR5-1 has an additional 15 amino acid section in the N-terminal region *(52)*. GluR5-2 has three further variants, termed GluR5-2a, -2b, and -2c, each of which varies in its C-terminal domain. Variation in the C-terminal domain also gives rise to the two splice variants of GluR7, GluR7a and GluR7b *(35)*. No alternative splicing has been reported for GluR6, KA1, or KA2 subunits.

AMPA receptor subunits (GluR1–4) contain an alternatively spliced cassette of 38 amino acids in the extracellular loop preceding the M4 transmembrane domain. Two variants of this cassette exist, termed "flip" and "flop" isoforms, which differ in their desensitization profiles. The flip isoform displays less desensitization after application of glutamate or AMPA than does the flop isoform *(53)*.

2.4.2. RNA Editing

When the genomic sequence for GluR2 receptor subunits was determined, a mismatch was discovered between the genomic sequence and the cDNA sequence. Whereas the

Table 3
Summary of the Multiple Isoforms of AMPA and Low-Affinity Kainate Receptor Subunits

Receptor subunits (splice variants in brackets)	Contains flip/flop cassette	Contains Q/R RNA editing site in M2	Contains R/G editing site preceding flip/flop cassette
GluR1	Yes	No	No
GluR2	Yes	Yes	Yes
GluR3	Yes	No	Yes
GluR4 (4c)	Yes	No	Yes
GluR5 (1, 2a, 2b, 2c)	No	Yes	No
GluR6	No	Yes	No
GluR7 (7a, 7b)	No	No	No

initially characterized cDNA sequence coded for an arginine in the middle of GluR2's M2 re-entrant loop, the genome codes for a glutamine. The genes for all AMPA and kainate receptor subunits code for a neutral glutamine (Q) at this position, however, RNA for GluR2, GluR5, and GluR6 can undergo site-specific posttranscriptional RNA editing that leads to the replacement of this amino acid with a positively charged arginine (R) *(54–56)*. This modification is highly significant; animals without Q/R editing have seizures and die young. Thus, the genome codes for a lethal mutation. Accordingly, editing from Q to R at this site in the GluR2 subunit is very efficient and is thought to be almost complete in rat brain *(54)*. The replacement of glutamine by arginine at the Q/R editing site in GluR2 subunits results in ion channels with low calcium permeability and linear current–voltage relationships *(57,58)*. Edited GluR2 subunits determine the channel properties when coassembled with other AMPA receptor subunits (which code for Q and are not edited). Because most native AMPA receptors have a GluR2 subunit, most AMPA receptors in the brain are not calcium permeable owing to the presence of edited GluR2 subunits.

Q/R editing is a result of the actions of ADAR2 (adenosine deaminase acting on RNA – 2) *(59)*. This enzyme recognizes a specially folded RNA structure and deaminates the critical adenosine to make an inosine. This changes the three-letter code from CAG (which codes for glutamine) to CIG, which codes for arginine. The ADAR2 knockout in mice is lethal, but not in mice where the GluR2 Q/R site is mutated at the genome to code for R *(60)*.

Q/R site editing of GluR5 and GluR6 subunits also results in lower Ca^{2+} permeability, although homomeric GluR6 edited receptors are not purely cation selective as they also permit anions through their channels *(55,58,61,62)*. GluR5 and GluR6 editing is less efficient with 35 and 75% of these subunits being edited, respectively *(61)*.

Another site of RNA editing is found in the segment immediately preceding the flip/flop site. Three of the AMPA receptor subunits, GluR2, GluR3, and GluR4, undergo editing of an arginine (R) to a glycine (G) at this site; this modification increases the rate of onset and the rate of recovery from agonist-induced desensitization in receptors containing these subunits *(63)*.

In the first transmembrane-spanning segment, M1, GluR6 receptors contain two further editing sites. An isoleucine/valine site is encoded by the gene and a tyrosine/cysteine site is encoded by the edited transcipt *(64)*. The Ca^{2+} permeability of these kainate receptors can vary depending on editing of both M1 and M2 regions *(61)*. Calcium-permeable AMPA

and kainate receptors have an inwardly rectifying current-voltage relationship, which is a result of a polyamine (spermine) block of the channels at positive potentials *(65)*.

2.5. Homomeric and Heteromeric Assemblies of AMPA and Kainate Receptors

Each of the AMPA receptor subunits, GluR1–GluR4, and the low-affinity kainate receptor subunits, GluR5–GluR7, can form functional channels when expressed homomerically. However, the ability of the subunits to form heteromeric complexes greatly increases the functional diversity of AMPA and kainate receptors. Several studies have demonstrated that changing the subunit composition slightly can dramatically change the pharmacology of receptors. For example, (*S*)-5-iodowillardiine (300 μ*M*) shows no activity at homomeric GluR6 or GluR7 receptors but elicits small currents in GluR6/KA2 and GluR7/KA2 heteromers *(66)*. Also, the reportedly GluR5-selective agonist ATPA gave an EC_{50} value of 2.1 μ*M* at recombinant GluR5 subunits and was inactive at GluR6 homomeric channels, yet when tested at heterologous subunit assemblies it, gave an EC_{50} of 6.3 μ*M* at GluR5/KA2 receptors and 84 μ*M* at GluR6/KA2 receptors *(67,68)*. Heteromeric assemblies of subunits also display different rates of desensitization than homomeric receptors. For example, in one study 10 m*M* glutamate currents in GluR6 homomers and GluR6/KA2 receptors gave desensitization times (τ_{des}) of 3.8 ± 0.2 ms and 2.3 ± 0.2 ms, respectively, and 30 m*M* glutamate currents gave τ_{des} times of 7.6 ± 0.53 ms in GluR7 homomers but 6.6 ±1.0 ms in GluR7/KA2 heteromeric receptors, *(66)*. (*S*)-5-Iodowillardiine currents displayed a τ_{des} of 8.9 ± 1.6 ms in GluR5 receptors, which was significantly reduced to 2.6 ± 0.2 ms in GluR5/KA2 heteromers *(66)*.

A recent study making use of the selective kainate receptor agonist dysiherbaine demonstrated that each type of subunit within a heteromeric kainate receptor can contribute a distinct conductance upon activation by agonist binding *(69)*. The authors reported how a long-lasting interaction between dysiherbaine and GluR5 subunits elicits a tonic current from GluR5/KA2 heteromers; then subsequent cooperative gating of the KA2 subunits can be elicited by another agonist, such as glutamate *(69)*.

Examples of both homomeric and heteromeric AMPA and kainate receptors have been detected *in situ*, although the subunit composition of most native receptors remains unknown.

2.6. Pharmacology of AMPA and Kainate Receptors

Only a brief introduction of the pharmacology of AMPA and kainate receptors is given here; for more in-depth reviews, *see* refs. *28,36,70*. For structures of the most useful pharmacological tools, *see* Figs. 3 and 4.

2.6.1. AMPA Receptor Agonists

Originally defined using the agonist quisqualate, AMPA was shown to be a more selective agonist for this receptor type in the 1980s *(71)*. This agonist is still widely used in the study of AMPA receptors, although the willardiine derivative (*S*)-5-fluorowillardiine is also a potent and selective AMPA receptor agonist *(72,73)*. As kainate-evoked AMPA receptor responses are nondesensitizing, this agonist is also widely used to activate AMPA receptors.

2.6.2. Kainate Receptor Agonists

The standard agonists at kainate receptors, kainate and domoic acid, have limited use owing to their activation of AMPA receptors *(28)*. More recently, however, ligands have been

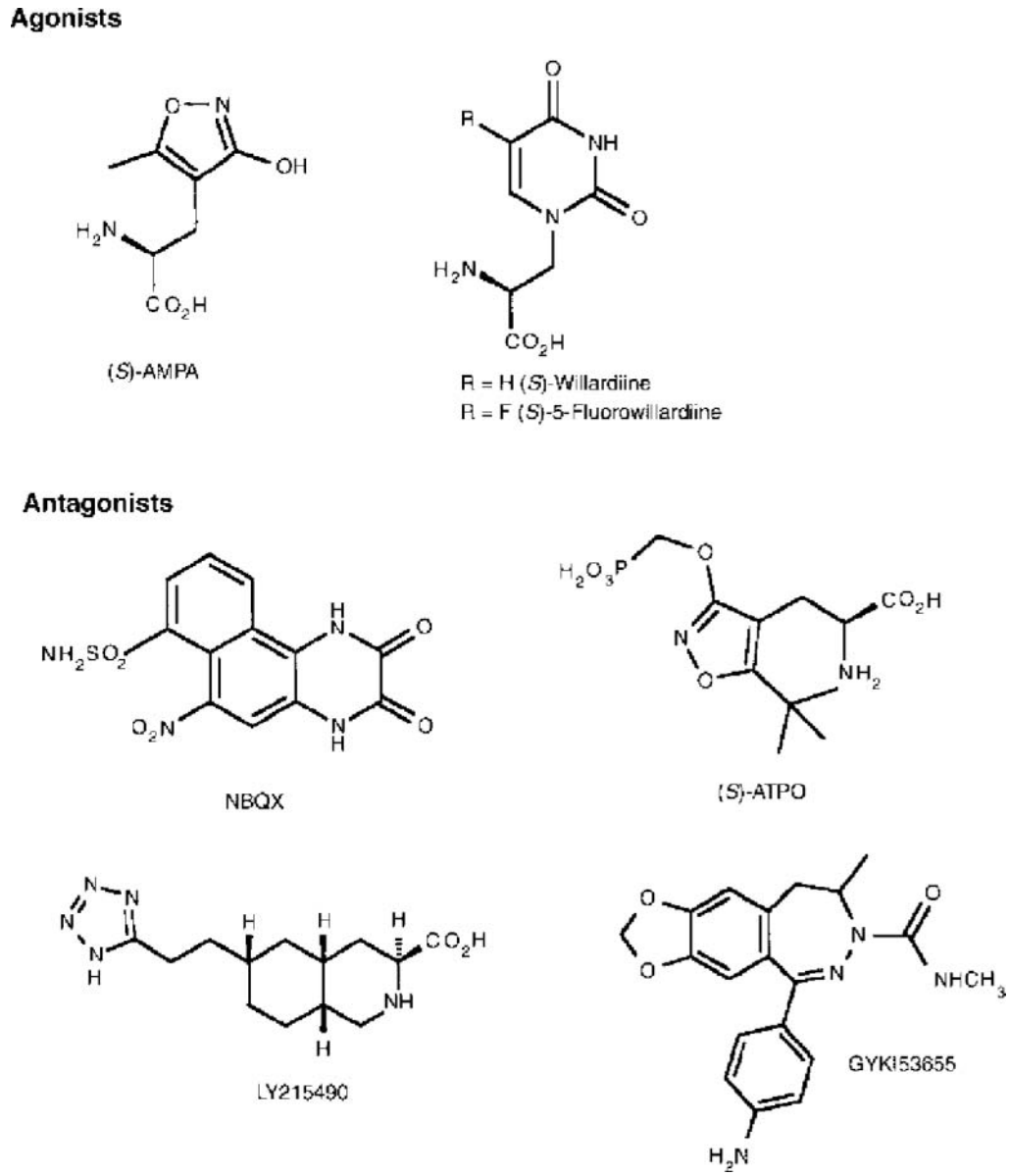

Fig. 3. Structures of key AMPA receptor compounds.

developed that show selectivity for kainate over AMPA receptors. (*S*)-5-Iodowillardiine and ATPA both show selectivity for the GluR5 kainate receptor subunit over other kainate or AMPA receptors *(67,72)*. (2*S*,4*R*)-4-methylglutamate shows potent agonist activity at both GluR5 and GluR6 receptors *(74)*, although the fast desensitization properties of this agonist often lead to its use as a functional antagonist of these receptors *(75)*.

2.6.3. AMPA Receptor Antagonists

The most commonly used competitive antagonists of AMPA receptors are the quinoxalinediones. Of these NBQX is the most selective, although it displayed only 30-fold selectivity for AMPA over kainate receptors in binding studies *(76)*. CNQX is less potent

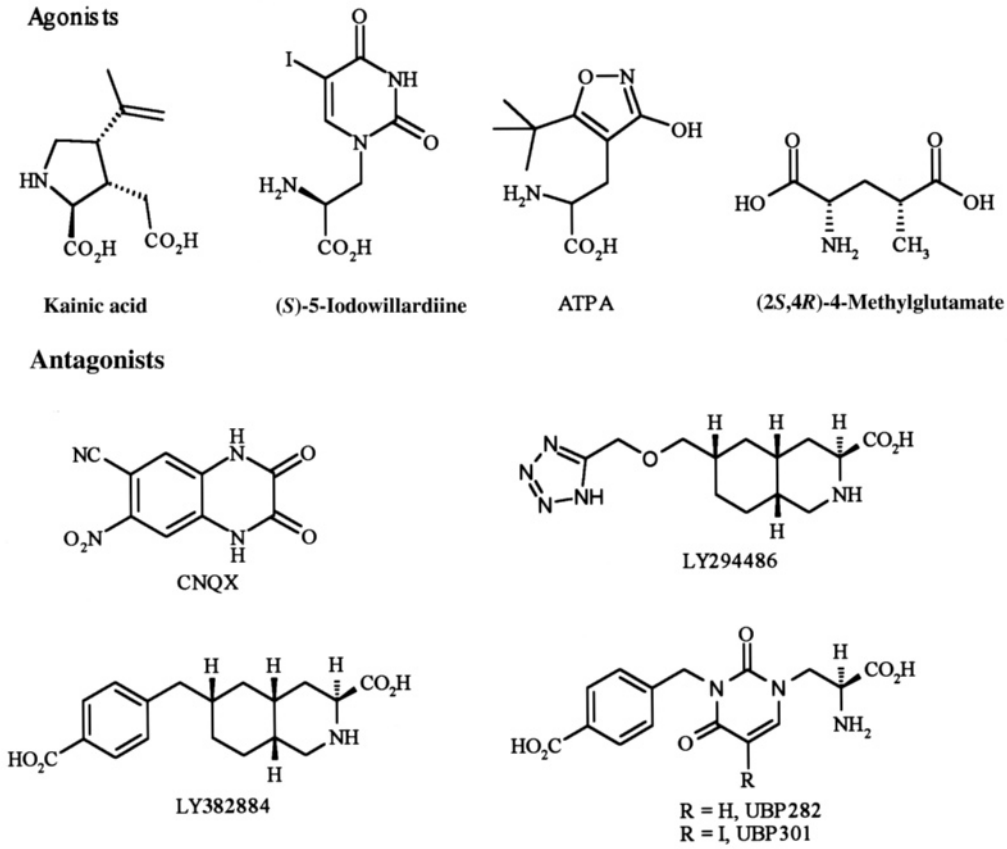

Fig. 4. Structures of key kainate receptor compounds.

and selective so its use is limited to studies where the nonselective antagonism of both AMPA and kainate receptors is required *(76,77)*.

A more selective pharmacological tool for blocking AMPA receptor responses is the noncompetitive antagonist GYKI53655 *(75,78)*. This 2,3-benzodiazepine and a related compound GYKI52466 have been used widely in physiological studies to selectively depress AMPA over kainate receptor responses *(28,36)*. They appear to bind to an allosteric site on the AMPA receptor acting as negative allosteric modulators *(79,80)*.

2.6.4. Kainate Receptor Antagonists

Until recently there was a paucity of selective kainate receptor antagonists, which limited information about the physiological roles of this receptor family. A series of decahydroisoquinolines have been developed as antagonists of AMPA and kainate receptors and several of these compounds, including LY382884, LY294486, and LY293558, show selectivity for the GluR5 kainate receptor subunit over other AMPA or kainate receptor subunits *(36,81)*. They have therefore been used to demonstrate the importance of the GluR5 receptor subunit in CNS functions. More recently, novel antagonists based on the structure of willardiine, such as UBP282 and UBP301, have been developed, the latter showing selectivity for the GluR5-containing kainate receptors present on neonatal dorsal root fibers over the AMPA receptors expressed on spinal motoneurons *(81)*. There is still a lack of antagonists with selective activity at GluR6 or GluR7 receptor subunits.

2.6.5. Allosteric Modulators

Several positive allosteric modulators of AMPA receptors have been reported, the most commonly used being the benzothiadiazide cyclothiazide (for a review, *see* ref. *28*). For kainate receptors, the lectin concanavalin A is often used to reduce receptor desensitization. These two allosteric modulators can be used to differentiate between physiological responses of AMPA or kainate receptors *(83)*.

2.7. AMPA and Kainate Receptor Radioligands

Radiolabeled ligands are available for both AMPA and kainate receptors. These are useful for determining the pharmacological properties, as well as distributions and densities of receptors in a variety of tissues or experimental conditions. As expected [^{3}H]AMPA labels AMPA receptors *(13)*. [^{3}H]5-fluorowillardiine is another AMPA receptor agonist that can be used to radiolabel AMPA receptors *(84)*. In addition, the AMPA antagonist [^{3}H]NBQX labels a larger population of AMPA receptors *(85)*. To label kainate receptors, there are two agonists available, [^{3}H]kainate *(14)* and [^{3}H]4-methylglutamate *(86)*. The high- and low- affinity sites labeled by [^{3}H]kainate can be distinguished by adding calcium, which inhibits binding to the high-affinity site *(87)*. Both AMPA and kainate receptors are labelled by L-[^{3}H]glutamate *(12,88)*, but this signal is relatively small owing to the labeling of NMDA receptors and thus the more selective radioligands are preferred.

2.8. Physiological Roles of AMPA and Kainate Receptors

2.8.1. Presynaptic Modulation by AMPA and Kainate Receptors

In addition to their role in mediating postsynaptic excitatory transmission in the CNS, AMPA and kainate receptors have also been reported to exist presynaptically, acting to regulate synaptic transmission (for reviews, *see* refs. *89* and *90*). Presynaptic kainate receptors reportedly modulate both GABAergic and glutamatergic terminals and in recent years there has been conjecture as to whether presynaptic kainate receptors act by ionic or metabotropic mechanisms *(89,90)*.

Studies have suggested that kainate causes a biphasic modulation of NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) on CA1 neurons in the hippocampus and this action is sensitive to the GluR5 receptor antagonist LY294486 *(91,92)*. The activation of presynaptic kainate receptors has also been shown to suppress glutamate release from primary afferent spinal cord neurons *(93)*.

In the hippocampus and other brain areas, it has been shown that kainate application, possibly acting at GluR5-containing kainate receptors, causes a decrease in evoked inhibitory postsynaptic current amplitude in the presence of GYKI53655 *(66,94–96)*. Most studies agree that the depression of GABAergic transmission by kainate is owing to a direct presynaptic effect *(97–99),* although some reports have suggested that depression of evoked GABA release is secondary to an enhancement of GABA release, with the GABA then acting on presynaptic $GABA_B$ regulatory receptors to downregulate release *(93,95)*.

In some cases kainate receptor agonists can also act to facilitate GABAergic transmission. Researchers have attributed this to a direct effect at presynaptic terminals of interneurons with kainate possibly acting at non-GluR5 receptors *(100,101)*. However, in a recent study, Braga and colleagues reported that low concentrations of GluR5 kainate receptor agonists could enhance GABAergic transmission whereas high concentrations depressed transmission in the basolateral amygdala *(99)*. The authors concluded that

both these effects were owing to a direct effect of the kainate receptor agonists at GABAergic terminals and the bidirectional effects of the agonists may be a result of activation of two receptor populations, each with different affinities for the agonists and mechanisms of action *(99)*. The development of more selective ligands for kainate receptors may help to clarify this.

Although fewer examples have been described, evidence for presynaptic AMPA receptors is also beginning to emerge. Presynaptic AMPA receptors have been reported to inhibit the release of GABA from GABAergic interneurons in the cerebellum *(102)*, yet to increase the release of GABA from cerebellar stellate cells *(103)*. A recent study has also reported the functional expression of AMPA receptors on the central terminals of dorsal root ganglion neurons which, when activated, inhibit the synaptic release of glutamate *(104)*.

The increase in reports of presynaptic AMPA and kainate receptors led to the question of how these receptors regulate transmitter release. Although many of the presynaptic effects can be explained by ionotropic mechanisms, there have also been reports of metabotropic functions. Rodriguez-Moreno and Lerma *(97)* reported a metabotropic function for the presynaptic kainate receptors on CA1 interneurons and a kainate receptor-mediated metabotropic function has also been reported in CA1 pyramidal cells where kainate exposure leads to an inhibition of an afterhyperpolarization potassium current (I_{sAHP}), which follows short bursts of action potentials *(105)*. Inhibition of this I_{sAHP} by excess glutamate may lead to hyperexcitability via a positive feedback loop of glutamate release *(97,105)*.

2.8.2. AMPA and Kainate Receptor Function: Information From Transgenic Mice

As there are few subunit selective pharmacological tools, researchers have used gene targeting to provide some information about the roles of different AMPA and kainate receptor subunits. Mice lacking the GluR2 subunit contain AMPA receptors with increased Ca^{2+} permeability, enhanced neuronal excitability, and increased synaptic plasticity in the CA1 region of the hippocampus *(106,107)*. Kainate-evoked AMPA receptor-mediated currents and AMPA receptor-mediated neurotoxicity were also increased in GluR2$^{-/-}$ neurons *(108)*. Transgenic mice overexpressing the AMPA receptor subunit GluR2-flip have been used to demonstrate the crucial role of these receptor subunits in the pathogenesis of focal hypoxic-ischaemic neuronal cell death *(109)*.

In GluR1 knockout mice associative long-term potentiation was absent in CA3 to CA1 synapses *(110)*. Knockout mice have also implicated the importance of GluR1-containing AMPA receptors in the synaptic plasticity in the basolateral nucleus of the amygdala, which underlies conditioned reinforcement *(111)*.

In one GluR5 mutant where all the Q/R sites are edited (i.e., encode arginine), fewer and smaller responses to the kainate receptor agonist domoate were recorded in isolated dorsal root ganglion neurons *(112)*. In GluR5 global knockout mice, there is a loss of kainate receptor-mediated potentiation of evoked excitatory synaptic transmission in perforant path inputs to CA3 neurons and loss of kainate-induced enhancement of mEPSC frequency in mossy fiber synapses, although overall neuroanatomy and general health is normal *(100,113,114)*.

In GluR6 global knockout mice the following are absent: small kainate-induced currents in CA1 hippocampal neurons, kainate receptor-mediated responses in CA3 neurons, high-affinity kainate binding in CA3 neurons or in the dentate gyrus, and synaptic activation

of kainate receptors in the mossy fiber pathway. There is also loss of kainate receptor-mediated depression of evoked excitatory synaptic transmission in both mossy fiber and associational-commisural inputs to CA3 neurons and loss of kainate receptor-mediated potentiation of evoked excitatory synaptic transmission in perforant path inputs to CA3 neurons. In addition, there is a reduction in mossy fiber LTP and GluR6$^{-/-}$ mice are less susceptible to seizures induced by kainate injection *(113–116)*. Mutant mice in which the Q/R site cannot be edited have been used to demonstrate that kainate-induced seizure susceptibility is inversely correlated with the degree of editing of the GluR6 subunit *(117)*.

In KA2 global knockout mice, there is a loss of the facilitatory effect of low doses of kainate at mossy fiber synapses and the depressant effect of kainate occurs at lower concentrations than in wildtype mice. The heterosynaptic facilitation of mossy fiber EPSCs is also absent in KA2 knockouts *(118)*.

3. NMDA RECEPTORS

3.1. NMDA Receptor Function: Physiological Properties

3.1.1. Voltage Dependency and Mg^{++} Blockade

NMDA receptors have a distinctive role in synaptic transmission because of several unique physiological and biochemical properties. Several years passed after identifying the first NMDA receptor antagonist before the first demonstration of an NMDA receptor-mediated synaptic response *(119)*. This delay occurred because NMDA receptors do not mediate the primary fast synaptic response in a glutamate-using synapse. Instead, they are more robustly activated under special conditions, such as high-frequency synaptic activation or with concurrent depolarization. Under normal physiological conditions, a single activation of a synaptic pathway results in an AMPA receptor-mediated synaptic response but with little detectable NMDA receptor-mediated component *(120)*. The apparent absence of a NMDA receptor synaptic component is owing to the voltage-dependent properties of the NMDA receptor. In contrast to most other ligand-gated ion channel receptors, NMDA receptor currents are both ligand gated and voltage gated *(121,122)*. Greater NMDA receptor responses occur when the cell is moderately depolarized from the resting membrane potential. Thus, partial depolarization results in larger NMDA-evoked currents, even though the voltage-gradient driving force responsible for these currents is reduced. The voltage dependency of NMDA receptors is because of the preferential blockade of NMDA receptor channels by Mg^{++} ions at negative membrane potentials *(123,124)*. Hence, at the normal negative resting membrane potential, physiological concentrations of Mg^{++} ions potently block NMDA receptor channels. However, if the cell has been previously depolarized, such as during high-frequency stimulation, Mg^{++} ions can no longer block the NMDA receptor channel and a larger current results. Thus, NMDA receptor responses are dependent upon the immediately preceding history of the cell, with larger NMDA receptor responses occuring if the cell is currently depolarized from a previous synaptic signal.

3.1.2. Calcium Permeability

NMDA receptor ion channels are highly permeable to calcium *(125)*. Whereas most ligand-gated cation channels are permeable to just Na^+ and K^+ ions, the NMDA receptor is also permeable to Ca^{++} ions. Calcium itself is a potent second messenger, able to regulate the functions of a large variety of intracellular signaling systems. It is this influx of calcium

that is thought to be responsible for many of the subsequent biological actions induced by NMDA receptor activation. With the combination of the two distinctive physiological properties, voltage dependency and calcium permeability, NMDA receptors are able to use calcium as a trigger for experience-dependent plasticity, most notably the phenomenon known as long-term potentiation (LTP).

3.1.3. Slow, Long-Lasting Channel Currents and Desensitization

The AMPA and kainate receptors can activate in less than a millisecond and desensitize in 1–10 ms. In contrast, NMDA receptor activation occurs much more slowly, peaking well after the AMPA receptor response has desensitized (20–30 ms; ref. *120*). Furthermore, the NMDA receptor response has a half-life of 100–5000 ms. Thus, at fairly low frequencies, some NMDA receptors are continuously activated.

Unlike AMPA and kainate receptors, NMDA receptors do not show as complete or rapid desensitization. They do, however, desensitize. At least three different mechanisms contribute to NMDA receptor desensitization: (a) Ca^{++}-dependent inactivation, (b) glycine/Ca^{++}-independent desensitization, and (c) glycine-sensitive desensitization (for review, *see* ref. *126*). The Ca^{++}-dependent inactivation is displayed by NR1a/NR2A and NR1a/NR2D receptors, but not by NR1a/NR2B and NR1a/NR2C receptors *(127)*. The glycine/Ca^{++}-independent desensitization is most robust with NR1a/NR2A, less apparent with NR1a/NR2B, and not seen in NR1a/NR2C *(127)*. Glycine-sensitive desensitization is reversed by saturating concentrations of glycine. The apparent desensitization is thought to be a result of glutamate binding causing a reduction in glycine affinity *(128)*.

3.1.4. Glycine or D-Serine Coagonist

Although NMDA receptors are termed glutamate receptors, they require the presence of another agonist, glycine or D-serine, to achieve channel activation *(129,130)*. Thus, L-glutamate or NMDA alone is insufficient to evoke an NMDA receptor-mediated response. Likewise, glycine alone is insufficient to evoke an NMDA receptor response, but glycine together with L-glutamate results in full receptor activation. In many preparations, the glycine-binding site appears to be saturated, or nearly saturated, in the absence of exogenous glycine or D-serine. Hence the receptor is responsive to L-glutamate. Because of the assumed tonic saturation of the glycine site, relatively few studies have examined the role of cellular mechanisms that modulate glycine levels in the synapse. However, there is some evidence that both glycine and D-serine may have their extracellular levels regulated at subsaturating concentrations, and hence may play a role in modulating the excitability levels of NMDA receptors *(131)*. In recent years, the role of D-serine has become an important topic and the enzymes that regulate its synthesis have been characterized *(132)*. A provocative finding is that some schizophrenics contain a genetic mutation in a protein that modulates the enzyme oxidizing D-serine—a finding consistent with the NMDA-receptor hypofunction hypothesis of schizophrenia *(133)* and the recent report of reduced D-serine in schizophrenics *(134)*. (*See* Chapter 7 for further discussion).

3.1.5. NMDA Receptor Cellular Functions

It is now well established that NMDA receptor activation is necessary for most forms of LTP that have been observed in brain tissue (for reviews, *see* refs. *135–137*). In LTP, high-frequency stimulation of an afferent input leads to long-lasting enhancement of the synaptic response when the afferent is tested later with a single stimulation. This phenomenon is thought to represent a cellular mechanism for learning and accordingly,

NMDA receptor blockade (or NMDA receptor knockout) can block some forms of learning *(138)*. Interestingly, NMDA receptor activation is also required for some instances of the opposing phenomenon of LTD. In LTD, the use of other stimulus paradigms leads to a depression of synaptic responses. Currently, it is thought that the entry of low levels of calcium causes LTD whereas higher levels of calcium influx lead to LTP *(139)*. Alternatively, we have presented evidence that the NMDA receptor subtypes triggering LTP and LTD are pharmacologically distinct *(140)*.

NMDA receptor activation is also key to several forms of experience-dependent plasticity wherein experience (e.g., visual stimulation) causes both the pruning and expansion of afferent terminals onto their target fields. The most well-characterized example is the formation of ocular dominance columns owing to binocular visual stimulation. For a review of this field, see ref. *139*. NMDA receptor activation is also required for somatosensory mapping of the whisker representations in cortex and trigeminal nucleus (the "barrels") *(141,142)*.

In addition to the above-mentioned plasticity mechanisms, NMDA receptor activation is required for plasticity related to pain enhancement in some model systems *(143,144)* and in the development of opiate tolerance *(145)*. In still other neuronal systems such as in the spinal cord, NMDA receptor activation is necessary for proper rhythm generation *(146)*. With the presence of NMDA receptors throughout the CNS, and a diversity of signaling systems with which NMDA receptors may be interacting, NMDA receptors are likely to have many other presently unknown functions in the CNS.

3.2. Molecular Properties of NMDA Receptors

3.2.1. NMDA Receptor Subunits

NMDA receptors are a multimeric complex composed of subunits derived from three related families: NR1, NR2, and NR3 subunits (for reviews, *see* refs. *147–149)*. Both NR1 and NR2 subunits are required for receptor function. The NR1 subunit contains the glycine-binding site whereas the NR2 contains the L-glutamate-binding site *(150)*. In contrast, the NR3 subunit appears to modulate receptor function in a limited number of situations *(151)*. Multiple lines of evidence suggest that there are two NR1 subunits and two NR2 in a single NMDA receptor complex. Thus NR1/NR2 complexes may exist as a tetramer *(152)*. However, the effect of including NR3 subunits upon the stoichiometry is unknown.

The NR1 subunit (also termed NMDAR1 for rat and ζ1 for mouse), is over 900 amino acids in length and displays 22–26% identity with AMPA and kainate receptor subunits *(153,154)*. The NR1 gene consists of 22 exons; exons 5, 21, and 22 can be alternatively spliced, resulting in eight distinct NR1 isoforms *(155,156)*. Exon 22 includes a stop codon, and hence has a different C-terminal than proteins that do not have exon 22.

Subsequent to the cloning of the NR1 subunit, the NR2 subunits were identified *(157–162)*. The four members of the NR2 subunit family (NR2A–NR2D for rat and ε1–ε4 for mouse) are the products of four separate genes. The physiological and pharmacological properties of native and recombinant NMDA receptors vary with the specific NR2 subunit present in the heteromeric complex *(160,163–165)*.

3.2.2. NR3 Subunits: When a Glutamate Receptor Is Not a Glutamate Receptor

The NR3A was initially termed χ − 1 *(166,167)*. Among various glutamate receptor subunits, NR3A has highest identity with NR1 and NR2 subunits (27%). When coexpressed with NR1/NR2B in oocytes, it reduces the magnitude of NMDA-evoked current responses. Further suggesting that NR3 is an NMDA receptor subunit is that NR1

subunits are required for the surface expression of NR3 subunits, that NR3 subunits are associated with both NR1 and NR2 subunits, and that NR3 subunits alter the channel properties of NR1/NR2 receptor complexes *(168)*. Intriguingly, in the NR3A knockout mouse *(151)*, NMDA receptor-mediated responses are larger and spine density is increased. More recently, an additional protein has been identified, NR3B, which in the human is 57% identical to NR3A *(169)* and in the mouse is 51% identical to NR3A *(170)*. This subunit is highly expressed in midbrain and lower regions *(171)*, particularly in motoneurons of the brainstem and spinal cord *(170)*.

A striking finding is that NR1/NR3 subunit complexes are functional when coexpressed in *Xenopus* oocytes *(172)*. Consistent with the presence of an NR1 subunit and the absence of an NR2 subunit, this receptor is activated by glycine and not by glutamate. Thus, this receptor is formed from two glutamate receptor subunits but is not a receptor for glutamate. This receptor complex is inhibited by D-serine, calcium impermeable, and insensitive to the classic NMDA receptor channel blockers MK-801 and Mg^{++}. There is evidence that these may exist in brain.

3.2.3. NMDA Receptor Subunit Structure

3.2.3.1. The Amino Terminal Domain

The N-terminal NMDA receptor subunits (the ATD or amino terminal domain; *see* Fig. 1) has homology to bacterial amino acid-binding protein LIVBP (leucine- isoleucine- valine-binding protein; ref. *173* and is the general site where modulators such as zinc and ifenprodil influence the desensitization properties of NMDA receptors *(174)*. As in the other glutamate receptor subunits, this region has been proposed to play a role in subunit assembly; however, functional receptors are formed even if this domain is removed *(175)*.

3.2.3.2. The S1/S2 Domain

The S1/S2 domain has homology to other bacterial amino acid-binding proteins (glutamine-binding protein QBP and lysine, arginine, ornithine binding protein—LAOBP; refs. *173,176*), and it is these domains on NR1 and NR2 that is thought to be the primary ligand binding sites for glycine and glutamate respectively *(40)*. The crystal structure of the S1/S2 region of the NR1 subunit has recently been described. This study provides detailed description of the mechanism by which glycine and 5,7-dichlorokynurenic acid bind to the NR1 subunit *(47a)*. As expected, this structure is very similar to the GluR2 crystal structure with the S1/S2 structure forming a "clamshell" bilobed structure that closes in the agonist-bound state.

3.2.3.3. The M2 Domain

NMDA receptor ion channels are thought to have the same general structure as postulated for AMPA and kainate receptors. Thus, the M2 re-entrant loop is the primary portion of the sequence lining the ion channel pore. Portions of M1 and M3 also appear to line the pore. An important difference between NMDA and non-NMDA receptors is that the amino acid at the critical Q/R site of AMPA and kainate receptors is an asparagine, which contributes to the calcium permeability of NMDA receptors *(177)*.

3.2.3.4. The C-terminal Domain: Site of Intracellular Protein–Protein Interactions

The C-terminal tail of NMDA receptor subunits, the region following the last transmembrane domain, is located intracellularly and is the primary site for intracellular

protein–protein interactions. For both the NR1 and NR2 subunits, a variety of proteins are known to be interacting with this domain. NR2 subunits with truncated C-termini form functional ion channels, but their ability to be properly localized within the cell is impaired. Furthermore, since NR1 and NR2 C-termini bind to various signaling and cytoskeletal proteins, various downsteam signals require the specific localization at the NMDA receptor ion channel. Mice expressing truncated NR2 subunits act much like mice missing the subunit altogether even though functional ligand-gated ion channels are formed *(178)*.

A key protein family that organizes many of these protein-protein interactions are the membrane-associated guanylate kinase proteins, of which PSD-95 (SAP90) is the prototype. The family of PDZ domain-containing proteins related to PSD-95 are characterized by having three N-terminal PDZ domains followed by an SH3 (src homology 3) domain and a guanylate kinase (GK) homology domain *(179)*. Each of these domains mediate specific protein–protein interactions. Specific PDZ domains interact with specific sequences in the C-terminal of proteins and specific SH3 domains bind to specific, proline-rich sequences in their target proteins. The GK domain is enzymatically inactive and binds to specific sequences in GKAPs (GK-associated proteins). The PSD-95 family includes PSD-95, SAP97, SAP102, and Chapsyn-110.

The C-terminal of the NR2B (and the NR2A) subunit ends with the sequence SIESDV, which preferentially interacts with the second PDZ domain (PDZ2) of PSD-95 *(180)*, SAP102 *(181,182)*, and potentially other PDZ-containing proteins. The NR2C and NR2D subunits end in SLESEV, which presumably slightly changes the PDZ-selectivity of the C-terminal. NR1-3a has been reported to bind to PSD-95 *(183)*. In addition to the roles of clustering and anchoring, these scaffolding proteins allow for spatially ordered signal transduction systems. Hence NMDA receptor activation can preferentially activate multiple calcium-activated processes by virtue of the localization of the calcium-responsive systems. For example, calcium-sensitive neuronal nitric oxide synthase *(184)*, and a Ca^{++}-calmodulin kinase II-phosphorylated neuronal ras-GAP *(185,186)* can also associate with PSD-95. Thus, NMDA receptor Ca^{++} influx can selectively modulate NO production and the ras effector pathways such as MAP kinase. In recent years, the list of identified proteins that interact with NMDA, AMPA, and kainate receptors has become quite long. For recent reviews, *see* refs. *187,188*.

3.2.4. NMDA Receptor Heteromeric Complexes

Most studies presently favor a tetrameric complex for the NMDA receptor *(152)*. This is consistent with evidence of two glutamate- and two glycine-binding sites *(189)* and evidence for two NR1 subunits in a complex *(190)*. A tetrameric structure is also supported by the construction of a functional receptor consisting of four subunits joined in tandem *(191)*. This work also suggests that the complex may form as a dimer of dimers in the arrangement NR1/NR1/NR2/NR2. However, there is other evidence that supports a pentameric structure *(192)*. For NMDA receptors that contain an NR3 subunit, there is presently no information regarding subunit stochiometry.

Because NR2 subunits confer distinct physiological and pharmacological properties to NMDA receptors, an important question is whether there are heteromeric complexes that contain more than one type of NR2 subunit. Most studies support the existence of heteromeric complexes. When coexpressed in *Xenopus* oocytes NR1/NR2A/NR2C subunits have properties consistent with a heteromeric structure *(193)*. Likewise, coexpressed

NR1/NR2A/NR2B *(194,195)*, coexpressed NR1/NR2B/NR2D *(196)*, and coexpressed NR1/NR2A/NR2D *(197)* form receptors, which have properties suggestive of heterotrimeric receptor complexes. Such heterotrimeric complexes may exist in brain. Some NR2A subunits are coimmunoprecipitated with NR2B antibodies *(198)*. Furthermore, from single channel analysis, NR1/NR2B/NR2D receptors appear to be found in cerebellar Golgi cells *(199)*.

3.3. Pharmacology of NMDA Receptors

3.3.1. Glutamate Recognition Site Agonists

L-Aspartate and L-glutamate are potent NMDA receptor agonists *(11)*. These typify the structural requirements for agonist activity: two negative charge groups (preferably both carboxys) separated by three or four carbon–carbon bond lengths (aspartate and glutamate, respectively); the α-carbon should be in the S- (or L)-configuration, and the ω-charge group should be a carboxy but can also be a sulfonic acid, or a tetrazole group.

Several rigid glutamate analogs have been constructed that are potent NMDA receptor agonists that provide insight into the optimal configuration of charges to obtain agonist activity. These compounds include homoquinolinate, (2S,3R,4S) 2-(carboxycyclopropyl)glycine (L-CCG-IV) *(200)*, (1R,3R) 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), and 1-aminocyclobutane-1,3-dicarboxylic acid (ACBD). The high potency of these structures suggests that L-glutamate is active in a folded conformation *(201)*. (For structures of key NMDA receptor compounds, *see* Fig. 5).

3.3.2. Glutamate Recognition Site Competitive Antagonists

Structure-activity studies indicate several features that are important for antagonist action at the glutamate recognition site of the NMDA receptor complex (for a detailed review, see ref. *202*). Antagonists have the same general structural requirements as do agonists, with the exception that antagonist activity occurs (a) by increasing the chain length between the two negative charges, (b) by replacing the S-chiral center (as in *s*-glutamate) with an R stereoisomer (as in *R*-AP5), and (c) by replacing the distal negative carboxy with a phosphate group (as in AP5 compared to amino-adipate). Experimentally useful antagonists include: The R isomers of AP5, AP7, CPP, CPPene, CGS19755, and CGP37849 *(69)*. A variety of other multiring structures and additional groups have also been shown to increase the antagonist potency of the basic AP5/AP7 structure. These compounds include EAB515 *(203)*, LY 274614 *(204)*, and PBPD *(196)*.

Because the NR2 subunit has a glutamate-binding site, the four different NR2 gene products might be expected to each contain pharmacologically distinct glutamate-binding sites. Indeed, studies have confirmed that four distinct pharmacological profiles can be seen for native and recombinant NMDA receptors containing the different NR2 subunits *(163,164,196,205)*. However, at the present, glutamate site antagonists only weakly discriminate between the different NR2 subunits. In general, AP5-like antagonists (e.g., AP5, CPP, CGS19755) display a NR2 subunit selectivity pattern of NR2A > NR2B > NR2C > NR2D (high to low affinity). Interestingly, the larger, multiring antagonists (e.g., EAB515, LY 274614, and PBPD) display varied patterns of NR2 selectivity *(196,206)*. We have recently identified the large multiring antagonist PPDA as a high-affinity antagonist that has significantly higher affinity at NR2C and NR2D subunits than at NR2A/NR2B *(140)*.

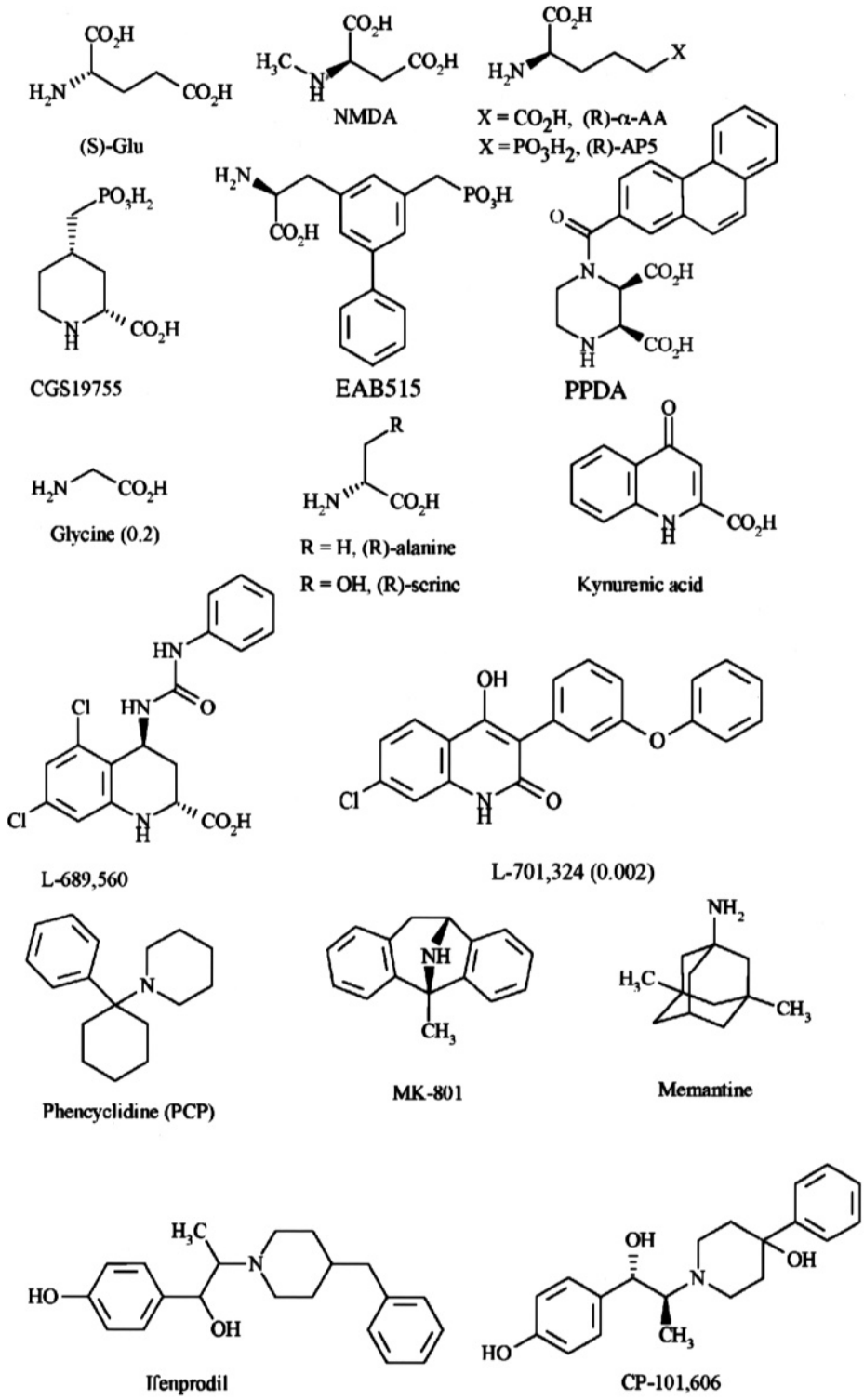

Fig. 5. Structures of key NMDA receptor compounds.

3.3.3. NMDA Receptor: Glycine Recognition Site Agonists

Glycine binds specifically to the NR1 subunit *(207)*, however, the NR2 subunits confer subtype-specific pharmacological properties to the glycine-binding site in a heteromeric receptor complex. Potencies for the agonists glycine, D-serine, D-alanine, and 1-aminocarboxycyclobutane are significantly lower at NR1/NR2A receptors than receptors composed of NR1/NR2B, NR1/NR2C, and NR1/NR2D (ranked in order of increasing potency; refs. *158,164,208–211*).

1-Amino-1-carboxycyclopropane (ACPC) is a selective agonist of the glycine binding site with an intrinsic activity of 92% *(212)*. ACPC has a structure that is similar to that of the amino acid agonists, while being incorporated into a cyclopropyl ring. Expanding the cyclopropyl ring of ACPC to a cyclobutyl ring results in 1-aminocarboxycyclobutane (ACBC), a partial agonist with low efficacy *(213)*. Increasing the size of the ring structure of ACBC to cyclopentane results in the amino acid derivative cycloleucine, a full antagonist of the NMDA glycine-binding site with weak potency *(214)*. HA-966 *(215)* and D-cycloserine *(216)* are also glycine site partial agonists with roughly 15% and 50% intrinsic activity, respectively. In contrast to NR1/NR2A and NR1/NR2B, D-cycloserine, at NR1/NR2C receptors, has higher efficacy than glycine itself *(217)*.

3.3.4. NMDA Receptor: Glycine Site Antagonists

One of the first glycine-binding site antagonists to be identified was kynurenic acid *(218)*. Kynurenic acid is a weak nonselective excitatory amino acid antagonist, the first selective glycine site antagonists are variants of this compound. They include 7-chlorokynurenic acid *(219)*, 5-7-dichlorokynurenic acid *(220)*, and 7-chloro-5-iodokynurenic acid (L-683,344) *(221)*. Other high-affinity antagonists include: (E)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1H-indole-2-carboxylic acid (MDL 105,519; ref. *222*), 7-chloro-4-hydroxy-3(3-phenoxy)phenyl-2(H)quinolone (L-701,324; ref. *223*), and (+/−)-4-(*trans*)-2-carboxy-5,7-dichloro-4-phenylamino-carbonylamino-1,2,3,4-tetrahydroquinoline (L-689,560, ref. *224*).

3.3.5. NMDA Receptor Channel Blockers

More than 20 yr ago, Lodge and colleagues discovered that ketamine and phencyclidine (PCP) can block NMDA receptor-mediated responses *(225)*. Since then, many compounds have been identified that block NMDA receptor action in an uncompetitive manner by binding to a site(s) within the open ion channel. NMDA receptor channel blockers are typified by the high-affinity compounds MK-801 (dizocilpine maleate), PCP, and TCP (1-[1-(2-thienyl)-cyclohexyl] piperidine). Each of these compounds display use-dependent and voltage-dependent blockade of the receptor complex. In both electrophysiological *(226)* and radioligand-binding *(227)* studies, channel blockade (or radiolabeled channel blocker binding) is dependent upon the activation of the receptor complex by agonist binding at both the glutamate- and glycine-binding sites.

3.3.6 Allosteric Modulatory Sites on the NMDA Receptor

3.3.6.1. Polyamines

Polyamines (e.g., putrescine, spermidine, and spermine) can modulate NMDA receptor activity. These compounds are found throughout the brain *(228)* and can be released following neuronal depolarization *(229)*. Polyamines have three effects on NMDA receptor activity: (a) glycine-dependent stimulation characterized by a polyamine-stimulated

increase in glycine affinity for its binding site, (b) glycine-independent stimulated increase in the maximal amplitude of NMDA receptor responses, and (c) voltage-dependent inhibition of NMDA receptor responses (for a review, *see* ref. *230*).

Polyamine sensitivity is subunit-dependent. Glycine-independent stimulation by spermine in recombinant receptors is inhibited by the N-terminal insert (exon 5) of the NR1 subunit *(231,232)*. In addition, the NR1 amino acid residue E342, is necessary for glycine-independent spermine stimulation *(233)* but has no effect on polyamine glycine-dependent potentiation or voltage-dependent channel block. Mutations at equivalent positions in NR2A and NR2B subunits had no effect on spermine stimulation.

The extracellular loop region between TM3 and TM4 of the NR1 subunit also participates in glycine-independent spermine stimulation as well as voltage-dependent channel block. Mutations in this region reduce glycine-independent polyamine potentiation and mutations of specific negatively charged amino acids in this sa me region on both NR1a and NR2B subunits reduced the voltage-dependent block by spermine *(234)*. Additionally, amino acids in a portion of the transmembrane-spanning regions of the NR1 subunits (TM1,2,3) are involved in spermine stimulation probably through allosteric effects or changes in gating processes *(235,236)*.

In addition to the NR1 subunit, the NR2 subunit also contributes to both the stimulatory and inhibitory effects of polyamines at NMDA receptors *(232,237,238)*. Polyamines cause glycine-independent stimulation at NR1a/NR2B receptors but not at NR1a/NR2A, NR1a/NR2C, or NR1a/NR2D receptors. However, glycine-dependent stimulation *(237)* and voltage-dependent inhibition *(239)* were seen at both NR1a/NR2A and NR1a/NR2B receptors. Taken together these data suggest that there are at least three distinct polyamine-binding sites on NMDA receptors.

3.3.6.2. Ifenprodil and Other NR2B Selective Compounds

A variety of other pharmacological agents bind and modulate NMDA receptor activity with a selectivity similar to the polyamines. Ifenprodil is an NMDA receptor antagonist *(240)* at a site separate from that of glutamate and glycine. Ifenprodil exhibits greater than a 100-fold selectivity for NR2B over NR2A-containing receptors *(165,241)* and very low affinity at NR2C- and NR2D-containing receptors *(238)*. A variety of other related compounds show NR2B selectivity; these include haloperidol *(242)*, CP-101,606 *(243)*, Ro 8-4304 *(244)*, and Ro 25-6981 *(245)*. Site-directed mutagenesis studies show that spermidine, haloperidol, and ifenprodil all have overlapping binding sites but that the specific molecular determinants required for high-affinity binding differ between each of these compounds *(194,241,242)*. These compounds have been useful for defining the actions of NR2B-containing receptors in brain.

3.3.6.3. Proton Inhibition

At low pH, NMDA receptor responses are inhibited *(246,247)*. Increased external protons suppress NMDA receptor currents by decreasing channel open probability. The proton site appears independent of agonist binding sites because proton blockade was noncompetitive with NMDA and glycine. Proton inhibition may represent an intrinsic mechanism to protect neurons from NMDA receptor excitotoxicity during pathological acidosis. The absence of the N-terminal insert of the NR1 subunit is required, like that of glycine-independent stimulation by spermine, for proton inhibition. Thus the presence of exon 5, and more specifically K211 in exon 5, potentiates NMDA receptor function through relief of

the tonic proton inhibition that is present at physiological pH *(248)*. Additionally, polyamine stimulation may be linked to the relief of tonic inhibition by protons suggesting that polyamines and protons share common molecular-binding determinants *(249)*, particularly within NR2B-containing receptors for which both are most selective.

3.3.6.4. Zinc

Zinc displays subunit-specific actions at recombinant NMDA receptors. At low concentrations, zinc (1 μ*M*) enhances homomeric $NR1_{OXX}$ (NR1 lacking the N-terminal insert) receptor responses while having no effect on homomeric receptors containing $NR1_{1XX}$ subunits *(155,250)*. At higher concentrations zinc inhibits both NR1 subunits with and without the N-terminal insert. Both of these phenomena occur without a change in the affinity for glutamate or glycine. The NR2 subunits also contribute to zinc's actions on NMDA receptors. Zinc displays a voltage-dependent inhibition of NMDA receptor responses in heteromeric NR1/NR2A and NR1/NR2B receptors and, at lower zinc concentrations, a voltage-independent inhibition of NR1/NR2A receptors *(251–253)*. This appears to account for the observation that the addition of heavy-metal chelators to buffer solutions significantly potentiates NR1a/NR2A, but not NR1a/NR2B, receptor responses *(252)*.

3.4. NMDA Receptor Radioligands

3.4.1. Glutamate Site Ligands

Radioligands represent a straightforward method of quantifying receptor density and distribution. Many different radioligands have been developed for the study of the glutamate-binding site of NMDA receptors: L-[^{3}H]glutamate, D-[^{3}H]AP5, [^{3}H]CPP, [^{3}H]CGS19755 *(12,19,254)*, and, of highest affinity, [^{3}H]CGP39653 *(255)*. Of these radioligands, only L-[^{3}H]glutamate labels all four NR2 subunits of native and recombinant NMDA receptors *(163,256)*, the other compounds (which are all antagonists) selectively label NR2A- and, to varying degrees, NR2B-containing receptors *(256)*. The agonist [^{3}H]homoquinolinate labels predominantly NR2B-containing NMDA receptors in rat brain *(257)*. Thus, there is still a need for subunit-selective radioligands for NR2C- and NR2D-containing receptors.

3.4.2. Glycine Site Ligands

The glycine-binding site on NMDA receptors can be labeled with a variety of radioligands. [^{3}H]Glycine itself labels NMDA receptors *(258)* as well as the antagonists [^{3}H]MDL 105,519 *(259)*, [^{3}H]5,7 dichlorokynurenic acid *(260,261)*, [^{3}H]L-689,568 *(262)*, and others. The glycine site antagonist ([^{3}H]CGP 61594) has been shown to display a high-affinity selectively for NR2B-containing receptors *(263)*. Glycine binding to the inhibitory glycine receptor, localized in the lower brainstem and spinal cord, can be distinguished from glycine binding to the NMDA receptor by using the inhibitory glycine receptor antagonist strychnine.

3.4.3. Channel Blocker and Polyamine Site Ligands

Many studies have used the radioligand [^{3}H]-MK801 to characterize the ion channel of NMDA receptors *(264)*. This agent has high affinity and is highly specific. [^{3}H]-TCP and [^{3}H]-PCP can also be used to label the NMDA receptor ion channel (though PCP is less specific, ref. *265*). An important factor to consider in using channel blocker radioligands is that these are usually slowly accessible to the closed NMDA receptor ion channel.

Thus the time required to achieve equilibrium reflects the degree of channel activation. This property can be useful, because channel blocker ligands can be used to measure channel activation/inhibition at short, nonequilibrium conditions or used under fully activated, equilibrium conditions to measure NMDA receptor density *(227)*.

Multiple radioligands have been described that can be used to examine the ifenprodil/polyamine binding site. These include [^{3}H]ifenprodil *(266,267)*, [^{3}H]Ro-25-6981 *(245)*, and [^{3}H]CP-101,606 *(268)*. These agents are selective for NR2B subunits.

3.5. NMDA Receptor Function: Information From Transgenic Studies

3.5.1. NR1 Knockouts

Each of the NMDA receptor subunits have been knocked out in mice. Additionally, some subunits have been over expressed or replaced with point mutations. The NR1 knockout is lethal neonatally *(269)*. In these animals, the brainstem barrel fields representing the whisker somatosensory map fail to develop *(141)*. The development of the cortical whisker representations were also found to be dependent upon NR1 when studied with the conditional NR1 knockout mouse in which NR1 is missing from excitatory cortical neurons *(142)*. Interestingly, mice expressing very low levels of NR1 display behavior consistent with schizophrenia and these behaviors are treatable with antipsychotics *(270)*. Consistent with extensive pharmacological evaluation, conditional knockout of CA1 hippocampal NR1 subunits blocks LTP in the hippocampus and blocks spatial learning *(271)*.

3.5.2. NR2 Knockouts

In the NR2A knockout, there is a reduction in hippocampal LTP and spatial learning *(272)* and a reduction in the conditioned eyeblink response *(273)*. Of the NR2 knockouts, only NR2B –/– mice do not survive, in part because of a loss of the suckling response *(274)*. These mice show a loss of LTD and NMDA receptor-mediated responses in the hippocampus and have impaired development of the barrel fields. NR2C knockouts show few effects *(275)*. NR2C subunits are predominantly found in the cerebellum where they are coexpressed with NR2A subunits *(160)*. When both NR2A and NR2C are knocked out there is a deficit in motor coordination *(276)*. As with NR2C, NR2D knockout effects are relatively subtle. NR2D knockout mice display reduced spontaneous activity *(277)*, reduced sensitivity to stress *(278)*, and a block of pain in a specific model of allodynia *(279)*.

3.5.3. NR3 Knockouts

Consistent with NR3 coexpression studies where NR3 expression inhibits NMDA receptor currents, NR3 knockout mice display an increase in NMDA-induced currents. The NR3–/– mice also display increased spine density, increased spine head length, and increased spine neck length in cortical neurons *(151)*.

4. CONCLUSIONS

The glutamate-gated ion channels underlie most of the fast excitatory synaptic transmission in the vertebrate CNS. The identification and cloning of these receptors have revealed extensive diversity in molecular structure owing to multiple subunits, alternative splicing, and RNA editing. With these diverse structures, glutamate receptors display a wide variety of channel kinetic properties, desensitization mechanisms, cellular localization mechanisms, and biochemical signal transduction mechanisms. Thus, instead of a simple fast-on, fast-off depolarizing signal, the ionotropic glutamate receptors display an array of highly specialized

physiological properties that can give complex and distinctive qualities to signaling in specific synapses. Furthermore, these specific signaling properties can be regulated by a variety of mechanisms. Consequently, in various CNS disease states, there is considerable potential for seemingly subtle alterations in receptor function that may have profound clinical implications.

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5
Metabotropic Glutamate Receptors

Krystyna Ossowska

1. INTRODUCTION

L-Glutamate, one of the main neurotransmitters in the central nervous system (CNS), acts on two groups of receptors: (a) a group of ionotropic receptors that includes *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), and kainate receptors, and *(2)* a group of metabotropic receptors (mGluRs). Ionotropic glutamate receptors, which are ligand-gated ion channels permeable for Ca^{2+}, Na^{+}, and K^{+}, are responsible for fast and relatively large changes in membrane conductance *(1,2)* and are covered in Chapter 4. In contrast, stimulation of mGluRs evokes a complex cascade of intracellular events that indirectly modulates neuronal excitability and produces delayed and slow synaptic currents *(1,2)*. Both groups of glutamate receptors are involved in several physiological and pathological functions including neuronal growth and plasticity, neurotoxicity, cognitive and motor behavior, depression, anxiety, drug abuse, epilepsy, and others. Some ligands of ionotropic receptors have already been introduced to clinical practice (e.g., amantadine, memantine, D-cycloserine), whereas mGluRs may be considered as an emerging target for the treatment of several diseases (e.g., Parkinson's disease, depression, epilepsy and others; *see also* Chapters 10, 19, 21, and 22).

2. MOLECULAR STRUCTURES

The metabotropic glutamate receptors were discovered in the mid-1980s, when Bardsley and Roberts *(3)*, Sladeczek et al. *(4)*, Nicoletti *(5–7)*, and others (for review, *see* ref. *2*) described glutamate-dependent phosphoinositide hydrolysis in the striatal and cerebellar cell cultures, as well as in brain slices, synaptosomes, and glial cells. Shortly thereafter, Sugiyama et al. *(8)* used for the first time the term "metabotropic" for receptors expressed in *Xenopus* oocytes transfected with rat brain mRNA. These receptors were preferentially activated by quisqualate, which triggered, via interaction with G protein, phosphoinositide hydrolysis leading to the formation of inositol 1,4,5-triphosphate (IP3) and mobilization of intracellular Ca^{2+} *(8)*.

Metabotropic glutamate receptors belong to a family 3 of heptahelix G protein-coupled receptors (GPCRs), which exhibits low-sequence homology (~12%) with classic rhodopsin-like family 1 *(9)*. Apart from mGluRs, the family 3 of GPCRs contains also

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

parathyroid Ca^{2+}-sensing receptor, γ-aminobutyric acid $(GABA)_B$ receptor, and putative olfactory, pheromone and taste receptors *(9)*. Until now eight mGluRs, composed of 501–1199 amino acids, coded by eight genes, have been identified in rats, mice, and humans, and cloned (cf. ref. *2*). They have been classified into three groups according to their sequence homology, signal transduction, and pharmacological properties. Group I contains two receptors: mGluR1 (five isoforms: a, b, c, d, e) and mGluR5 (two isoforms: a, b). Group II includes two receptors: mGluR2 and mGluR3; and group III comprises four receptors: mGluR4 (two isoforms: a, b), mGluR6, mGluR7 (two isoforms; a, b), and mGluR8 (three isoforms: a, b, c) (cf. ref. *2*). These receptors are composed of an exceptionally long N-terminal extracellular domain of approx 560 amino acids, a seven-transmembrane (TM) domain, and a C-terminal intracellular domain, that is submitted to alternative splicing, which leads to formation of the above-mentioned isoforms *(9–11)*. MGluR1e is an exceptional splice variant (N-terminally truncated 578 amino acid protein) coded by a gene having an additional exon inserted before the seven-TM domain, which contains an in-frame stop codon *(11)*. N-terminal extracellular and seven-TM domains in other isoforms are separated by a cystein-rich region *(9,11)* (Fig. 1). Receptors belonging to one group exhibit 70% sequence homology, whereas homology between groups amounts to only 45%. *(11)*.

Glutamate, agonists, and competitive antagonists bind to the extracellular domain, which forms two globular domains separated by a cleft *(9,12,13)*. Such a flytrap configuration of this domain closes glutamate that leads to changes in conformation of the TM domain region and to alterations of intracellular signaling *(9)* (Fig. 1). In contrast, allosteric binding sites for positive modulators (allosteric enhancers) or noncompetitive antagonists are formed by the TM helices in mGluR1 *(14,15)*, mGluR5 *(14)*, mGluR2 *(16)*, and mGluR4a *(17)*. The second and the third intracellular loops and C-terminal tail are responsible for coupling with G proteins (Fig. 1). The least conservative second loop determines selectivity, whereas the third, highly conservative one is decisive for activation of G protein *(9)*. All mGluRs form homodimers, which is important for receptor activation *(9)*.

3. G PROTEINS AND SECOND MESSENGERS

3.1. Group I mGluRs

Receptors belonging to group I mGluRs (mGluR1 and mGluR5) couple to pertussis toxin (PTX)-insensitive G protein ($G_{q/11}$), which activates phospholipase Cβ (PLCβ) (cf. refs. *11,18*). This enzyme catalyzes phosphoinositide hydrolysis with a production of IP3 and diacylglycerol (DAG) (Fig. 2). Stimulation of PLC by mGluR1a activation is also partly dependent on the PTX-sensitive G_i/G_o protein (cf. ref. *11*). Moreover, agonist binding at mGluR1a in certain cell lines but not in neurons or astrocytes stimulates adenylate cyclase (cf. ref. *11*). Furthermore, some mGluR1 responses, e.g., activation of a nonreceptor tyrosine kinases belonging to Src family, may be independent of G proteins *(19)*.

Stimulation of the group I mGluRs increases an intracellular calcium ($[Ca^{2+}]_i$) concentration (Fig. 2). However, kinetics of this process differ depending on the activated receptor. Activation of mGluR5a induces oscillations of $[Ca^{2+}]_i$ *(20)*, whereas stimulation of mGluR1a produces a single peak followed by a plateau of $[Ca^{2+}]_i$ level *(9,21)*. Mobilization of intracellular $[Ca^{2+}]$ induced by stimulation of these receptors depends on at least three processes: (1) activation of PLCβ leading to a production of IP3. IP3 induces Ca^{2+} release from internal stores via activation of specific receptors (IP3Rs)

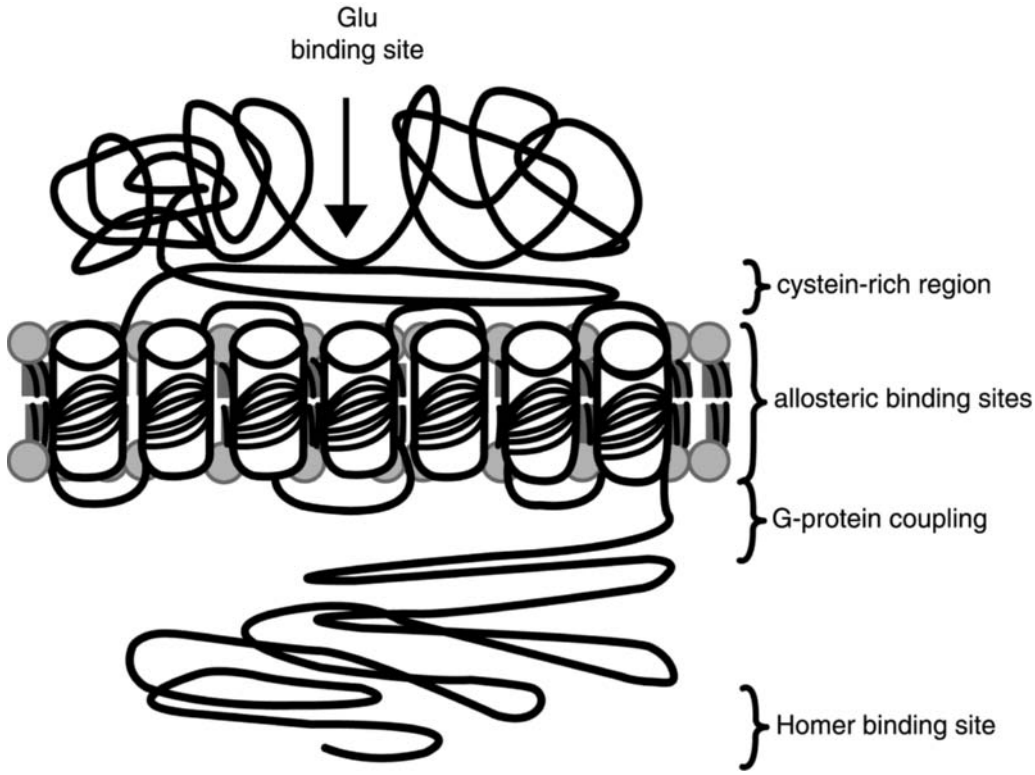
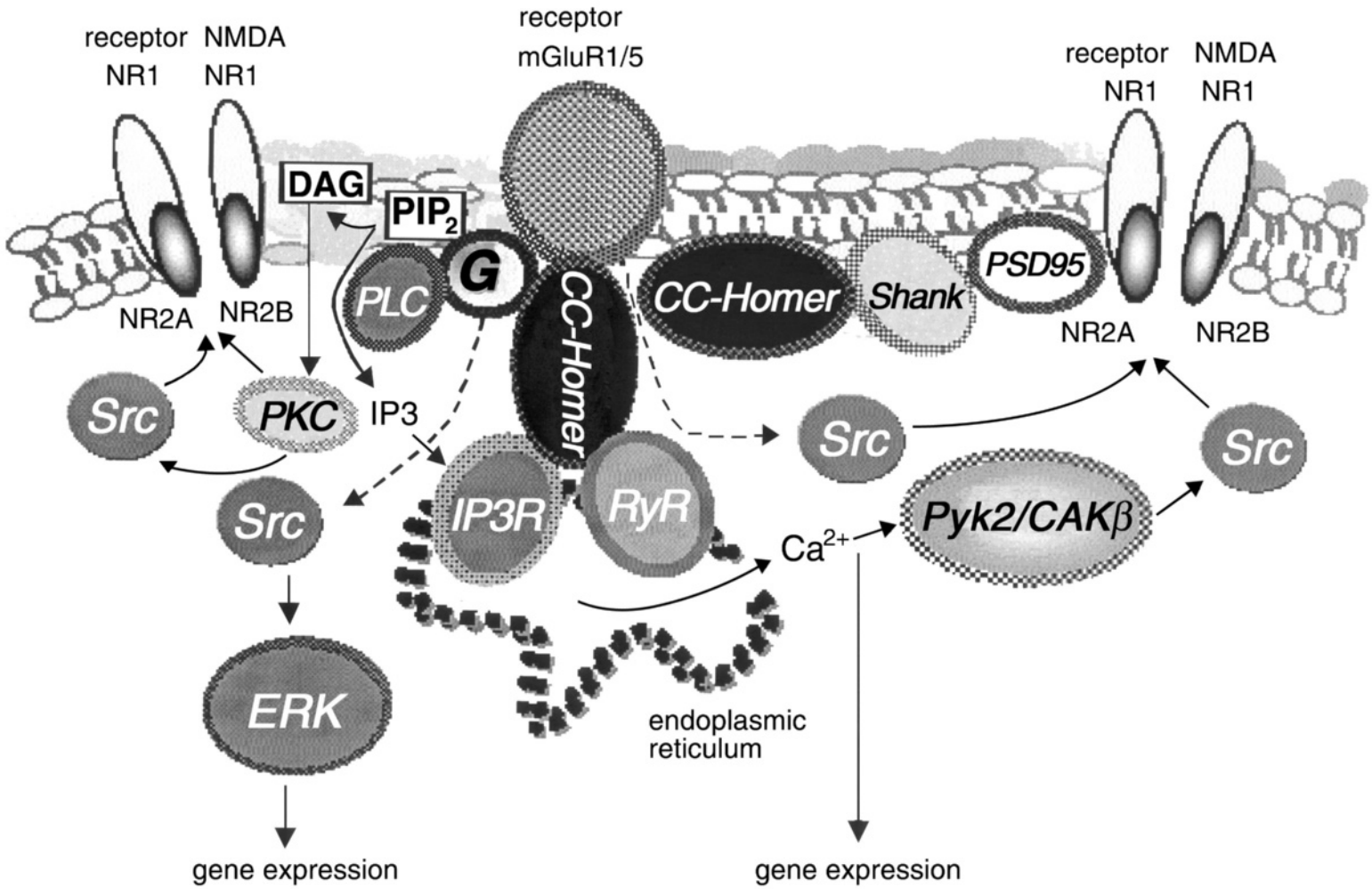


Fig. 1. A model of metabotropic glutamate receptor.

associated with endoplasmic reticulum *(10)* (Fig. 2); (2) mobilization of Ca^{2+} from internal stores via ryanodine-sensitive receptors (RyRs) (Fig. 2) *(10)*. These receptors colocalize with IP3Rs on endoplasmic reticulum, but it is not known whether RyRs-sensitive stores constitute the same calcium pool as those sensitive to IP3Rs; (3) Ca^{2+} influx through plasma-membrane voltage-dependent L-calcium channels. This process is also ryanodine-sensitive *(10)*.

Homer proteins may be involved in all the above-mentioned processes contributing to an increase in $[Ca^{2+}]_i$. These proteins constitute a physical link between mGluRs and Ca^{2+} internal stores (Fig. 2) *(10,22)*. Homer proteins form dimers (CC-Homers) by coiled-coil interaction of C-terminal regions that contain a leucine-zipper motif. Their N-terminals recognize, in turn, a proline sequence (PPXXFR) of the distal region of C-terminal domain of mGluR1a or mGluR5a/b, on one side, and the same sequence of IP3Rs or RyRs, on the other *(22)*. Immunochemical studies have indicated that Homer proteins 3, 1b/1c co-immunoprecipitate with mGluR1a and IP3Rs, which suggests that they form a complex with these receptors *(22)*. A family of Homer proteins includes also Homer 1a, which is a product of an immediate early gene (IEG), transiently induced by physiological synaptic stimuli. This protein is devoid of leucine-zipper sequence and does not form dimers. Therefore, in spite of the fact that Homer 1a binds either to mGluRs or to IP3Rs, it cannot crosslink mGluRs and internal Ca^{2+} stores. Contrariwise, a competition of Homer 1a with CC-Homers for common binding sites disrupts the link induced by the latter proteins and inhibits the mGluRs-evoked Ca^{2+} release *(22)*.


receptor NMDA
NR1 NR1
receptor
mGluR1/5
receptor NMDA
NR1 NR1
DAG
PIP2
G
PLC
CC-Homer
CC-Homer
Shank
PSD95
NR2A
NR2B
NR2A
NR2B
Src
PKC
IP3
Src
IP3R
RyR
Src
Src
Pyk2/CAKβ
Ca2+
ERK
endoplasmic
reticulum
gene expression
gene expression

Group I mGluRs (mGluR1 and mGluR5) couple also, independently of Ca^{2+}, to another intracellular signaling pathway, viz. the extracellular signal-regulated kinase (ERK) cascade (Fig. 2). ERKs are a subgroup of the family of the mitogen-activated protein kinases (MAPKs), which are key regulators of gene expression, cell proliferation, differentiation, and cell survival. It has been found that stimulation of either mGluR1a or mGluR5a phosphorylates ERKs, which leads to their activation *(23,24)*. This process is dependent on a G protein (Gi/Go for mGluR1a, Gq for mGluR5a) and nonreceptor tyrosine kinases (Src) (Fig. 2) *(24)*. Tyrosine receptor kinase seems to be also involved in activation of ERK cascade by mGluR1a *(24)*.

3.1.1. Regulation of Group I mGluR Function by Protein Kinases

Several recent studies indicate that the function of mGluRs may remain under positive or negative control of protein kinases. It has been found that an increase in $[Ca^{2+}]_i$ induced by mGluR1a is dependent on protein tyrosine kinases. It has been hypothesized that these kinases may phosphorylate tyrosine residues of either G protein, or the receptor itself, and in this way may activate IP3/Ca^{2+} signaling *(18,25)*. However, the most recent study has shown that phosphorylation of mGluR5 by protein tyrosine kinases does not influence phosphoinositide hydrolysis *(26)*.

In contrast, phosphorylation of serine and threonine residues of mGluR1 and mGluR5 by protein kinase C (PKC), which is an enzyme activated by DAG (Fig. 2), leads to desensitization of these receptors and a drop of IP3 production and $[Ca^{2+}]_i$ level *(9,20,23)*. In accordance with this view, the previously described mGluR5-induced oscillations of $[Ca^{2+}]_i$, result probably from a sequence of activations and PKC-dependent inactivations of this receptor *(20)*. On the other hand, PKC-induced phosphorylation of mGluR5 is inhibited by calmodulin, which binds to C-terminal domain of this receptor in a Ca^{2+}-dependent manner *(27)*.

G protein-coupled receptor kinases (GRKs) may be additionally involved in the desensitization of mGluRs *(21)*. Such a GRK-induced process has been described for mGluR1a. In the presence of agonist, this receptor is phosphorylated by GRK4. Then, arrestin1a and dynamin, which act as adaptors between the phosphorylated receptors and components of endocytotic machinery, bind to mGluR1a. This results in the receptor uncoupling from G

Fig. 2. Complex intracellular processes triggered by stimulation of mGluR1/5 receptors, and their interactions with *N*-methyl-D-aspartate (NMDA) receptors. MGluR1/5 receptors couple with G protein and activate phospholipase C (PLC), which catalyzes hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) with a production of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). DAG activates protein kinase C (PKC), whereas IP3 stimulates specific receptors (IP3R) localized on the endoplasmic reticulum, thereby leading to Ca^{2+} release from internal stores. MGluR1/5 receptors may also stimulate Ca^{2+} release via stimulation of ryanodine receptors (RyR). MGluR1/5 are physically linked with IP3R and RyR via CC-Homer proteins. Moreover, these receptors crosslink NMDA receptors via CC-Homers and/or postsynaptic density proteins, Shank, and PSD-95. Stimulation of group I mGluRs activates different tyrosine kinases belonging to Src family, in a manner dependent on or independent of G protein, which results, e.g., in activation of NMDA receptors (phosphorylation of NR2A/NR-2B subunits) or activation of extracellular signal-regulated kinase cascade (ERK). NMDA receptors may be phosphorylated and activated also by PKC or Ca^{2+}- and calmodulin-activated kinases. Sometimes activation of Src kinases may depend on PKC, or Ca^{2+} and Pyk2/Cell adhesion kinase (CBK)β. For further details, *see* text.

protein, internalization, and a decrease in phosphoinositide hydrolysis and Ca^{2+} level *(21,28)*. The previously mentioned data show that a prolonged stimulation of these receptors by specific agonists leads to their homologous desensitization via activation of PKC and GRKs *(9,21)*. However, the most recent study carried out on slices of rat globus pallidus has indicated that in some cases desensitization of mGluR1 may need coactivation of mGluR5 *(29)*. This is a heterologous desensitization that involves PKC *(29)*. ERK pathway becomes also desensitized as a result of continuous stimulation of mGluR5. However, the basic mechanism of this process is unknown because it does not involve PKC activation *(23)*.

3.2. Group II and III mGluRs

It is generally accepted that both groups II and III mGluRs couple to PTX-sensitive Gi/Go proteins (cf. ref. *30*). Stimulation of these receptors inhibits adenylate cyclase activity and decreases cyclic adenosine monophosphate (cAMP) level (cf. ref. *30*). However, this process does not seem to be the sole alteration in second messenger systems evoked by activation of these receptors. Stimulation of recombinant mGluR2 in CHO (Chinese hamster ovary) cells has been reported to activate PLC and phosphoinositide hydrolysis via $G_{\alpha 15}$ subunit, a member of the G_q protein family *(31)*. However, in hippocampal slices selective agonists of group II mGluRs: (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate (LY 354740) and (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (2R,4R-APDC) increase phosphoinositide hydrolysis only indirectly via enhancing the effect of group I mGluRs *(32,33)*. Similarly, activation of mGluR7 via G_o in cultured cerebellar granule cells or via $G_{\alpha 15}$ in cell lines has been found to stimulate PLC/IP3/$[Ca^{2+}]_i$ cascade *(34,35)*.

Quite a different signal transduction pathway is stimulated by mGluR6 in depolarizing bipolar cells of retina, which are activated by cyclic guanosine monophosphate (cGMP) *(36)*. In these neurons, mGluR6 activates cGMP phosphodiesterase which, in turn, hydrolyses cGMP to 5′-GMP and in this way inhibits membrane conductance *(36)*.

3.2.1. Regulation of Group II and III mGluRs Function by Protein Kinases

The function of receptors belonging to group II and III is modulated by protein kinases: PKC and/or cAMP-dependent protein kinase (PKA). PKC has been found to phosphorylate mGluR2 *(37)* which leads to uncoupling of this receptor from G protein and reversal of its effects on cAMP and neuronal activity *(38–40)*. Regarding group III mGluRs, a direct phosphorylation of mGluR7 by PKC, which inhibits its functioning, has also been reported *(39,41,42)*. This PKC-induced phosphorylation of mGluR7 is diminished by binding of calmodulin to C-terminal domain of this receptor *(42)*.

PKA phosphorylates serine residue (Ser^{843}) of the intracellular C-terminus of mGluR2, and uncouples this receptor from $G_{\alpha 15}$. The latter process results in a decrease in phosphoinositide hydrolysis and in reversal of presynaptic inhibition induced by this receptor stimulation *(31,38)*. PKA does not phosphorylate mGluR7 *(42)*.

4. LOCALIZATION

4.1. Group I mGluRs

MGluRs belonging to group I are widely distributed in the CNS. The highest concentration of mGluR1 is present in Purkinje cells and molecular layer of the cerebellum, in glomeruli of olfactory bulb, and in CA1 region of the hippocampus *(43)*. Moderate levels

of these receptors have been found in the basal ganglia (globus pallidus, islands of Calleya, caudate-putamen and nucleus accumbens, subthalamic nucleus), midbrain (substantia nigra, superior colliculus, ventral tegmental area), other regions of cerebellum (stellate cells, basket cells, etc.), pyriform and cingulate cortex, amygdala, CA3 region, and dentate gyrus of the hippocampus *(43)*.

In spite of the fact that regional distribution of mGluR5 mostly overlaps that of mGluR1, their densities in distinct brain structures differ markedly. The highest concentration of mGluR5 has been detected in the basal ganglia (caudate–putamen, nucleus accumbens, olfactory tubercle) and CA1, CA3, and dentate gyrus of the hippocampus whereas their moderate and low levels occur in the globus pallidus and the substantia nigra, respectively (cf. ref. *44*). In all these structures the density of mGluR5 is considerably higher than that of mGluR1. In contrast, Purkinje cell layer of the cerebellum, which is enriched of mGluR1, is devoid of mGluR5 (cf. ref. *44*). Moreover, mGluR1 and mGluR5 may be differentially distributed on neuronal subpopulations, e.g., in the cerebral cortex. In this structure, somatostatin neurons exhibit almost four times higher immunoreactivity of mGluR1 than mGluR5, whereas the opposite relationship is characteristic of neurons stained for glutamic acid decarboxylase 67 (GAD67) or parvalbumin *(45)*.

MGluR1a and mGluR5 are mainly postsynaptic receptors. Their highest density is observed in perisynaptic annulus, located at the edge of both axo-spineous and axo-dendritic synaptic junctions, which surrounds the postsynaptic density of so-called type 1 synapses. Smaller numbers of these receptors are localized extrasynaptically on dendrites and somatic membranes *(43,46–48)*. These receptors have never been found in the main body of the postsynaptic density *(43,47)*. Immunohistochemical methods discovered also presynaptic mGluR1 and mGluR5 on axon terminals in the cerebral cortex, striatum, CA1 region of the hippocampus, or substantia nigra pars reticulata *(46,49,50)*. MGluR5 has also been found on astrocytes *(51)*.

4.2. Group II and III mGluRs

Immunohistochemical as well as binding studies using a selective antagonist—[^{3}H]-(2S)-2-amino-2-[(1S,2S)-2-carboxycyclopropan-1-yl]-3-(xanth-9-yl) propionic acid ([^{3}H]LY 341495)—or agonist—[^{3}H]LY 354740 of group II mGluRs—revealed that densities of these receptors varied throughout the brain *(52–55)*. Their highest density was identified in the forebrain: cerebral cortex, hippocampus, caudate-putamen, nucleus accumbens, olfactory bulb. Medium levels were found in hypothalamus, cerebellum, amygdala, thalamus, superior colliculus, whereas their densities in the globus pallidus, pons, and medulla were low *(52–55)*.

Group II (mGluR2/3) mGluRs are localized mainly in the terminal zone of axons. MGluR2 and mGluR3 are present in preterminal axonal region, and in extrasynaptic membrane of axon terminals and only rarely in presynaptic membrane *(53,56)*. They are not associated with the presynaptic junction sites. Glial processes have also been reported to be immunopositive for mGluR3 *(56)*.

Distribution of individual members of group III mGluRs in the brain also varies considerably. Intense mGluR7 immunoreactivity was seen in olfactory bulbs, anterior olfactory nucleus, islands of Calleya, olfactory tubercle, piriform and entorhinal cortices, amygdala and hippocampus, layer I of the neocortical regions, globus pallidus, superior colliculus, locus coeruleus, medulla, and spinal cord *(57)*. MGluR4 are most prominently

expressed in the cerebellum, basal ganglia, the sensory relay nuclei of the thalamus, and some hippocampal regions *(58)*. MGluR6 receptors show a unique distribution. They are present only in depolarizing bipolar cells of retina (cf. ref. *11*). In contrast to other receptors, the highest expression of mGluR8 is localized in the pontine nuclei and reticulotegmental nucleus followed by reticular thalamic nucleus, olfactory bulb, basal amygdaloid nucleus, and cerebral cortex *(34)*. Low expression of this receptor has been found in molecular layer of the cerebellum *(59)* and hippocampus *(56)*.

In contrast to mGluR2/3 receptors, mGluR4, mGluR7, and mGluR8 are abundant in the active zone of the presynaptic membrane of both asymmetric (glutamatergic) and symmetric (GABAergic) synapses *(56–58,60)*. Therefore, they may act as glutamatergic autoreceptors, and heteroreceptors that influence GABA release.

MGluR2, 3, 4, and 7 receptors have also been found postsynaptically on dendritic shafts and somatic membrane *(48,52,53,61,62)*.

5. THE ROLE OF mGluRs IN SYNAPTIC TRANSMISSION

5.1. Postsynaptic and Presynaptic Effects of Group I mGluRs

Postsynaptic mGluRs belonging to group I are mainly excitatory receptors. They are activated by glutamate at submicromolar and low micromolar concentrations *(1)*. Activation of these receptors by repetitive electrical stimulation or selective agonists increases neuronal excitability (depolarization, slow excitatory synaptic current, inward current) in slices of various brain regions and cell cultures *(61,63–68)*. Depending on the brain structure or neuronal subpopulation, these effects result from stimulation of either mGluR1 (e.g., substantia nigra pars compacta) or mGluR5 (e.g., subthalamic nucleus) or both receptors (e.g., cholinergic striatal interneurons) *(25,67,69)*. Sometimes, however, stimulation of these receptors does not induce any synaptic currents but enhances that produced by stimulation of NMDA receptors (e.g., mGluR5 localized on striatal medium spiny neurons) *(70)*. The excitatory effects induced by group I mGluRs have been suggested to result from inhibition of K^+ channels (cf. refs. *11* and *29*), increased permeability of nonselective cationic channels for Na^+, K^+, and Cs^+ (cf. refs. *29, 63,* and *71*), or Cl^- efflux through Ca^{2+}-activated Cl^- channels *(66)*. The contribution of specific signal transduction pathways to these effects is unclear. Some studies have shown that they are dependent on G protein *(25,63,72)* and Ca^{2+} level *(63,66)*. However, other data have not confirmed any involvement of G protein *(71)*, PLC/IP3/Ca^{2+} pathway, or PKC in these processes *(25,64,71,72)*, but postulated a significant contribution of non-Src protein kinases *(25)*.

Stimulation of mGluR1 on the dopaminergic neurons of the substantia nigra or ventral tegmental area produces a more complex effect: first hyperpolarization (outward current) mediated by activation of Ca^{2+}-dependent K^+-channels, which is followed by depolarization (inward current) *(65)*. It has been speculated that the first inhibitory effect of mGluR activation becomes quickly desensitized and replaced by excitatory response *(73)*.

Presynaptic mGluRs belonging to group I may also regulate glutamatergic transmission. Both mGluR1 and mGluR5 receptors have been found to facilitate glutamate release from presynaptic terminals *(74–76)*. However, the contribution of each receptor to that effect may depend on an examined region; e.g., glutamate release in forebrain or cerebral cortex has been found to be stimulated by mGluR5 but not mGluR1 receptors *(75,76)*. These data suggest that presynaptic mGluR1 and mGluR5 may not colocalize on the same terminals or they act differently. Moreover, Grillner and Mercuri *(77)* and

Katayama et al. *(65)* have reported an opposite effect of stimulation of presynaptic group I mGluRs in the substantia nigra pars compacta. They have found that depolarization of dopaminergic neurons induced by electrical stimulation is diminished by an agonist of group I mGluRs—(S)-3,5-dihydroxyphenylglycine (S)-3,5-DHPG)—which may suggest a decrease in glutamate release as a result of stimulation of these receptors *(65,77)*. Similar effect has been postulated for mGluR1 in the substantia nigra pars reticulata *(78)* and subthalamic nucleus *(79)*. The previously mentioned opposite effects of presynaptic receptors of group I observed in different structures may be explained by desensitization of these receptors, which results in functional switch from facilitation to inhibition of glutamate release *(80)*. In fact, such a sequence of effects has been found in cerebrocortical nerve terminals *(80)*.

5.2. Presynaptic Effects of Group II and III mGluRs

Stimulation of group II and III by their selective agonists does not induce any postsynaptic current in different brain regions *(29,61,65,70,71)*. In contrast, activation of both these groups of receptors has been reported to inhibit excitatory postsynaptic currents induced by electrical stimulation, which suggests their depressive influence on presynaptic terminals *(39,41,61,65,77,81–83)*. This suggestion has been supported by a number of studies that indicate that activation of group II and III inhibits glutamate release from presynaptic terminals *(41,84–86)*. That process seems to result from inhibition of L- and N-type voltage-dependent Ca^{2+} channels (group II mGluRs), or P/Q-type of Ca^{2+} channels (group III mGluRs) (cf. refs. *11*, *35*, and *87*). The facilitating effect of group II and III mGluRs on K^+ channels *(88–90)*, or a direct modulation of release machinery has also been postulated *(81)*. Inhibition of adenylate cyclase induced by these receptors does not seem to contribute to the previously mentioned presynaptic inhibition and neurotransmitter release *(31,41)*. In contrast, this process seems to involve Ca^{2+}-dependent calmodulin binding to C-terminal domain of mGluR7 *(91)*.

Glutamate concentration in the synaptic cleft appears to be in a milimolar range (cf. ref. *30*). This neurotransmitter binds to mGluR7 with low affinity (at almost milimolar concentrations) (cf. ref. *30*). In comparison, group II mGluRs are much more sensitive and activated by low micromolar concentrations of this amino acid (cf. ref. *30*). It has been hypothesized that because mGluR7 is present in the active zone of the presynaptic membrane, it is stimulated by glutamate released during normal physiological synaptic activity and mediates feedback inhibition. In contrast, group II mGluRs, which are localized peri- or extrasynaptically, can be activated only when the terminal is overstimulated and synaptic level of glutamate is excessively elevated *(84,85,92)*. This is also the reason why receptors of group II are particularly sensitive to glutamate whose concentration drops with an increasing distance *(30)*. Therefore, receptors belonging to group II seem to inhibit excessive glutamatergic transmission, which may lead to pathological disturbances.

5.3. Interactions Between Group I mGluRs and NMDA Receptors

Metabotropic glutamate receptors belonging to group I may also influence synaptic transmission by complex and multifarious interactions with NMDA receptors. A number of studies have shown that stimulation of both mGluR1 and mGluR5 enhances the NMDA-induced excitatory (depolarization, inward currents) responses in brain slices,

neuronal cultures or cell lines *(70,93–99)*. Several mechanisms have been postulated to be involved in this process. Group I mGluRs are localized in a proximity of NMDA receptors and may couple to these receptors via CC-Homers and postsynaptic density proteins: Shank and PSD-95 (Fig. 2) *(100)*. Moreover, because CC-Homers bind also to IP3Rs and RyRs, they may constitute a physical link between mGluRs, NMDA receptors, and intracellular Ca^{2+} stores *(100)*.

Facilitation of NMDA-induced responses by stimulation of mGluRs has also been suggested to depend on phosphorylation of protein subunits of NMDA receptor, which leads to (1) removal of Mg^{2+} block and opening of the NMDA-gated channel (cf. ref. *10*), or (2) delivery of new NMDA channels to the plasma membrane by regulated exocytosis *(66)*. NR2A and NR2B subunits of NMDA receptors may be phosphorylated by PKC, Ca^{2+}- and calmodulin-activated kinases, and tyrosine Src kinases *(99,101–104)* (Fig. 2). A role of PKC in mGluR1/5-induced activation of NMDA receptors has been postulated by some authors *(93,98,99,102–104),* but not by others *(97,105)*. Moreover, a cascade of processes relaying signals from mGluRs to NMDA receptor, which finally leads to its phosphorylation by Src-kinases, has been defined for mGluR1a in *Xenopus* oocytes, frog spinal motoneurons, or cortical neurons. This chain of reactions includes: (1) coupling to G protein, (2) activation of PLC, (3) mobilization of intracellular Ca^{2+}, (4) activation of calmodulin, (5) activation of proline-rich cell adhesion kinase 2 (Pyk2/CAKβ), (6) activation of Src kinases, and (7) phosphorylation of tyrosine residues of NR2A and NR2B subunits of NMDA receptor (Fig. 2) *(99,101,106)*. Similarly, stimulation of mGluR5 in CA3 region of the hippocampus enhances NMDA-induced current in a way dependent on both G protein and Src kinases *(95)*. Some studies, however, which also pointed to the significant role of Src kinases in potentiation of mGluR1-evoked NMDA receptor responses in CA3 region, have shown that this process is independent from G protein *(95)*. Moreover, facilitative influence of group I mGluRs on NMDA-induced responses independent of phosphoinositide hydrolysis and intracellular Ca^{2+} level has also been reported by others *(66,94)*.

Interaction between mGluRs and NMDA receptors is not limited to facilitation of responses of the latter receptors. Thus, stimulation of group I mGluRs has been reported to inhibit NMDA-induced currents in cultures of hippocampal, striatal, cortical, and cerebellar neurons *(107–109)*. This effect, which may result from internalization of NMDA receptors *(108)*, has been reported to be G protein-dependent but independent of Ca^{2+} homeostasis and PKC activation *(109)*. In addition, the NR2C subunit of the NMDA receptor may be involved in this process, at least in the cerebellum *(107)*.

NMDA receptors may also reciprocally influence group I mGluRs in a complex manner. It has been shown that lower concentrations of NMDA enhance, whereas higher ones inhibit responses of mGluRs *(110–113)*. The former phenomenon has been suggested to result from the NMDA-induced dephosphorylation of those sites of mGluR5, which are normally phosphorylated by PKC. Activation of phosphatases (phosphatase 2B or calcineurin) is involved in this process *(114)*. Because PKC-induced phosphorylation of group I mGluRs leads to their desensitization, NMDA seems to reverse that process *(114)*. On the other hand, a precise mechanism of inhibition of mGluR5 by high concentration of NMDA is not known. Phosphorylation of tyrosine residues of mGluR5 by stimulation of NMDA receptors has been reported; however, no functional significance of that process has been discovered so far *(26)*.

6. THE ROLE OF mGluRs IN BRAIN PATHOLOGY

6.1. The Role of mGluRs in Neurodegeneration

Apart from its role of a neurotransmitter, glutamate may also exert neurotoxic effects in some pathological states. When the extracellular level of this amino acid is excessively elevated as a result of an increased release or impaired reuptake, glutamatergic receptors are overactivated, which may lead to the so-called excitotoxicity and neuronal death by apoptosis and/or necrosis. Sensitivity of glutamate receptors, activity of ion channels, and intracellular mechanisms triggered by glutamate (e.g., activation of Ca^{2+}-dependent enzymatic pathways, increased generation of intracellular free radicals, and glutathione depletion) have been suggested to be involved in excitotoxic effects of glutamate. Stimulation of ionotropic glutamate receptors (NMDA, AMPA/kainate) plays a crucial role in this process (cf. ref. *115*). Several recent in vitro and in vivo studies have indicated that mGluRs may also make a substantial contribution to the glutamate-induced neurotoxicity.

MGluRs belonging to group I are the first candidate supposed to be involved in neurotoxicity since they are mainly excitatory receptors that cooperate with NMDA complex, and increase $[Ca^{2+}]_i$ level. In accordance with this view, stimulation of these receptors by a nonselective (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid [(1S,3R)-ACPD] or selective (S)-3,5-DHPG and (R,S)-2-chloro-5-hydroxyphenylglycine [(R,S)CHPG] agonists (Table 1) have been found to produce neuronal degeneration and increase neurotoxic effect of NMDA in different in vitro and in vivo models *(116)*, (for review, *see* ref. *117*). However, a number of studies have shown an opposite, i.e., neuroprotective, effect of stimulation of group I receptors in in vitro models (hippocampal slices and neuronal culture, cerebellar granule cells, spinal motor neurons) exposed to NMDA, kainate, hypoxia, or hypoglycemia *(107,118–122)*, as well as in in vivo studies of focal cerebral ischemia *(123)*.

Several hypotheses have been presented to explain the above-mentioned discrepancies. Opposite effects of stimulation of group I mGluRs may result from: (1) different state of mGluRs activity, (2) different subunit composition of NMDA receptors present in the examined tissue, and (3) the presence or the lack of astrocytes. It has been found that first (S)-3,5-DHPG administration to cortical cultures increases NMDA-induced toxicity, whereas the second treatment affords neuroprotection. These effects parallel an enhancement and diminution of the NMDA-induced glutamate release, respectively *(118)*. Therefore, it has been hypothesized that, at least in cortical neurons, group I mGluRs switch from the state of facilitation to inhibition of both glutamate release and neurotoxicity *(118)*. On the other hand, group I mGluRs localized on other neurons, where their stimulation induces only protective effect, may be endogenously switched on attaining a "neuroprotective mode" *(118)*.

According to the second concept, the presence of the NR-2C subunit in the NMDA receptor complex may be crucial for the group I mGluR-induced inhibition of glutamate excitotoxicity in cerebellar granule cells *(107)*. When this subunit is depleted, the depressing effect of group I agonists is reversed and these compounds show a tendency to potentiate glutamate toxicity *(107)*. In some pathological conditions, for example, during experimental ischemia, the expression of NR-2C is increased in the hippocampus or cerebral cortex *(124)*. In such a situation, the neuroprotective action of group I mGluRs agonists may be of special importance.

Table 1
Selected Ligands of Metabotropic Glutamate Receptors According (If Not Stated Otherwise) to Schoepp et al. *(2)*

Subtype		AGONISTS				ALLOSTERIC ENHANCERS	COMPETITIVE ANTAGONISTS		NON-COMPETITIVE ANTAGONISTS
Group I	mGluR1	1S,3R-ACPD		(S)-3,5-DHPG t-ADA		Ro 67-7476 *(15)* Ro 01-6128 *(15)* Ro 67-4853 *(15)* Xanthene and thioxanthene derivatives *(227)*	(S)-MCPG	AIDA LY 367385	CPCCOE1 PHCCC BAY 36-7620[a] *(226)* Substituted pyrroles *(230)*
	mGluR5				CHPG				MPEP*# *(132)* SIB-1757 SIB 1893*# *(132)*
Group II	mGluR2			DCG-IV[+] L-CCG-I 2R,4R-APDC LY 354740* LY 389795* LY 379268* LY 404040* *(221)*		substituted sulfonamides *(228,229)*		LY 341495*	
	mGluR3								
Group III	mGluR4		L-AP4 L-SOP (RS)-PPG *(212)*	ACPT-1 *(222)*		PHCCC *(17)*	MSOP DCG-IV[+] *(189)* MAP4		
	mGluR6			L-CCG-I *(189)*					
	mGluR7								
	mGluR8			(R,S)-3,4-DCPG*[+] *(224)* (S)-3,4-DCPG *(224)* L-CCG-I *(189)*					

[a](R)-isomer-antagonist of AMPA receptors.

*Active in vivo after systemic administration; + at higher concentrations, an agonist of NMDA receptors.

[#]at concentrations higher than 20 μ*M*, an antagonist of NMDA receptors.

t-ADA, *trans*-Azetidine-2,4-dicarboxylic acid; BAY 36-7620, (3aS,6aS)-6a-naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopenta[c]furan-1-on; MAP4, (S)-2-amino-2-methyl-4-phosphonobutanoic acid; (S)-MCPG, (S)-α-methyl-4-carboxyphenylglycine; MSOP, (RS)-α-methylserine-*O*-phosphate; PHCCC, *N*-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide; Ro 67-7476, (S)-2-(4-fluoro-phenyl)-1-(toluene-4-sulfonyl)-pyrrolidine; Ro 01-6128, diphenylacetyl-carbamic acid ethyl ester; Ro 67-4853, (9H-xanthene-9-carbonyl)-carbamic acid butyl-ester.

As mentioned earlier, astrocytes express mGluR5 *(51)*. Their role in excitotoxic effect of group I mGluRs has been supported by the finding that their supplement to cultured granule cells switches the neuroprotective effect of (S)-3,5-DHPG to exacerbation of excitotoxicity *(117)*.

In contrast to agonists, antagonists of both mGluR1 and mGluR5 exhibit uniformly neuroprotective effects in several models. Selective antagonists of mGluR1—(RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA), (S)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY 367385), and 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt) (Table 1)—have been found to diminish neuronal degeneration induced by NMDA, hypoxia, glucose deprivation, mechanical injury in cortical culture, or hippocampal slices *(125–128)*, and injury caused in vivo by transient global ischemia, trauma *(127–130)*, or intrastriatal NMDA application *(125,126)*. Selective antagonists of mGluR5—2-methyl-6-(phenylethynyl)pyridine (MPEP), 6-methyl-2-(phenylazo)-3-pyridinol (SIB-1757), and 2-methyl-6-(2-phenylethenyl)pyridine (SIB-1893) (Table 1)—are neuroprotective against toxicity induced by NMDA or by β-amyloid peptide in cortical cultures in vitro, and against focal cerebral ischemia or NMDA/quinolinic acid-induced injury in vivo *(117,123,125,131)*. MPEP and SIB-1893 administered systemically selectively block mGluR5 *(2)*. However, when applied in vitro at concentrations higher than 20 μ*M*, they act as NMDA receptor antagonists, which may make some contribution to their neuroprotective effects observed in the above models *(132)*. The mechanism of neuroprotection afforded by mGluR1 (but not mGluR5) antagonists has been suggested to involve also an enhancement of GABAergic transmission *(125,128)*.

Stimulation of group II and III mGluRs has been expected to be neuroprotective because these receptors reduce glutamate release and inhibit voltage-dependent Ca^{2+} channels (cf. refs. *11, 41,* and *84–87*). Moreover, mGluR3 receptors are localized on astrocytes and stimulate synthesis and release of a putative neuroprotective factor (transforming growth factor β = TGF-β) *(117,133)*. In accordance with this view, selective agonists of group II—(2R,4R)-APDC, LY 354740, (1R,4R,5S,6R)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268) and (1R,4R,5S,6R)-2-(thia-4-aminobicyclo[3.1.0hexane-4,6-dicarboxylate (LY 389795) (Table 1)—have been evidenced to afford neuroprotection against neuronal degeneration induced by NMDA/kainic acid in vitro and in vivo *(129,134,135)*. Moreover, LY 354740 and LY 389795 administered systemically protected the brain against transient global but not focal ischemia (129,135,136). In contrast to the previously cited data, Behrens et al. *(137)* did not find any neuroprotective effect of LY 354740 against NMDA-induced toxicity or ischemia in vitro and in vivo.

Group III mGluRs agonists do not penetrate across the blood–brain barrier and therefore, the only available information about their neuroprotective effects derives from in vitro studies or in vivo experiments after intracerebral administration. These compounds—(RS)-4-phosphonophenylglycine ((R,S)-PPG), L-(+)-2-amino-4-phosphonobutyric acid (L-AP-4), and L-(+)-2-amino-4-phosphonobutyric acid (L-SOP) (Table 1)—provided protection against toxic pulse of NMDA and mechanical injury in cortical or cerebellar cultures *(138–142)*, or quinolinic acid-induced striatal lesions *(141)*. Moreover, L-AP-4 and L-SOP exhibit antiapoptotic activity against neurotoxicity induced by β-amyloid peptide *(143)*. In contrast, (R,S)-PPG administered icv was ineffective in

focal cerebral ischemia in mice and global cerebral ischemia in gerbils or rats *(144)*. The above-mentioned compounds do not differentiate subtypes of group III mGluRs. However, these compounds have been found to be ineffective in mGluR4-deficient mice, which suggests a contribution of this receptor to neuroprotection *(138)*.

6.2. The Role of mGluRs in Parkinson's Disease

6.2.1. Neuronal Mechanisms Involved in Pathophysiology of Parkinson's Disease

Parkinson's disease (PD) is a relatively common chronic neurodegenerative disease that is characterized by the following primary symptoms: akinesia (bradykinesia), muscle rigidity, and tremor. It is generally accepted that parkinsonian symptoms result from degeneration of dopaminergic neurons of the nigrostriatal pathway whose cell bodies are localized in the substantia nigra pars compacta and axon terminals in the striatum. That lesion leads to dramatic losses of dopamine in the latter structure *(145)*. Although the role of dopaminergic deficiency in PD has been known for years, neither pathological factor that induces this disease nor its appropriate therapy has been discovered so far.

Degeneration of nigrostriatal pathway results in a number of secondary functional alterations affecting interconnections of the basal ganglia. First of all, the lack of striatal dopamine leads to activation of striatal cholinergic interneurons, which is involved in the well-known disturbance in the dopaminergic–cholinergic equilibrium *(146)*. Furthermore, an imbalance between the two main GABAergic striatal efferents—the striopallidal ("indirect"—leading to the lateral part of the globus pallidus) and strionigral ("direct"—leading to the substantia nigra pars reticulata) pathways—has been suggested to occur in PD *(147)*. These pathways exert an opposite influence on the GABAergic neurons of the substantia nigra pars reticulata, which project to the thalamus. The strionigral pathway "directly" inhibits nigrothalamic neurons, whereas the striopallidal one activates them "indirectly" via a sequence of pallido-subthalamo-nigral projections. It has been hypothetized that in the course of PD the balance is shifted toward activation of the striopallidal pathway. In normal individuals, this pathway is inhibited by dopamine via D2 receptors, and stimulated, via NMDA and AMPA receptors, by glutamic acid released from the cortico-thalamo-striatal terminals *(147,148)*. Therefore, the lack of striatal dopamine in PD results in activation of this pathway and a release of GABA in the lateral globus pallidus, which inhibits the next link—the GABAergic pallidosubthalamic pathway. This effect leads, in turn, to disinhibition of glutamatergic subthalamonigral neurons and to an increase in glutamatergic input, via NMDA and AMPA receptors, to nigral efferent neurons *(149)*. Because in PD the "direct" strionigral GABAergic pathway (normally activated by dopamine via D1 receptors) is inhibited, the excitatory influence predominates in the substantia nigra pars reticulata, which results in overactivation of the GABAergic nigrothalamic output pathway. GABA released from nigrothalamic terminals inhibits, in turn, glutamatergic thalamocortical neurons, which indirectly leads to activation of the glutamatergic corticostriatal pathway closing the previously mentioned neuronal circuit *(147)* (Fig. 3).

Several lines of evidence support the view that glutamate-induced excitation of neurons in the striatum, subthalamic nucleus, and substantia nigra pars reticulata plays a significant role in development of parkinsonian symptoms (cf. ref. *150*). First of all, the blockade of NMDA receptors by antagonists and the inhibition of subthalamic neurons by high-frequency stimulation have been found to exert therapeutic effects in parkinsonian patients and in animal models of PD (cf. refs. *150* and *151*). Recent studies have

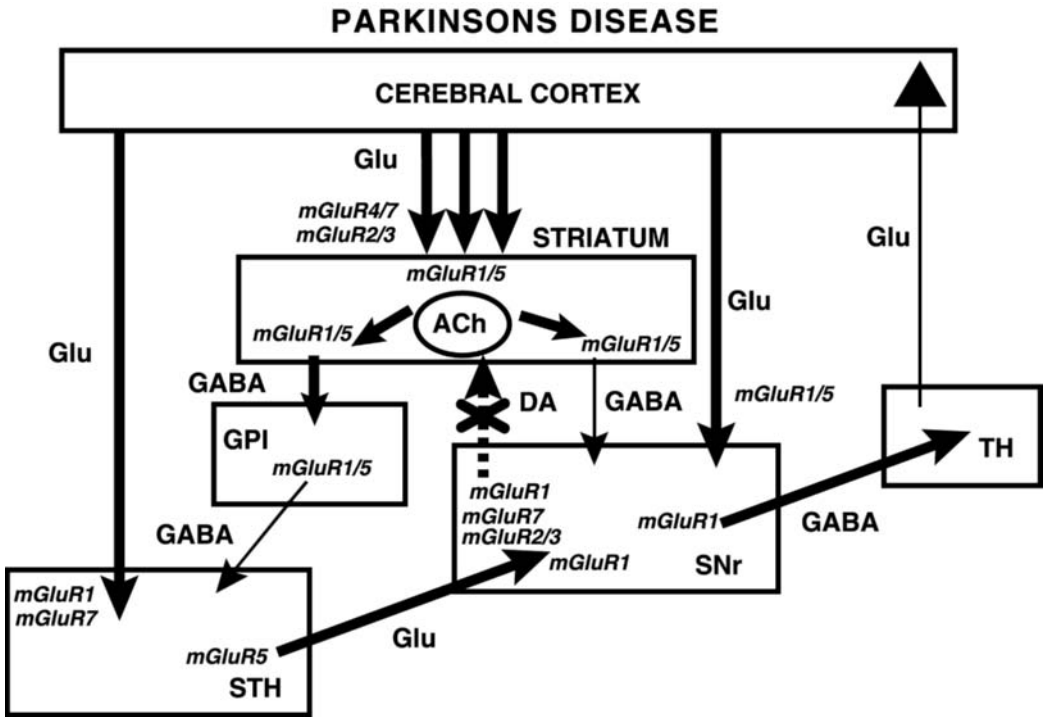


Fig. 3. Neuronal pathways engaged in development of Parkinson's disease symptoms. The activated pathways are drawn as thick arrows, whereas the inhibited ones are marked by thin arrows. Lesioned dopaminergic pathway is marked by broken line. ACh, cholinergic interneuron, GABA, (γ-amino-butyric acid)-ergic pathway; Glu, glutamatergic pathway, GPl, globus pallidus, lateral part, mGluR1,2,3,4,5 and 7, subtypes of metabotropic glutamate receptors localized either postsynaptically (bigger letters) or presynaptically (smaller letters); SNr, substantia nigra pars reticulata; STH, subthalamic nucleus; TH, thalamus.

indicated that mGluRs seem to be also involved in generation of parkinsonian symptoms and may constitute a target for potential antiparkinsonian therapy.

MGluRs belonging to group I (mGluR1 and mGluR5) contribute to stimulation of neuronal pathways involved in expression of parkinsonian symptoms: the striopallidal, subthalamonigral, and nigrothalamic ones. Stimulation of mGluR5 potentiates the NMDA-induced membrane depolarization and inward current in striatal efferent neurons *(70)*, and increases striatal proenkephalin mRNA level *(152)*. Because expression of enkephalin, which selectively colocalizes with GABA in striopallidal neurons, parallels their activity, it seems that mGluR5 may actually stimulate the striopallidal pathway. Furthermore, this pathway is also activated by striatal cholinergic interneurons *(146)* (Fig. 3). Although expression of mGluR1 and mGluR5 on these neurons is low, they are activated by both these receptors (Fig. 3), which leads to acetylcholine release *(61,67,153)*. In this way, mGluR1 and mGluR5 may indirectly activate the striopallidal pathway via their influence on cholinergic interneurons.

Electrophysiological studies have indicated that subthalamonigral and nigro-thalamic neurons are also activated by mGluR5 and mGluR1, respectively *(69,154)*. Moreover, presynaptic receptors, mGluR1 and mGluR5, localized on GABAergic terminals in the

substantia nigra pars reticulata (Fig. 3) may decrease GABA release and in this way cooperate with postsynaptic receptors in activation of the nigrothalamic pathway *(154)*. However, stimulation of some mGluR1 receptors may also induce an opposite effect; i.e., they may counteract the glutamate-induced stimulation of the above-mentioned subthalamonigral and nigro-thalamic pathways. Such an effect has been described for presynaptic mGluR1 localized on glutamatergic terminals in both the substantia nigra pars reticulata and subthalamic nucleus *(78,79)* (Fig. 3). Moreover, mGluR1 localized postsynaptically in the globus pallidus (Fig. 3) activates neurons of this structure *(29)*. Because at least some of them are GABAergic neurons projecting to the subthalamic nucleus, their stimulation by glutamate may indirectly inhibit a subsequent glutamatergic subthalamonigral pathway.

As mentioned above, group II and III mGluRs of the basal ganglia act predominantly as presynaptic receptors (Fig. 3), which inhibit glutamate-induced neuronal excitation. They have been found to inhibit glutamate release in the striatum *(84–86)* and diminish excitatory postsynaptic currents/potentials induced by stimulation of afferents in this structure *(82,83)*, as well as in the subthalamic nucleus (only group III mGluRs) *(79)* and substantia nigra pars reticulata *(155,156)*. Presynaptic group III mGluRs diminish additionally inhibitory GABAergic transmission in the lateral globus pallidus and substantia nigra pars reticulata *(156,157)*. The role of individual receptor subtypes in all the above-mentioned structures is difficult to establish, because of a poor selectivity of agonists of group II [L-(+)-2-amino-4-phosphonobutyric acid (DCG-IV), 2R,4R-APDC, LY 354740] or III (L-AP-4 (Table 1) used in these studies. However, the level of effective concentration of L-AP-4 seems to suggest that presynaptic effect of this compound in the subthalamic nucleus or substantia nigra pars reticulata results from stimulation of mGluR7 *(156,157)* (Fig. 3).

6.2.2. Symptomatological Effects of mGluRs Ligands

The previously described effects of mGluRs in the basal ganglia have advanced the conclusion that antagonists of group I, or agonists of group II or III, which predominantly inhibit glutamate-induced excitation of striatal, subthalamic, or nigral neurons, may alleviate parkinsonian symptoms. Unfortunately, only a few selective compounds that penetrate well through the blood–brain barrier are available so far (Table 1).

It has been found that systemic (LY 354740) or intraventricular (DCG-IV) administration of selective group II agonists inhibits parkinsonian-like muscle rigidity and catalepsy induced by haloperidol *(155,158,159)* or akinesia induced by reserpine in rats *(160)*. These effects seem to be owing to stimulation of nigral but not striatal receptors since DCG-IV administered directly into the substantia nigra produces similar effects *(160)*, whereas intrastriatal injections of other group II agonists [2R,4R-APDC or (2S,1′S,2′S)-2-(carboxycyclopropyl)glycine = L-CCG-I] (Table 1) do not reverse the haloperidol-induced deficits *(161–163)*.

Several studies have also supported the role of group I mGluR blockade in antiparkinsonian effects. Systemic treatment of rats with a single dose of MPEP, a noncompetitive antagonist of mGluR5, has been reported to reduce the haloperidol-induced catalepsy, hypolocomotion, and muscle rigidity *(163,164)*. Moreover, Breysse and coworkers *(165)* have reported that akinesia induced by a lesion of dopaminergic neurons is reversed by chronic but not acute treatment with this compound. However, according to the latter authors MPEP does not inhibit the haloperidol-induced catalepsy *(165)*.

Selective antagonists of mGluR1 available so far cross the blood–brain barrier poorly. However, a representative of these compounds, AIDA, administered directly into the striatum has been shown to inhibit parkinsonian-like muscle rigidity *(162,163)* and catalepsy induced by haloperidol *(166)*. Antiparkinsonian-like effects of both AIDA and MPEP may be attributed, at least partly, to inhibition of the striopallidal pathway since these compounds reverse the haloperidol-induced increase in striatal proenkephalin mRNA expression *(166,167)*. AIDA is a low-potency antagonist of mGluR1 whose selectivity is limited *(2)*. However, other more potent and selective antagonists of this receptor—LY 367385 and CPCCOEt *(2)* (Table 1)—administered intrastriatally induce even stronger inhibitory effect on the haloperidol-increased proenkephalin expression *(167)*.

The scarcest information is available on the potential role of agonists of group III mGluRs. None of these compounds crosses the blood–brain barrier; however, the most recent study of Marino and coworkers *(157)* has shown that L-AP4 administered intraventricularly decreases both the reserpine-induced akinesia and haloperidol-induced catalepsy.

6.2.3. Neuroprotective Effects of mGluRs Ligands

MGluRs may also be involved in producing lesions of dopaminergic nigrostriatal neurons in PD (*see also* Chapters 19, 21, and 22). Although a pathogenic factor that triggers this degeneration has not been discovered yet, experimental studies with the use of dopaminergic toxins—1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and methamphetamine—suggest that excitotoxic effect of glutamate may contribute to this process (cf. ref. *150*). Dopaminergic nigrostriatal neurons are activated via NMDA and AMPA receptors by glutamatergic projections originating from the subthalamic nucleus and cerebral cortex *(65,77)*. In addition, dopaminergic neurons express considerable numbers of mGluR1 and low amounts of mGluR5 *(62)*. The mGluR1 has been found to contribute to activation of these neurons *(65,168)*, inducing their hyperpolarization, which is rapidly desensitized and followed by depolarization *(65)*. Potential role of mGluR1 in degeneration of these neurons has been proven by a protective action of AIDA administered intraventicularly against MPTP-induced toxicity *(169)*. MGluR5 seems also be involved in this process since its selective blockade by MPEP or SIB 1893 administered systemically reduces toxic effect of methamphetamine (170–172). Moreover, a reduction of excitatory, glutamatergic input to the striatum by stimulation of group II mGluRs by DCG-IV *(82)* has also been found to protect striatal dopaminergic terminals against 1-methyl-4-phenyl-pyridinium (MPP^+)-induced neurotoxicity *(173)*.

6.3. The Role of mGluRs in Antidepressant Action

Neuronal mechanisms underlying depressive symptoms, as well as those responsible for antidepressant drug action, are largely unknown. They seem to involve alterations in noradrenergic, serotonergic, and dopaminergic neurotransmission. Recently some role of reduced tone of glutamatergic transmission in antidepressant action has been postulated (*see also* Chapter 10). First of all, chronic treatment with clinically effective antidepressants induces an adaptive subsensitivity of NMDA receptor complex. Moreover, antagonists which bind to recognition, phencyclidine, and allosteric glycine sites of this receptor are effective in animal screening tests for antidepressant activity (for review, *see* ref. *174*).

Animal studies seem to indicate that antidepressant therapy may also produce adaptive changes in group I mGluRs, and that these receptors may constitute a target for new potential antidepressant drugs. Chronic treatment of rats with a classic antidepressant, imipramine, or electroconvulsive shocks reduces excitatory influence of group I mGluRs (increase in population spike amplitude, depolarization, inhibition of after-hypolarization) on hippocampal neurons of CA1 region *(175–177)*, which may suggest functional subsensitivity of these receptors. Several reports have been published that corroborated the contribution of the previously mentioned effect to antidepressant action. It has been found that the blockade of mGluR5 by an acute or chronic (14 d) treatment with their noncompetitive antagonist, MPEP (Table 1), induces antidepressant-like effects in tail-suspension test in mice *(178)*, or in bulbectomized rats *(179)*. On the other hand, chronic administration of imipramine or electroconvulsive shocks increases expression of mGluR1 and mGluR5 in CA1 and CA3 regions of the hippocampus *(180–182)*. Such elevated synthesis of both these receptors may be a compensatory response to their functional inhibition induced by antidepressant treatment.

In contrast to the above-mentioned antagonist of mGluR5, which inhibits glutamatergic transmission at the level of postsynaptic mGluRs, stimulation of group II mGluRs by their selective agonist, LY 354740, has not been found to produce any antidepressant-like effect in tail-suspension test or behavioral despair test *(183)*. However, the latter observation does not conclusively exclude the role of this group of mGluRs in antidepressant drug action since chronic imipramine treatment has been found to upregulate mGluR2/3 expression and their signal transduction in rats *(181)*.

6.4. The Role of mGluRs in Anxiety

Stimulation of GABAergic transmission via benzodiazepine receptors is the main mechanism responsible for therapeutic effects of anxiolytic drugs. However, other neurotransmitter systems (e.g., serotonergic or noradrenergic) are also involved in this process. A number of experimental studies show that anxiolytic-like effect in animal models of anxiety may be achieved by a decrease in glutamatergic transmission induced by the blockade of NMDA receptors (for review, *see* refs. *174* and *184* and Chapter 12). Moreover, the most recent studies indicate that ligands of mGluRs, which inhibit glutamate-induced neuronal excitation at the level of pre- (group II and III) or postsynaptic (group I) receptors, may also be useful for anxiolytic therapy.

The first study that reported anxiolytic action of mGluRs ligands was published in 1997 *(185)*. This study has shown that (S)-4-carboxy-3-hydroxyphenyl-glycine (S-4-C3HPG)—which is an antagonist of group I and agonist of group II mGluRs—administered into CA1 region of the hippocampus exhibits anxiolytic-like effects in the Vogel conflict test in rats *(185)*. Shortly afterward, it appeared that both these mechanisms, that is, the blockade of group I and stimulation of group II mGluRs, may be involved in that effect. This conclusion has been supported by the fact that LY 354740, an agonist of group II, administered systemically or intrahippocampally induced anxiolytic-like effects in a number of the so-called conditioned (fear-potentiated startle response, Vogel conflict test, four-plate test) and unconditioned (elevated plus maze) tests *(183,186–188)*. A similar, but approx 100 times stronger effect in the fear-potentiated startle assay has also been reported after a systemic administration of a newly synthetized agonist of these receptors—a rigid version of LY 354740—(2S,1′S,2′S,3′R)-2-(2′-carboxy-3′-methylcyclopropyl)glycine *(189)*. Moreover, a blockade of mGluR5 by an acute, or chronic

systemic administration of a noncompetitive antagonist of these receptors, MPEP was effective in conditioned responses (Vogel test, four-plate paradigm, Geller–Seifter test) and unconditioned tests (elevated plus maze task, social exploration, stress-induced hyperthermia, marble burying) in rats and mice *(178,179,190)*. The blockade of mGluR1 by a selective noncompetitive antagonist, CPCCOEt, administered directly into CA1 region of the hippocampus has also induced anticonflict effect in the Vogel test *(188)*.

Only a few data are available on the contribution of group III mGluRs to anxiety and anxiolytic effects. L-SOP, an agonist of these receptors, administered into the hippocampus induced anticonflict effect in rats *(188)*. Although L-SOP is not selective for specific subtype of receptors belonging to group III, its anxiolytic effect may involve at least mGluR8. This suggestion is based on the fact that mGluR8-deficient mice exhibit higher anxiety level than wildtype mice in the elevated plus maze test *(191)*.

6.5. The Role of mGluRs in Antipsychotic Action

It is generally supposed that overactivity of subcortical dopaminergic and cortical serotonergic transmissions is involved in psychotic symptoms but some contribution of glutamatergic system dysfunction to these symptoms has also been suggested *(192)*. The latter view is based mainly on the finding that phencyclidine (PCP), which is an uncompetitive NMDA receptor antagonist, induces both positive and negative psychotic symptoms in humans *(193)*. On the other hand, PCP and ketamine induce an increase in glutamate release in the prefrontal cortex, as a compensatory effect to the blockade of NMDA receptors *(194)*. PCP is suggested to be the best model compound to study neuronal mechanisms underlying psychotic symptoms and to screen putative antipsychotic compounds in animals.

Data regarding the role of mGluRs in schizophrenia and in antipsychotic drug action are rather scarce and inconclusive. This may be because of a complexity of psychotic symptoms and a lack of their appropriate animal equivalents, which may be a reason for diverse effects of mGluR ligands in different models. Stimulation of group II mGluRs has been postulated to exert antipsychotic effects by a reversal of glutamate release induced by stimulation of 5-HT2A receptors, or by a blockade of NMDA receptors. In accordance with this view, LY 354740 and LY 379268 inhibit excitatory postsynaptic currents (EPSCs) induced by stimulation of 5-HT2A receptors in slices of medial prefrontal cortex *(195,196)*, as well as glutamate release induced by PCP or ketamine in this structure in vivo *(194,197)*.

Some studies seem to support the aforementioned view. First, an atypical neuroleptic, clozapine, also inhibits glutamate release in the frontal cortex in vivo *(198)*. Moreover, chronic treatment with clozapine or another atypical neuroleptic, olanzapine, increases the expression of mGluR2 and mGluR3 mRNA in this structure *(199)*. Furthermore, according to some studies, LY 354740, LY 379268, and L-CCG-I decrease the PCP- or (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyprohepten-5,10-imine maleate (MK-801)-induced locomotion and stereotypy *(194,200–203)*, as well as working memory deficits (estimated in delayed alternation task) *(194)*, in a manner dependent on the presence of mGluR2 *(202)* and similar to clozapine *(203)*. However, other authors did not find any influence of LY 354740 or its racemic form, LY 314582, on (1) the PCP-induced deficit in sensorimotor gating *(204,205)*, (2) the PCP-increased locomotor activity *(206)*, (3) an impairment of working memory induced by MK-801, a selective antagonist of NMDA receptors *(204)*, or (4) discriminating effects of PCP *(205)*. Moreover, no

association has been found between schizophrenia and mGluR2 *(207)* or mGluR3 gene polymorphisms *(208)*.

MGluR5 receptors have also been suggested to play a role in schizophrenia. In contrast to mGluR2/3, however, an association of allele frequency of gene coding for mGluR5 with this disease has been found *(209)*. Moreover, mGluR5-deficient mice exhibit sensorimotor gating deficit *(210)*, and MPEP administered in rats enhances the deficit induced by PCP, as well as PCP-increased locomotor activity *(206)*. The above results seem to suggest that agonists of mGluR5, rather than antagonists, may be therapeutic in psychoses. Unfortunately no such compounds that cross the blood–brain barrier have been discovered yet.

6.6. The Role of mGluRs in Drug Addiction

Ligands of mGluRs have also been reported to have a beneficial impact in the treatment of drug addiction (*see also* Part VIII). Agonists of group II mGluRs, DCG-IV, injected icv, or LY 354740 administered systemically, inhibit nicotine- or morphine-withdrawal symptoms in rats *(183,211–214)*. Similarly, a knockout of mGluR5 receptors in mice and their blockade by MPEP in rats inhibit acquisition and expression of addiction, measured by inhibition of the morphine-induced conditioned place preference or cocaine self-administration *(215,216)*. Moreover, chronic administration of cocaine has been found to induce subsensitivity of group II and III mGluRs in nucleus accumbens and amygdala *(217,218)*.

6.7. Antiepileptic Effects of mGluRs Ligands

Antiepileptic drugs may be searched for among ligands of all groups of mGluRs. In general, agonists of group II and III, or antagonists of group I may be therapeutic. Systemically active agonists of group II (LY 354740, LY 379268, LY 389795) have been shown to be anticonvulsive when administered alone, or to increase anticonvulsant activity of conventional antiepileptic drugs in different models *(219,220)*, (for review, *see* ref. *221*). Similar effects have been reported for agonists of mGluR4 [(1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid = ACPT-1] *(222)* (Table 1) and mGluR8 [PPG, (RS)-3,4-dicarboxyphenylglycine = (R,S)-3,4-DCPG] *(223,224)* (Table 1) or antagonists of both mGluR5 (for review, *see* ref. *44*) and mGluR1 *(225,226)*.

7. CONCLUSIONS

The review of a current knowledge about the role of mGluRs in brain functions indicates that these receptors are involved in various physiological and pathological processes. Ligands of these receptors may be useful as neuroprotective agents in Parkinson's disease, Alzheimer's disease, ischemia, hypoxia, or hypoglycemia. Moreover, they may possess anxiolytic, antidepressive, antipsychotic, antiepileptic, antiparkinsonian, and other therapeutic properties. Unfortunately only a few such compounds that cross the blood–brain barrier are available so far. They are agonists of group II (LY354740; LY389795; LY379268; (+)-4-amino-2-thiabicyclo[3,1,0]hexane-4,6-dicarboxylic acid 2-oxide = LY 404040), and III [(R,S)-3,4-DCPG, an agonist of mGluR8, whose R isomer is an antagonist of AMPA receptors], and antagonists of mGluR5 (SIB-1893, MPEP). Animal studies seem to indicate that these compounds, administered in therapeutic doses, may be devoid of serious side effects such as ataxia or memory dysfunctions

(179,186). At present one of them, namely LY 354740, is in phase II clinical trials for the treatment of panic disorder and/or anxiety *(221)*.

ACKNOWLEDGMENT

The study was supported by the statutory funds of the Institute of Pharmacology, Polish Academy of Sciences. I am grateful to Professor Wolfarth for assembling the reference materials for this review and for his kind critical comments on the manuscript.

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III SCHIZOPHRENIA

6
Dopamine and Schizophrenia

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1. INTRODUCTION

Schizophrenia is a severe and pervasive illness afflicting approx 1% of the general population. The symptoms of schizophrenia have classically been subdivided in positive and negative symptoms. Positive symptoms refer to features that occur as a result of the disease, and include hallucinations (especially auditory) and delusions. Negative symptoms are features that are normally present but are reduced or absent as a result of the disease and include avolition, anhedonia, inattentiveness, and social withdrawal *(1)*. In more recent years it has become clear that this division in positive and negative symptoms is too simplistic. Using a factor analytical approach, Liddle investigated the symptomatology of stable schizophrenic patients and found three independent clusters of symptoms *(2)*: (1) *reality distortion*, which includes hallucinations and delusions; (2) *psychomotor poverty*, which includes poverty of speech, flat affect, and decreased spontaneous movements, and (3) *disorganization*, which include disorders of the form of thought and inappropriate affect.

Schizophrenia usually develops around or shortly after puberty, with a somewhat younger onset of age in males than in females. In some, though not in all cases, females have a second smaller peak shortly after menopause sets in *(3)*. This strongly suggests that hormones, such as estrogens, might have a protective influence. This would also explain why the course of schizophrenia in young females is usually somewhat more benign. With respect to the development of positive and negative symptoms, there is evidence that the negative symptoms develop prior to the positive symptoms. Thus, young children usually show disturbances in attention and social behavior several years prior to the development of the positive psychotic symptoms *(4,5)*.

2. THE ETIOLOGY OF SCHIZOPHRENIA

Although the etiology is not yet fully elucidated, there is ample evidence that genetic factors play an important role. This is illustrated by family, twin, and adoption studies *(6)*, where there is a clear correlation between concordance rates and the percentage of genes shared with an individual with schizophrenia. However, these studies have also shown that the genetics of schizophrenia are highly complex and cannot be described with simple Mendelian inheritance. Moreover, in spite of many molecular genetic studies,

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

the gene (or genes) involved in schizophrenia have not been identified. It would be beyond the scope of this paper to analyze all the genetic linkage studies, but linkage has been shown between schizophrenia and regions on chromosomes 1q, 5q, 6p, 8p, 10p, 13q, and 22q *(7,8)*. However, many other studies have failed to replicate this *(9)*. In a recent study, for example, the entire genome of 301 families with at least two schizophrenic family members was screened using 396 polymorphic markers. This led to scan with an average spacing of 10 centiMorgans. In spite of this very extensive genetic analysis, only one region with specific linkage to schizophrenia was found [on chromosome 10 (10p14)], which had not been identified in other genome-wide scans before *(10)*. The lack of a single gene that is clearly and unequivocally linked to schizophrenia suggests that more than one gene is involved. Moreover, it is highly likely that schizophrenia is a heterogeneous disease, with different subtypes that may be linked to different genes, making replication studies difficult.

In spite of the large amount of evidence that genes play a role in schizophrenia, there is also ample evidence that such factors only induce a predisposition and cannot, by themselves, explain the occurrence of schizophrenia. This is most clearly illustrated in the concordance rate of monozygotic twins, which is approx 50%, thus much lower than 100%. This implies that nongenetic factors must also play a role in ultimately determining the occurrence of schizophrenia. In recent years many epidemiological studies have been performed to try and elucidate these environmental factors. It appears that both early and late environmental factors can increase the risk of developing schizophrenia. Among the early environmental risk factors are *prenatal stress factors* such as famine *(11)*, unwantedness of a pregnancy *(12)*, and death of a spouse during pregnancy *(13)*, *perinatal stress factors*, such as obstetric complications, especially low Apgar scores *(14)*, and *early postnatal factors*, such as rearing in an urban environment *(15)*, immigration *(16,17)*, and parental loss *(18)*. In addition to these early environmental factors, there is evidence that environmental factors later in life may increase the risk of developing schizophrenia, including stressful life events *(19,20)* and cannabis use *(21–23)*.

Thus, schizophrenia seems to be a result of a combination of genetic and early-life and late-life environmental factors, and it appears to be the *interaction* between genes and environment that ultimately leads to the development of this severe disease. Mednick, for instance, studied genetic high-risk subjects and found that one of the most important factors predicting the outbreak of schizophrenia was early maternal separation *(24)*. Likewise, obstetric complications seems to occur especially in high-risk subjects *(25)*.

3. THE PATHOLOGY OF SCHIZOPHRENIA

As with the etiology, the pathology of schizophrenia is still an enigma. In general, the brains of patients with schizophrenia are smaller, with larger ventricles and gyri and smaller cortical volumes *(26,27)*. In addition to these more global deficits, a number of specific, though more subtle neuropathological findings have been reported. These focus predominantly on the hippocampal formation *(28–31)* and the prefrontal cortex *(32–34)*. Deficits have also been described in many other brain areas, including the cerebellum, basal ganglia, thalamus, and cingulate cortex *(26)*.

4. DOPAMINE AND SCHIZOPHRENIA

The most prominent neurochemical entity related to schizophrenia is, without any doubt, dopamine. In fact, the dopamine hypothesis consists of two separate parts: (1) *the*

dopamine hypothesis of schizophrenia and (2) *the dopamine hypothesis of antipsychotic drugs*. The first states that the symptoms of schizophrenia are owing to an increased dopamine transmission, whereas the second states that the therapeutic effects of antipsychotic drugs result from their inhibitory action on the dopamine transmission. Even though these arguments are often considered to be two sides of the same coin, there is no *a priori* reason for this. It is quite possible that the primary disturbance in schizophrenia is located upstream of the dopaminergic terminal regions (such as the aforementioned prefrontal cortex or the hippocampus), but that this disturbance can be modified at this lower level by dopamine antagonists. For that purpose the two hypotheses will be discussed separately in the remainder of this chapter.

4.1. The Dopamine Hypothesis of Antipsychotics

Although it is often suggested that the dopamine hypothesis of antipsychotic drugs was originally proposed by Carlsson and Lindqvist in 1963, this is actually not correct. In fact, in their original biochemical study these authors found an increase in both dopamine and noradrenaline metabolites after the administration of chlorpromazine and haloperidol. Indeed, the authors concluded that antipsychotics work through an interactions with the catecholamines, dopamine, and/or noradrenaline *(35)*. In fact, it was van Rossum in 1966 who showed that all antipsychotics were able to reverse the behavioral effects of levodopa, and he therefore suggested that the therapeutic effect of antipsychotics is related to their dopamine receptor-blocking properties *(36)*. About 10 yr later two independent studies were published showing that there was a good correlation between the dopamine blockade and the therapeutic dose of antipsychotic drugs *(37,38)*. Although these results have generally been taken to "prove" that the therapeutic effects of antipsychotics are indeed solely because of blockade of dopamine D_2 receptors, there is also evidence that the D_2 receptor alone cannot explain the effectiveness of antipsychotics.

Especially the introduction of the so-called atypical antipsychotics has challenged the validity of the dopamine receptor hypothesis. These drugs, such as clozapine, risperidone, olanzapine, and quetiapine, induce much less extrapyramidal (parkinsonian-like) side effects than the classical antipsychotics such as chlorpromazine and haloperidol. Because these side effects are directly related to the blockade of D_2 receptors in the caudate–putamen, this suggests that the atypical antipsychotics do not induce an overall blockade of D_2 receptors. These findings have led to the regional selectivity hypothesis, which states that classical and atypical antipsychotics have a differential effect on the various dopaminergic systems. Electrophysiological studies on clozapine, haloperidol, and various other compounds indeed suggested that classical antipsychotics block dopaminergic activity in both the mesolimbic and nigrostriatal system, whereas atypical antipsychotics only affect the mesolimbic system *(39,40)*. Although the hypothesis appears attractive, and would leave the overall dopamine hypothesis of antipsychotics intact, there are a few problems. First of all, it has been suggested that the differences between haloperidol and clozapine are an artifact, as it could not be observed in nonanesthetized animals *(41,42)*. Moreover, in freely moving rats, haloperidol and clozapine did not differentially affect dopamine release in the terminal regions of the nigrostriatal and mesolimbic system *(43)*. Finally, most of the novel atypical antipsychotics, such as olanzapine, ziprasidone, and risperidone, fail to show this regional selectivity *(44)*.

The dopamine hypothesis of antipsychotic drugs predicts that in therapeutically effective doses all antipsychotics should block the D_2 receptors to a similar extent. With the advent

of the position emission tomography (PET) scan technology, it has become possible to investigate this in living patients, and the results appear to be in violation of this prediction. Most antipsychotics need approx 60–80% D_2 receptor occupancy to be therapeutically effective. However, the atypical antipsychotics clozapine and quetiapine were found to be therapeutically effective at doses that blocked only about 25–35% of the D_2 receptors *(45)*. It seems difficult to explain this with the hypothesis that only the dopamine D_2 receptors are relevant for the therapeutic effects. It seems much more likely that at least for clozapine and quetiapine other receptors are also involved in the therapeutic effects. Indeed, the atypical antipsychotics are known to bind to a large number of different receptors *(46)*.

Recently an alternative theory again focusing exclusively on the role of dopamine receptors has been proposed *(47)*. The authors proposed that the essential difference between classical and atypical antipsychotics is the speed with which the atypical antipsychotics detaches from the dopamine receptor (k_{-1}). This can be calculated with the formula $K_A = k_1/k_{-1}$, in which K_A is the affinity constant and k_1 is the association constant, i.e. the speed with which the drug binds to the receptor. In general this k_1 is more or less constant for most drugs, including all antipsychotic drugs. This implies that k_{-1} is dependent only on the affinity. Drugs with a low affinity will have a high $k_{-1}$and thus will rapidly dissociate from the receptor. Kapur and Seeman argue that this explains the apparent low level of binding of clozapine and quetiapine in PET scan studies. Moreover, they argue that because of this rapid dissociation, the risk for inducing extrapyramidal side effects is lowered because these drugs do not induce a permanent blockade of the D_2 receptor. However, there are several arguments against this hypothesis. First of all, the fast dissociation rate of atypical antipsychotics (and hence the low affinity) is compensated for by increasing the dose, which should lead to an equally strong blockade as with the more potent classical antipsychotics. Secondly, it would imply that all atypical antipsychotics have a low affinity and that all antipsychotics with a low affinity are atypical. Neither of these assumptions appear to be correct. Atypical antipsychotics such as sertindole and risperidone have a high affinity for the D_2 receptors. Likewise, classical antipsychotics such as chlorpromazine have a very low affinity for the D_2 receptors. Finally, the hypothesis is unable to explain why the low-potency drug clozapine is effective in patients resistant to higher-potency antipsychotic drugs *(48)*.

In summary, although there is clear evidence for a role of dopamine in the therapeutic effects of antipsychotics, it is difficult to explain the available data solely on the basis of the blockade of D_2 receptors. Especially the finding that some patients are resistant to one antipsychotic yet respond favorably to others, strongly suggests that nondopamine receptors also play a role. At present it is unclear which receptor(s) this could be.

4.2. The Dopamine Hypothesis of Schizophrenia

One of the first indications that schizophrenia may be related to an increased activity of the dopaminergic system came from the seminal work of Connell on amphetamine-induced psychosis *(49)*. His results clearly showed that humans can develop schizophrenia-like symptoms when they receive amphetamine. Because amphetamine is (predominantly) an indirect dopamine agonist, enhancing release and blocking reuptake, this suggested that an increased dopamine transmission was somehow responsible for the schizophrenia-like symptoms. Later studies showed that other dopamine agonists like levodopa *(50)*

and methamphetamine *(51)* can also induce psychotic symptoms in nonschizophrenia patients. In addition to the effects in nonpsychotic patients, amphetamine also exacerbates existing symptoms in schizophrenic patients *(52)*, suggesting that an increased dopamine transmission is somehow related to the occurrence of psychotic symptoms.

Over the years, the dopamine hypothesis has been modified many times. The most important modification came with the observation that dopamimetics tend to induce only positive symptoms and are less effective in inducing negative symptoms. Likewise antipsychotics have only limited effect against the negative symptoms. This led to the idea that only the positive symptoms are related to dopamine *(53)*. A further modification came with the realization that negative symptoms can even *improve* with dopamimetics *(54–56)*. This led to the idea that negative symptoms may be related to a reduction in dopamine. Since positive symptoms (related to an increased dopaminergic transmission) and negative symptoms (related to a decreased dopaminergic transmission) can co-occur in the same patients, this implies that different dopaminergic systems must be involved in these symptoms.

The central question is, therefore, is there evidence for an increased and a decreased dopamine transmission in schizophrenic patients? The simplest way to measure this is by analyzing postmortem tissue. This material is most easily accessible and allows a detailed neurochemical analysis, with a very high spatial resolution. An important confounding factor is that virtually all patients with schizophrenia have at one point in time or another been treated with antipsychotics, and most of them have been treated with these drugs for a prolonged period of time, often up to many years. Because antipsychotics affect the dopaminergic system (as mentioned earlier), this might lead to erroneous conclusions. Moreover, postmortem studies give a static picture, and will never be able to give information about the dynamics of the dopaminergic system. Finally, people with schizophrenia develop the disease at a relatively young age and can live with it for 30–50 yr or more. In other words, postmortem changes will also reflect adaptation of the body to many decades of the disease. In vivo measures of dopaminergic activity would be able to circumvent most of these problems, especially if they could be done in drug-naive, first-episode patients. Because it is impossible to describe all the studies that have investigated the dopaminergic system in schizophrenia, we will focus on the most important results that have been obtained.

4.2.1. Is There Evidence for a Hyperdopaminergic State in Schizophrenia?

Figure 1 gives a schematic representation of the dopaminergic synapse, showing the different levels at which alterations in dopaminergic transmission can occur. Both pre- and postsynaptic processes may contribute to the development of a hyperdopaminergic state. Increased levels of dopamine have been described in several regions of postmortem brains of schizophrenic patients, including the caudate nucleus *(57)*, the nucleus accumbens *(58)*, and the amygdala *(59)*. In addition tyrosine hydroxylase (TH, the rate-limiting enzyme in the dopamine synthesis) levels were increased in the caudate putamen *(60,61)*. Moreover, there is in vivo evidence of an increased activity of the other synthesizing enzyme dopa-decarboxylase in schizophrenia patients *(62–64)*. Differences have also been observed in the capacity of dopaminergic cells to reuptake released dopamine. Thus both the K_M and the V_{max} of the high-affinity dopamine transporter (DAT) system in synaptosomes were significantly increased in the nucleus accumbens, but not the frontal

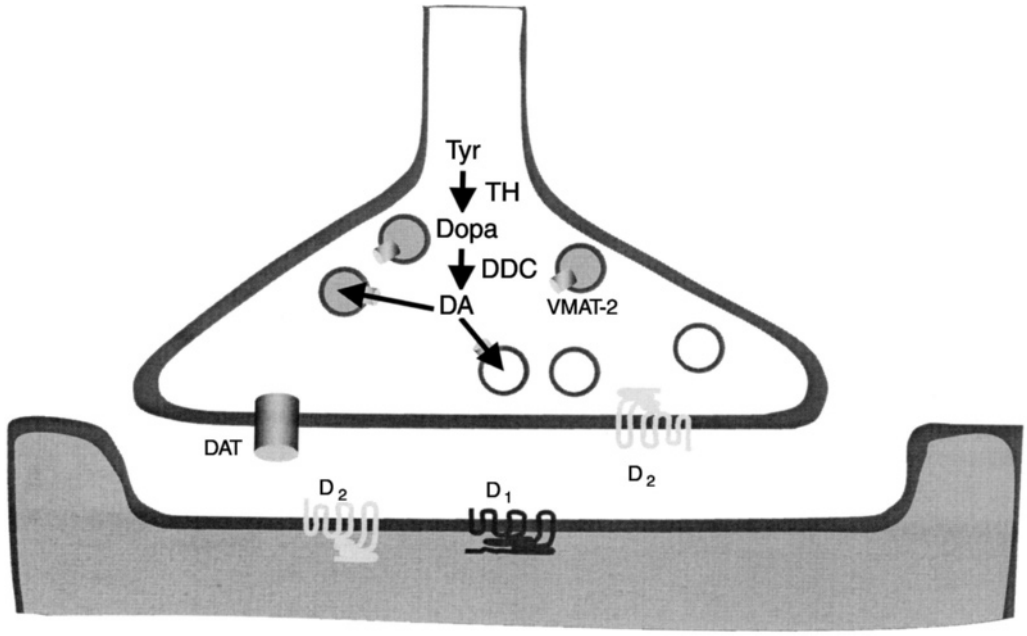

Fig. 1. A simplified representation of the dopaminergic synapse. The dopaminergic receptors are designated as families: D_1 (encompassing the D_1 and D_5 receptor) and D_2 (encompassing D_2, D_3 and D_4) Tyr; tyrosine; DA; dopamine; TH; tyrosine hydroxylase; DDC; dopa-decarboxylase; DAT; dopamine transporter; VMAT-2; vesicular monoamine transporter-2.

cortex of schizophrenic patients *(65)*, suggestive of an increased reuptake in schizophrenic patients. On the other hand, no change *(66)* or decreases *(67)* in the total number of reuptake sites in the striatum have also been described. In vivo studies using the PET technique failed to find alterations in DAT binding *(68,69)*. Interestingly, when using the ligand [^{11}C]DTBZ (dihydrotetrabenazine) a small but significant increase was found in the brainstem of schizophrenic patients. DTBZ specifically labels the vesicular monoamine transporter (VMAT-2), responsible for uptake of the monoamines into the storage and release vesicles *(70)*. Although it is not yet clear whether this is related to dopaminergic or noradrenergic neurons, it was shown many years ago that the [^{3}H]dopamine uptake in platelet storage granules was significantly increased in acute schizophrenic patients *(71)*. Because this effect could be reversed by reserpine, it suggests that this uptake carrier may be similar to the vesicular transporter in the brain. This might imply that also in the brain of schizophrenic patients more dopamine is taken up in storage vesicles, and hence more dopamine may be released on stimulation of the cells.

With respect to the involvement of postsynaptic processes in the development of hyperdopaminergia, most of the studies have concentrated on the dopamine receptors in various brain regions. Dopamine is known to bind to at least five different receptors belonging to two families. The D_1 family is composed of the D_1 and the D_5 receptors, whereas the D_2 family consists of the D_2, D_3, and D_4 receptors. Moreover, the D_2 receptor can exist in at least three different forms depending on the size of the third intracellular loop: D_{2short}, D_{2long}, and $D_{2longer}$ *(72)*. Unfortunately, selective ligands for the specific

receptor subtypes are not yet available. Therefore most studies have been limited to investigating the D_1 or the D_2 family of receptors, without being able to distinguish between the individual subtypes.

In several recent meta-analyses, the dopamine receptor–binding studies were evaluated *(73–75)*. These studies all concluded that there was support for the assumption that D_2 receptors are elevated in a subgroup of schizophrenic patients. However, they also provided evidence that postmortem studies usually led to larger differences between schizophrenics and controls than in vivo measures, suggesting that some of the increase in dopamine D_2 binding is owing to antipsychotics treatment. Alternatively, one might speculate that the number of D_2 receptors increase with progression of the illness, since most in vivo studies have been performed on drug-naive patients in an early stage of the disease. According to the meta-analysis, brain region and ligand used also significantly affect the outcome of dopamine-binding studies. The effect of brain region points to a nonhomogenous increase in D_2 binding in the brain of schizophrenic patients. Indeed, athough there is ample evidence that D_2 receptors' density is increased in the caudate putamen of schizophrenic patients, especially in postmortem tissue *(61,76,77)*, increases in other brain regions are much less evident. Thus Ruiz and colleagues did not find alterations in [^{3}H]raclopride binding in the frontal cortex *(76)*. The situation is even less clear in the nucleus accumbens. Whereas some find an increase in D_2 binding *(66)*, others find no differences *(78,79)*. The effect of choice of binding ligand on the effect size points to a differential distribution of different types of dopamine receptors in schizophrenic patients. Indeed, some have reported upregulation of D_4 receptors in schizophrenia *(79,80)*, though others have failed to be able to replicate this *(81)*. Recently an increase in mRNA for one of the dopamine D_2 receptors ($D_{2longer}$) has been described in the frontal cortex *(72)*. Because neither the second messenger system nor the function of this D_2 receptor subtype has yet been elucidated, it is unclear what the functional consequences of this increase in mRNA is.

The development of selective PET ligands for the dopamine receptors has led to a large number of studies in antipsychotic-free or even antipsychotic-naïve patients. The first papers using PET showed that drug-free and drug-naïve patients with schizophrenia have increased number of D_2 receptors in the caudate putamen *(82,83)*. Since then many more papers have been published using a variety of ligands. The results have been mixed, though most have been unable to find a difference between schizophrenic patients and controls *(84–87)*. Interestingly, the D_2-binding data of the individual patients in the last study seemed to fall into two groups, one with D_2 levels in the normal range and one with levels above the normal range. This suggests that there may be a subgroup of patients with increased D_2 levels within the schizophrenic population.

A distinct disadvantage of the first generation of PET ligands, such as [^{11}C]methylspiperone or [^{11}C]raclopride, is that they did not bind strongly to the D_2 receptors. Thus they were unable to detect dopamine receptors outside of the basal ganglia. The development of more specific ligands such as FLB457 has made it possible to also investigate extrastriatal dopamine receptors. However, so far, no clear-cut increases in extrastriatal D_2 receptors have been observed.

A final approach for studying the dopaminergic system is by using challenges to activate this system. Such studies are especially useful in investigating the dynamicity and reactivity of the dopaminergic cells. The previously mentioned increased activity of dopa-decarboxylase in combination with the increased activity of the VMAT-2 suggests

that the presynaptic dopaminergic terminals of schizophrenic patients contain more releasable dopamine than normal. One way to evaluate this is by treating patients and controls with amphetamine followed by the administration of a positron-emitting D_2 ligand, such as [^{11}C]raclopride. If amphetamine induces a stronger release of dopamine in schizophrenic patients, one would expect to see a more rapid reduction in raclopride binding, as more endogenous ligand competes with this PET ligand. Such an increased presynaptic release of dopamine has indeed been observed in the caudate–putamen in at least three different studies *(88–90)*. Interestingly, this increased responsiveness of the dopaminergic system was observed at the onset of the illness and during acute exacerbations but not when the patients were in remission *(91)*. A recent study provided strong evidence that this increased release of dopamine was present not only after stimulation with amphetamine, but also at baseline *(92)*. The authors pretreated controls and antipsychotic-free/naïve schizophrenic patients with α-methyl-para-tyrosine (αMpT), which blocks the TH activity thereby selectively depleting the cells of dopamine. In addition, they used the single photon emission computerized tomography (SPECT) to visualize the striatal D_2 receptor occupancy. The authors showed that, although the D_2 binding between the controls and the schizophrenic patients was not different at baseline, the increase in binding after αMpT was more than twice as large in schizophrenic patients compared to controls (19% vs 9%). Thus, all these data strongly suggest that there is an increased presynaptic dopaminergic activity and release in the caudate putamen of schizophrenic patients.

Studies using direct dopamine agonists, such as apomorphine, provided evidence of an increased sensitivity of the postsynaptic receptors. Thus the apomorphine-induced increase in plasma levels of growth hormone was much stronger in schizophrenic patients than controls *(93)*. Likewise apomorphine activated the regional cerebral blood flow in the anterior cingulate cortex *(94)* and decreased the glucose utilization in the caudate–putamen *(95)* to a much stronger degree in schizophrenic patients than in healthy volunteers. Interestingly, not all effects of apomorphine are upregulated. The apomorphine-induced increase in plasma ACTH and cortisol appears to be blunted in schizophrenic patients *(96,97)*.

Taking all these data together there is now, approx 45 yr after the original papers on the induction of psychotic symptoms after amphetamine use, direct evidence of a hyperfunctioning on the dopaminergic system in schizophrenia. This is most evident at the subcortical level, predominantly at the level of the basal ganglia. However, one should be aware of the fact that extrastriatal dopaminergic systems have not been investigated in any great detail yet. It might therefore be possible that other areas (including the nucleus accumbens) may also exhibit signs of hyperdopaminergia.

4.2.2. Is There Evidence for a Hypodopaminergic State in Schizophrenia?

As discussed above, several authors have linked the occurrence of negative symptoms to a reduced activity of the dopaminergic system. This would imply that the brains of schizophrenic patients should also show signs of hypodopaminergia. Because positive and negative symptoms can co-occur within the same patients *(1)*, the dopaminergic hypoactivity should be located outside of the basal ganglia.

Postmortem analysis of the brains of schizophrenic patients indeed found signs of a hypodopaminergic state, especially in cortical regions. Reductions in TH immunoreactivity

were found in area 9 of the prefrontal cortex, especially in layer 6 *(98)*, as well as in the entorhinal cortex *(99)*. In addition, there is a reduction in the number of DATs in the prefrontal cortex *(98)*. Less evidence has been obtained with respect to reductions in dopamine receptors. Whereas some authors found a reduction in D_1 binding in the prefrontal cortex *(100,101)*, this was not confirmed by others *(102)*. In addition, there were no differences in mRNA levels for D_1 receptors *(103)*, and a recent in vivo studies also failed to show differences in D_1 binding *(104)*. Recently a decrease in levels of the DARPP-32 protein was found in the prefrontal cortex of schizophrenic patients *(105)*. DARPP-32 is specifically localized in neurons containing dopamine receptors and controls the physiological characteristics of these neurons, as stimulation of dopamine D_1 receptors phosphorylates (and activates) DARPP-32 and stimulation of D_2 receptors dephosphorylates (and deactivates) DARPP-32. Whether this reduction is the result of a reduction in the number of dopamine-containing neurons or in the amount of peptide per cells remains to be investigated. With respect to other receptors, a reduction in mRNA levels for the D_3 and D_4 receptors has been observed in the orbitofrontal cortex *(103)*. Likewise, using [^{11}C]FLB457, a reduction in D_2 binding was observed in antipsychotic-naïve schizophrenic patients in the anterior cingulate cortex, as well as a strong tendency for a reduction in the thalamus *(106)*.

In summary, although much less investigated, the brains of schizophrenic patients also shows signs of hypodopaminergia, especially in frontal and temporal cortical regions, including the prefrontal, the anterior cingulate, and the entorhinal cortex.

5. INTEGRATION

Although the relevance of dopamine for schizophrenia has long been recognized, it was not until recently that hard biochemical evidence for a dysregulation of the dopaminergic system has been demonstrated in schizophrenic patients. Although there is still some controversy and much more confirmatory work needs to be done, the overall consensus is that schizophrenic patients have both a hyperactive subcortical dopaminergic system and a hypoactive cortical dopaminergic system. One important question that has not been solved yet is whether all patients suffer from this dopaminergic imbalance or whether some patients have predominantly a hyperactivity and others primarily a hypoactivity. As was mentioned above, the hyperactivity is primarily related to the occurrence of positive symptoms and the hypoactivity to the negative symptoms. Because both can occur in the same patients, both a hyperactivity and a hypoactivity should co-occur, though so far this has not been investigated.

There is, however, ample animal evidence that these two states can co-occur within the same rat. Already in 1980, Pycock and colleagues showed that lesioning of the prefrontal cortical dopaminergic system led to an upregulation of the subcortical dopaminergic system, including the nucleus accumbens and the striatum *(107,108)*. Since then, these findings have been replicated and extended many times, and all data point to a tonic inhibitory control of prefrontal dopamine on subcortical dopaminergic terminal fields. Removing this inhibitory control leads to an enhanced accumbal dopaminergic response to stress *(109)*, an effect predominantly mediated via cortical D_1 receptors *(110)*. Moreover, partial lesions also enhance the responsiveness to naturally reinforcing stimuli, such as highly palatable food and sex-related olfactory cues *(111)*. Thus, the data clearly

indicate that a reduction in cortical dopamine can co-occur with an increase in subcortical dopamine. It is not clear, however, whether these two are always causally related to each other. Given the independence of negative and positive symptoms in schizophrenia, it should be assumed that a reduction in prefrontal dopamine can also occur independent of an increase in subcortical dopamine.

Overall the data clearly point to a dysregulation of the dopaminergic system in schizophrenia, especially a hyperreactive striatal dopaminergic system. The in vivo studies clearly have shown that both the basal dopamine release (measured by the binding of raclopride after treatment with the TH inhibitor αMpT), as well as the amphetamine-induced dopamine release is enhanced in schizophrenic patients. In this respect it is important to realize that there are different pools of dopamine within the terminal region *(112)*. In general, a distinction is made between the so-called readily releasable pool (stored in vesicles close to the plasma membrane), and the so-called storage pool (stored in vesicles farther away from the plasma membrane; *see also* Fig. 1). Because both TH and dopa-decarboxylase occur in the cytosol, dopamine also occurs freely in a so-called cytosolic pool *(113)*. Newly synthesized dopamine accumulates preferentially in the readily releasable pool, which explains why this pool is so sensitive for αMpT *(114)*. The storage vesicles are thought to contain a much larger amount of dopamine, and this pool is more sensitive to reserpine *(112)*. Reserpine binds to the VMAT-2, which is responsible for sequestering dopamine into the vesicles. As mentioned above, schizophrenic patients are more sensitive to the effects of αMpT *(92)*, indicating a larger readily releasable pool of dopamine in these patients. These data would fit with the increased activity of both TH and DOPA-decarboxylase in schizophrenic patients. Moreover, it might also explain the higher sensitivity to amphetamine. Amphetamine is known to have multiple action of the dopaminergic system *(115)*. First of all, it binds to the DAT, where it works as a false substrate and is taken up into the presynaptic terminal. This stimulates a process called reverse transport (RT), where the DAT transports cytosolic dopamine out of the cell, instead of into it. In addition amphetamine can enter the presynaptic terminal through diffusion. Following the entry of amphetamine into the cell, it also enters the vesicles where it causes a change in pH, which subsequently leads to a leakage of dopamine from the vesicles into the cytosol, which can act as a substrate for RT. It is not clear whether amphetamine depletes both vesicular pools, but the work in DAT knockout mice shows that the readily releasable pool is certainly affected *(115)*. In summary, these data seem to indicate that especially the readily releasable and cytosolic compartment of the dopaminergic system are hyperactive in schizophrenic patients. Whether the storage pool is also altered has not been investigated in great detail yet, though some studies showed increases in the amount of VMAT-2 in schizophrenic patients *(70,71)*.

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7

Glutamate and Schizophrenia and the *N*-Methyl-D-Aspartate Receptor Hypofunction Hypothesis

Stefan Bleich and Johannes Kornhuber

1. INTRODUCTION

1.1. Prevalence and Symptoms of Schizophrenia

Schizophrenic psychoses are severe mental disorders over the course of which hallucinations, changed perception, cognitive disturbances, as well as social withdrawal and lack of drive may occur. There is an increased familial incidence of schizophrenia. Whereas the risk of disease is around 1% in the average population, it increases to over 40% in children of two schizophrenic parents *(1)*. In the meantime, numerous family, twin, and adoption studies have demonstrated that genetic factors make a major contribution to the etiology of schizophrenic psychoses *(2,3)*. Despite these genetically related influences on the development of endogenous psychoses, the genesis of this disease has yet to be explained. Thus, although genetic factors appear to have been confirmed for the etiopathogenesis of schizophrenic psychoses, the results of other research work have been all the more heterogeneous over the past years.

2. DOPAMINE RECEPTORS

2.1. Biochemical Hypotheses on the Pathogenesis and Pathophysiology of Schizophrenic Psychoses

With the development of the neuroleptics and proof that the antipsychotic effect of the neuroleptics is based on a blockade of dopaminergic receptors, the as yet best-known biochemical hypothesis (dopamine hypothesis) was put forward to explain the pathogenesis and pathophysiology of schizophrenic psychoses, as first formulated by Carlsson and Lindquist *(4)*: The genesis of the schizophrenic psychoses has to do with a hyperfunction of the dopaminergic system, and the antipsychotic efficacy of the neuroleptics is based on a blockade of dopaminergic receptors. The dopamine antagonism in the so-called mesolimbic/mesocortical pathways (A_{10}), which lead from the mesencephalon (ventral tegmental area) to the nucleus accumbens, to the corpus amygdaloideum, to the prefrontal cortex and other structures of the lymbic system, is attributed in detail to the antipsychotic effect of the neuroleptics. In contrast, the blockade of the nigrostriatal

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

dopamine pathways (A_9) is held responsible for the typical side effects all the neuroleptics, the extrapyramidal disturbances. Neuroleptics also block serotonin, α-adreno, histamine, and muscarinergic acetylcholine receptors, as well as other receptor systems to a lesser degree *(5)*. Although clinical experience of the therapeutic effect of dopamine antagonists in schizophrenias, the occurrence of psychotic symptoms upon administration of dopamine agonists (e.g., amphetamine), as well as the demonstration of elevated dopamine receptor density (D2 and D4 receptors) in postmortem brain tissue (striatum/basal ganglia) of schizophrenia patients suggest an involvement of the dopaminergic system *(6,7)*, these results could not be replicated in other studies with a different methodological background (e.g., positron emission tomography [PET] studies) *(8,9)*. A primary change in dopaminergic transmission has thus yet to be demonstrated beyond doubt in patients with schizophrenic psychoses *(10)*, although it takes on a rational position of importance within the context of the so-called equilibrium hypothesis. The good antipsychotic effect of clozapine, a substance with a broad pharmacological spectrum of action, with a low dopaminergic affinity for dopamine receptors, has led to the search for alternative hypotheses to the dopamine hypothesis. Besides the dopamine hypothesis, many other biochemical hypotheses have now been put forward, which include changes in the area of the endorphine system *(11)*, disturbances of prostaglandin metabolism *(12,13)*, and a postulated involvement of viral infections in the etiopathogenesis of schizophrenias *(14)*. Apart from the dopamine hypothesis, Table 1 shows some of the equilibrium hypotheses based on the dopaminergic system, as well as more recent hypotheses that are largely independent of the dopaminergic system. Various review articles deal with the hypotheses mentioned in detail *(15–17)* and the topic is also covered in Chapter 6; however, the glutamate hypothesis of schizophrenia and glutamatergic neurotransmission are described below.

3. GLUTAMATE AND SCHIZOPHRENIA

3.1. The Glutamate Hypothesis

The first study to make a connection between glutamate and the schizophrenic psychoses was described by Kim and coworkers *(18)*. Significantly reduced cerebrospinal fluid (CSF) glutamate levels were found in patients with schizophrenic psychoses compared with controls in this investigation, and the hypothesis of a glutamatergic hypofunction in schizophrenia was formulated.

Put in a simplified way, the glutamate system is the counterpart of the γ-aminobutyric acid (GABA) system. Glutamate is just as widespread a neurotransmitter as GABA, and accordingly is found in around 30% of all synapses. The cells of origin of the glutamate system are located in particular in the entire cortex and in the hippocampus. It is projected primarily into the limbic system, the basal ganglia and, in turn, into the entire cortex. The glutamate system has an excitatory effect on succeeding systems, and glutamate is undoubtedly the most important messenger substance of the association pathways and of the corticofugal pathways. The effect of glutamate influences almost all other neurotransmitter systems, whereby glutamate can also have an inhibitory effect via other mechanisms. Glutamate thus activates, for example, neuron systems that have an inhibitory effect on the dopaminergic system in the basal ganglia, so that elevated glutamatergic activity results in reduced dopaminergic activity. Together with dopamine, glutamate plays an important role in cognitive processes and memory functions. In simple terms, the glutamate hypothesis rests on the assumption of an equilibrium between dopaminergic

Table 1
Brief Overview of the Biochemical Hypotheses on the Pathogenesis and Pathophysiology of Schizophrenic Psychoses According to Kornhuber and Weller *(17)*

Hypotheses	References
Dopamine hypothesis	*4,77*
Equilibrium hypotheses related to the dopaminergic system	
Glutamate hypothesis	*18–20,47*
Cholinergic hypothesis	*78,79*
GABA hypothesis	*80*
Serotonin hypothesis	*81*
Adenosine hypothesis	*82*
CCK hypothesis	*83*
Opioid hypothesis	*84*
Neurotensin hypothesis	*85*
Hypotheses not directly dependent on the dopaminergic system	
Toxic effects on NMDA antagonists	*86*
Sigma hypothesis	*87*
Changes in signal transduction	*88*

GABA, γ-aminobutyric acid; CCK, cholecystokinin; NMDA, *N*-methyl-D-aspartate.

and glutamatergic neurotransmission (Fig. 1), whereby dopamine receptor antagonists have an antipsychotic effect and partial glutamate agonists probably also have an antipsychotic effect *(18–21)*. The model of a cortico-striato-thalamo-cortical control system was extended over the past few years essentially on the basis of animal pharmacological behavioral observations, in order to include noradrenergic mechanisms in addition to the glutamatergic, GABAergic and dopaminergic ones *(22,23)*.

Over the past years, interest in research into schizophrenia has constantly grown with regard to excitatory neurotransmitters (glutamate, aspartate, homocysteine) and glutamate receptors. As already mentioned, markedly reduced CSF glutamate levels were found in patients with schizophrenic psychoses compared with controls *(18)* and the hypothesis was put forward that *N*-methyl-D-aspartate (NMDA)-receptor function is possibly of aetiological importance in the schizophrenias. Reduced glutamate levels were later also found in postmortem brain tissue of schizophrenic patients *(24)*, although these results could not be confirmed in other studies *(25,26)*. In addition, elevated *N*-acetylaspartylglutamate (NAAG) levels and a reduced concentration of NAAG-associated dipeptidase, which cleaves NAAG into glutamate and aspartate, were found in postmortem brain tissues of schizophrenic patients *(24,27)*. These results may point to a hypofunction of glutamatergic synapses, since the latest findings show NAAG to be a partial agonist at the NMDA receptor, with low affinity and low intrinsic activity. There is also evidence in support of the NMDA receptor hypofunction hypothesis from a recent study observing reduced levels of D-serine in patients suffering from schizophrenia *(28)*. D-Serine modulates the strychnine-insensitive glycine sites of the NMDA receptor and is enriched in the forebrain. In general, the following circumstances suggest a participation of the glutamatergic system in the pathogenesis of the schizophrenias:

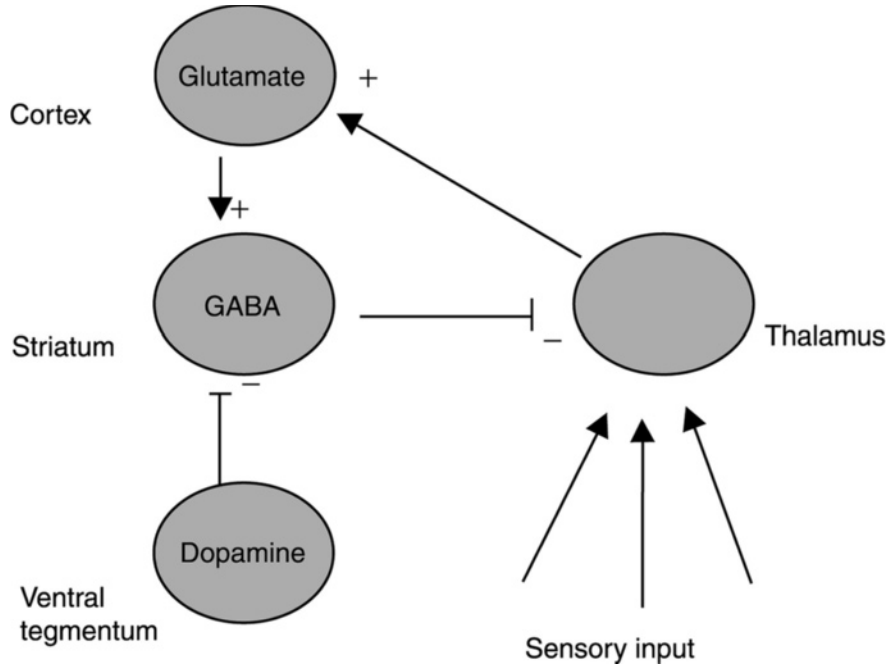

Fig. 1. The glutamate hypothesis of the schizophrenias according to Kornhuber et al. *(20)* (illustrated on the left): The hypothesis postulates an equilibrium between inhibitory dopaminergic and activating glutamatergic neurons. The model of a cortico-striato-thalamo-cortical feedback loop *(76)* integrates the glutamate hypothesis with regional-anatomical hypotheses (thalamic filter model according to ref. *89*) on the pathophysiology of schizophrenic psychoses. A hypofunction of the glutamatergic cortico-striatal pathway would indirectly open the thalamic filter, leading to an uncontrolled flow of sensory information to the cortex and in this way to psychotic experience. This model merges biochemical (glutamate, dopamine) and anatomical (frontal cortex, thalamus) hypotheses into a cortico-striato-thalamo-cortical control system.

1. Various receptor channel blockers can induce pharmacotoxic psychoses. A classical example of this is phencyclidine (PCP) psychosis, whereby the tolerability of channel blockers depends decisively on the affinity to the PCP-binding site. In contrast to PCP, other NMDA channel blockers, such as memantine, have as a whole a relatively low potential for inducing pharmacotoxic psychoses *(29,30)*. One of the main supports for the glutamate hypothesis of schizophrenia is derived from the effect of the substance PCP. In PCP abuse ("angel dust"), psychotic symptoms very similar to those of schizophrenia are frequently observed, which is why this is currently regarded as the best pharmacological model for the schizophrenias *(31,32)*. The psychotomimetic effect of PCP is not only more rapid and stronger than that of the amphetamines, PCP also causes negative symptoms in addition to the productive–psychotic symptoms. In addition, pre-existing schizophrenias can be exacerbated under PCP intake. At the lowest concentrations in which PCP triggers these effects, this substance interacts selectively with the NMDA receptor *(32)*.
2. In postmortem brain tissues of schizophrenic patients, the number and density of NMDA receptors is increased (e.g., prefrontal cortex, hippocampus, basal ganglia), which may suggest an inductive effect (compensatory upregulation of NMDA receptors) owing to reduced glutamatergic neurotransmission *(33,34)*. What is more, other investigations suggest the presence of dysfunctions at other glutamate receptors: The receptor subunit (GluR2) at the α-amino-3-hydroxy-5-methylisoxazole-4-proplonate (AMPA) receptor, which also provides for the integrative function of the NMDA receptors, is reduced in the postmortem brain tissue

of schizophrenic patients *(35)*, which increases the sensitivity to glutamatergic stimulation and may in this way potentiate neurotoxic effects.

3.2. Glutamate Receptors: Genetic Studies

The importance of reduced NMDA receptor-mediated glutamatergic transmission for the induction of schizophrenic behavior has also been shown in animal experiments. Schizophrenia-like behavior such as increased motor activity, stereotypy, and anomalies in social and sexual behavior were shown by mice with a genetic knockdown of the NR1 subunit with 5% expression of the normal NR1 subunits *(36)*. The behaviors could be improved by administration of haloperidol and clozapine *(36)*. Furthermore, mice carrying homozygous point mutations in the glycine-binding site of the NMDA NR1 subunit (*Grin1*) exhibited a fivefold reduction in receptor glycine affinity, displayed moderate deficits in long-term potentiation (LTP) induction and spatial learning, and exhibited increased startle reactivity but normal locomotor activity and prepulse inhibition (PPI) *(37)*. The severity of this NMDA receptor hypofunction was insensitive to antipsychotics *(37)*. Further effects were shown for mice with a knockout of the NR2A subunit *(38)*. NR2A knockout mice exhibited increased locomotor activity in a novel environment and an impairment of latent learning in a water source-finding task *(38,39)*. The increased locomotor activity was found neuroleptic-sensitive and could be reduced by treatment with haloperidol and risperidone *(39)*.

Investigators who examined levels of glutamate receptors on the basis of the implication of altered glutamate receptor function in schizophrenia found a dysregulation *(40)*. However, inferences about receptor activity are difficult to draw from a study of receptor density. A reduced number of receptors might be considered to be a molecular response to an increase in receptor activity, but could equally be interpreted as a cause of decreased receptor function. In the light of this difficulty in providing a conclusive explanation, it is worth reviewing the studies of glutamate receptors in schizophrenia. Several investigators have reported that the expression of AMPA and kainate receptor subtypes is reduced in the hippocampus, an area considered to play an important role in schizophrenia *(34,40)*. Some studies have shown the AMPA subunits GluR1 and GluR2 to be decreased in the hippocampus and the parahippocampal gyrus *(35,41,42)*. Consistently with this, ligand binding to AMPA receptors was decreased *(43)*. The kainate receptor subtypes GluR6 and KA2 were also significantly reduced in the schizophrenic hippocampus *(44)*. Studies on kainate receptor density, conducted with radiolabeled kainate, demonstrated a decrease in the hippocampus, as well as an increase in the cortex *(43,45,46)*. Data obtained for the NMDA receptor indicate abnormal expression in the cortex and putamen in schizophrenia *(40)*. The number of NMDA receptors is increased in the putamen *(33,47)*, whereas the number is increased and the composition of the NMDA receptor subunits is altered in the cortex *(48–50)*. However, as might be expected from human postmortem studies, investigators conducting research on schizophrenia did not always find the same changes in the expression of ionotropic glutamate receptors *(51,52)*. However, certain observations still appear to be more or less consistent among different research groups. These include the abnormally low expression of AMPA/kainate receptors in the hippocampus and the increase in the number of NMDA receptors in putamen and cortex *(40)*. The increase in NMDA receptor levels may be because of decreased NMDA receptor function, whereas AMPA receptors may be reduced in brain areas with secondary glutamate elevation. Thus, although schizophrenia is accompanied

by a dysregulation of ionotropic glutamate receptors, the pathological basis and consequences vary across brain areas, and are still subject to interpretation. The expression of the inhibitory subunit NR3 has yet to be analyzed in postmortem schizophrenic brains. However, it will be important to explore the expression of these receptor subtypes in schizophrenia in relation to NMDA receptor hypofunction. Levels of expression of the NR3A subunit are a matter of particular interest because NR3A has been found in areas of the brain relevant to schizophrenia, such as cortical areas and the thalamus *(53)*.

Another focus of schizophrenia research are mGluRs. Animal experiments have provided evidence suggesting that Group II mGluRs (mGluR2/3) play a functional role in schizophrenia. Group II mGluRs reverse the behavioral, locomotor, and cognitive effects of PCP in rats *(54)*, and they appear to interfere with PPI *(55)*. However, evidence of structural or quantitative differences of any mGluRs in schizophrenia is scant. The expression of mGluRs (mGluR1-5, -7, and -8) has been examined in various brain areas (i.e., prefrontal cortex, hippocampus, thalamus), but was found to be unchanged in schizophrenia *(56–58)*. An association of a genetic polymorphism with schizophrenia has been reported for mGluR5 *(59)*, whereas gene polymorphisms for mGluR2 (4, 7, and 8) did not demonstrate any significant association with schizophrenia *(60–62)*.

3.3. Future Directions: Pharmacotherapeutic Influence of Glutamatergic Neurotransmission

The complex nature of the glutamate system is reflected in the combination of neuroplasticity and neurotoxicity experienced with conventional antipsychotic drugs. The neuroplasticity induced by antipsychotic drugs appears to be important for the therapeutic benefits, whereas the generation of neurotoxicity is detrimental. Considerable biochemical, pharmacological, and clinical evidence is available to show that the glutamate system is abnormal in schizophrenia. A primary episodic malfunctioning of the glutamate system can be used to explain several different models of schizophrenia, such as the neurodevelopmental and the progressive neurodegeneration models, the overactive dopamine or the hypoactive GABA system models. This malfunction is possibly based on genetic abnormalities and may be exacerbated by stress and environmental factors. In view of the role played by glutamate in the pathology of schizophrenia, a pharmacological stabilization of the glutamate system may make it possible for us to prevent psychotic episodes and neurotoxicity. It is interesting that mGluR5 potentiates NMDA receptor activity, which might suggest that mGluR5 agonists have a therapeutic potential in schizophrenia *(63,64)*. In an animal phencyclidine model of schizophrenia targeting mGluR2-3 (group II) with LY354740 attenuated the disruptive effects of phencyclidine on working memory, stereotypy, locomotion, and cortical glutamate efflux *(65)*.

The therapeutic benefit for the treatment of schizophrenic psychoses may therefore lie in the development of (partial) glutamate agonists as antipsychotics. The affinity of such substances should, on the one hand, be so low that they leave the ion channel quickly after physiological activation of the NMDA receptor and, on the other, so high that they do not leave the ion channel upon low-grade depolarization and glutamate concentrations in the micromolar range. These properties are of particular importance, because a strong agonistic stimulation of the NMDA receptor would otherwise lead to so-called excitotoxicity, i.e., to neurotoxic cell damage. The therapeutic intake of glycine or preferably D-cycloserine substances would appear to be very promising, the latter substance

overcoming the blood–brain barrier, thus increasing the cerebral glycine concentration. Glycine is an indirect agonist at the NMDA receptor and first study results of treatment of schizophrenic patients with high oral glycine dosages showed good efficacy especially on the productive symptoms of schizophrenic psychoses *(66,67)*. Recently, it has been shown that high-dose glycine treatment significantly improved negative symptoms of schizophrenia *(68)*. Furthermore, D-cycloserine, an antitubercolosis drug and a partial agonist at the glycine-binding site of the NMDA receptor, led to an exacerbation of productive symptoms in uncontrolled studies *(69)*, but had a favorable influence on the negative symptoms of schizophrenia *(70–73)*. In placebo-controlled trials, D-cycloserine (50 mg/d) also improved significantly negative symptoms and is effective when added to olanzapine or risperidone *(74,75)*. In conclusion, enhancement of glutamatergic neurotransmission with drugs having agonistic activity at the glycine site of the NMDA receptor is an innovative pharmacological approach for treatment of schizophrenia. Inhibitors of glycine reuptake are active in certain experimental models predictive of antipsychotic properties. Taken together, distinct "subtypes" of glycine B site-bearing NMDA receptor may fulfill differential roles in psychotic states and blockade of certain populations of NMDA receptor may be of use in the management of schizophrenia (for review, *see* ref. *76*).

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8
Role of Glycine in Schizophrenia

Beate D. Kretschmer

1. INTRODUCTION

The glutamate (GLU) hypothesis of schizophrenia was first published in 1980 and was based on reduced Glu levels in the cerebrospinal fluid (CSF) of schizophrenic patients *(1)*. Although it took a while, this negative correlation between Glu levels and schizophrenic symptoms has recently been confirmed *(2)*. Further evidences are also in concert with a hypoactive glutamatergic system as one but not solely underlying mechanism in schizophrenia (*see* Chapter 7 by Bleich and Kornhuber) and one of these major findings is going back to the late 1950s when schizophrenia-like symptoms were described after administration of phencyclidine (PCP) in humans *(3)*. However, a link between PCP-induced effects and the glutamatergic system was drawn not earlier than 20 years later when an interaction of PCP with the GluR and more specifically with the *N*-methyl-D-aspartate (NMDA) receptor was shown *(4–6)*. Further antagonists that block the NMDA receptor in a competitive or noncompetitive manner induce schizophrenia-like symptoms as well when they are given to humans and also to animals *(7–11)*. More support for the GLU hypothesis derived from postmortem studies showing an increase of NMDA receptor density in several brain areas *(12–13)* most probable as a consequence of lowered GLU release in these regions although differences regarding the NMDA subunits can be seen *(14–15)*. Adaptation in the density of other GluRs as α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainate-, and metabotropic receptors has also been described, but the findings are less concise and depend on the respective subunit of the receptor and on the anatomical structures *(14,16–17)*.

The glycine-binding site is one of several other binding sites (i.e., polyamine, zinc, magnesium, and noncompetitive) that are expressed in the NMDA receptor complex (*see also* Chapter 4). Altogether they modulate the function of the NMDA receptor and depending on the composition of the NMDA receptor complex the pharmacological characteristics of the whole complex change significantly.

Glycine binds to the glycine-binding site as a coagonist and is a positive modulator of the NMDA receptor complex. The presence of glycine is prerequisite for the activation of the NMDA receptor through GLU or NMDA (*18–22*; for review, *see* ref. *24*). Interest in the glycine-binding site in schizophrenia came up when Ishimaru et al. *(12)* described lower levels of glycine-binding sites in the cortex of schizophrenic patients and when

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

therapeutic consequences were drawn from the GLU hypothesis. Because a therapeutic approach via direct GluR-agonists is not feasible owing to the risk of neurotoxicity, stimulation of a hypoactive glutamatergic system through the coagonistic glycine-binding site that bears no risk of neurotoxicity seemed therefore to be smarter from the functional point of view.

There are at least two opportunities to increase glycinergic transmission and by this to induce functional GLU agonism:

1. The glycine site can be modulated directly through agonists and indeed several agonists have been described. Variations that lead to modified chemical structures are limited and are very often accompanied by the loss of agonistic properties because glycine itself is a very small molecule. Although this approach does not bear the risk of neurotoxicity, it bears the risk of tolerance development in a chronic treatment schedule. However, many studies in humans and animals indicate that pathophysiological conditions can be improved by the direct agonists (*see* Section 3).
2. The glycine site can be modulated indirectly via blockade of the glycine transporter (GlyT). This transporter is located in the vicinity of glycinergic transmission and controls the glycine concentration in the synaptic cleft. Two subtypes have been described, GlyT1 (with three isoforms) and GlyT2 (two isoforms). GlyT2 is present in presynaptic elements in spinal cord and brainstem and regulates the strychnine-sensitive glycinergic transmission *(24)*, whereas GlyT1 is present in glia cells in frontal cortex, hippocampus, striatum, thalamus, and also on the level of brainstem and spinal cord. This latter one is colocalized with the NMDA receptor complex and controls the glycine concentration in the NMDA receptor complex *(25)*. Blockade of the GlyT1 would therefore extend the time of glycine available in the synaptic cleft after release from its storage sites. Moreover, this approach is without any interaction with the glycine site itself and without a risk of tolerance development and neurotoxicity. Indeed, an increase of glycine concentration in a schizophrenia-related structure (ventral hippocampus) has recently been shown in rats treated with the GlyT1 inhibitor ORG 24598 *(26)*. To evaluate the role of glycine in schizophrenia, behavioral effects of glycine site antagonists and agonists are summarized in the following paragraphs. If an impaired glutamatergic transmission is one underlying mechanism in schizophrenia, (1) glycine site antagonists should have behavioral effects resembling to those of NMDA receptor antagonists and (2) glycine site agonists or GlyT 1 inhibitors should improve schizophrenic symptoms in animals and patients.

2. EVIDENCE FROM ANIMAL STUDIES

Schizophrenia is characterized by positive symptoms with hallucinations, delusions, attention deficits, loose associations, disorganized thoughts, and psychomotor stimulations, and by negative symptoms with flat affect, social impairment, and impaired memory and cognition (*see also* Chapter 6). In animals schizophrenia-like symptoms can be induced by pharmacological or by neurodevelopmental manipulations (i.e., neonatal hippocampus lesion, isolated rearing, neonatal PCP administration), however only particular aspects of behavioral dysfunctions can be generated (i.e., psychomotor stimulation with perseverative behavioral aspects like hyperlocomotion, stereotyped behavior, and head weaving; attention or sensory gating deficits with deficits in prepulse inhibition (PPI) and latent inhibition; social impairment; and cognitive disturbances).

2.1. Psychomotor Stimulation

In parallel to NMDA receptor antagonists, glycine site antagonists induce psychotomimetic effects although less pronounced and sometimes less concise between the individual compounds. For example, systemic administration of the partial agonist

(R,+)-HA-966—which acts predominantly as an antagonist—or of the full antagonists L-701,324, MRZ 2/576, and ACEA 1021 do not increase locomotor behavior but rather induce sedation in higher doses when given to rodents *(27–33)*. In contrast, stereotyped sniffing behavior and head weaving can be induced by systemic administration of (R,+)-HA-966, L-687,414, L-701,324, and ACEA 1021. The latter one has, however, a tight dose–response relationship although the induced behavior can be blocked by the glycine site agonist DCS *(28,30,34,35)*. The overall intensity of the psychotomimetic effects induced by these substances is lower than that of direct NMDA receptor antagonists. This is supported by the finding that glycine site antagonists do not substitute for the discriminative stimuli of noncompetitive NMDA receptor antagonists *(36,37)*.

The dichotomy of the effects of glycine site antagonists on locomotion and stereotyped behavior is also reflected by studies investigating the antagonist 7-chlorokynurenate (7-CLKYN), which does not penetrate the blood–brain barrier and needs to be administered directly into the brain. Infusion of 7-CLKYN into the third ventricle of rats dose-dependently enhances stereotyped sniffing behavior but has no effect on locomotion *(38,39)*. In parallel to systemic applications, higher doses generate sedation and strong muscle relaxation and by this reduce motor activity *(39)*. When 7-CLKYN is infused into the dorsal striatum or the nucleus accumbens—structures that are directly involved in stereotyped behavior and locomotion—the same results are obtained. The antagonist induces stereotyped sniffing behavior after infusion into both structures but dose-dependently only after infusion into the dorsal striatum. However, locomotor behavior is once again not affected by this antagonist *(40)*.

Thus, blockade of the glycine-binding site induces at least some psychomotor stimulatory symptoms that are described for direct NMDA receptor antagonists. The symptomatology is less pronounced after systemic administration of the glycine site antagonists. However, it resembles to the symptomatology of the noncompetitive NMDA-antagonists after local infusion into structures directly linked to the respective symptom. As mentioned earlier, a low brain penetration and/or bioavailability of glycine receptor antagonists may be reasons behind the differences.

Glycine agonists have been less intensively characterized. So far no effect on sniffing behavior and locomotion has been found after administration of D-serine and glycine *(41)*. Also, the partial agonist DCS with an agonistic profile in doses up to 20–30 mg/kg also does not modulate sniffing behavior and locomotion in rats *(29,31,42,43)*.

Other partial agonists such as (R,+)-HA-966, ACPC, L-687,414, cycloleucine, and S18841 with an intrinsic activity between 10 and 92% *(23)* are in vivo mainly characterized by antagonistic effects even when they have a high intrinsic activity in vitro. They are commonly used as antagonists.

Some more interest was given to the effects of glycine agonists in pathophysiological models such as pharmacologically or environmentally induced hyperlocomotion and stereotyped sniffing behavior via PCP, (+)MK-801, amphetamine, apomorphine, or impaired neurodevelopment (neonatal hippocampus damage, isolated rearing).

Neonatal ventral hippocampal damaged rats are characterized by one prominent symptom that they share with schizophrenic patients; they show a postpupertal onset of symptoms especially a hypersensitivity to PCP, (+)MK-801, and amphetamine, and also changed responses to novelty *(46–49)*. Glycine itself is able to attenuate the effects on novelty- and amphetamine-induced hyperlocomotion *(46)*.

However, glycine site agonists give controversial results in pharmacologically induced models. Whereas amphetamine- or apomorphine-induced hyperlocomotion cannot be antagonized by glycine or the GlyT1 inhibitors (GDA and its derivates, ORG 24461, NFPS) *(50–52)*, the effects on PCP- and (+)MK-801-induced psychotomimetic effects are less clear. In several studies alanine, D-serine, D-cycloserine (DCS), glycine, and GlyT1 inhibitors (ORG 24461, NFPS) are able to attenuate PCP-mediated behavior *(50–55)*, (+)MK-801-induced effects are however potentiated by DCS *(42,56)* and antagonized by D-serine *(57)*. Although this latter finding looks puzzling, there is a previous study pointing to a false-positive effect of D-serine. *(42)*. In this study, it is found that (+)MK-801-induced locomotion was reduced by coadministration of DCS; sniffing stereotypy was however increased at the same time *(42)*. Thus, in the combination of (+)MK-801 and DCS there is a shift in the behavioral dominance toward a focused hyperactivity, a shift that is precisely described for higher doses of (+)MK-801 and also for amphetamine; hyperlocomotion over a large area of the cage at low doses and hyperactivity in one location of the cage with stereotyped sniffing at higher doses *(58,59)*. Thus, a decrease of (+)MK-801-induced behavior after coadministration of D-serine *(57)* seems to be the result of a focused stereotypy and in this respect, (+)MK-801-mediated psychotomimetic actions are not antagonized but rather potentiated by glycine site agonists. Even surprising on the first view, it can be explained by the functional interaction of the glycine-binding site and the noncompetitive binding site at the NMDA receptor complex. From electrophysiological experiments, it is well known that activation of the glycine-binding site enhances the binding affinity of the NMDA receptor although it increases the frequency of the channel openings *(21,60)*. Because the binding of noncompetitive NMDA receptor antagonists like PCP and (+)MK-801 strictly depends on an open state of the ion channel, the activation of the glycine-binding site increases the binding probability of the noncompetitive NMDA receptor antagonists and potentiates the behavioral effects of these antagonists. Thus, the potentiated behavior of noncompetitive NMDA receptor antagonists supports the benefit of glycine site agonists as functional GLU agonists even if it is indirect.

2.2. *Sensorimotor Gating and Glycine*

Apart from psychomotor stimulation, attention or sensory gating deficits can also be observed in schizophrenic patients and animals pharmacologically stimulated or impaired in neurodevelopment. The sensory gating deficit can be measured as a PPI deficit. It is widely accepted as a model with excellent predictive, face, and construct validity *(61,62)*. More recently, however, reports of inhomogeneous presence of PPI deficits in schizophrenics and relatives took some of the convincing power of this model in schizophrenia. In fact, a PPI deficit does not solely predict the latent or manifested presence of schizophrenia, a PPI deficit does not appear in all schizophrenics, and nor does the intensity of a PPI deficit correlate with the intensity of negative and positive symptoms *(62–66)*. Moreover, PPI deficits can also be found in other psychiatric and neurodegenerative diseases, like obsessive compulsive disorder, Huntington's disease, noctural enuresis, attention deficit disorder, Tourette syndrome, blepharospasm, nonepileptic seizure, and to a lesser extent posttraumatic stress disorder *(62)*. Thus, a PPI deficit has some convincing associations and correlations to schizophrenia but it cannot be taken as the ultimate indicator for this disease.

In animal studies the predictive validity of PPI experiments is more concise and this model is commonly used to identify new antipsychotic drugs and to elucidate the underlying mechanism of sensory gating under normal and maladapted conditions. The glycine site antagonists (R,+)-HA-966, L-701,324, ACEA 1021, MRZ 2/576, and MDL 105,519 have, in contrast to noncompetitive NMDA receptor antagonists, no effect on PPI when they are given systemically in rats *(30,32,67–72)*. The antagonists also have no synergistic or inhibitory effects on PCP- or apomorphine-induced PPI deficits *(32,68)*.

The situation differs when the glycine site antagonists 5,7-diCLKYN or 7-CLKYN are applied into the third ventricle or nucleus accumbens, respectively *(73,74)*. Both antagonists show a marked PPI deficit and that of 7-CLKYN can be attenuated by a glycine site agonist *(74)*. Although this behavioral modulation via the nucleus accumbens should be surprising considering the negative effects on locomotion *(40)*, a later study suggests that a PPI deficit is mediated via an anatomical pathway different from that of locomotion *(75)*. The higher local concentration of the antagonists in structures directly linked to PPI may be the reason why these antagonists are able to disrupt PPI after local but not systemic administration.

Studies of glycine site agonists on PPI are rare; DCS and glycine have been studied but they have no effect on PPI themselves *(70,74)*. However, in a neurodevelopmental approach *(44,76)* where the animals develop a PPI deficit postpuperal, glycine itself and the GlyT1 inhibitor ORG 24598 attenuate the lesion-induced deficit *(26)*. Thus, sensory gating seems to be sensitive to a manipulation of the glycine-binding site.

2.3. Cognitive Deficits and Glycine

Cognitive deficits in schizophrenia are dominated by strategic sequencing and planning deficits and working memory deficits that are subserved in the dorsolateral part of the prefrontal cortex *(77)*. In healthy volunteers, the noncompetitive NMDA receptor antagonist ketamine induced deficits resembling those of schizophrenics using, for example, the Wisconsin Card Sorting Test and delayed word-recall tests *(11,78–80)*. Moreover, these symptoms can be exacerbated in stable schizophrenic patients by treatment with ketamine *(79,81)*. In animals, not only NMDA receptor antagonists but also the glycine site antagonists; (R,+)-HA-966 *(83)* and its less selective racemate (±)-HA-966 impair working memory in a PFC-related operant delayed matching-to-position task *(84)*. In contrast, ACEA 1021 has no effect in a delayed nonmatching -to-sample task *(85)*. Up to now glycine site agonists or antagonists have not yet been tested on schizophrenia-like cognitive deficits in neurodevelopmental and pharmacological models. Interestingly, DCS treatment is able to improve cognitive impairments in Alzheimer's patients when given for 4 wk *(86)*, as well as in hippocampal-damaged rats when given acutely *(87)*.

2.4. Transgenic Mice and Glycine

Pharmacological manipulations are commonly used to characterize a particular transmitter or modulator system. The respective drugs, however, are administered acutely and the behavioral outcome after this approach does not reflect the pathophysiological situation where a system is in a chronic maladapted condition. To circumvent this missing correlation, transgenic animals with a permant overexpression or downregulation of particular genes are closer to the situation of chronic diseases like schizophrenia. However, missing genetic information can induce compensatory mechanisms during the embryonic and

postembryonic development with an unknown outcome on the whole transmitter balance. To bypass this uncertain situation, "induceable" transgenic animals that develop an overexpression or downregulation during adulthood can be used. They are free of a developmental compensation but are not easy to create and are less commonly used. Nevertheless, the results available from transgenic animals are valuable and they may give a deeper insight into chronic conditions, although they very often confirm data that have been previously established with respective antagonists (e.g., *see* refs. *88* and *89*). Thus, it is necessary to keep in mind that results from transgenic animals as well as those from pharmacological studies are excellent tools but they have limits.

Transgenic mice lacking the NR1 or NR2A subunit, and therefore having a malfunction in the glycine-binding site, show the phenotype of PCP- or (+)MK-801-treated animals with hyperlocomotion, stereotypy, social withdrawal, cognitive deficits, and a hyperactive monoaminergic system in striatum and frontal cortex; effects that are sensitive to treatment with antipsychotics like haloperidol, clozapine, and risperidone *(90–92)*. Moreover, mice carrying a point mutation in the glycine-binding site of the NR1 subunit show a reduced glycine affinity instead of reduced density that is accompanied by deficits in long-term potentiation (LTP) induction, spatial learning, and an increase in startle reactivity with, however, normal locomotion and PPI *(93)*. A mouse line with a 2-point mutation in the glycine-binding site also exhibits impaired LTP induction and dopamine and serotonin hyperfunction. These mice are insensitive to (+)MK-801 treatment, are supersensitive to startle stimuli without a defect in PPI, and show prominent hyperactivity and stereotyped behavior that do not habituate. The stereotyped behavior, but not the hyperactivity, is sensitive to clozapine, haloperidol, or M100907 treatment, however only at higher doses that are already sedative in wildtype mice *(94)*. Thus, the overall more pronounced effects of mutations in the glycine site may result from the reduced function of the NMDA receptor complex in contrast to the marked loss of NMDA receptor subunit density that may induce compensatory changes and by that reduces the symptomatology (*see* ref. *94*).

3. EVIDENCE FROM HUMAN STUDIES

From clinical trials in indications where a blockade of the glutamatergic system may be of benefit (e.g., stroke, epilepsy, pain), psychotomimetic effects in volunteers are well-known results of uncompetitive and competitive NMDA receptor antagonists *(3,8,81,95–100)*. In contrast to dopamine agonists, blockade of the glutamatergic system induces positive as well as negative symptoms and more important exacerbates psychotic symptoms, most prominently those already present in the schizophrenic patients *(80,81)*. Glycine site antagonists gained interest in GLU-dependent diseases because they seem to have fewer side effects. They were tested for efficacy in stroke, head trauma, epilepsia, pain, and neuropathic pain (*see* ref. *101*). In general, the glycine antagonists are described as well tolerated and safe in humans. In clinical phase II studies on stroke and head injury, the antagonist ACEA 1021 generates in one and two out of six patients (in a medium-dose group) visual hallucinations and transient memory disturbances, respectively. Thus, for ACEA 1021 there is an ascending risk of psychotomimetic side effects with increasing doses *(100)*. In contrast, in clinical studies with the antagonist GV 15526, no psychotomimetic side effects have been reported *(102–104)*. So far, none of the glycine site antagonists has reached the market. The development of almost all glycine site antagonists discontinued because the therapeutic

window between beneficial and mechanism-related side effects was too small. Thus, from the profile of the antagonists, the glycine site in humans bears also the risk of generating psychotomimetic effects; however more clinical data are needed for a final assessment.

In schizophrenia, glycine agonism as a treatment option emerged with the GLU hypothesis. Since bioavailability and brain penetration of glycine itself are limited, high doses are needed. Nevertheless, its efficacy was tested in a couple of trials in schizophrenic patients. The most surprising outcome was that in most studies glycine and also DCS treatment mainly affect negative symptoms and leave positive symptoms unaffected *(105–117)*. Although other reports show comparable efficacy of D-serine on positive, negative, and cognitive symptoms *(118)*, the negative outcome on positive symptoms was a drawback for the therapeutic use of glycine agonism as antipsychotic. The reason behind this may be found in the co-medication used at that time. Until the late 1990s mainly classical neuroleptics were used as gold standards in schizophrenia. They are known to be more effective on positive than negative symptoms. It is therefore not surprising that patients who are already well controlled on positive symptoms may be less sensitive to a further improvement by the co-medication with glycine site agonists. An additional aspect comes from the predominance of negative symptoms induced by the GLU/glycine receptor antagonists and it seems obvious to conclude that therapeutic effects of glycine agonists are directed toward negative symptoms. These two aspects are further supported by recent studies that directed more attention to the type of co-medication. It was found that D-serine treatment is ineffective in combination with the atypical neuroleptic clozapine *(119)*, whereas the combination of glycine plus clozapine or olanzapine—another atypical antipsychotic—still ameliorates negative symptoms but with a less pronounced effect as in patients under classical neuroleptics *(120–122)*. Moreover, symptoms worsened when DCS was combined with clozapine *(112,123)* but still improved in combination with risperidone *(117,124)*, however once again less prominently as in combination with classical neuroleptics *(123,125)*. This outcome in general is not astonishing when we consider the higher efficacy of the atypical neuroleptics on negative symptoms *(111)*. Furthermore, there are studies revealing that especially clozapine acts as a functional GLU/glycine agonist since it increases serum GLU levels *(126)* and enhances NMDA receptor-mediated responses in the prefrontal cortex *(127)*.

A risk of neurotoxicity obvious for direct GLU agonists is unlikely from the receptor function and was not present when glycine or DCS was given in a chronic treatment regime of 4 d to healthy subjects; there was no effect on cognition or other behavioral parameter (schizophrenic, anxiety, sadness, panic) *(128)*. A longer treatment period with high doses (1–5 mo; 1 g/kg/d or 5 g/kg/d) of glycine in rats was free of neurotoxic effects in neuronal and glia cells, but was, however, accompanied by a reduction of class B, N-type Ca2+ channels in parietal cortex and hippocampus after 3 and 5 mo of continuous treatment without functional implications *(129)*.

By summarizing the efficacy of glycine site agonists in schizophrenic patients, it is obvious that the agonists themselves as well as in combination with classical neuroleptics are mainly effective on negative symptoms whereas in combination with atypical neuroleptics they have no further benefit. A more prominent benefit for the patients may result from a fine titration of the different treatment options in relation to the preponderance of symptoms.

4. CONCLUDING REMARKS AND FUTURE DIRECTIONS

Summarizing the findings of glycine ligands in animal models of schizophrenia and in schizophrenic patients reveals that (1) downregulation of glycinergic transmission by antagonists induces symptoms associated with schizophrenia and (2) upregulation of glycinergic transmission by agonists or GlyT inhibitors ameliorates symptoms associated with schizophrenia. Thus, a dysfunction in glycinergic transmission needs to be taken into account as one mechanism involved in schizophrenia. Of course, data from additional studies indicate that schizophrenia is a multifactorial disease and that other systems than the glutamatergic/glycinergic are involved. Dopamine, serotonin, and GLU are the most frequently discussed transmitter systems in this regard. It is not clear, however, whether a single defect in one transmitter system is responsible for the disease or if defects in other transmitter systems are the consequence of an initial defect in one system. It has been discussed that a critical period for the development of schizophrenia seems to be in the neonatal phase *(49)*. Support for this comes from animals with neonatal damage in the ventral hippocampus—a region rich in glutamatergic innervation and transmission. These animals show schizophrenia-like symptoms not earlier than postpuberal *(48–49)*, a phenomenon also known from schizophrenic patients. Apart from the hippocampus other structures rich in Glu have been identified as sensitive to manipulation that leads to schizophrenic symptoms. Evidences are found for the prefrontal and frontal cortex, amygdala, and entorhinal cortex (*see* refs. *77,130,* and *131*). Because these glutamatergic efferents terminate in structures that are dopamine-driven and are involved in emotional information processing, the implications of the dopaminergic and serotonergic system are not surprising. A deficit in information processing rather than a defined anatomical or neurochemical deficit as an underlying mechanism in schizophrenia seems therefore more probable and fits with the missing neuropathology in schizophrenia.

A speculation on upcoming treatment options in schizophrenia needs to consider that family and twin studies show a role of genes in determining the susceptibility to schizophrenia. It is, however, evident that multiple gene loci are involved. Nevertheless, several candidate genes have been identified that may offer new opportunities for the development of new drugs *(132–134)*. Whether a concert of gene defects or a single gene defect in combination with other events are responsible for the development of schizophrenia is unclear for now. Interestingly, among others several genes have been identified that seem to have link to glutamatergic/glycinergic transmission. Future research is needed to show if the genes DISC-1 (disrupted-in-schizophrenia-1), dysbindin, neuregulin-1, G72, and PRODH *(132)* provide a new class of targets in drug development and if the treatment of schizophrenic patients is improved by these new approaches.

ACKNOWLEDGMENT

Supported by the Deutsche Forschungsgemeinschaft (SFB 307 and KR 831/3-1).

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IV Depression

9
Dopamine and Depression

Phil Skolnick

1. INTRODUCTION

Monoamine-based theories of major depressive disorder (MDD) have dominated thinking in biological psychiatry for over 40 yr. These theories were largely grounded on the principle of "reverse engineering." In this case, the demonstrable effects of "first generation" antidepressants (e.g., tricyclics, such as imipramine) on the reuptake of norepinephrine and serotonin *(1,2)*, and the observation that drugs depleting these biogenic amines lower mood *(3)*.

A role for dopamine in depression was first hypothesized in the mid- to late-1970s *(4,5)*, well after the link between norepinephrine, serotonin, and depression had been established. In addition to the difficulties inherent in promulgating a new hypothesis, interest in exploring the role of dopamine (and other transmitters) in MDD was dampened by the demonstration that selective serotonin reuptake inhibitors (SSRIs) were effective antidepressants. The commercial success of the SSRIs focused attention on the serotonergic synapse in the etiology of MDD and as a target for the development of new antidepressants. Despite several seminal publications appearing in the early 1980s (e.g., refs. *6* and *7*), studies to explore the role of dopamine in MDD were, with few notable exceptions, considered out of fashion. However, the contribution of anhedonia to depressive symptomatology, and the recognition that dopaminergic transmission is critical to reward and motivational processes, refocused attention on the role of the dopaminergic synapse in MDD. Although it is naive to view a single transmitter as responsible for the constellation of symptoms that comprise MDD (*see* Chapter 10; *see* refs. *8* and *9* for review), this chapter overviews preclinical and clinical evidence linking dopamine and the pathways subserved by this transmitter to MDD and antidepressant action.

2. "HYPODOPAMINERGIA" IN MDD

There is an extensive literature dating back more than 30 yr *(10)* that links activation of mesocorticolimbic dopaminergic pathways to rewarding events and incentive-driven, goal-oriented behaviors (reviewed in refs. *11* and *12*; *see* Chapter 14). It is this literature that provides the framework linking dopaminergic pathways to MDD. Anhedonia (the inability to experience pleasure) and diminished interest in all (or almost all) activities are central to a diagnosis of MDD. The link between anhedonia and dopaminergic pathways

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

stems from the "dopamine hypothesis of reward" *(6)*. Wise *(6)* reported that neuroleptics delayed impairment of operant reinforcement maintained by diverse reinforcers, including food, water, drugs, and intracranial self-stimulation. Wise *(6)* concluded that neuroleptics (and by implication blockade of dopamine receptors) specifically impair primary reinforcement, and that this action is dissociable from an effect on performance. Wise *(6)* viewed this effect of neuroleptics as impairing the pleasurable effects of rewarding stimuli (i.e., anhedonia), and hypothesized that the hedonic properties of reward are effected through dopamine.

Although the dopamine hypothesis of reward has been refined and reinterpreted over the past two decades (e.g., refs. *13* and *14*), there is general agreement that activation of mesocorticolimbic dopaminergic pathways is pivotal in the selection and orchestration of both goal-directed behaviors and reward-related learning. An in-depth treatment of this topic is provided by Beninger and Gerdjikov (*see* Chapter 14).

An animal model of an affective disorder such as depression cannot be fully validated. Nonetheless, the chronic mild stress (CMS) paradigm developed by Willner and colleagues exhibits considerable face, construct, and predictive validity (reviewed in ref. *15*). In this model, rats are exposed to daily sessions of uncontrollable, inescapable stressors (e.g., cage tilt, stroboscopic light, wet bedding). The primary behavioral expression of this model, subsensitivity to a reward (e.g., the availability of a palatable solution of sucrose/saccharin; the opportunity to respond for intracranial self-stimulation [ICSS]), may reflect a diminished ability to experience pleasure. Several weeks of CMS are required to elicit this apparent subsensitivity to reward, and antidepressants, administered over a period of weeks, can reverse this phenomenon (reviewed in refs. *15* and *16*). Several studies have documented that the CMS model alters mesocorticolimbic dopaminergic pathways to produce a functional hypodopaminergia. Thus, Papp et al. *(17)* reported a reduction in radioligand binding to D2/D3 receptors in the nucleus accumbens (but not the striatum) of rats subjected to CMS. This effect was reversed by chronic administration of imipramine. Expression of mRNA-encoding D2 receptors is also reduced following an extended period of CMS *(18)*. This reduction is observed in the shell and core of the accumbens, as well as in lateral aspects of the caudate. CMS also appears to reduce expression of D2 mRNA-in cell body-rich areas including the substantial nigra and lateral (but not medial) aspects of the ventral tegmentum. By contrast, expression of mRNA-encoding D1 receptors was largely unaffected by CMS. These studies complement a more extensive literature describing the effects of antidepressants on dopaminergic pathways detailed in Subheading 3.

CMS has also been reported to blunt the rewarding (evaluated in a conditioned place preference paradigm) and motor stimulant properties of quinpirole, a D2/D3 agonist *(19)*. The latter observation should be viewed in the context of a large body of evidence (described in Subheading 3) that chronic (but not acute) antidepressant treatments *enhance* locomotor responses to dopamine agonists, including quinpirole. CMS does not appear to alter basal dopamine content in dialysates from the nucleus accumbens *(20)*. At face value, this would seem at variance with a clinical literature indicating decreased levels of the dopamine metabolite homovanillic acid (HVA) in the cerebrospinal fluid (CSF) of depressed individuals. However, in response to presentation of rewarding stimuli, dopamine levels increase in the nucleus accumbens (and prefrontal cortex) (reviewed in ref. *14*). This increase in dopamine, elicited by presentation of palatable food, is *blunted*

(in both regions) in rats subjected to CMS, and restored by chronic treatment with desipramine *(20)*. Further, in control rats, the response to an aversive stimulus (in this case, tail pinch) is a *reduction* in dopamine with a probe implanted in the nucleus accumbens. In rats subjected to CMS, there is a significant *increase* in dopamine output. These latter findings may be viewed as consistent with clinical studies indicating there is a reduction in dopamine turnover in MDD.

Much of the evidence for a reduced turnover of dopamine in depressed individuals has been in the literature for more than a quarter century (reviewed in ref. *7*). This evidence is grounded on reports that levels of HVA, the major metabolite of dopamine, are lower in CSF of depressed individuals compared to controls. This interpretation is predicated on the assumptions that both dopamine reuptake and CSF flow are unchanged in these depressed individuals, with HVA concentrations proportional to dopamine release. Many, but not all studies report (reviewed in refs. *7* and *21*) this reduction in depressed individuals. A more consistent picture emerges in individuals administered probenecid, a drug that blocks acid transport out of the CSF. Reduced CSF HVA levels have been reported in the majority of depressed individuals in these studies. However, interpretation of these data as they relate to a hypodopaminergia in MDD is not straightforward. Most CSF HVA likely emanates from the caudate nucleus, owing to both its size and relative proximity to the ventricular system. Thus, alterations in CSF HVA levels are more likely to reflect changes in activity of nigrostriatal rather than mesocorticolimbic DA function. This interpretation is consistent with: (1) reports that low CSF HVA levels are associated not only with depression, but also with Parkinson's and Alzheimer's disease *(22)*; (2) that CSF HVA levels are generally elevated in mania (reviewed in ref. *23*), and (3) the observation that CSF HVA levels are lowest in patients with marked psychomotor retardation (reviewed in ref. *7*). *In toto*, these data are consistent with the hypothesis that CSF HVA levels may more accurately reflect motor activity rather than depressed mood. Nevertheless, several double-blind studies indicate that among depressed subjects who improved following antidepressant treatment, those patients with the lowest levels of CSF HVA levels (and by inference, the most profound hypodopaminergia) responded best. Jimerson and Post (reviewed in ref. *24*) reported a significant negative correlation ($r = -0.66$; $p < 0.05$) between these measures. The antidepressants used in both of these studies are dopaminergics, piribedil (a dopamine agonist), and nomifensine *(25)*, a dopamine/norepinephrine reuptake blocker *(26)*. Although the number of patients in both studies were small, Van Scheyen et al. *(25)* did not observe a similar relationship between CSF HVA levels and individuals responding to chlomipramine.

Bowden et al. *(27)* reported no significant differences in dopamine and HVA concentrations in caudate, putamen, and nucleus accumbens in suicide victims with a documented history of depression compared to controls, although there was a trend for HVA concentrations to be lower in suicides. Lower concentrations of the dopamine metabolite dihydroxyphenylacetic acid (DOPAC) were reported in caudate nucleus of those suicides free of antidepressants. These data are consistent with a decreased turnover of dopamine in depression in view of reports of either no change in ligand binding to dopamine transporters in suicide victims *(28)* or a decrease in transporter binding "potential" (using the position emission tomography [PET] ligand, [^{11}C]RTI-32) in the striatum of depressed individuals *(29)*.

Although these clinical studies may be viewed as consistent with a hypodopaminergia in MDD, this association is far from causal. The information in Subheadings 3 and 4

detail both preclinical and clinical studies consistent with the hypothesis that MDD is associated with a hypodominergia is mesocorticolimbic structures.

3. ALTERED RESPONSES TO DOPAMINE AGONISTS FOLLOWING CHRONIC ANTIDEPRESSANT TREATMENTS

A fundamental inconsistency in biogenic-amine-based theories of depression is the lack of a temporal relationship between increases in synaptic concentrations of biogenic amines and an antidepressant action. Thus, in most double-blind, placebo-controlled studies, several weeks (usually ≥3) of antidepressant treatment are required to produce clinically meaningful improvement in depressive symptomatology. In contrast, changes in biogenic amine disposition are readily demonstrable both in vitro and following acute treatment. This so-called "therapeutic lag" is generally viewed as a period of antidepressant-induced molecular and cellular adaptation(s) that must precede symptom relief. The pioneering work of Vetulani and Sulser *(30)* marked the beginning of studies aimed at understanding the molecular bases for the adaptive process(es) responsible for this lag. During the past decade, several of the cellular adaptations produced by chronic antidepressant treatments have been shown to extend well beyond the aminergic synapse (reviewed in refs. *8, 31,* and *32*). Nonetheless, in preclinical studies, sensitization of mesolimbic dopamine receptors is perhaps the most consistent change produced by chronic antidepressant treatments. This sensitization is produced by structurally diverse antidepressants, as well as nonpharmacological interventions including electrocerebral silence (ECS) and rapid eye movement-sleep deprivation (reviewed in ref. *33*).

Serra et al. *(5)* first described changes in behavioral responses to the dopamine agonist, apomorphine, following chronic antidepressant treatments. These investigators observed a potentiation of the motor-stimulant effects of apomorphine, and a reduction in the hypomotility produced by lower doses of this drug. The motor stimulation produced by high doses of apomorphine has been attributed to stimulation of postsynaptic receptors, whereas its inhibitory effects have been linked to stimulation of dopamine autoreceptors that would inhibit dopamine release (reviewed in ref. *33*). The robust nature of the former phenomenon is supported by the demonstration that enhancement of motor activity following chronic (but not acute) antidepressant treatments is observed not only with apomorphine, but also with other, subtype selective dopamine agonists (e.g., quinpirole, 7-OHDPAT) *(34–36),* as well as amphetamine *(37)*. Further, these effects have been observed following chronic treatment with many agents (e.g., fluoxetine, imipramine, desimipramine, citalopram, mianserin, oxaprotiline, mirtazepine). In contrast, chronic antidepressant treatment does not appear to enhance the stereotypy produced by either direct (e.g., apomorphine, quinpirole) or indirect (e.g., amphetamine) acting dopaminergics *(34,37)*. These observations, when taken together with the ability of chronic antidepressants to enhance the motor stimulant properties of quinpirole and amphetamine injected directly to the nucleus accumbens *(38,39)*, indicate a selective perturbation of mesolimbic dopaminergic neurons. Because mesolimbic dopaminergic neurons play a key role in the control of motivation and reward-related behaviors that appear dampened in MDD (reviewed in ref. *40*), it can be hypothesized that the several weeks of antidepressant treatment required to produce this increased sensitivity to dopaminergic stimulation may contribute to the therapeutic lag common to biogenic-based antidepressants.

Despite the robust nature of this phenomenology, there have been several laboratories (e.g., ref. *41*) unable to demonstrate an increase in the motor-stimulant properties of dopaminergic agonists following chronic antidepressant treatments. These latter findings may be related to the mechanism(s) by which chronic antidepressants increase the behavioral sensitivity to dopamine agonists. Taken together with the multiple variables[1] in these studies and the tendency of laboratories not to strictly replicate, but rather modify and embellish, the number of reports confirming the ability of chronic antidepressant treatments to alter the behavioral responses to dopaminergic stimulation is remarkable. There is also evidence for a pharmacodynamic interaction between the antidepressant used for chronic treatment and the dopaminergic compound employed as the challenge. For example, in a study examining the locomotor responses of several dopamine agonists following chronic mirtazepine treatment, Rogoz et al. *(42)* reported the locomotor effects of amphetamine, but not quinpirole or 7-OHDPAT, were potentiated. This is perhaps expected in view of the potential number of intracellular targets affected by antidepressants *(31,43,44)*.

It has been more difficult to reproduce the initial observation made by Serra et al. *(5)* that chronic antidepressants prevent the hypomotility evoked by low doses of dopamine agonists. In some reports, this phenomenon was not observed in the presence of an increased locomotor response to higher doses of these same agents (e.g., refs. *34* and *37*). However, there have been behavioral studies confirming this phenomenon (e.g., ref. *45*; discussed in ref. *33*), as well as electrophysiological data *(46)* consistent with these findings. Nonetheless, the difficulty in reproducing this finding should not be viewed as suprising given the relatively narrow dose range for many of these drugs to produce a hypomotility (and the difficulties inherent in measuring a "floor" effect), the number of dependent variables in designing such a study, and the possibility that specific antidepressants perturb a subset of potential targets.

Behavioral studies with selective dopamine receptor agonists like quinpirole and 7-OHDPAT indicate that, at minimum, chronic treatment with most antidepressants alter the responsiveness of D2/D3 receptors, and that these antidepressant-induced changes appear largely confined to the mesocorticolimbic system. Studies using *in situ* hybridization and receptor autoradiography are consistent with the hypothesis that chronic antidepressants can increase the expression of mRNA encoding D2 and/or D3 receptors and radioligand binding to these receptors. Whereas early studies using [^{3}H]raclopride and other antagonists failed to demonstrate antidepressant-induced changes in radioligand binding to dopamine receptors (reviewed in ref. *33*), Rogoz and Dziedzicka-Wasylewska *(47)* reported chronic treatment with imipramine, citalopram, and mianserin increased [^{3}H]quinpirole but not [^{3}H]raclopride binding to both caudate nucleus and nucleus accumbens. Although these findings indicate that [^{3}H]agonists but not antagonists are capable of detecting antidepressant-induced changes in dopamine receptors, in a subsequent study using different antidepressants (tianeptine and fluoxetine), this group reported increases in both [^{3}H]quinpirole and raclopride binding to the caudate nucleus and the core of the nucleus accumbens *(48)*. Ainsworth et al. *(49)*

[1]Consider some of the variables in such a study: antidepressant, dose and dosing regimens, rat strain, challenge dose(s) of dopamine agonists, agonist employed (and dopamine receptor selectivity of this agent), and method of measuring behavior.

reported that chronic (14-d) treatment with fluoxetine and desipramine increased "D2-like" binding (i.e., binding to $D_{2,3, \text{ and/or } 4}$ receptors) to the shell of the nucleus accumbens, whereas a higher dose of fluoxetine also increased ligand binding to the core of the nucleus accumbens. The monoamineoxidase (MAO) inhibitor tranylcypromine did not affect radioligand binding to the nucleus accumbens, but reduced ligand binding to the ventromedial and dorsolateral striatum. In the same study, Ainsworth et al. *(49)* measured levels of mRNA, encoding D1 and D2 receptors. None of the antidepressants affected expression of D1 mRNA whrereas all three compounds increased D2 mRNA expression in the shell of the nucleus accumbens. The ability of tranylcypromine to increase D2 mRNA but not ligand binding may reflect the difference in specificity between the radioligand (that binds to D2, D3, and D4 receptors), and the mRNA probe. Alternatively, temporal differences between changes in the expression of mRNA and receptor protein could account for this apparent discrepancy following tranylcypromine.

Lammers et al. *(50)* examined the expression of mRNA encoding D3 receptors following administration of several antidepressants for up to 42 d. With the exception of fluoxetine, by 21 d each of the antidepressants (at a single-dose level) increased the expression of D3 mRNA, but apparently in a region-selective fashion. All of the compounds (desipramine, imipramine, amitryptiline, tranylcypromine) except fluoxetine increased expression in the nucleus accumbens shell, whereas desipramine increased expression in frontal cortex, septum, olfactory tubercle, and the Islands of Callejo. Similar, drug × region interactions were observed following 21 d of treatment with the other antidepressants. It is noteworthy that fluoxetine decreased D3 mRNA expression in nucleus accumbens, which can be contrasted with its effect on expression of D2 receptors *(49)*. Further, when drug-induced effects on D3 mRNA expression are compared over time, different temporal patterns emerge among the brain regions examined. If radioligand binding to D3 receptors was used as the dependent variable, a different drug × duration of treatment × region interaction emerges *(50)*. Of note is the observation in the Lammers et al. *(50)* study that ligand binding in the control group appeared to decrease in a time-dependent fashion; by 42 d of saline injection, ligand binding to D3 receptors in accumbens was significantly lower compared to values at 10 d. Fluoxetine-induced reductions in D3 mRNA expression at 21 d had returned to control values by 42 d of treatment, whereas ligand binding to the shell of the accumbens actually increased at this time point compared to controls. The Lammers et al. *(50)* study amply illustrates how a snapshot (i.e., examination of a drug-induced change at one time point [or dose, or brain region]) may not adequately portray either the effect(s) of a particular drug or model the clinical situation.

A number of other studies (e.g., refs. *18, 36, 48, 51,* and *52*) have also reported that chronic antidepressants increase either radioligand binding and/or expression of mRNA encoding D2/D3 receptors in mesolimbic structures. Most of these studies used a fixed treatment duration or dose of drug; several of these studies merit special comment. For example, Dziedzicka-Wasylewska et al. *(18)* reported that chronic (5-wk) treatment with imipramine and fluoxetine increased the expression of mRNA encoding D2 (but not D1 receptors) in the shell of the nucleus accumbens. No effects on D2 (or D1) mRNA expression were observed in the core of the nucleus accumbens. Increases in D2 mRNA expression were also present in the lateral but not medial portions of the caudate

putamen. In these studies, imipramine but not fluoxetine significantly elevated D2 mRNA expression in the medial and lateral ventral tegmenal area. Both regimens were sufficient to restore sucrose intake in a parallel group of animals subjected to CMS and, as discussed earlier, this regimen of CMS (sufficient to produce significant reductions in sucrose consumption) significantly reduced in D2 mRNA in the shell of the nucleus accumbens—an effect partially restored by both imipramine and fluoxetine.

Rogoz et al. *(42)* reported chronic treatment with mirtazepine potentiated the locomotor stimulant effects of amphetamine whereas the stimulant effects of both 7-OHDPAT and quinpirole were unchanged. No changes in either radioligand-binding to dopamine receptors or mRNA expression were observed in mesolimbic areas. Mirtazepine affects multiple aminergic systems (it has indirect 5-HT_{1A} receptor-stimulating properties and appears to function as an $\alpha 2$ and 5-HT_2 receptor antagonist), but is not a classical reuptake blocker. Berendsen et al. *(53)* have shown that acute treatment of mirtazepine modulates the behavioral effects of haloperidol, inhibiting its cataleptic action and enhancing its ability to inhibit apomorphine-induced climbing. This latter report indicates the ability of mirtazepine to affect dopamine receptor function following acute administration may preclude the long-term changes in postsynaptic dopamine receptors observed after other, biogenic-amine-based antidepressants. Nonetheless, the increased sensitivity to amphetamine (but not to either quinpirole or 7-OHDPAT) produced by chronic mirtazepine administration indicates this antidepressant does enhance dopaminergic tone, albeit in a manner different than reuptake inhibitors.

In toto, this body of preclinical evidence indicates chronic antidepressant treatments do enhance dopaminergic "tone" in mesocorticolimbic pathways. Given the potential number of downstream targets impacted by biogenic-amine-based antidepressants *(8,31,43)*, it is perhaps not surprising that these agents produce multiple changes in dopaminergic pathways in an apparent drug-, dose-, region-, and time-dependent fashion. The few clinical studies in this area do not provide definitive corroborative evidence of antidepressant-induced changes in dopamine receptors. Ebert et al. *(54)* reported no changes in the binding of the single-photon emission computed tomography (SPECT) ligand IBZM to striatal dopamine receptors between nondepressed and depressed individuals. Further, antidepressant treatment did not alter IBZM-binding in the depressed cohort as a whole. However, if the depressed group were divided into responders and nonresponders, antidepressant therapy reduced ligand binding in the (five) improved patients. The authors interpret this reduction as the result of an antidepressant-induced increase in the tonic release of dopamine, an interpretation compatible with data from preclinical studies (e.g., refs. *49* and *55*). Using radioligand binding to measure D1 and D2 receptors, Bowden et al. *(56)* reported no differences in receptor densities in postmortem samples of the caudate, putamen, and nucleus accumbens of suicide victims with a diagnosis of depression (and had been antidepressant-free for at least 3 mo) compared to matched controls. Increased densities of D2 receptors were noted in all of these brain regions from the suicide victims who had been treated with antidepressants. Although these investigators argue that the increased density of D2 receptors could be attributed to concurrent treatment with neuroleptics, these findings are also compatible with many of the preclinical studies described in this section. A more recent study examining D2 receptors in the caudate nucleus of depressed suicide victims *(57)* found no evidence for changes in the B_{max} of [^{3}H]raclopride, but did report a significant reduction in affinity of

this radioligand in a subgroup of individuals. Clearly, additional clinical studies are needed to determine if antidepressant-induced changes in dopaminergic pathways documented in preclinical studies are relevant to the human condition.

4. PHARMACOLOGY OF DOPAMINERGIC DRUGS IN MDD

Clinical studies indicate that an increase in dopaminergic "tone," produced either by blockade of dopamine transporters or via direct stimulation of postsynaptic dopamine receptors, is sufficient to produce an antidepressant action. For example, bupropion (Wellbutrin®) is a dopamine reuptake inhibitor *(26,58)* that is as effective as SSRIs in the treatment of MDD (reviewed in ref. *59*). However, bupropion is not a high-affinity inhibitor of dopamine reuptake *(26)*, nor is it selective for the dopamine transporter. Bupropion has been reported to act as a nicotinic antagonist *(60)*, and inhibits norepinephrine reuptake *(26,58)*. Because selective inhibition of norepinephrine reuptake is sufficient to produce an antidepressant action (reviewed in ref. *61*), this latter action could either contribute to or explain the antidepressant effects of bupropion. In preclinical studies, the potency of bupropion to inhibit firing of noradrenergic neurons in locus coeruleus (13 mg/kg, ip, rats) approximates its ED_{50} in the forced swim test (10 mg/kg) *(58)*. The forced swim test, although lacking the face and construct validity of a true model of depression, is an excellent predictor of clinically effective antidepressants *(62,63)*. Further, in the Cooper et al. *(58)* study, inhibition of midbrain dopaminergic neurons was observed only at fourfold higher doses of bupropion. At face value, the nicotinic antagonist properties of bupropion (which may well contribute to its use in smoking cessation) would not contribute to its antidepressant properties. Thus, nicotine appears to mimic the actions of antidepressants in both preclinical (e.g., ref. *64*) and clinical *(65)* studies.

Perhaps more compelling evidence that activation of dopaminergic pathways can produce an antidepressant action is derived from clinical studies demonstrating that direct-acting dopamine agonists are antidepressant. There have been several double-blind trials comparing the dopamine (D2/D3 receptor-preferring) agonist bromocriptine to tricyclic antidepressants (imipramine and amitriptyline) in depressed patients. Although these trials *(66–68)* are small by contemporary standards, in each instance, bromocriptine appeared as effective as a tricyclic in reducing Hamilton Depression rating scale scores. Nausea was the most prominent side effect in these studies. There have been a number of open trials using bromocriptine (reviewed in ref. *69*) with small numbers of patients; most of these trials report an antidepressant response to bromocriptine. At face value, these data support the hypothesis that dopamine receptor activation is sufficient to effect an antidepressant action, thereby implicating dopamine receptors in depression. It should be noted that the PDSP database (http://.crwu.edu/pdsp.asp) indicates that bromocriptine also binds with nM affinities to a number of serotonin receptor subtypes (e.g., 5HT1A, 6, and 7) that may contribute to its therapeutic effects.

The antidepressant properties of the D3 receptor-preferring agonist pramipexole have also been examined in a double-blind, placebo-controlled trial. In this study, three doses of pramipexole were compared with a standard dose of fluoxetine and placebo. By end point (8 wk), patients receiving an intermediate dose of pramipexole (1 mg/kg) significantly improved compared to placebo in the three depression rating scales employed Hamilton Rating Scale for Depression (HAM-D), Montgomery–Asberg Depression Rating Scale (MADRS), and Clinician's Global Impressions-Severity of Illness (CGI-SI). The

most dramatic improvement was manifested in the high-dose pramipexole group (5 mg/kg), although the dropout rate at this dose precluded statistical comparisons *(70)*. Pramipexole has also been shown to significantly reduce MADRS scores and a patient self-rating scale in an open-label study of Parkinson's patients receiving levodopa-(L-dopa) *(71)*. The daily dose of L-dopa was significantly reduced during this period, which may have contributed to the improvement in mood. Nonetheless, these data are consistent with the report of Corrigan et al. *(70)* that pramipexole has antidepressant properties.

When used in a combination strategy with "traditional" antidepressants *(72)*, dopaminergic agents have been reported to improve depressed mood in patients, including those patients either resistant to, or exhibiting only a partial response to serotonin and/or norepinephrine reuptake inhibitors. Several studies have reported that addition of bupropion, most often to SSRIs such as paroxetine and fluoxetine resulted in greater symptomatic improvement than when either drug was used alone *(73–75)*. The ability of bupropion to inhibit norepinephrine reuptake does not permit an unequivocal assignment of this effect to its inhibition of dopamine reuptake. However, in one study *(75)*, bupropion-enhanced responses combined with venlafaxine, a dual-uptake inhibitor. Nonetheless, bupriopion inhibited the *O*-demethylation of venlafaxine, which further confounds interpretation of this study.

Perhaps more compelling evidence that dopaminergic receptor activation augments the effects of traditional antidepressants derives from studies using dopamine agonists. One preclinical study is of particular interest in this context. Maj and coworkers *(76)* demonstrated that pramipexole had a synergistic action in the forced swim test when combined with dual-uptake inhibitors (amitryptiline, imipramine). Further, SSRIs (which are generally reported as inactive in the rat variant of this procedure) such as fluoxetine potentiate the antidepressant-like actions of pramipexole in the forced swim test *(76)*. In the more realistic CMS model, dopamine agonists (quinpirole and bromocriptine and pramipexole), like other antidepressants *(15)*, restored stress-induced deficits in sucrose consumption.

In open trials, Koyama and coworkers *(77,78)* used bromocriptine and pergolide in patients resistant to (but concurrently maintained on) traditional antidepressants. In both studies, clinical improvement was noted in a significant proportion of patients following addition of a dopamine agonist. Lattanzi et al. *(79)* examined the effects of adding pramipexole to traditional antidepressants in patients classified as drug-resistant. In this 4 mo study using inpatients (both unipolar and bipolar depression), highly significant reductions in MADRS and clinical global impression were obtained, with 67.7% considered responders on MADRS, and 74.2% on CGI, respectively. Perugi et al. *(80)* examined the effects of pramipexole or ropinirole in treatment-resistant bipolar disorder. In this open study, dopamine agonists were added to conventional antidepressants and mood stabilizers; for inclusion in this study patients had not responded to this combination of drugs for at least 8 wk. Eight patients (44.4%) were considered responders (four pramipexole and four ropinerole, respectively) with five patients exhibiting a marked improvement (CGI = 1), and three moderate improvement (CGI = 2), respectively. Based on retrospective chart review, Sporn et al. *(81)* reported that adjunctive use of pramipexole improved 40% and 50% respectively of patients with unipolar and bipolar depression based on marked to moderate improvement in the CGI-I (improvement) scale. *In toto*, this body of clinical literature indicates that increasing dopaminergic tone improves response to conventional antidepressants in a refractory subpopulation of patients with

both unipolar and bipolar depression. However, the clinical studies described here are generally quite small (<20 patients) and have an open design. In the ideal, double-blind, controlled studies (that are appropriately powered) will be required to rigorously test the hypothesis that increasing dopaminergic tone augments the effect of conventional agents.

5. THE "BROAD SPECTRUM" ANTIDEPRESSANT: COMBINING DOPAMINE, NOREPINEPHRINE, AND SEROTONIN REUPTAKE BLOCKADE IN A SINGLE MOLECULE

The efficacy of the prototypic tricyclic, imipramine, had a profound influence on the development of pharmacotherapies for MDD. Follow-on agents (e.g., desmethylimipramine, nortryptyline, amitryptyline), produced by modification of the tricyclic structure, constitute a family of dual-uptake inhibitors, albeit with different relative potencies at serotonin and norepinephrine transporters *(26,82)*. Selective reuptake inhibitors (e.g., SSRIs, such as fluoxetine, paroxetine, and citalopram) have, in large part, supplanted tricyclic antidepressants as the standard of care because, as a group, SSRIs are safer and easier to use. Nonetheless, there is evidence, although not unequivocal, that dual-uptake inhibitors are more effective than SSRIs, particularly in the treatment of severely depressed individuals. A "second generation" of dual-reuptake inhibitors (e.g., venlafaxine and duloxetine) with a "cleaner" side-effect profile than tricyclics may well replace SSRIs as the drugs of choice for MDD.

These newer compounds, although safer and easier to use than the tricyclics, do not offer clearly demonstrable advantages with respect to either speed of onset or efficacy.[2] Given the preclinical and clinical findings described in the previous sections, drug development strategies directed at simultaneously increasing synaptic concentrations of dopamine, norepinephrine, and serotonin could result in a more rapid onset of relief and/or greater efficacy than single-or dual-uptake inhibitors. In theory, there are a number of strategies that may be employed to accomplish this goal *(83)*. Among biogenic-amine-based approaches, a compound capable of inhibiting the reuptake of norepinephrine, serotonin, and dopamine is perhaps the most straightforward. Such compounds have been termed "broad spectrum antidepressants" *(83)*. Because the dopamine, serotonin, and norepinephrine transporters belong to a gene family of 12 transmembrane transporters *(84)*, the design and synthesis of a triple-reuptake inhibitor appears, at face value, straightforward. However, the design of *bioavailable*, *safe*, and *well-tolerated* molecules active at all three transport proteins represents a formidable synthetic challenge.

Substituted azabicyclo[3.1.0]hexanes (exemplified by sibling molecules DOV 21,947 and DOV 216,303) have been identified as orally available, triple-reuptake inhibitors *(85,86)*. Phase I studies with the more advanced compound, DOV 216,303 (manuscript in preparation), have demonstrated that this compound is safe and well tolerated. This compound is currently in a Phase II trial for the treatment of MDD. In HEK 293 cells expressing a recombinant form of the corresponding human transporter protein, DOV 216,303 inhibits [^{3}H]norepinephrine and [^{3}H]serotonin uptake with equal potency, and is approximately four-fold less potent as an inhibitor of [^{3}H]dopamine uptake (Table 1) *(85)*.

[2]The term *efficacy* in this context can reflect a variety of outcome measures, such as an increase in the percentage of patients with a significant reduction in depressive symptomatology, an increase in the percentage of patients achieving remission, and/or a decrease in the percentage of individuals relapsing.

Table 1
DOV 216,303 Inhibits [^{3}H] Biogenic Amine Uptake

	[^{3}H]5-HT	[^{3}H]DA	[^{3}H]NE
DOV 216,303	13.8 ± 1.5	78 ± 15	20.3 ± 6.1
Fluoxetine	7.3 ± 2.9	10^5	1020 ± 18
Imipramine	8.0 ± 2.3	$>10^5$	70 ± 21
Desmethylimipramine	64 ± 17	$>10^5$	4.2 ± 1.1

Human recombinant neurotransmitter transporters were expressed in HEK-293 cells exactly as described in Eshleman *(26)*. [^{3}H]Serotonin (5-HT), dopamine (DA), and norepinephrine (NE) were used to measure reuptake at the human serotonin, dopamine, and norepinephrine transporter, respectively exactly as described in Eshleman *(26)*. Values (IC_{50}, nM) represent the X ± SEM of at least three independent experiments for DOV 216,303 *(85)*. Values for the other antidepressants are shown for comparison; these data are from Eshleman *(26)*.

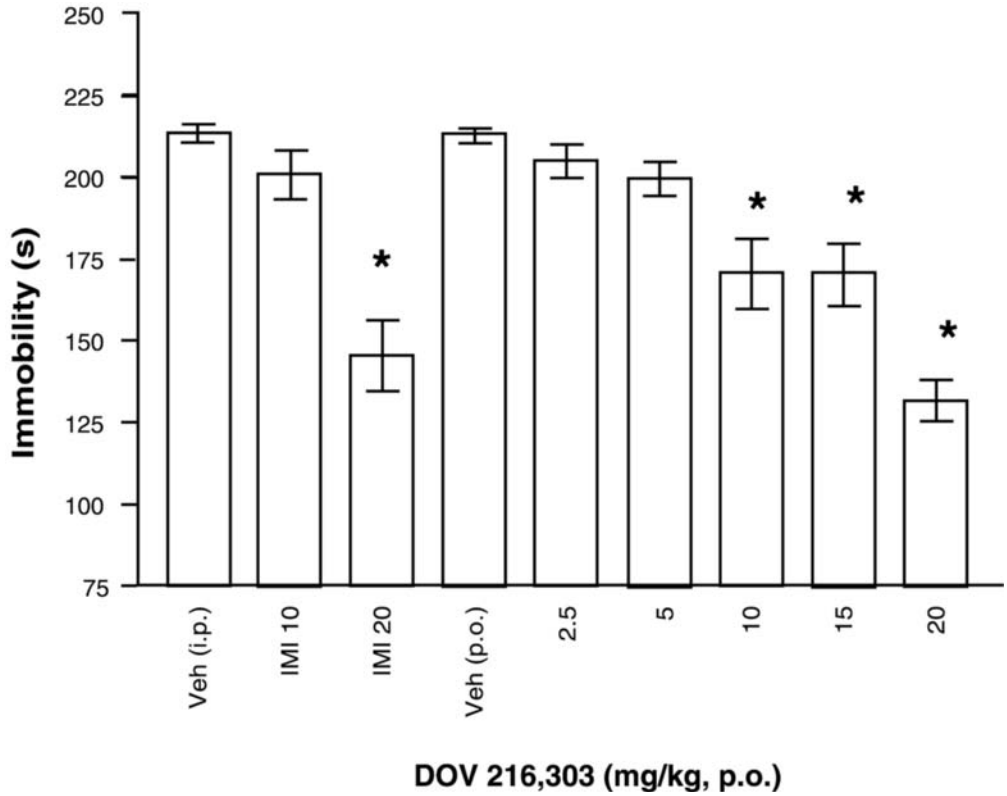

Fig. 1. Effect of DOV 216,303 in the forced swim test. Imipramine (intraperitoneal), DOV 216,303 (oral), or vehicle were administered to male, Swiss albino mice 60 min prior to testing. The duration of immobility was measured for the last 4 min of a 6-min test as described *(89)*. Values represent X ± standard error of mean of ≥6 mice/group. Symbol: *, $p < 0.001$, Dunnett's multiple comparison test. These data are from Skolnick *(85)*.

The optimum potency ratios for inhibiting uptake at the three transporters are unknown. However, among currently used "single" and "dual" reuptake inhibitors, the potency ratios (serotonin IC_{50}:norepinephrine IC_{50}) span several orders of magnitude, ranging from citalopram at one extreme (~3000-fold more selective as an inhibitor of serotonin

reuptake) to milnacipran, which is about equipotent as an inhibitor of norepinephrine and serotonin reuptake *(61,82)*. Nonetheless, based on in vitro potencies in recombinant human receptors expressed in HEK 293 cells (Table 1), the plasma levels of DOV 216,303 attained in Phase I studies would be sufficient to significantly inhibit uptake of all three biogenic amines (ref. *86* and manuscript in preparation). Further, based on the potency of azabicyclo[3.1.0]hexanes in behavioral despair models *(85,86)* these compounds must readily cross the blood–brain barrier.

DOV 216,303 and DOV 21,947 are orally active and potent *(85,86)* in behavioral despair models such as the forced swim *(87)* and tail suspension *(88)* tests (Fig. 1). Like clinically active antidepressants, these compounds reduce immobility in both procedures at doses that do not stimulate motor activity *(85)*. These behavioral despair procedures, although highly predictive of antidepressant activity in humans *(62,63)*, do not yield useful information about either onset of action or efficacy. Although preclinical and clinical data indicate that such a broad-spectrum antidepressant will be superior to serotonin and/or norepinephrine reuptake inhibitors, the ultimate test of this hypothesis will be in the clinic.

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10
Glutamate and Depression

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1. INTRODUCTION

1.1. Prevalence and Symptoms of Depressive Disorders

There are two principal types of mood disorder: major depression and bipolar disorder. Recurring episodes of major depression constitute unipolar depression whereas individuals who alternate periods of mania and depression are manic-depressive and suffer from bipolar disorder. Depression is a disease with a prevalence of 3–5% in developed countries and a lifetime morbidity of approx 15–18%. The disease is more prevalent in females than in males. Prevalence values are not, however, very precise because many depressed patients are still neither diagnosed nor treated. Clinical symptoms include depressed mood and loss of interest in almost everything, anhedonia and fatigue, as well as sleep disturbances, low self-esteem, guilty feelings, and suicidal tendencies. Other somatic symptoms such as gastrointestinal or cardiovascular disorders are also often present. The symptoms of mania are almost the exact opposite of those of depression. In major depressive disorders, there is a high risk not only for suicide but also for life-threatening effects on multiple organ systems, so it is considered that the mortality risk engendered by major depression is similar to that of the more severe cardiac and cerebrovascular diseases (reviewed in refs. *1* and *2*). It is supposed at present that depressive syndromes are the result of a combination of susceptibility genes and multiple environmental factors. The search for genetic substrates underlying depressive disorders has not as yet resulted, however, in any universally accepted finding.

1.2. Initial Theories on Depression: The Monoaminergic Hypothesis

Early theories on the pathogenesis of depressive disorders have been entirely based on the mechanism of action of antidepressant drugs (*see also* Chapter 9). Because of the absence of animal models for a disease involving higher human emotions, it has been accepted that understanding the mechanisms underlying antidepressant treatment would provide substantial advance in the interpretation of pathological changes in depression. The initial biogenic amine hypothesis of depression was based on the effects on monoamine levels of reserpine, an antihypertensive drug, and antidepressants. Reserpine induces monoamine depletion as well as marked sedation and depressive symptoms, whereas clinically effective antidepressants increase monoamine levels and reverse reserpine-induced

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

sedation. Most therapeutically useful antidepressants block the monoamine transporters providing increased extracellular levels of serotonin (5-HT) and/or noradrenaline (NA) or, alternatively, prevent monoamine degradation by monoamineoxidase (MAO) or act on presynaptic auto/heteroreceptors controlling monoamine release (Table 1). Lithium salts are of much value in the prophylaxis of bipolar disorder and electroconvulsive treatment (ECT) is still widely used in the treatment of depression. Another more recent physical therapy is rapid transcranial magnetic stimulation, which appears to improve mood in depression *(3)*. There is, however, a significant proportion of patients who do not respond to any antidepressant treatment and there is also a lag time of some weeks for the therapeutic effect of these agents, not correlated with the rapid increase in the availability of monoamines, suggesting that slower adaptive mechanisms, related or not to the monoaminergic systems, could be involved in the antidepressant effect (*see also* Chapter 9). In this regard, the so-called β-adrenoceptor downregulation hypothesis *(4,5)*, which assumed that suppression of signaling through β-adrenoceptors after chronic antidepressant treatment was indispensable for clinical efficacy, was the first widely accepted approach in the search for adaptive changes induced by chronic antidepressant treatment. However, this hypothesis was challenged since some more recently introduced antidepressants, such as the selective serotonin reuptake inhitors, did not downregulate β-adrenoceptors and some of them, such as citalopram, even produced the opposite effect *(6)*.

2. MORPHOLOGICAL CHANGES IN DEPRESSION

2.1. Neuroimaging and Neuropathological Studies in Depressed Patients

A consistently observed neuroanatomical change in unipolar major depression has been the volume loss in the hippocampus. Reductions in hippocampal volume, evaluated using magnetic resonance imaging, were nearly 20% in some reports, and apparently dissociated from antidepressant medication or ECT; these reductions have been correlated with the total lifetime duration of depression *(7,8)*. Neuronal atrophy and cell death have been reported not only in the hippocampus but also in the prefrontal cortex of depressed patients. In brain-imaging studies, a decreased volume of the subgenual prefrontal cortex along with a reduced blood flow was found *(9)*. Decreased number and size of neurons, as well as decreased glial cells in the prefrontal and orbitofrontal cortex have been also reported *(10,11)*.

Other positron emission tomography studies have revealed increased cerebral blood flow and glucose metabolism, positively correlated with depression severity, in the amygdala *(12)*, one of the brain regions mediating emotional and stress responses. Antidepressant treatment producing symptom remission decreased amygdala metabolism, supporting the notion that chronic antidepressants have an inhibitory effect on amygdala function *(12)*. Conversely, areas that appear to inhibit emotional expression, such as the posterior orbital cortex, suffer histopathological abnormalities in depression. Excellent reviews on neuroimaging and postmortem studies in depression have been published in recent years *(11–13)*.

2.2. Stress-Induced Neuroanatomic Changes

It is known that a significant percentage of major depression patients display some form of hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis, such as hypercortisolemia and lack of feedback inhibition, and increased release of corticotropin-releasing factor (CRF). A high percentage of patients with Cushing disease also manifest

Table 1
Representative Antidepressant Drugs Acting on the CNS 5-HT and NA Systems

NA/5-HT reuptake inhibitors
Imipramine
Amitriptyline
Clomipramine
Venlafaxine (low anticholinergic side effects)
Selective 5-HT reuptake inhibitors
Fluoxetine
Paroxetine
Sertraline
Citalopram
Selective NA reuptake inhibitors
Desipramine
Reboxetine
Miscellaneous (non-MAO inhibitors)
Tianeptine (enhances 5-HT uptake)
Mirtazapine (antagonist at α_2-adrenoceptors controlling monoamine release)
MAO inhibitors
Phenelzine
Tranylcypromine

CNS, central nervous system; 5-HT, serotonin; NA, noradrenaline; MAO, monoamine oxidase.

depressive symptoms as well as hippocampal atrophy *(1,14,15)*. The reduced hippocampal volume may be a consequence of the increased release of glucocorticoids induced by stress, so depression may be ultimately considered as a stress-related disorder. Stress and glucocorticoids make certain neuronal populations more vulnerable to the neurotoxic effects of ischemia, hypoglycemia, and excitatory amino acids *(15)*. Chronic stress or repeated glucocorticoid administration induce in rodents dendritic atrophy in hippocampal neurons of the CA3 region, a suppression of the normal production of granule cells in the dentate gyrus, and even neurotoxic effects on pre-existing hippocampal neurons (reviewed in refs. *16* and *17*). It remains to be established with certainty whether stress is an epiphenomenon of depression or is rather critically involved in the pathophysiology of depression (cf. ref. *2*), although there is abundant evidence suggesting that there is a causal link between stressful experience and depression *(18)*.

Glucocorticoids, secreted during stress, contribute to neuronal atrophy in the hippocampus through two major mechanisms. One of them, shown not only in fat cells but also in cultured hippocampal neurons, is a decreased glucose uptake *(19)* that could result in increased sensitivity to other neurotoxic insults. The other major mechanism is an enhanced activation of glutamatergic transmission. It is known that an elevation of glucocorticoid levels from the low basal range to those typically excitotoxic increases glutamate levels by fourfold *(17)*. Excessive stimulation of glutamate receptors, in particular of the *N*-methyl-D-aspartate (NMDA) ionotropic receptor, may result in cell death through increased intracellular Ca^{2+} levels (Fig. 1).

Antidepressant
PKA
pCREB
BDNF
trkB
AMPA
exocytosis
Enhanced synaptic connectivity
Ras
Raf
MEK
ERK
RSK-2
pCREB
Bcl-2
Cortisol
GR
glucose uptake
Stress Depression
Glutamate
NMDA
Ca^{2+}
ROS
Neuroplasticity
BDNF
trkB
PI3K
Akt
GSK-3
Bad
Bcl-x
Genetic vulnerability

Expression of the neurotrophin brain-derived neurotrophic factor (BDNF) is also downregulated by stress in different hippocampal subfields *(20)*. In the hippocampus and in other brain regions, BDNF influences neuronal survival, differentiation, and synaptic strength so reduced levels of this neurotrophic factor may also contribute to the atrophy and decreased function of different populations of hippocampal neurons. Like other neurotrophic factors, BDNF activates the mitogen-activated protein (MAP) kinase-signaling pathway, which inhibits cell death through different mechanisms, notably through an increased expression of the antiapoptotic protein Bcl-2 (ref. *21*; *see* Fig. 1). In the context of the present review, it is of interest that BDNF and NMDA receptor antagonists share protective effects on stress-induced neurotoxicity. Whereas NMDA antagonists prevent the reduction by corticosterone of cell proliferation in the adult dentate gyrus *(22)*, BDNF prevents neuronal cell death induced by corticosterone *(23)*.

2.3. *Antagonism of Stress-Induced Changes by Antidepressants*

Standard treatments for depression such as administration of antidepressant drugs or ECT have effects on the hippocampus that should counter those found in major depression, such as stress-induced retraction of dendritic processes in CA3 pyramidal neurons *(24)* or reduction of neurogenesis in the adult dentate gyrus *(25)*. Stress-induced changes in neural plasticity of the hippocampus can be prevented by representative antidepressants, such as imipramine and fluoxetine, and also by ECT *(26)*. In a model of psychosocial stress in primates, it was found that tianeptine, an antidepressant with an unconventional mechanism of action (*see* Table 1), prevented many of the morphological changes associated to stress, including the inhibition of cell proliferation in the hippocampus *(27)*. The ability of the NMDA antagonist MK-801 to prevent corticosterone-induced decrease of proliferating cells in the dentate gyrus *(22)*, suggests the possibility of common mechanisms for antidepressants and NMDA antagonists (*see* Section 5).

Fig. 1. Effect of stress and antidepressant treatment on the regulation of neuroplasticity and cell survival in affective disorders. Cellular plasticity and survival depend on genetic factors. Stress associated to depression increases cortisol and glutamate levels. Stimulation of glucocorticoid receptors reduces glucose uptake, increasing the sensitivity to neurotoxic insults. Excessive stimulation of *N*-methyl-D-aspartate receptors induces cell death through increased Ca^{2+} levels and formation of reactive oxygen species. Stress also decreases brain-derived neurotrophic factor (BDNF) levels with the consequent attenuation in the PI-3K/Akt pathway, which promotes cell survival through inhibition of glucogen synthase kinase activity and reduced expression of the proapoprotic proteins Bad and Bcl-x. Different classes of antidepressants stimulate the cyclic adenosine monophosphate-protein kinase A signaling system and activate the transcription factor CREB (cAMP response element-binding protein). One of the target genes of CREB is BDNF, which inhibits cell death by activating the extracellular signal-regulated kinase mitogen-activated protein (MAP) kinase pathway and promoting the expression of the antiapoptotic protein Bcl-2. Antidepressants may also reduce NMDA receptor function and induce the membrane insertion of α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors, leading to enhanced synaptic connectivity and activation of the antiapoptotic mechanisms of the MAP kinase pathway. GR, glucocorticoid receptors; NMDA, *N*-methyl-D-aspartate; ROS, reactive oxygen species; BDNF, brain-derived neurotrophic factor; GSK-3, glucogen synthase kinase; PKA, protein kinase A; CREB, cAMP response element-binding protein; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein ERK, RSK, ribosomal subunit kinase-2.

3. GLUTAMATERGIC TRANSMISSION DYSFUNCTION IN DEPRESSION

3.1. Changes in Glutamate Levels

Glutamate in mood disorders has been studied using magnetic resonance spectroscopy. Although preliminary, some of these studies appear to reflect regionally specific alterations in glutamate turnover rates associated with mood disorders, such as reduced glutamate in the anterior cingulate cortex *(28,29)*.

The reported deficit of glial cells in mood disorders (*see* Subheading 2.1.) could cause complex changes in glutamate neurotransmission. Because glial glutamate uptake is critical for removing glutamate from the synapse, the reduced number of glial cells may produce toxic accumulation of extracellular glutamate *(30)*. In response to AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptor stimulation, glial cells release D-serine, which stimulates the glycine site of the NMDA receptor *(31)*. Glial deficits may consequently produce glutamatergic hyperactivity. Because glial cells also release trophic factors that participate in the development of synaptic networks, abnormalities in glial function could contribute to the pathophysiology of mood disorders *(32)*.

The effect of antidepressants on glutamate release has been analyzed in some studies. One of them *(33)* showed that desipramine enhanced the spontaneous vesicular release of glutamate in cultured hippocampal neurons. In contrast, it was found in an ex vivo study *(34)* that imipramine markedly blunted glutamate overflow in the prefrontal cortex but not in the striatum, although similar effects were found after acute or chronic antidepressant treatment. Because antidepressants are only efficacious on chronic treatment, the significance of these findings is unclear.

Stress increases extracellular levels of glutamate in the prefrontal cortex, nucleus accumbens and hippocampus *(35–37)*. In adrenalectomized rats, this effect is reduced in the prefrontal cortex or blocked in the hippocampus, indicating that corticosterone is involved in the stress-induced elevation of extracellular glutamate levels in brain regions *(36,37)*. Further studies on the effect of chronic antidepressant treatment on enhanced extracellular glutamate levels induced by acute or chronic stress would be no doubt of interest at the time of assessing the neuroprotective effect of antidepressants on stress-induced neurotoxicity. It is of note that in a single magnetic resonance spectroscopy study, a decreased caudate glutamate resonance was found following paroxetine treatment for obsessive-compulsive disorder *(38)*.

It has been suggested that inhibition of glutamate release could be a valid approach in the treatment of depression. Repeated administration of lithium, the prototype mood stabilizer, promotes glutamate uptake and reduces glutamate receptor function *(39)*. Lamotrigine, an anticonvulsant agent that among other effects, reduces glutamate release has antimanic and antidepressant efficacy *(40)*. Clinical trials for the efficacy in major depression of riluzole, another inhibitor of glutamate release that is used for the treatment of amyotrophic lateral sclerosis, are in progress.

3.2. Changes in Glutamate Receptor Function

NMDA receptor dysfunction has been studied in postmortem samples from suicide victims, many of whom could have been depressed patients. A reduced binding to the glycine site of the NMDA receptor complex was found in suicide victims as compared to sudden-death controls *(41)*. This study has been questioned, however, as diagnoses of the

suicide victims, as well as previous pharmacological treatments, were in general unknown. In another postmortem study on suicide victims with a firm diagnosis of depression, no change in the binding characteristics of the noncompetitive NMDA receptor antagonist, ^{3}H-MK-801, was found *(42)*.

Postmortem studies in the striatum of patients with major depression or bipolar disorder revealed only minimal changes in mRNA expression of the different NMDA and AMPA receptor subunits, the only significant change being a reduced GluR1 mRNA expression in bipolar disorder *(43)*. The striatal expression of excitatory amino acid transporters was also analyzed in mood disorders. A decrease in neuronal EAAT3 and EAAT4 mRNA in bipolar disorder and a reduced EAAT4 mRNA in major depression was found *(44)*.

Exposure to stress has been shown to increase mRNA levels of NR1 and NR2 subunits of the NMDA receptor and the GluR1 subunit of the AMPA receptor in the rat hippocampus, as well as the expression of NR1 and GluR1 in the ventral tegmental area *(45,46)*.

4. ANTIDEPRESSANT TREATMENT AND SYNAPTIC PLASTICITY

Recent hypotheses on the pathophysiology of depressive disorders involve adaptive plasticity of neural systems. As proposed by Duman and colleagues *(47)*, depression could result from an inability to make the appropriate responses to stress as a consequence of a dysfunction of the normal mechanisms underlying neural plasticity. It has been supposed for some time that plastic changes should be involved in antidepressant actions since there is a lag time of several weeks for the therapeutic effect of antidepressants whereas acute effects on monoamine transporters or monoamine-inactivating enzymes are of rapid onset (*see also* Chapter 9). Indeed, abundant evidence indicates that antidepressants exert key effects on cell-signaling pathways regulating neuroplasticity and cell survival.

4.1. Effect of Antidepressant Treatment on the Cyclic Adenosine Monophosphate Signaling System

Chronic treatment with different classes of antidepressants, including selective 5-HT and NA reuptake inhibitors, upregulates the cyclic adenosine monophosphate (cAMP) transduction cascade leading to the activation of cAMP-dependent protein kinase A (PKA), which phosphorylates proteins with a key role in cell signaling *(47–49)*. One of them is the transcription factor CREB (cAMP response element-binding protein), which mediates many of the actions of the cAMP system on cell signaling (Fig. 1). The time course for the induction of CREB is consistent with the lag time for therapeutic effectiveness of antidepressant treatment *(50)*. Furthermore, overexpression of CREB in the rat dentate gyrus produced an antidepressant-like effect in the forced swim and learned helplessness tests *(51)*. Consistent with these data, it was found in a postmortem study that CREB was decreased in the temporal cortex of depressed patients and this effect was reversed by antidepressant treatment *(52)*.

4.2. Effect of Antidepressant Treatment on BDNF Expression

Among the target genes of CREB is the BDNF, which contributes to cellular processes underlying neuronal plasticity and cell survival. Chronic administration of antidepressants with different primary mechanisms of action increases BDNF mRNA and its receptor trkB in the rat hippocampus and blocks the downregulation of BDNF mRNA in the hippocampus in response to restraint stress *(53–55)*. It is of interest that BDNF induces

antidepressant-like effects in two widely used animal models of depression such as the learned helplessness and forced swim test *(56)*. The notion that BDNF may be regulated by antidepressant treatments is supported by postmortem studies in the hippocampus of depressed patients. An increased BDNF expression was found in dentate gyrus, hilus, and supragranular regions of subjects receiving antidepressant medication *(57)*. Consistent with this finding, decreased serum BDNF levels were found in major depressed patients *(58)*. The mechanisms that underlie BDNF inhibition of cell death include activation of the MAP kinase cascade, which leads to phosphorylation of CREB and to increased expression of the antiapoptotic protein Bcl-2 and inactivation of the apoptotic protein Bad *(59)*. Activation of trkB receptor by BDNF also enhances cell survival through the phosphatidylinositol-3 kinase (PI-3K)/Akt pathway (ref. *60; see* Fig. 1). Interestingly, it was recently reported that chronic antidepressant treatment is also able to increase the intensity of Bcl-2 immunostaining in rat hippocampus *(61)*.

4.3. Effect of Antidepressants on Neurogenesis in Adult Brain

Neurogenesis has been demonstrated in the adult mammalian brain from different animal species, including humans *(62)*. Neurogenesis is restricted to two brain areas, the olfactory bulb and the dentate gyrus of the hippocampus. The new neurons are derived from the subventricular zone or from the subgranular zone of the hippocampus. Stress activates the HPA axis with the consequent release of glucocorticoids, which downregulate neurogenesis in the hippocampus through downstream actions on NMDA receptors *(63)*. Glutamate, by acting at NMDA receptors, suppresses neurogenesis whereas NMDA antagonists, such as MK-801, enhance it *(64)*. Exposure to stress, including learned helplessness, a paradigm of inescapable stress, decreases neurogenesis *(63)* and, conversely, exposure to an enriched environment *(65)* and chronic, but not acute, treatment with different classes of antidepressants, and also with the mood-stabilizing agent lithium, increases the neurogenesis of dentate gyrus granule cells *(25,66)*. Both the cAMP signaling system and BDNF, which are upregulated by chronic antidepressant treatment *(50,53,67)*, play a role in the regulation of neurogenesis. It has been suggested *(68)* that the lag time of several weeks for the therapeutic effectiveness of antidepressants is consistent with the time taken by newly born dentate gyrus neurons to migrate and to become integrated into the existing brain circuitry.

5. INVOLVEMENT OF NMDA RECEPTORS IN ANTIDEPRESSANT ACTIONS

5.1. Physiological and Pathophysiological Role of NMDA Receptors

Excitatory synaptic transmission is mediated by three distinct classes of ionotropic receptors—NMDA, AMPA, and kainate—and by the three groups of metabotropic glutamate receptors (*see* Chapters 4 and 5). It is widely accepted that excitatory amino acid receptors are involved in numerous aspects of both normal and abnormal brain function. Activation of NMDA and AMPA receptors appears to underlie the vast majority of fast excitatory transmission in the central nervous system (CNS). Synaptically released glutamate results in a two-component EPSC on binding to NMDA and AMPA receptors. Activation of AMPA receptors mediates a component of rapid onset and decay, whereas the activation of NMDA receptors is more prolonged probably owing to the higher affinity of glutamate for NMDA than for AMPA receptors, at least one order of magnitude. NMDA

receptors are highly permeable to Ca^{2+}, whereas AMPA receptors are only permeable when they have no GluR2 subunits.

The elevation of cytosolic Ca^{2+}, leads to the activation of a variety of enzymes, including kinases with a critical role in paradigms of synaptic plasticity, such as long-term potentiation (LTP) in the hippocampus *(69)*, a long-lasting enhancement in the strength of synaptic connections between neurons that represents a widely accepted model for learning and memory. NMDA receptors are critical for the induction of LTP. In the expression and maintenance of LTP, there is an increase in AMPA receptor function, so a sequential activation of the two major classes of glutamate ionotropic receptors is necessary for this paradigm of synaptic plasticity. The effects of exposure to various types of uncontrollable stress on glutamate-mediated hippocampal synaptic plasticity has been studied. In general, LTP in rodent hippocampus is impaired by behavioral stress *(70)*, including manipulations such as the inescapable stress of learned helplessness, an animal model for depression. It is not clear however that antidepressant treatment can restore stress-induced impairment in hippocampal synaptic plasticity.

Excessive activation of ionotropic glutamate receptors can precipitate seizures and induce acute neuronal injury (excitotoxicity) and may also underlie some chronic neurodegenerative disorders. A significant proportion of the neuronal death associated with intense glutamate exposure is mediated by NMDA receptor activation, probably because lethal amounts of Ca^{2+} influx are induced more rapidly than in the case of AMPA or kainate receptor activation. Sustained elevation in intracellular Ca^{2+} initiate toxic cascades that ultimately result in neuronal cell death through free-radical production and lipid peroxidation, activation of nitric oxide (NO) synthase, and release of NO, which interacts with reactive oxygen species to generate peroxynitrite and uncoupling of mitochondrial electron transport enhancing production of free radicals *(71)*.

5.2. Modulation of NMDA Receptors by Antidepressants

Acute and chronic treatment with antidepressants affects NMDA receptors. Chronic antidepresant treatment inhibits the binding of the uncompetitive NMDA antagonist ^{3}H-MK-801 to mouse brain membranes *(72)* and reduces NMDA-induced behaviour *(73)*. Chronic, but not acute, administration of most clinically effective antidepressants down-regulates the strychnine-insensitive glycine site of the NMDA receptor in cortical membranes *(74)*. A transcriptional mechanism has been suggested for this down-regulation, as antidepressants such as citalopram and imipramine are able to produce after chronic administration to mice a region-specific altered expression of mRNA for NMDA receptor subunits *(75)*. In the latter study, a reduced NR1 subunit mRNA expression was found in different cortical areas, including the frontal cortex, and in subcortical regions, including striatum and amygdala. In the hippocampus, NR1 expression was not altered by antidepressant treatment but the expression of NR2 subunits was reduced to a varying extent in hippocampal fields by the two antidepressants tested. These studies suggest that chronic antidepressant treatment would be abating NMDA receptor function through a reduction in the proportion of active glycine sites *(76)*. To our knowledge, no electrophysiological study has been however performed as yet to confirm such assertion.

NMDA receptor antagonists exert a protective effect on multiple neuronal insults *(71)*. By reducing NMDA receptor expression, chronic antidepressants should exert also

neuroprotective actions. Furthermore, chronic antidepressants promote the expression of the neurotrophin BDNF with trophic and neuroprotective properties. A link between NMDA receptors and BDNF was found in a study on primary neuronal cultures where BDNF reduced NR2A and NR2C mRNA levels with a concomitant decrease in NMDA-induced Ca^{2+} entry *(77)*. On this basis, it has been suggested *(78)* that, by promoting BDNF formation and by antagonizing NMDA receptors, antidepressants reach an identical functional end point, which results in a protection of vulnerable neurons.

5.3. Antidepressant-Like Actions of NMDA Receptor Antagonists

As previously indicated, NMDA receptor activation is required for LTP in the hippocampus and inescapable stress impairs the induction of LTP. On the basis of the ability of antidepressants to antagonize the syndrome of learned helplessness induced by inescapable stress, an animal paradigm that models aspects of depression, Skolnick and colleagues first suggested the possible utility of NMDA receptor antagonists as antidepressants. In their initial studies using the so-called "behavioral despair" models (forced swim and tail suspension tests), a dose-dependent reduction in immobility was found with a competitive NMDA antagonist (AP-7), a glycine partial agonist (ACPC), and MK-801, an uncompetitive channel blocker *(79)*. Studies from this and other groups have later reported similar effects in various animal models with different NMDA antagonists such as memantine, a low-affinity uncompetitive NMDA antagonist, CGP-39551 and CGP-37849, competitive NMDA antagonists, and eliprodil an NR2B-selective antagonist (refs. *80–82*; Table 2). Synergistic effects of weak uncompetitive NMDA receptor antagonists (memantine and amantadine) with different antidepressants (imipramine, fluoxetine, venlafaxine) also have been reported *(83)*, suggesting the potential utility of these combinations in treatment-resistant depressed patients. It is of interest that one of the principal events in the neurotoxic cascade following NMDA receptor activation is NO production. NO synthase inhibitors also significantly reduce the immobility time in the forced swim test *(84)*, suggesting that an antidepressant-like effect can be obtained by any interruption of the NMDA receptor signaling cascade (cf. ref. *78*). However, a 5-HT-dependent mechanism also appears to be involved in the antidepressant-like effects of NO synthase inhibitors *(85)*.

5.4. Effects of NMDA Antagonists in Depressed Patients

There have been few clinical studies with NMDA antagonists in depression (Table 2). In one of them *(86)*, ketamine, an intravenous dissociative anaesthetic that uncompetitively blocks the NMDA receptor channel, was given on a double-blind basis to a cohort of patients unresponsive to conventional antidepressants and a significant reduction in the scores of the Hamilton Depression Rating Scale was found. Positive results in clinical studies have been also obtained with other low-affinity NMDA receptor antagonists, such as metapramine, amantadine, memantine, and also D-cycloserine, a partial agonist/antagonist, at the glycine site of the NMDA receptor (*see* reviews in refs. *29* and *78*). Further clinical studies with memantine in major depression are in progress, as well as clinical trials in treatment-resistant bipolar depression with the anticonvulsant felbamate, an NMDA antagonist at the glycine site. Because highly potent NMDA receptor antagonists such as MK-801 or ketamine elicit a number of psychotomimetic side effects *(87)*, more subtle approaches aimed at dampening NMDA receptor function are probably necessary.

Table 2
Preclinical and Clinical Data on the Antidepressant Efffect of Modulators of Glutamatergic Neurotransmission[a]

Preclinical studies			
Compound	Forced swim test (rats, mice)	Tail suspension test (mice)	Chronic mild stress (rats)
NMDA antagonists			
MK-801	+	+	+
AP-7	+	–	
ACPC	+	+	+
CGP 37849	+		+
CGP 40116			+
CGP 39551	+		
Eliprodil	+	–	
Memantine	+		
AMPA potentiators			
LY 392098	+	+	
LY 404187	+	+	
LY 451616	+	+	

Clinical trials		
Compound	Indication	Development status
NMDA antagonists		
Ketamine	Major depression	Phase II
Memantine	Major depression	Phase III
Felbamate	Resistant bipolar disorder	Phase II
Glu release inhibitor		
Riluzole	Major depression	Phase II

[a](+) Significant effect; (–) nonsignificant effect.
NMDA, *N*-methyl-D-aspartate; AMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate.

6. INVOLVEMENT OF AMPA RECEPTORS IN ANTIDEPRESSANT ACTIONS

6.1. Physiological Role of AMPA Receptors

AMPA receptors mediate most of the fast excitatory neurotransmission in mammalian brain and are an important target for mechanisms controlling synaptic strength. As already mentioned (Subheading 5.1.) an increase in AMPA receptor function is necessary for the expression and maintenance of LTP, a lasting enhancement in the strength of synaptic connections between neurons. Another main mediator of synaptic plasticity is the neurotrophin BDNF, which is induced by AMPA receptor activation, an effect found initially in vitro *(88)* and also in vivo *(89)*. Because chronic antidepressant treatment increases BDNF in the hippocampus, probably through activation of the cAMP transduction pathway and phosphorylation of the transcription factor CREB, there appears to be a close relationship between the effects of antidepressants and AMPA receptor activation, suggesting the interest of these ionotropic receptors in the search for new antidepressants.

6.2. Effect of Antidepressants on AMPA Receptors

Antidepressant treatment may potentiate AMPA-mediated transmission. It has been found that repeated electroconvulsive treatment increases GluR1 mRNA expression in different fields of the rat hippocampus *(90)*. Fluoxetine increases phosphorylation of the GluR1 subunit, preferentially at the Ser-845 PKA site *(91)*, a change that contributes to maintaining AMPA receptors at the synapses. We have found that chronic antidepressant treatment with either paroxetine, a selective 5-HT reuptake inhibitor, or desipramine, which is a more selective noradrenaline reuptake inhibitor, produced an increased expression of the AMPA receptor subunits GluR1 and GluR2/3 in rat hippocampus *(92)*. This effect was observed after chronic antidepressant treatment for 21 d but not after acute treatment and was restricted to membrane extracts and not to total protein extracts from rat hippocampus, suggesting a trafficking of these subunits from intracellular pools to synaptic sites. Changes in phosphorylation systems induced by chronic antidepressant treatment may account for the membrane insertion of AMPA receptors. Among the changes in phosphorylating enzymes, an increase of calcium/calmodulin-dependent protein kinase II (CaMKII) activity at postsynaptic sites *(93)* may result in the incorporation of GluR1-containing AMPA receptors into the synaptic membrane, where the upregulation of protein kinase A (PKA) by chronic antidepressants *(67)* contributes to prevent endocytosis of the membrane-inserted receptors. The increased number of AMPA receptors at the synapses may be a mechanism to enhance the strength of synaptic transmission. In subsequent immunoprecipitation studies (Frechilla et al., unpublished results), we found that desipramine increased the interaction of the GluR2/3 subunits of the AMPA receptor with the *N*-ethylmaleimide sensitive factor (NSF), which plays a critical role in protein trafficking, and also of the GluR1 subunit with the protein SAP 97, involved in the synaptic insertion of AMPA receptors, providing a mechanism for the enhanced expression of AMPA receptor subunits in hippocampal membranes. Because repeated antidepressant administration is required for increased expression of BDNF and its receptor trkB in the hippocampus and also for regulating AMPA receptor insertion into the synapses, the sequential correlation between both effects remains to be established *(55)*. AMPA receptor activation promotes BDNF expression but, reciprocally, BDNF increases the surface expression of AMPA receptor subunits *(94)*. The interplay between both molecular effectors probably represents a major contribution to the enhanced synaptic plasticity in the hippocampus induced by chronic antidepressant treatment.

6.3. AMPA Receptor Potentiators

AMPA receptor potentiators or Ampakines are compounds able to increase AMPA-mediated excitatory postsynaptic responses and to reduce the rate of desensitization/deactivation of the ligand-gated ionic channel *(95)*. Several classes of Ampakines have been identified, including benzothiazides, such as cyclothiazide, pyrrolidones with nootropic effect, such as piracetam and aniracetam, benzoylpiperidines (CX-516, CX-614) and, more recently, biarylpropylsulfonamide derivatives (LY392098 and LY404187). Different Ampakines (cyclothiazide, CX-614, LY392098, LY404187) are able to potentiate the AMPA-stimulated increase in the expression of BDNF or to potentiate BDNF expression by themselves. This effect has been found in vitro and also in vivo, after daily administration for 5–7 d, in hippocampal subfields, notably in the dentate gyrus *(88,96,97)*. Increased BDNF mRNA is blocked by selective antagonists, of

AMPA receptors, such as NBQX, but not by NMDA receptor antagonists, such as MK-801 *(96)*. Chronic administration of LY451646 also increased, like clinically effective antidepressants, progenitor cell proliferation in the rat dentate gyrus in a dose-dependent manner *(98)*. Ampakines are not able to affect AMPA channel opening in the absence of glutamate or other AMPA receptor agonist, so it is to be supposed that these compounds also augment glutamate levels in neuronal cultures. AMPA receptor-mediated increase in BDNF expression has been linked to activation of voltage-sensitive L-type calcium channels because this increase can be blocked by nimodipine, a typical calcium channel blocker *(77)*. Increase in $[Ca^{2+}]_i$ can then activate BDNF expression through multiple mechanisms including the activation of calcium response elements located in the promoter region of the BDNF gene *(99)*. Activation of a MAP kinase pathway that may be activated by Lyn kinase (a member of the Src family of protein tyrosine kinases) may be also involved in the increased BDNF expression induced by AMPA receptor potentiators *(77,100)*.

Ampakines not only promote, like chronic antidepressants, BDNF expression but the biarylpropylsulfonamides LY392098 and LY404187 are also effective in animal models of depression with high predictive validity such as the forced swim test and the tail suspension test (refs. *77* and *101*; *see* Table 2). The antidepressant-like effect of this class of compounds can be blocked by the noncompetitive AMPA receptor antagonist LY300168. The rapid antidepressant-like effect of Ampakines in these behavioral despair tests do not appear in principle to be linked to a significant increase in BDNF levels. Some studies have shown however that BDNF can be induced as an immediate early gene, in only 30 min, in response to behavioral manipulation *(102)*. Potentiation of AMPA-mediated glutamatergic transmission may consequently exert an antidepressant-like effect, probably mediated through an increased expression of the neurotrophin BDNF.

7. METABOTROPIC GLUTAMATE RECEPTORS AND ANTIDEPRESSANT TREATMENT

Metabotropic glutamate receptors form a family divided into three subgroups. Group I includes the subtypes mGlu1/5 coupled to PI hydrolysis, group II includes mGlu2/3 coupled to G_i proteins, and group III includes mGlu4/6/7/8, also coupled to G_i proteins in heterologous expression systems (*see* Chapter 5). It has been shown that repeated imipramine treatment attenuates the neuronal responsiveness to the selective group 1 mGlu receptor agonist dihydroxyphenylglycol (DHPG) in the CA1 field of rat hippocampus with a time course correlated with the delayed therapeutic effect of antidepressants in humans *(103)*. It is of note that in this study the attenuation of the response to DHPG was still detectable 1 wk after imipramine withdrawal. Chronic imipramine, and also chronic ECT, enhanced the expression of group I mGlu receptors, located postsynaptically and generally connected with the enhancement of glutamatergic transmission, in different rat hippocampal fields. The most pronounced effects were the increased expression of the splice variants mGluR1a in CA3 and mGluR5a in CA1 *(104)*. An antidepressant-like activity has been found with a mGlu5 receptor antagonist, MPEP, although it is possible that this effect is rather related to the additional interaction of this compound with the serotonergic system *(105)*.

An upregulation of mGlu2/3 receptor protein in the hippocampus, cerebral cortex, striatum, and nucleus accumbens was found in rats chronically, but not acutely, treated

with imipramine *(106)*. Some functional effects associated with mGlu receptors such as the amplified PI response to combined activation of group 1 and group 2 mGlu was also enhanced by the chronic antidepressant treatment. It has been proposed that endogenous activation of group II mGlu receptors negatively modulate the activity of the HPA axis *(107)*. Accordingly, these findings suggest that agonists at these receptors would oppose the effects of stress.

8. CONCLUSIONS AND FUTURE DIRECTIONS

The monoaminergic hypothesis of depression has provided the basis for extensive research into the pathophysiology of mood disorders and has been of great significance for the development of effective antidepressants. Current antidepressant treatments not only increase serotonin and/or noradrenaline bioavailability but also originate adaptive changes increasing synaptic plasticity. Novel approaches to depression and to antidepressant therapy are now focused on intracellular targets that regulate neuroplasticity and cell survival. Accumulating evidence indicates that there is an anatomical substrate for such a devastating neuropsychiatric disease as major depression. Loss of synaptic plasticity and hippocampal atrophy appear to be prominent features of this highly prevalent disorder. A combination of genetic susceptibility and environmental factors make hippocampal neurons more vulnerable to stress.

Abundant experimental evidence indicates that stress causes neuronal damage in brain regions, notably in hippocampal subfields. Stress-induced activation of glutamatergic transmission may induce neuronal cell death through excessive stimulation of NMDA receptors. Both standard antidepressants and NMDA receptor antagonists are able to prevent stress-induced neuronal damage. NMDA antagonists are effective in widely used animal models of depression and some of them appear to be effective also in the few clinical trials performed to date.

Chronic antidepressant treatment increases the expression in hippocampal subfields of the neurotrophin BDNF, which promotes processes underlying neuronal plasticity and cell survival. Antidepressants also increase AMPA receptor insertion into synapses of the hippocampus, a mechanism contributing to enhanced synaptic strength and to increased BDNF expression. The interplay between AMPA receptors and BDNF appears to be a key factor in the enhanced synaptic plasticity induced in the hippocampus by chronic antidepressants. In this context, the development of AMPA receptor potentiators, which promote BDNF expression and show antidepressant-like effects in animal models, may represent a novel approach to the treatment of mood disorders.

We are still far from understanding the complex cellular and molecular events involved in mood disorders. Yet, there appears to be an emerging role for glutamate neurotransmission in the search for the pathogenesis of major depression. Mechanisms for potentiation of AMPA-mediated neurotransmission, for attenuation of NMDA receptor function, and for increased neurotrophic factor signaling appear to be promising targets in the search for a more effective antidepressant therapy.

ACKNOWLEDGMENT

The research work of the authors reported here was supported by a grant from Ministerio de Ciencia y Tecnología, Spain (BFI 2001-1602).

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V Stress and Aggression

11
Dopamine, Glutamate, and Aggression

Klaus A. Miczek and Eric W. Fish

1. INTRODUCTION

Dopamine (DA) and glutamate are part of intricate neurobiological mechanisms mediating different kinds of aggressive behavior, involving the canonical amines and acids, neuropeptides, and neurosteroids in corticomesolimbic circuits *(1)*. Beginning with invertebrates, the critical role of serotonin (5-HT) for aggression-inhibiting mechanisms has emerged, and it is important to understand how other transmitters, such as DA and glutamate, interact with serotonergic mechanisms. The two major sources of information for delineating the role of glutamate and DA in aggressive behavior are (1) neurobiological studies of preclinical model systems, mostly in rodents and cats, and (2) investigations into the mechanisms for pharmacotherapeutic interventions in clinical settings. The epidemiological statistics on criminal violence, emergency room visits, and public health records of treating violent individuals and victims of violence document the magnitude and urgency of the problem and the need for increasing the understanding the neurobiological basis of these adaptive and pathological behaviors. Although most acts of violence are committed by individuals who are not in mental health settings, and although most mentally ill patients are not violent, the rate of injury resulting from violent acts is higher for mental health staff than injurious accidents for heavy construction and mining professions *(2,3)*. Up to 22% of psychiatric inpatients committed aggressive acts within the last 2 wk *(4)*.

Pharmacotherapeutic management of patients with violent outbursts profoundly changed in the 1950s with the introduction of neuroleptic agents, and current practices continue to resort to compounds with potent antagonistic effects at DA D2 receptors *(5,6)*. The discovery of chlorpromazine and haloperidol initiated the modern era of an effective pharmacotherapeutic approach to calming aggressive patients with diagnoses that range from psychotics, depressives, schizophrenics, mentally retarded, nonpsychotic character-disordered delinquents, amphetamine abusers, and alcoholics or patients suffering from organic brain syndrome *(7–14)*. Many studies from the 1960s and 1970s documented that all types of phenothiazines, from either the amino-alkyl, piperidyl-alkyl, or piperazine-alkyl groups, thioxanthenes, or butyrophenones are effective in the management of aggressive patients. The effectiveness of chlorpromazine and haloperidol in reducing aggressive behavior continues to serve as benchmark in the evaluation of novel

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

compounds. Whereas the first-generation neuroleptics were critiqued as a form of "chemical restraint," subsequently developed compounds increasingly improved the profile of action, and most importantly, reduce the incidence of tardive dyskinesia and extrapyramidal symptoms in the course of continued treatment *(15)*.

Excitatory amino acids (EAAs), such as glutamate, and inhibitory amino acids, such as γ-aminobutyric acid (GABA), began to be implicated in the neurobiological basis for violent behavior when the mechanisms for seizure-related violence were explored *(16)*. The link between seizures and aggressive and violent behavior is highlighted by the large proportion of epileptic patients committing injurious acts *(16,17)*. Excessive neuronal discharges (i.e., ictal responses) and the display of episodic violent behavior (i.e., the dyscontrol syndrome) share a common mechanism, presumably glutamatergic activity in the temporal lobe *(16,18)*. The evidence for aggressive and violent outbursts during ictal events is extremely limited, with rare cases of ictal rage and aggression *(19)*, whereas cases of interictal violence and aggression are more frequent *(20)*. The relationship between seizure disorders and aggression remains a controversial area, and in particular the role of glutamate in such a link awaits systematic investigation. More direct support for glutamatergic mechanisms in aggressive behavior derives from neurobiological and molecular biology studies, and those will be the focus of our review.

2. FUNCTIONALLY DIVERSE AGGRESSIVE BEHAVIORAL PATTERNS

A key contribution from the behavioral neurobiology of aggression is the differentiation of aggressive behavior patterns that serve diverse functions, ranging from adaptive to destructive modes of behavior. The term "aggression" refers to behaviors that are intended to harm another individual. There are several forms of aggression, each with specific environmental triggers, and behavioral and temporal sequences, serving different functions. Descriptions of aggression across phyla and orders reveal that these behaviors have evolved to promote the survival of an individual and, in social species, to establish and maintain the social organization of a species *(21,22)*. These kinds of adaptive, species-typical forms of aggression can be quantified in laboratory animals using several experimental procedures *(23,24)*. Clinicians and public health officials, however, are most concerned with aggressive behaviors that have detrimental or maladaptive consequences to the individual or the social group *(25)*. Therefore, it is increasingly important for preclinical researchers to differentiate the mechanisms that make aggression adaptive from those who render it destructive and maladaptive. The ethical dilemma of aggression research in animal models comprises the concurrent observation of the principle to reduce harm and injury, as well as the principle to increase the validity of the model system by increasing the potential for harm *(26)*.

2.1. Species-Typical Aggressive Behavior

Aggression has been delineated into different types, such as offensive and defensive behavior in dominance and territorial confrontations, maternal aggression during the postpartum phase, play fighting in juveniles, and predation *(23,27)*. Each type can be distinguished on the basis of its distal, proximal, and triggering antecedents *(26)*, the behavioral topography itself *(28,29)*, as well as its biological function. Though each form is an important component of a species' behavioral repertoire, the distinctions between offensive and defensive aggression are perhaps the most relevant to preclinical psychopharmacology.

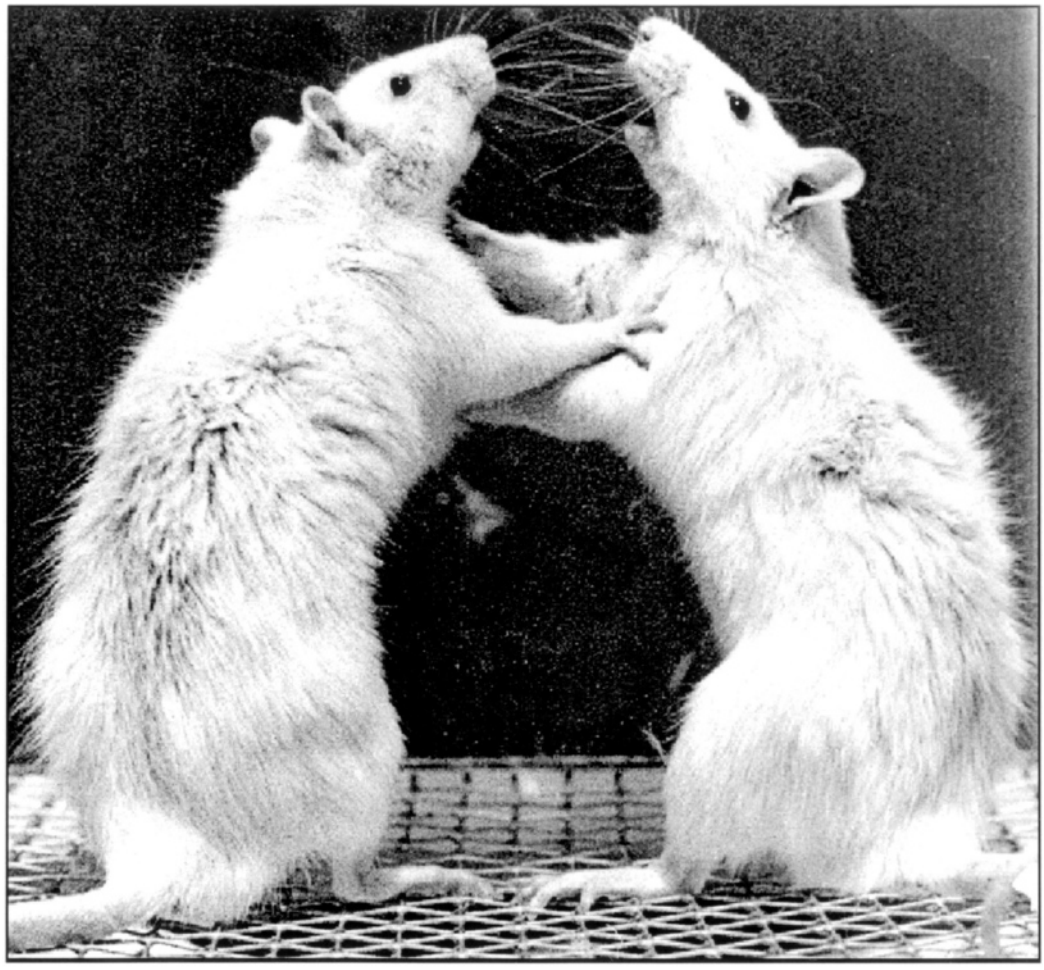

Fig. 1. Mutual upright posture in rats. Both rats assume concurrently a defensive posture with head angled upward and forepaws moving up and down.

Defensive–aggressive behavior refers to those responses that occur in reaction to a painful or noxious stimulus as, for example, pain-induced aggression *(30,31)*, reactivity to handling *(32–34)*, electrically evoked "affective defense" in cats *(35,36),* and aggression toward an interspecies threat *(37)* (Fig. 1). In general, these forms of aggression involve a rapid and sudden attack toward the noxious stimulus, which can be followed by fleeing or attempted escape. Defensive bites are not preceded by anogenital investigation or threat postures and are typically directed toward the face of the opponent *(38,39)*. Maternal aggressive behaviors (i.e., attacks by a lactating female toward male or female intruders) peak in the first week postpartum and share similarities with defensive aggression *(40,41)*, but escape attempts and ritualized displays are uncommon *(39,42,43)*.

Offensive aggression, on the other hand, is a pattern of behavior that is characterized by an intricate sequence of pursuits, threats, and attacks. Offensive aggression occurs in "bursts," series of rapid behaviors that are separated by pauses *(44,45)*. In territorial species such as mice, offensive aggression functions to disperse rival males from a territory that is marked and patrolled by one breeding male, several females, and the prepubertal

Fig. 2. Attack bite by a resident rat and evasive action by the intruder rat.

offspring *(46)*. Behaviorally, the most salient aggressive behaviors in mice are the sideways threat, a rotation of the body toward that of the opponent, and the attack bite, which is generally delivered to the hindquaters *(28)*. Pursuit can also occur in response to the opponent's fleeing. Tail rattling, "mincing," and "pussyfooting" are characteristic of highly aroused mice before initiating an attack *(47)*. In colonial animals, such as rats and primates, aggression serves the purpose of maintaining dominance hierarchies. Threat displays, such as the sideways threat by a dominant rat or the facial and postural displays in primate species, may prevent confrontations from escalating into tissue-damaging attacks *(48–50)* (Figs. 2 and 3).

2.2. *Escalated Types of Aggressive Behavior*

In contrast to the species-typical norms of behavior, exaggerated, intense levels of aggression can occur under the influence of alcohol *(45,51,52)*, after the omission of an expected reward *(53–55)*, in response to brief aggressive interactions *(56,57)*, at social instigation *(55,58,59)*, when aggression serves as a reinforcer *(60)*, or when genetically predisposed *(61)*. The levels of attack engendered by these manipulations exceed the species-typical behavior by two- to threefold, the pattern consists of less ritualized forms of displays, and these types of aggressive behavior may more closely model human injurious aggression. Changes in the patterns and sequences of aggression may also prove to be particularly important to understanding deviant forms of aggression *(61)*. When a corticosterone deficit is pharmacologically induced in previously aggressive rats, they respond to a submissive male with defensive rather than offensive behaviors *(62)*. Alcohol not only increases the amount of aggressive behavior, but also disrupts the sequence of aggression by prolonging bursts of attack *(45)*. When under the influence of alcohol,

Fig. 3. Resident rat (*right*) displaying the sideways threat posture toward an intruder (*left*) in the defensive upright posture.

individuals also appear to be less able to inhibit aggression in response to submissive behaviors of their opponent. Interestingly, prolonged bursts of aggression are seen in rats that behave "impulsively" on measures of cognitive performance *(63)*.

2.3. Importance of Aggressive Experience

The first display of aggressive behavior attracts attention, and without knowledge of an individual's complete developmental history, it is difficult to isolate the critical determinants for the very first occurrence. Once aggressive behavior is established in the repertoire of an individual, its likelihood of appearance becomes more predictable,

particularly when studying laboratory models of aggressive behavior, which typically involves placid domesticated species. Accruing experiences in aggressive confrontations lead to genomic and nongenomic changes in cellular activity in mesocorticolimbic circuits *(64,65)*. The experience of offensive aggressive behavior or defeat results in altered neural activity that is relevant to the pathogenesis of stress disorders such as posttraumatic stress and psychosis, and also of drug abuse *(66–68)*. Aggressive and defeat experiences prompt transcriptional and translational changes in cells of the mesocorticolimbic circuits, and several of these neuroadaptive changes appear to mediate the sensitized response to challenges *(69–71)*. Behavioral sensitization is seen in rats and mice that have experienced repeated aggressive episodes, and this form of neuroadaptation characterizes the individual who defends, submits, and flees rather than the individual who prevails. Thus, two types of neuroadaptation are triggered in parallel when repeated social stress is experienced in an aggressive confrontation, one comprising habituation and tolerance to some features of stress responses, and the other encompassing behavioral and neural sensitization. Blockade of *N*-methyl-D-aspartate (NMDA) receptors by dizolcipine and mGluR5 receptors with 2-methyl-6-(2-phenylethenyl) pyridine (MPEP) during the experience with aggressive confrontation prevents the expression of sensitized behavioral response to a challenge with a low 1 mg/kg amphetamine dose weeks later *(72)*. The critical anatomical site for this dizolcipine effect is the NMDA receptor on DA neurons in the ventral tegmental area, gating DA transmission *(73)* (Fig. 4).

3. NEURAL SYSTEMS FOR AGGRESSION

DA and glutamate are transmitters that are integral to the neural circuits subserving different kinds of aggressive behavior, in concert with other amines, acids, peptides, and steroids. It is useful to differentiate the neural mechanisms contributing to the initiation of aggressive acts from those that prompt its termination. DA and glutamate assume critical roles in the initiation and execution of diverse aggressive behavior patterns, as evidenced by neurochemical assays and pharmacological studies.

3.1. Neural Mechanisms for Initiating Aggression

3.1.1. Dopamine

The initiation of offensive aggressive behavior, like other motivated activities, depends on intact DA neurons in the mesocorticolimbic pathways *(74,75)*. Evidence from progressively more detailed neurochemical and pharmacological studies has identified the ascending dopaminergic projections from the ventral tegmental area, particularly to the ventral striatum, including the nucleus accumbens, and to the prefrontal cortex as critical for the initiation of different kinds of aggressive behavior. By contrast, destruction of these dopaminergic systems via a neurotoxin, such as 6-hydroxydopamine, results in exaggerated defensive rage-like reactions *(76,77)* (Fig. 1).

3.1.1.1 Neurochemical Correlates

Early postmortem tissue assays revealed that mice and rats that had displayed aggressive behavior showed elevated DA levels in the frontal cortex and in the ventral striatum, including the nucleus accumbens *(78–81)*. These correlations between aggressive behavior and increased mesocorticolimbic DA activity, based on postmortem tissue measurements, pointed to a potentially important link. Based on their correlative nature, however, these assay data cannot distinguish between dopaminergic activity *prior* to an aggressive

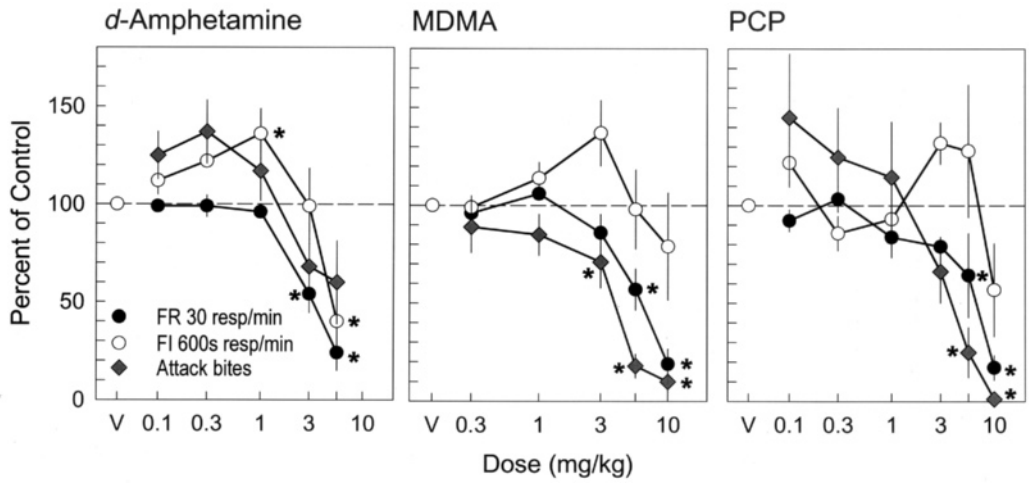

Fig. 4. Dizolcipine (0.1 mg/kg, ip) given prior to each of four social defeat experiences during confrontations with an aggressive opponent reverses the behavioral sensitization, as assessed in response to a cocaine challenge (10 mg/kg, ip) 10 d after the last defeat experience.

encounter relative to the activity *following* the confrontation. Cortical DA may be involved in processing the salient stimuli originating from the intruder (e.g., pheromones, movement patterns, vocal threats), as it is for other types of relevant life events that command attention *(82)*.

Evidence from in vivo microdialysis studies informs on the activity of dopamine in discrete brain areas during the initiation, execution, and termination of aggressive confrontations, both in resident rats that engage in offensive aggressive behavior and in intruder rats that show defensive and submissive responses. Fifty to 60% increases in extracellular concentrations of prefrontal cortical and accumbal DA are measured in intruder rats when they are exposed to an aggressive opponent and react with defensive upright postures, while being protected by a wire mesh screen (Fig. 5) *(83)*. In contrast to the persistent elevation of DA in prefrontal cortex and nucleus accumbens, no significant changes were seen in the striatum, although the intruder rat actively engaged in upright defensive postures with quick torso movements directed toward the threatening resident rat. In particular the elevated DA levels in the prefrontal cortex during the exposure to a threatening opponent is reminiscent of similar increase in DA in rats that are subjected to inescapable experimental stress, such as novel environments, foot shock, or restraint *(84,85)*. These findings question the often reiterated interpretation of the mesocorticolimbic DA pathway as a reward system.

Ostensibly aversive events such as social defeat stress trigger DA release in the terminal regions of this system that is comparable to that seen during intensely rewarding activity. Moreover, increased accumbal DA activity characterizes an intruder rat that had been repeatedly defeated during four preceding brief daily confrontations. These social-defeat experiences sensitize these rats and subsequently they acquire intravenous cocaine self-administration twice as fast as nonstressed control animals *(86)* (Fig. 6).

Particularly instructive are the adaptive changes in accumbal DA during the course of repeated confrontations in the resident aggressive individual. In a series of experiments,

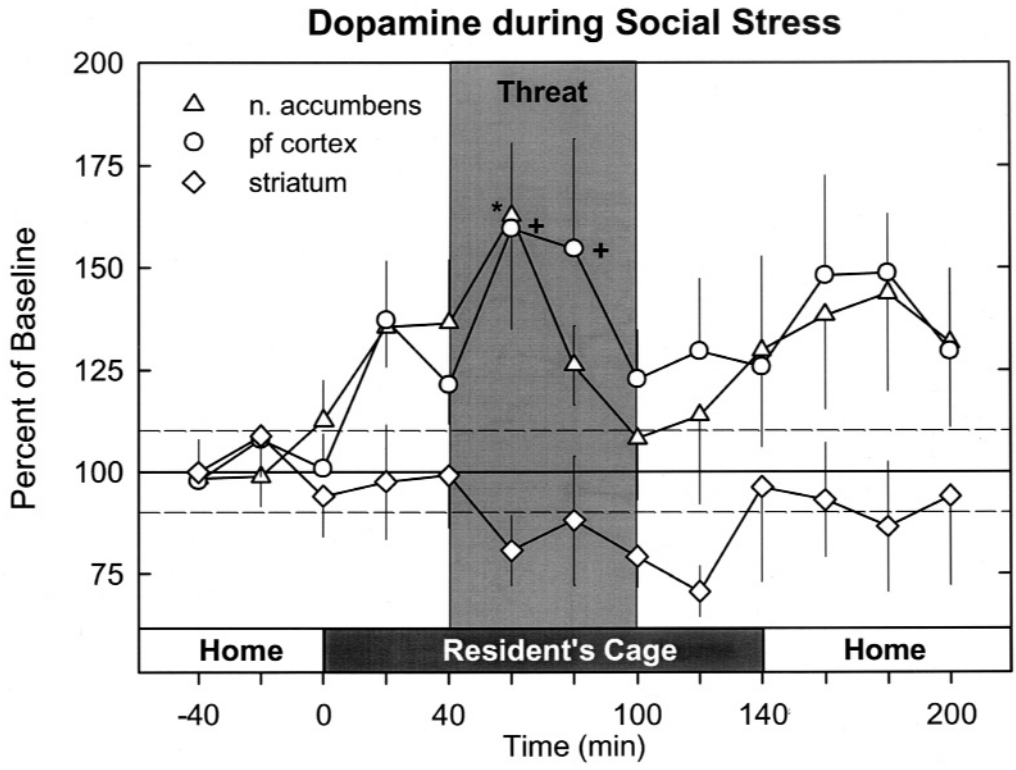

Fig. 5. Extracellular dopamine (DA) concentrations in the nucleus accumbens, prefrontal cortex, and lateral striatum of socially defeated rats before (*left*), during (*center*), and after (*right*) the threat of social defeat. DA concentrations are expressed as percent of baseline, obtained while in the home cage. Samples are collected every 20 min. Crosses indicate $p < 0.05$ compared to baseline, and asterisks indicate $p < 0.05$ between groups. (Adapted from ref. *83*.)

aggressive resident rats confronting an intruder daily were assayed for extracellular DA during the very first or after the 10th confrontation via in vivo microdialysis *(87)*. Confirming earlier evidence, the intensely arousing first confrontation was characterized by a very large tachycardia, as assessed via telemetry, and a rise in extracellular DA levels in nucleus accumbens that outlasted the confrontation. Significantly, once resident rats experienced these confrontations during 10 consecutive days at precisely the same time, DA levels rose on the 11th d in advance of the anticipated confrontation. As a matter of fact, even though the confrontation on the 11th d was omitted, accumbal 5-HT levels decreased after the usual time (Fig. 7). These data provide evidence for entraining or conditioning of monoamine release in anticipation of a salient life event, possibly preparing the individual for action.

3.1.1.2. Pharmacological Manipulations

Although clear changes occur in the DA system in preparation for, and as a result of aggressive behavior, there is less evidence that one of the DA systems specifically modulates aggression. Increasing dopaminergic activity by administration of low to moderate doses of amphetamine or apomorphine can increase the aggressive behavior of isolated mice or rats after omission of a scheduled reward. Higher doses of amphetamine increase

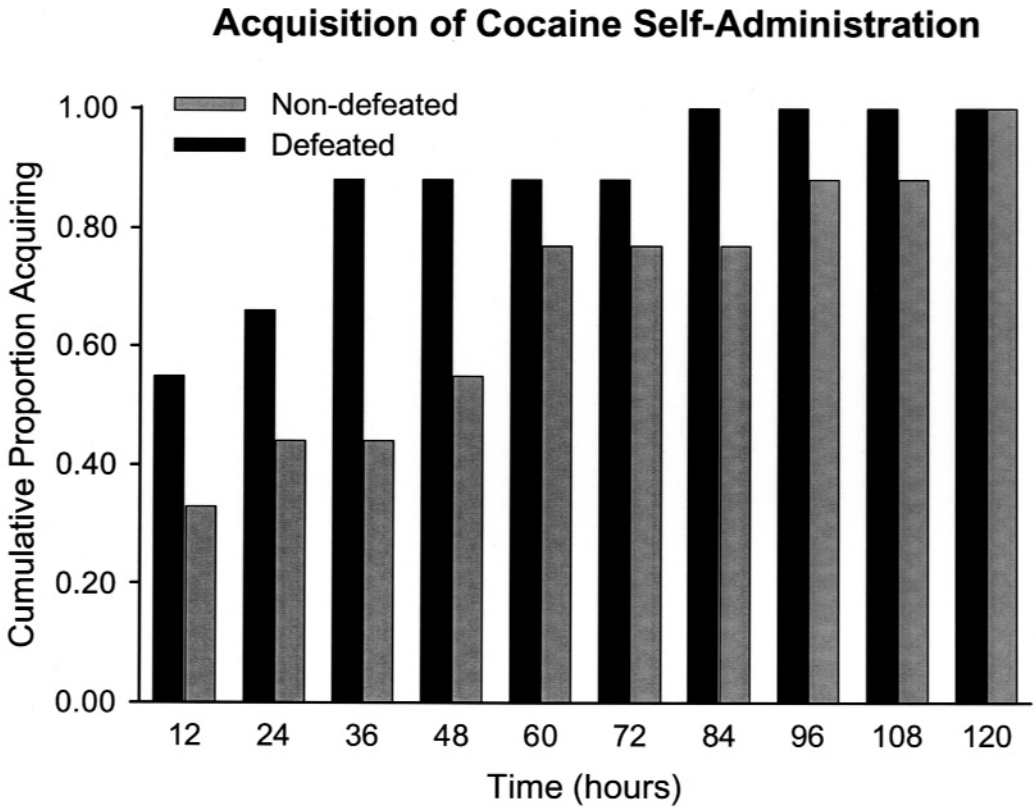

Fig. 6. Histogram of cumulative proportion of rats acquiring cocaine self-administration after they have been previously defeated (solid bars) or not (gray bars). Data for each group were examined in 12-h bins. (Adapted from ref. *86*.)

the defensive responses of rats reacting to electric shock or to the attacks by an opponent—behavioral changes that are likely to be owing to changes in general stimulus reactivity or arousal *(88–94)*. Amphetamines may also increase aggressive behavior secondarily, by preventing fatigue, particularly during extended fights *(95)*. However, when these agents are given to mice or monkeys that have extensive aggressive experiences, they disrupt both aggressive and social behaviors *(96–99)* (Fig. 8).

It is likely that neuroadaptations in dopaminergic neurons as a result of previous experience with aggression determine whether amphetamine and apomorphine enhance or disrupt aggressive behaviors. It will be significant to identify how these experiential factors not only regulate DA release concurrent with the initiation of an aggressive episode, but prompt up- and downregulation of DA receptor subtypes, and alter second messenger function and phosphorylation. There is evidence that brief defeat experiences in an aggressive confrontation profoundly increase the expression of c-fos in brainstem and limbic structures, and these changes persist for several months *(68,70,71,100,101)*. When undergoing withdrawal from morphine, a state with profound neurochemical sequelae including suppressed dopaminergic activity *(102,103)*, amphetamines enhance aggression in mice and rats *(104–106)*. The behavioral and pharmacological history of the individual has emerged as a critical determinant of psychomotor stimulant effects on

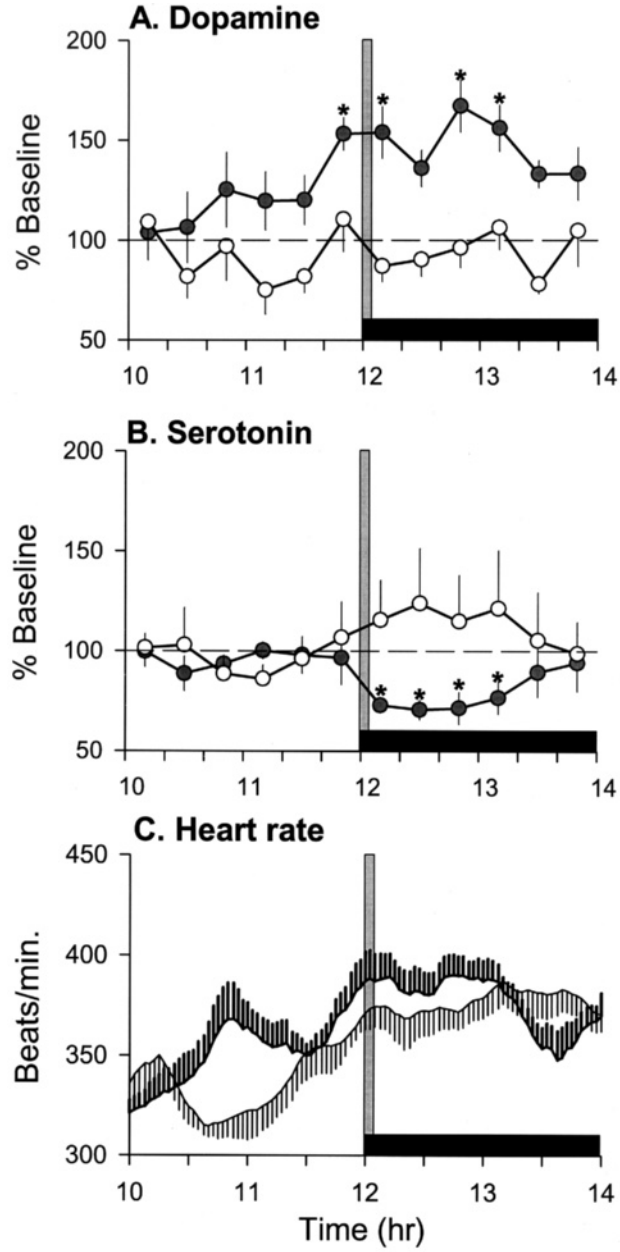

Fig. 7. Extracellular dopamine **(A)** and serotonin **(B)** concentrations in the nucleus accumbens, and heart rate **(C)**, 24 h after 10 d of regularly occurring aggressive confrontations. On this day, no confrontation took place. The time at which the confrontation was scheduled on previous days (12:00 h) is indicated by a gray vertical bar. The dark phase of the light–dark cycle is indicated by a horizontal bar. In **A** and **B**, data are expressed as a percentage of baseline and are presented as group means ± standard error of mean (SEM). Closed symbols depict data from rats that confronted an intruder during the previous 10 d (n = 7). Open symbols depict the light-entrained control group (n = 7). $^{*}p < 0.05$ vs light-entrained animals. **(C)** Changes in heart rate on Day 11, measured simultaneously with microdialysis sample collection. Bold lines depict the previously aggressive group. Thin lines depict the light-entrained group. Data are presented as group means ± SEM. A significant difference was observed in the maximum peak in heart rate, at 12:21 h in the previously aggressive group vs 13:12 h in the light-entrained group. (Adapted from ref. *87*.)

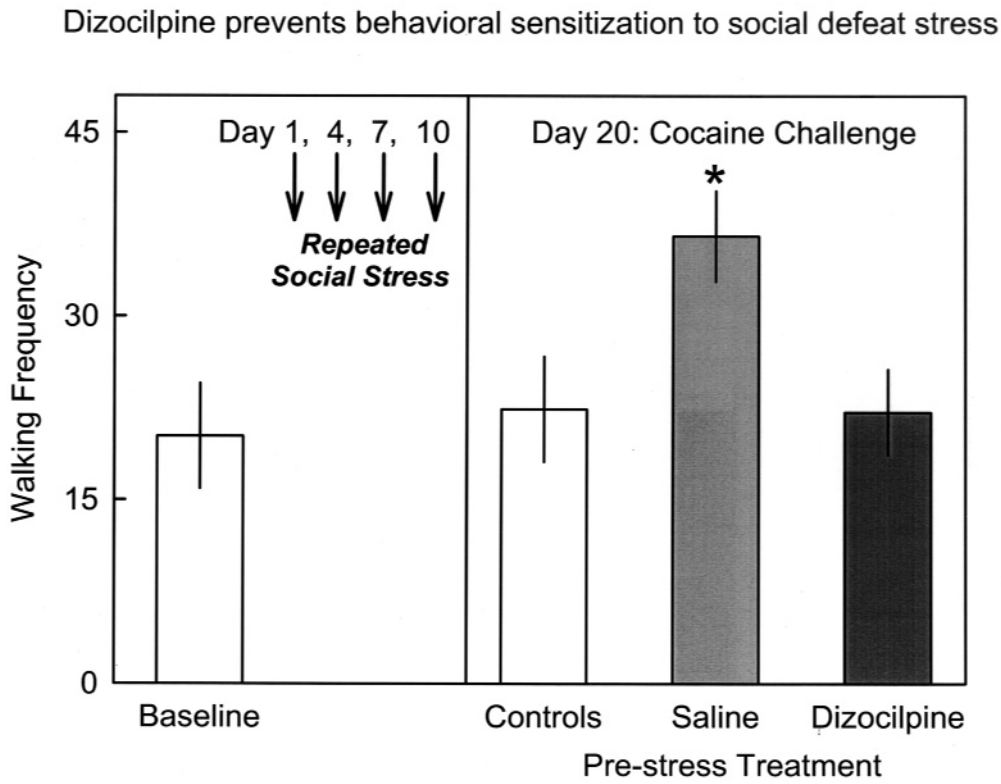

Fig. 8. *d*-Amphetamine, 3,4-methylenedioxy-*N*-methylamphetamine, and phenylcyclidine effects on response rates during the fixed ratio 30 (*black circles*) and fixed interval 600 s (*gray circles*) components of a multiple schedule of reinforcement, and on attack bites (*diamonds*), of resident male mice. Vertical bars in each data point identify ± standard error of mean. Asterisks denote measures that are significantly different from saline control. ($p < 0.05$). (Adapted from ref. *99*.)

aggressive behavior, and it can be hypothesized that these experiential factors are based on molecular changes in dopaminergic neurons.

The role of the two DA receptor families in aggressive behavior is only beginning to be delineated, with the exception of D2 receptor antagonists, which have been studied for decades. Ever since their introduction, "typical" neuroleptics proved effective in reducing aggressive behavior in both humans and laboratory rodents *(107–113)*, implicating the D2 receptor as a critical site of action. One of the hallmark features of behavioral pharmacological studies of aggressive behavior is the concurrent assessment of the specificity of antiaggressive effects. The sedative and motor-incoordinating effects of neuroleptics indicate the nonselective nature of their antiaggressive effects, which highlights the urgency to develop superior pharmacotherapeutic alternatives.

From a pharmacological perspective it appears problematic that in many animal species, including mice, D2 receptor agonists, such as quinpirole, and antagonists, such as raclopride, both decrease aggressive and motor behaviors *(114–117)*. More comprehensive and detailed behavioral analyses are required to distinguish the agonist and antagonist action. It appears that D2 receptor antagonists slow motor activities *(118)*, including those that are part of the aggressive behavioral repertoire, whereas D2 agonists

fragment and disrupt complex behavioral sequences as required for aggressive behavior patterns *(119)*.

So far, studies on aggressive behavior with agents acting on receptors in the D1 receptor family have engendered a pattern of results that is perplexing. Both the D1 receptor agonist SKF 38393 and the antagonist SCH 23390 reduce aggressive, schedule-controlled, and unconditioned motor behavior *(114,120)*, as do the D3 agonists 7-OH-DPAT and PD128907 and the antagonist U-99194A maleate *(121,122)*. The agonists and antagonists may be affecting aggression through distinct mechanisms. For example, the D2 receptor antagonists may reduce aggression by impairing and slowing down the necessary motor systems, whereas the D2 receptor agonists may reduce aggression by slowing the motor output and by affecting the motivation to initiate the behavior. It will be important to study the effects of DA receptor agonists and antagonists in procedures that distinguish the behaviors during the initiation phase of a fight from the actual execution of aggressive acts itself in order to dissociate the role of functionally separate DA receptor pools in the striatal, limbic and cortical terminal regions.

"Atypical" neuroleptic drugs, such as clozapine or olanzapine, have emerged as the treatment of choice for aggressive and nonaggressive schizophrenic patients. Maintenance therapy with clozapine has resulted in fewer assaults on mental hospital staff, significant reductions in aggressive acts, and fewer patients committing aggressive and violent acts *(123–125)*. Particularly important is the success with neuroleptic nonresponsive patients who show less aggressive behavior after clozapine treatment *(126–128)*. Reductions in aggressive behavior of schizophrenic and geriatric patients are also evident with olanzapine treatment *(129,130)*. Atypical neuroleptics may achieve their antiaggressive effects via action on serotonergic or histaminergic receptors rather than dopaminergic receptors. Olanzapine has a much greater affinity for the 5-HT_{2A}receptor than for the D2 receptors and also binds to muscarinic and H1 receptors. To what degree the anti-aggressive effects of "atypical" neuroleptics can be attributed to dopaminergic receptors remains to be determined.

The actual empirical support for a role of mesocorticolimbic dopamine and its receptor families in neural circuits that mediate specifically aggressive behavior is relatively weak. The clinical success in managing violent patients with neuroleptic drugs that target the D2 receptor family is compromised by the lack of behavioral specificity of the antaggressive effects. There has been better success with "atypical" neuroleptics, but their antiaggressive effects in schizophrenic patients may derive from action at nondopaminergic receptors. The future agenda should include a better understanding of the molecular events in dopaminergic cells that result from accruing aggressive or submissive experiences. These neuroadaptive changes may involve important interactions between DA and glutamate.

3.1.2. Glutamate

Surprisingly little is known about the importance of glutamate neurotransmission for aggressive behavior. Clinically, several disorders that alter glutamate are associated with symptoms of aggressive behavior. Seizure disorders are particularly noteworthy because anticonvulsant drugs can also reduce symptoms of aggressiveness *(131–133)*. Historically, seizure disorders have been associated with injurious acts *(16,17)*, but this has been difficult to confirm and causally link to the seizures themselves *(134,135)*.

In animal models of seizure disorder, where cats or rats have been periodically stimulated with trains of electrical pulses (i.e., kindled) or repeatedly injected with kainic acid in amygdaloid and hippocampal sites, there are long-lasting changes in emotional reactivity *(136–138)*. This reactivity can manifest itself as increased defensive aggression. Rats that have been kindled by repeated electrical stimulation of amygdaloid or hippocampal sites, but not of the caudate, are more resistant to capture by an experimenter and more reactive to prodding, 24 h after the last stimulation *(34)*. When kindled rats are tested in a resident–intruder procedure that allows for measurement of both defensive and offensive displays, they assume more defensive upright postures, escape more readily, and display fewer aggressive postures while confronting dominant resident males *(139)*. Kindling also can induce long-term changes in the defensive reactions of cats when exposed to a live rat or playback of recorded howling *(140)*. Whereas nonkindled cats typically responded by approaching and striking the rat, kindled cats withdrew from the rat. In response to the recorded howling, the kindled cats spent a longer time in a defensive posture. Changes in reactivity occur after exposure to the neurotoxin trimethyltin. Trimethyltin can increase components of glutamate neurotransmission *(141–144)* and causes a behavioral syndrome characterized by a persistent increase in seizure susceptibility and reactivity toward handling *(145)*. These studies suggest that excessive neural activity in critical limbic regions, like the amygdala, increases the probability of an exaggerated response toward threatening stimuli.

Excessive neuronal discharges (i.e., ictal responses) and the display of episodic violent behavior (i.e., the dyscontrol syndrome) share a common mechanism, presumably involving glutamatergic activity in the temporal lobe (e.g., refs. *16* and *18*). This excessive activity can result from an increased firing rate of temporal lobe neurons themselves or from impairment of the neurons normally inhibiting temporal lobe activity, descending projections from the prefrontal cortex. Violent offenders have reduced activity and volume *(146,147)* as well as function *(148)* of the prefrontal cortex. In children with temporal lobe epilepsy, only those with a history of aggression had reduced prefrontal cortex gray matter *(149)*, suggesting that it is prefrontal cortical mediation of limbic structures rather than excessive temporal lobe activity itself that contributes to aggressive behavior. Identifying the neurochemistry of the prefrontal projections that inhibit aggression would be an important step to understanding how glutamate elicits the symptoms of the dyscontrol syndrome.

One function of glutamate may be to exaggerate the excitability of the neural systems responsible for aggressive behavior particularly when aggression is intense. One may hypthesize that glutamate may be sensitizing an individual to become more aggressive. Blockade of the glutamate receptors could prevent this sensitized response. Of glutamate's ionotrophic (i.e., NMDA, AMPA, and kainate) and metabotrophic (mGluR1-8) receptors, the NMDA receptors are the most promising targets for the pharmacotherapeutic management of aggressive behavior. Low- and high-affinity uncompetitive receptor antagonists, such as dizocilpine (MK-801), memantine, MRZ 21579, have been shown to alter aggressive behaviors in animals, but their effects appear to depend upon an individual's history of aggressive behavior. In a preliminary study, hospitalized children given amantadine, a very low affinity receptor antagonist that also affects the DA system, to treat impulsivity, aggression, and/or hyperactivity were judged by hospital staff to have fewer of these symptoms *(150)*.

In preclinical studies, NMDA receptor antagonists have mixed effects on aggression and can be quite sedative. The individual's prior history and baseline of aggression are important for understanding the effects of these antagonists. Consistent with some reports in humans, phencyclidine (PCP) and dizocilpine (MK-801) have been reported to increase levels of aggression. These increases occurred when isolated mice confronted an intruder for the first time *(151–153)* or fought at low levels *(154,155)*, and when rats were sleep deprived *(156)*. These studies suggest that individuals with very little fighting experience may be more susceptible to the aggression-heightening effects of NMDA receptor antagonists.

Conversely, in mice and rats with a robust repertoire of aggressive behavior and particularly those that fight at very high, escalated levels, PCP and dizocilpine (MK-801) have been shown to decrease aggression *(99,157–159)*. The therapeutic potential of the low-affinity NMDA receptor channel blocker is evident from two studies on mouse aggression. When given to isolated, aggression-experienced male mice, the only effect of memantine (1–30 mg/kg) and MRZ 2/579 (0.3–10 mg/kg) was motor impairment at the highest memantine dose *(159)*. However, these drugs dose-dependently reduced aggression that had been heightened by morphine withdrawal at doses that were two- to threefold lower than those that impaired motor activity *(160)*.

Additional support for the regulation of excessive aggression by glutamate comes from studies on the threshold to elicit electrically stimulated "defensive rage" in cats. A glutamatergic pathway from the amygdala and the hypothalamus to the periagueductal grey matter (PAG) has been proposed to mediate the electrical stimulation of "defensive rage" *(36)*. Whereas microinjection of NMDA receptor antagonists MK-801 or AP-7 into several brain regions increases the amount of current needed to elicit the defense reaction *(161)*, NMDA receptor stimulation by itself is not sufficient to elicit the reaction except for in the PAG *(36,162,163)*. These results suggest that glutamate's function is to increase the sensitivity of the "defensive rage" pathway, leading to an exaggerated response to stimulation. Experiments comparing the effects of glutamate receptor antagonists on species-typical vs heightened or escalated aggressive behaviors could help address whether glutamate preferentially mediates the escalated form of the response. As the low-affinity NMDA receptor antagonists become more frequently prescribed therapies for dementia and Alzheimer's disease, it will be important to determine their efficacy in treating the symptoms of aggressive behavior.

Glutamate receptor genes have also been linked to aggressive behavior. Brodkin et al. *(164)* identified the glutamate receptor *AMPA3* gene as a candidate quantitative trait locus in a study on aggression by mice directed toward "dangled" intruder mice *(30)*. However, the importance of this or any other single gene in the glutamate system remains to be supported by studies on "knockout mice" *(165,166)*. Given the widespread nature of glutamate in the nervous system it is likely that the most significant contributions of glutamate receptor genes occur within the discrete pathways that mediate aggressive behavior. Studies using antisense oligonucleotides for glutamate receptors in areas, such as the prefrontal cortex, amygdala, and hypothalamus, might be more valuable approaches for understanding the importance of glutamate receptor genes in aggression.

Whereas a key role of glutamate is well established for several disease states, such as dementia, neurotoxicity, seizure susceptibility, and psychosis, there is less evidence for a specific role of glutamate in aggressive behavior. The data from electrical brain stimulation and kindling studies strongly suggest that glutamate is important for the genesis of

defensive reactions. Whether glutamate is also essential for species-typical and escalated offensive–aggressive behaviors awaits detailed ethopharmacological studies. The few studies using antagonists at the NMDA receptor indicate that these compounds may be useful for managing aggressive outbursts. However, early studies with the high-affinity NMDA receptor antagonists, PCP and MK-801, reveal anti-aggressive effects as part of nonspecific changes in motor activity, and suggest that an individual's behavioral history can interact with the actions of glutamatergic drugs. The low-affinity NMDA receptor antagonists, such as memantine, may offer more behaviorally specific effects on aggression by modifying escalated, rather than basal, levels of excitation. NMDA receptor antagonists may be particularly important in the treatment of aggressive symptoms associated with opiate and alcohol withdrawal. Both for the offensively aggressive and for the defensive animal accruing experiences in aggressive confrontations are correlated with profound neuroadaptive changes *(71)*. The neural changes in rats that have been sensitized by repeated defeat experiences include glutamatergic mechanisms as evidenced by the protective effects of NMDA and mGluR5 receptor antagonists (Covington, Gale, and Miczek, unpublished data). It is feasible that escalating offensive aggressive experiences may also be based on neuroadaptive changes involving glutamatergic mechanisms.

DA and glutamate interactions may be relevant for aggressive behavior, as has been suggested for disorders such as schizophrenia *(167)*, drug taking *(168)*, seizure disorders *(169)*, and Parkinson's disease *(170)*. Prefrontal cortical glutamate projections excite cells in the ventral tegmental area (VTA) that release DA *(171–174)*. These projections synapse on dopaminergic cells that ascend back to the prefrontal cortex and onto GABAergic cells that in turn feed back to the nucleus accumbens *(175)*. This circuit may be essential for regulating the rise in extracellular dopamine in frontal cortex that occurs during aggressive behavior *(176),* as well as other salient experiences *(83,84,177,178)*. Some of the behavioral effects of systemically administered NMDA receptor antagonists, such as motor activity, may be related to their effects on the DA system. PCP, ketamine, MK-801, and memantine have been shown to increase extracellular concentrations of dopamine in the nucleus accumbens *(179–181)* and prefrontal cortex *(182–184)*. It is tempting to hypothesize that these indirect effects on dopamine are relevant to aggressive behavior.

DA, in turn, regulates the activity of glutamate. In the prefrontal cortex, DA projections from the VTA synapse on glutamate containing cells *(185)*, and DA depletion in this area disrupts behavior such as cognitive performance (e.g., ref. *186*). The atypical neuroleptics, such as clozapine, binding to both D2 and D4, as well as other receptors *(187)*, are particularly effective at attenuating the behavioral effects of NMDA receptor antagonists *(188,189)*. Although D4 receptors have not been extensively studied regarding aggressive behavior, they are expressed in the prefrontal cortex *(190)* and there is evidence that they attenuate the activity of NMDA receptors *(191)* to reduce neuronal excitability *(192)*. It remains to be tested whether D4 receptor antagonism restores glutamatergic function in prefrontal cortex to reduce aggressive behavior.

DA and glutamate interact at both the cellular and neurocircuit levels to control a range of behaviors. Individually, they influence the expression of aggressive behaviors, and it remains to be determined how critical these interactions are for aggression. Developing therapies that target DA and glutamate interactions could promise more effective and behaviorally selective treatments for aggressive behavior.

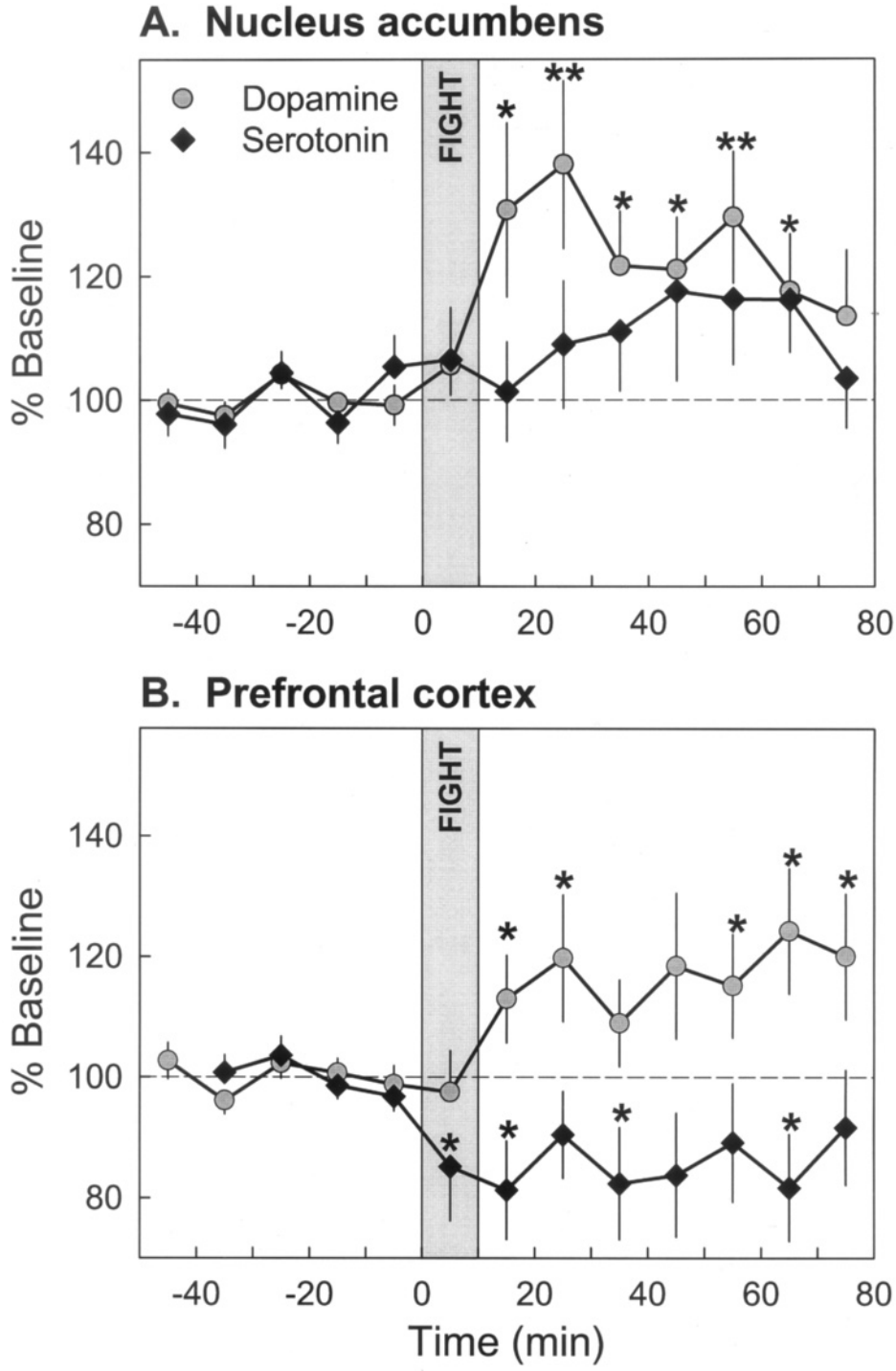

Fig. 9. Extracellular dopamine and serotonin concentrations in the nucleus accumbens (**A**; $n = 18$) or prefrontal cortex (**B**; $n = 15$) of male resident rats. Ten-minute samples were collected 50 min before, during, and 80 min after a confrontation with a smaller male intruder. The vertical gray bar indicates the 10-min period of actual physical confrontation. *Filled diamonds*, serotonin; *open circles*, dopamine. *Asterisks* indicate a significant change from baseline levels, as assessed by planned paired *t*-tests ($^{*}p < 0.05$; $^{**}p < 0.01$). (Adapted from ref. *65*.)

4. NEURAL MECHANISMS FOR INHIBITING AGGRESSION

DA and glutamate interact with the primary inhibitory system for aggressive behavior, namely the ascending dorsal and medial 5-HT projections from the raphe nuclei in the brain stem to striatal, limbic, and cortical terminal sites. Considerable anatomical, electrophysiological, and neurochemical evidence shows that glutamate interacts with serotonin in the raphe nuclei via ionotropic and metabotropic receptors *(193–195)*. An intricate pattern of glutamatergic influences on 5-HT neurotransmission comprises excitatory actions of glutamate via NMDA and AMPA receptors in the dorsal raphe nucleus and via metabotropic receptors in the PAG and frontal cortex, but little effect on the median raphe nucleus *(193,194,196)*. Substantial evidence documents how the 5-HT and DA systems interact as demonstrated by neurochemical and behavioral-pharmacological studies (e.g., refs. *197–199)*.

The inhibitory role of 5-HT systems in circuits mediating escalated forms of aggressive behavior represents the prevailing interpretation of the data that correlate low-CSF (cerebrospinal fluid) 5-HIAA levels with a life history of aggressive behavior *(200,201)*. The relationship between CSF 5-HIAA to 5-HT cell bodies in the dorsal and median raphe nucleus as well as their projections to corticolimbic and striatal terminals remains indirect, and it has been more instructive to learn about the role of serotoninergic cells in discrete corticolimbic cells of aggressive individuals. Imaging and metabolic studies highlight the significance of cells and terminals in the frontal lobe in violent individuals *(202,203)*.

Of particular interest for future studies are the dopaminergic and serotonergic projections to the prefrontal cortex as part of a circuit that is dysfunctional in individuals showing escalated aggression *(176)* (Fig. 9). As mentioned above, the microdialysis probes in the prefrontal cortex of aggressive resident rats show a decrease in extracellular serotonin and the increase in DA that are evident once an aggressive confrontation has been initiated and persist even after the termination of the fight. It would be instructive to learn whether glutamatergic activation in the prefrontal cortical region of aggressive animals precedes or follows the contrasting changes in dopaminergic or serotonergic neurons. It can be hypothesized that glutamatergic feedback from prefrontal cortex to accumbal and tegmental neurons regulates ascending transmission in individuals during an aggressive confrontation, as illustrated for other stress experiences *(204)*.

5. CONCLUSIONS

The monoamines, EAAs, and GABA are at the core of neural systems that mediate aggressive behavior. Because glutamate, DA, and several of their receptor subtypes are critically involved in neuroadaptive processes, such as sensitization and tolerance, it can be hypothesized that the long-term consequences of accumulated aggressive or defeat experiences depend on these dopaminergic and glutamatergic mechanisms. It will be valuable to learn how experiential factors in repeated aggressive confrontations depend on cellular activity in mesocorticolimbic DA and glutamate neurons. As is evident from the summary of research literature, there are urgent needs to investigate the effects of selective agonists and antagonists of DA and glutamate receptor subtypes in an anatomically specific manner. Mesocorticolimbic DA may be of particular significance in the reinforcing features of aggressive behavior, as it is for other intensely reinforcing activities. Future studies will have to determine which of the DA and glutamate mechanisms distinguish the initiation from the inhibition of aggressive behavior.

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VI Anxiety

12
Glutamatergic Systems and Anxiety

David N. Stephens

1. ANXIETY DISORDERS

Anxiety is a normal emotion experienced by humans and other mammalian species. However, anxiety also exists in pathological forms, and anxiety disorders are the most prevalent of psychiatric disorders. Prevalence rates vary with the diagnostic tools used to estimate them, and with study design, but the most extensive studies suggest that within the United States, 15.7 million people are affected yearly and 30 million at some point in their lives *(1)*. In a US study, 6% of men and 13% of women had suffered from an anxiety disorder in the previous 6 mo *(2)*.

According to current classification in the *Diagnostic and Statistical Manual* (DSM–IV) *(3)* major anxiety disorders include phobias, obsessive-compulsive disorder (OCD), posttraumatic stress disorder (PTSD), and generalized anxiety. Although the specific symptomatology and etiology of these disorders varies, as does the recommended psychotherapeutic and pharmacological treatment, all of these disorders are characterized by at least three core clusters of symptoms: autonomic arousal, avoidance, and cognitive disturbance. Arousal of the autonomic nervous system involves sympathetic activation with associated tachycardia, sweating, shortness of breath, dry mouth, and other concomitants of preparation for a "fight-or-flight" response to a real or perceived threat. Avoidance involves physical or psychological distancing from threatening environments or events. Anxiety-related cognitive disturbance focuses on thoughts and feelings about the perceived threat and includes such symptoms as intrusive thoughts (as in OCD and PTSD), difficulty concentrating, vigilance, and excessive worry. Although there are similarities in core symptomatology across anxiety disorders, and with normal anxiety, there are also differences in the symptoms of each individual disorder. Accompanying the core symptoms of arousal, avoidance, and cognitive disturbance present in generalized anxiety and fear are alterations in the neurochemical environment within the brain, and many workers in the field would argue that what distinguishes "normal" anxiety from the anxiety disorders is that the latter reflect a neurobiological disorder of the central nervous system (CNS).

2. NEUROBIOLOGY OF ANXIETY

Emotional behaviors have long been ascribed to the "limbic system," the large relative size of the human limbic areas prompting Donald Hebb, a figure better known in a quite

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

different context in the glutamate field, to point out that the evolution of intelligence had not led to a reduction in the importance of emotions, and to speculate that humans are the most emotionally developed animals (cited in ref. *4*). The term "limbic system" is difficult to sustain in the subsequent development of functional neuroanatomy, and these early ideas have been superseded by more specific hypotheses regarding neuronal structures involved in anxiety. Central among such hypotheses are those identifying the amygdala and its connections as the core of a system subserving fear conditioning (e.g., refs. *5–8*), the septo-hippocampal hypothesis of Gray *(9,10),* which posits that neural systems in the hippocampus and related areas, underlying behavioral inhibition, lie at the heart of anxiety mechanisms, whereas systems identified as mediating flight from immediate threat, and including the periaqueductal gray matter of the midbrain and its related hypothalamic circuits, represent the fundamental systems serving fear and panic reactions *(11)*. Each of these complementary hypotheses requires consideration of the role of glutamatergic transmission that might have implications for potential treatments.

2.1. Amygdala and Conditioned Fear

The amygdala has long been implicated in the expression of fear and anxiety. Early work on the Kluver–Bucy syndrome described how amygdala lesions in monkeys resulted in animals that showed little fear of objects and people that were treated as threatening by normal animals. More recently, activation of amygdala during panic attacks *(12,13)* or anticipatory anxiety *(14)* has been cited as evidence for involvement of the amygdala in clinical anxiety. Congruent findings that PTSD (but not panic disorder or OCD) patients show increases in blood flow in the right amygdala when exposed to anxiety-provoking stimuli have also been reported *(15–17)*, whereas in a functional magnetic resonance imaging (fMRI) study *(18)*, social phobic patients (but not controls) showed heightened activation of the amygdala bilaterally in response to presentation of emotionally neutral faces previously associated with an aversive odor.

Animal experimental work has also identified amygdala circuitry as being of central importance in processing of information during fear conditioning, and in the fear-potentiated startle paradigm (e.g., refs. *5, 6,* and *19*). Much of the work evaluating the role of the amygdala in mediating emotions has been the subject of recent excellent reviews (e.g., *19* and *20*). In particular, the amygdala appears to be of central importance in the formation of associations between discrete environmental events and aversive stimuli, and the expression of fear reactions through its projections to brainstem structures governing behavioral, autonomic, and endocrine responses to threat. Formation of associations between environmental contexts (i.e., the entire complex of cues provided by any environment) and aversive stimuli additionally requires the involvement of hippocampal systems projecting to amygdala nuclei. It is of note that both the thalamo-amygdala pathways and afferents from temporal cortex synapse on to lateral amygdala neurons bearing both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors *(21)*.

There is currently some discussion regarding the roles of amygdala nuclei in processing fear-related information. Although both Ledoux and Davis emphasize the lateral and basolateral part of the amygdala as the area that receives input regarding both aversive events and associated cues, and hold that these areas then provide inputs to the central nucleus, recent studies suggest that the central nucleus may also function independently of the lateral nuclei, receiving highly processed sensory input from entorhinal cortex and related areas (*see* ref. *20* for a review).

2.1.1. Intra-Amygdalar Pathways

Within the amygdala, information regarding at least simple acoustic cues reaches the central nucleus either directly *(7)* or from the lateral amygdala, which itself is thought to receive information from sensory, including auditory, pathways *(5)*. The lateral amygdala projects to the central nucleus both directly, and via relays in the basal and accessory basal amygdala. The lateral amygdala also receives information regarding nociceptive events, whereas the accessory basal nucleus receives input from the spinothalamic tract via the posterior thalamus *(22)* and the central nucleus, both indirectly via the parabrachial area *(23)* and directly from spinal cord *(24)*. The amygdala is therefore well-fitted to integrate information regarding aversive events and environmental stimuli that predict them. Certain lateral amygdala neurons fire in response to both nociceptive stimulation and auditory input *(25)*, offering the possibility of integration of auditory with nociceptive information by associative long-term potentiation (LTP) in the auditory input pathway.

2.1.2. Output Pathways

The central nucleus of the amygdala projects to other areas (*see also* Chapter 3)controlling the expression of fear responses, and lesions of the central nucleus disrupt the expression of the behavioral, autonomic, and endocrine responses of conditioned fear. Lesions in these projection areas are able to disrupt selectively parts of the fear response, so that damage to the lateral hypothalamus prevents blood pressure, but not freezing responses, whereas lesions of the midbrain central gray disrupt freezing, but not blood pressure responses *(26)*. Similarly, selective disruption of the conditioned release of pituitary-adrenal stress hormones is achieved by stria terminalis lesions *(27)*.

2.1.3. Learning Mechanisms in the Amygdala

LTP has been proposed as a mechanism whereby synaptic transmission is facilitated as a result of use. In the hippocampus CA1 region, the mechanism whereby repeated activation of synapse results in facilitated transmission has been demonstrated to depend on glutamate acting at α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors to depolarize the postsynaptic membrane, a consequence of the membrane depolarization is the expulsion of Mg^{2+} ions from the lumen of NMDA receptor-gated channels, allowing glutamate acting at these receptors to trigger Ca^{2+} flux through the channel. Ca^{2+} influx triggers a number of intracellular events that lead to enhancement of the fast AMPA receptor-mediated component of synaptic transmission *(28,29)* arising from increased concentration of AMPA receptors within the synapse, and, consequently, increased excitatory postsynaptic potential (EPSP) magnitude in the postsynaptic element following presynaptic activity. This basic mechanism may form the basis for the formation of associations; if a postsynaptic element (say, a spine) has synapses with two presynaptic inputs, then activity in one of them may provide the necessary depolarization to remove the Mg^{2+} block in neighboring synapses, thus allowing NMDA receptor-mediated transmission through the second synapse, and increased probability of presynaptic activity in the second synapse resulting subsequently in activation of the postsynaptic element. If synapse 1 carries information regarding an aversive event (the unconditioned stimulus [US]), and synapse 2 information regarding an environmental event (conditioned stimulus [CS]) occurring contemporaneously with the US, then, following synaptic strengthening, activation of the synapse carrying information about the CS may have similar postsynaptic consequences as activationg the synapse carrying information

regarding the aversive US did before strengthening occurred. Thus, a form of "associative LTP" may in principle underlie simple conditioning. Whether it indeed does so requires further evidence, but it is of considerable interest that prior fear conditioning increases the magnitude of EPSPs in amygdala slices *(30)*. Furthermore, LTP is found in the pathway from medial geniculate body to the lateral nucleus of the amygdala, which is thought to mediate conditioning of fear responses to acoustic stimuli, and tetanic stimulation of the medial geniculate body also results in a long-lasting potentiation of a field potential in the lateral amygdala elicited by a naturally transduced acoustic stimulus *(31,32)*. The stimulation coincidence parameters that are necessary for induction of LTP in the lateral amygdala closely resemble those required for the formation of associations between CS and US in fear-conditioning experiments *(33)*. Taken together, these experiments suggest that that LTP-like mechanisms underlie amygdala-mediated fear conditioning.

2.1.4. Glutamatergic Transmission in Amygdala Circuits

The neural bases of LTP have been most extensively studied in the well-characterized pathways of the hippocampus, and it is not clear whether the same mechanisms underlie LTP in amygdala pathways. Although NMDA receptor-dependent LTP has been demonstrated in pathways from cortex to amygdala *(34,35)*, and some pathways within the amygdala *(36,37)*, NMDA-independent LTP has also been suggested *(38)*. In the thalamo-amygdala pathway, NMDA-independent LTP may be mediated by Ca^{2+} influx through L-type voltage gated Ca^{2+} channels *(39)*. A further possible difference between hippocampal LTP and amygdala LTP (at least in the lateral amygdala) is in the presumed locus of plasticity. Although it is widely accepted that postsynaptic changes are responsible for the increased synaptic efficiency seen in hippocampal CA1 LTP, some forms of amygdala LTP may depend upon presynaptic changes *(40)*. Furthermore, synaptic facilitation resulting from low-frequency activation of the pathway from external capsule to lateral amygdala is independent of both NMDA receptors, and L-type calcium channels, and depends upon Ca^{2+} flux through kainate receptor-operated channels *(41)*. This form of LTP may not require alterations in AMPA receptor location or density within the synapse, and may implicate presynaptic mechanisms, including facilitated glutamate release *(41)*. The facilitation of transmission is also not limited to the synapse carrying the signal leading to the LTP (homosynaptic LTP) but spreads to neighboring synapses (heterosynaptic LTP). Inasmuch as these neighboring synapses may be involved in the processing of different environmental events, this latter property may result in generalization of conditioned fear to other stimuli that have not been specifically associated with a fearful event. This might be a mechanism underlying pathological conditions in which anxiety or fear are triggered inappropriately by innocuous stimuli *(41)*.

2.1.5. Glutamatergic Pharmacology of Amygdala-Mediated Fear Conditioning

The work outlined above suggests that fear conditioning may be amenable to manipulation by several drugs acting at glutamate ionotropic receptors. In keeping with the proposed role of NMDA receptors in the formation of LTP, NMDA antagonists given during acquisition of the conditioned fear response should prevent conditioned fear, and indeed, infusion of 2-amino-7-phosphonopentanoic acid (APV) into the basolateral amygdala (BLA) during acquisition blocked fear conditioning, whereas APV infusions prior to testing (when NMDA receptors may not be required for expression of the plasticity) had no effects *(42,43)*. Though others *(44,45)* have found NMDA blockade in the

right BLA to interfere with both acquisition, and expression of conditioned fear responses, the blockade of expression may be explained by the involvement of NMDA receptors in normal synaptic transmission within amygdala accessory pathways (e.g., ref. *45*). This explanation would also account for the effectiveness of intra-BLA infusions of NMDA antagonists in nonassociative measures of anxiety, such as the plus-maze *(46)* and social interaction tests *(47)*.

In keeping with the notion that expression of conditioned responses may depend on upregulation of non-NMDA mediated transmission, local infusion of the AMPA/kainate antagonists CNQX and NBQX into either central or basolateral amygdala blocks expression of fear-potentiated startle *(48,49)*.

2.1.6. A Wider Role of the Amygdala in Affective Behavior

In addition to its well-known role in mediating anxiety and fear, the amygdala also plays a central role in learning about appetitive events. The BLA appears to play an essential role in the attribution of affective value to environmental events that predict either aversive or appetitive events. Although animals may be able to learn about the predictive nature of such cues following lesioning of the lateral amygdala, the cues acquire no affective value of their own. In other words, stimuli associated with fear-producing situations may inform the animal of an imminent aversive event, but the stimulus will not evoke an emotional response. In the case of appetitive conditioning, rats with BLA lesions fail to learn new instrumental responses to obtain a cue previously associated with food or a drug reward. Current theories thus hold that the BLA functions to allow animals to utilize cues associated with primary reinforcers, whether positive or negative, to assess their affective properties, and to use that representation to alter their behavioral response *(50,51)*. Although largely developed to account for data acquired from appetitive conditioning, essentially similar functions are likely to apply to aversive conditioning. According to the model of Everitt and colleagues *(50)*, the affective value of the CS is processed by the BLA, but the consequences for behavioral output depend on the information being conveyed to the accumbens *(52–54)*. This approach predicts that disruption of BLA function might then reduce the organism's ability to assess the affective significance of cues conditioned to motivationally significant events—both positive and negative.

2.1.7. AMPAergic Transmission in Basolateral Amygdala

In the BLA, AMPA receptors mediate fast excitatory postsynaptic potentials in response to activation of glutamatergic inputs from both cortical and subcortical regions *(55,56)*. The BLA contains two major classes of neuron: (1) spiny pyramidal projection neurons and (2) sparsely spined, nonpyramidal local circuit neurons, most of which are γ-aminobutyric acid-(GABA)ergic *(57)*. It is the synaptic contacts of these GABAergic neurons that are likely to be the means by that benzodiazepine anxiolytics infused into the BLA achieve anxiolytic-like effects in rodent models of anxiety such as the Vogel punished licking test *(58)*. The GABAergic local circuit neurons differ from the pyramidal cells in their AMPAergic inputs. Whereas the GABAergic interneurons possess marked immunoreactivity to GluR1 subunits, the pyramidal cells exhibit only light GluR1 immunoreactivity *(59)*. Conversely, although GluR2/3 immunoreactivity has been reported in some interneurons, it is largely limited to pyramidal neurons *(59–61)*, and He and colleagues *(61)*, using a selective GluR2 antibody, conjecture that many

AMPA receptors on interneurons may not contain GluR2. This interpretation is consistent with electrophysiological evidence indicating that, whereas the AMPA component of the synaptic current at inputs to pyramidal cells is independent of calcium (the underlying receptors thus contain GluR2 subunits), in contrast, AMPA receptors on inhibitory interneurons show high permeability to calcium, indicating a low representation of GluR2 *(62)*. This complex arrangement makes it difficult to predict whether drugs acting at AMPA receptors are likely to give rise to anxiolytic or anxiogenic effects, because they will interact with both inhibitory and excitatory inputs to BLA pyramidal cells. However, animals with targeted deletions of GluR1 subunits should differ from mice with deletions of GluR2 or GluR3 subunits. Because GluR1 subunits represent by far the major component of AMPAergic receptors in the GABAergic interneurons, it is likely that targeted deletion of GluR1would result in a profound reduction in their excitability, with a consequent disruption of firing patterns of BLA pyramidal output neurons to which they normally provide an inhibitory control. Inasmuch as BLA neurons are involved in anxiety, one might then expect that GluR1 knockout mice would show increased anxiety as a consequence of reduced activation of GABAergic interneurons, whose outputs are presumably the site of anxiolytic action of benzodiazepines administered into the BLA. We have observed an increased tendency to thigmotaxis in an open field, and reduced open-arm exploration in the plus maze in GluR1 knockouts, as well as increased fear conditioning in a conditioned emotional response measure (Ripley, Mead, and Stephens, unpublished observations).

In the absence of GluR2 subunits in most receptors, the high calcium permeability of AMPA receptors in synaptic contacts onto BLA interneurons may make such synapses especially sensitive to plastic modification. Tetanic stimulation of inputs to BLA inhibitory neurons results in increased synaptic efficacy, which is independent of NMDA receptor activation, and is reflected in an increase in GABAergic inhibitory currents in pyramidal neurons *(62)*. Deletion of the gene-encoding GluR1 subunits can thus be expected not only to reduce the extent to which the inhibitory interneurons modulate pyramidal cell activity, but also to remove the substrate whereby plastic changes in the inhibitory control of pyramidal cell excitatory outputs (including those to accumbens; refs. *63–65*) occur during learning. In principle, this action may account for the loss of the ability of mice in which GluR1 subunits have been deleted to attribute affective properties to environmental cues associated with positive reinforcement *(66,67)*.

An alternative account of these findings might thus be that deletion of GluR1 leads to an impairment of the glutamatergic input from BLA to the ventral striatum *(64,65)* or orbitofronal cortex *(68,69)*, because the medium spiny neuron targets of this amygdala-accumbens pathway also express GluR1 subunit-containing AMPA receptors *(70)*.

The foregoing paragraphs illustrate the complexity of transmission within the amygdala glutamatergic circuits and much remains to be discovered before potential therapeutic agents based on interactions with glutamate systems can be rationally designed.

2.1.8. Dopamine–Glutamate Interactions in Amygdala

The schema outlined above suggests that the amygdala may influence behaviors related to anxiety by two rather separate mechanisms. First, outputs from the central nucleus to assorted brain areas may be responsible for both behavioral responses, such as flight or fight, mediated through hypothalamus and central gray of the midbrain, and

endocrine (via paraventricular nucleus) and vegetative consequences of fear-provoking events. Second, motivational consequences of fear-related stimuli may be organized through outputs to the ventral striatum and orbitofrontal cortex. This latter system offers a substrate for interactions between glutamate and dopamine systems paralleling those involved in appetitive motivation.

A third possible interaction is suggested by the observation that dopamine neurons arising from substantia nigra and ventral tegmental areas of the midbrain provide a rich innervation of the amygdala, and such projections are activated during presentations of conditioned fear stimuli. Blockade of these pathways by administration of either a D1 antagonist (SCH23390) into basal or lateral areas of the amygdala, or a D2 (quinpirole) antagonist into the ventral tegmental area (VTA; both of which treatments result in decreased D1 receptor activation at the amygdala target neurons) decreases freezing to a cue paired with a fear stimulus *(71,72)*. Similarly, either SCH23390 or the D2 antagonist, raclopride, administered into amygdala blocks the acquisition of fear-potentiated startle *(73,74)*, and a D2 antagonist, eticlopride, administered into amygdala attenuates conditioned freezing to a tone presented 24 h later, implicating D2 receptors in acquisition of fear conditioning *(75)*. In these experiments, injections were directed at lateral and basolateral aspects of the amygdala, and although there may have been some spread of the drug to neighboring areas, it seems likely that most of these effects are indeed attributable to these nuclei. A possible explanation of these observations holds that synaptic plasticity in the BLA requires not only coincidence of a sensory-related synaptic input (perhaps the CS) and one that causes a postsynaptic depolarization (perhaps the US), but also dopamine release *(76)*. Dopamine is known to enhance signal-to-noise ratio of strong inputs into postsynaptic elements bearing dopamine receptors, so that it can be hypothesized to enhance neuronal excitability, maximizing the association of the CS and US, while suppressing less significant inputs not related to the task. In particular, DA receptor activation in BLA potentiates the electrophysiological response evoked by electrical stimulation of sensory association cortex, while attenuating spikes elicited by stimulating prefrontal and mediodorsal thalamic inputs to the BLA *(77)*. Dopaminergic systems might thus play a facilitatory role in acquisition of conditioned fear *(78)*.

A further source of interaction between BLA dopamine and glutamate systems derived from the BLA's outputs to prefrontal cortex and nucleus accumbens. Accumbens medium spiny GABA neurons receive glutamatergic inputs from cortico-limbic areas, including prefrontal cortex, hippocampus, and amygdala, and dopamine systems may be important in biasing the selection of particular inputs to influence behavioral output through activation of the medium spiny neurons *(79,80)*. Glutamatergic afferents from the BLA form synapses in close proximity to dopamine terminals, and afferent activity from BLA increases dopamine efflux, which may then act to facilitate processing of further glutamatergic input from BLA *(79)*. The BLA may also affect dopamine release in the accumbens indirectly; BLA glutamatergic projections to medial prefrontal cortex activate feedback mechanisms to the VTA, which regulates firing of dopamine neurons *(81)*.

Dopamine is released in accumbens shell following exposure to both unconditioned and conditioned aversive and stressful events *(82,83)*, though the increased dopamine release may depend on fear conditioning *(83,84),* even in the case of apparently unconditioned experimental situations *(85)*. Consistent with a role of dopamine in fear conditioning, dopamine depletion in the accumbens disrupts aversive conditioning *(86)*.

Dopaminergic–glutamatergic interactions in BLA and accumbens are thus likely to play complex roles in processing of stimuli signaling aversive, as well as rewarding events. Consistent with this account, antipsychotic drugs, including clozapine, haloperidol, and raclopride *(87)* and dopamine D1 antagonists *(87)* given systemically block the acquisition (though not the expression) of conditioned fear in rodents.

Despite such evidence from animal studies, antipsychotic drugs are not recognized by prescribing agencies for the treatment of anxiety disorders, though they have a tradition of use in the control of anxiety associated with psychoses, and in the elderly, and are sometimes used by general practitioners for other forms of anxiety.

2.2. *Output Systems: Fight-and-Flight Systems in the Periaqueductal Gray*

As already outlined, amygdala outputs to the central gray may be important in mediating behavioral responses to cues conditioned to aversive events. The main excitatory input into the central gray is glutamatergic and NMDA receptors are widely distributed within the structure *(88,89)*. Injections of NMDA antagonists into the periaqueductal gray give rise to anxiolytic-like effects in the elevated plus-maze *(90–93)*. Similarly, injection of the glycine antagonist 7-chlorokynurenic into the dorsal periaqueductal gray blocked the anxiogenic effects of penetylenetetrazol in the elevated plus-maze *(94)*. More recently, anxiolytic-like effects of AP7 following injection into the dorsolateral or ventrolateral columns of the central gray in the Vogel punished licking test have been described *(93)*. Although these observations are in a general sense consistent with a role of glutamatergic systems within the periaqueductal gray in anxiety, it is unfortunate that further observations are not available in tests with more face validity as models of flight or of panic.

2.3. *The Septo-Hippocampal Hypothesis of Gray*

Gray and McNaughton *(10)* dispute that anxiety may be equated with conditioned fear, partly on the grounds that conventional anxiolytic drugs are ineffective against fear in animal models in which flight is the predominant response to the threat, whereas they are active in models in which the threat can be avoided passively. Although *panic* attacks may resemble flight behavior (and thus depend on neural circuitry engaged in flight reactions), other anxiety disorders do not engage these systems (located in a hierarchical defence system involving periaqueductal gray, medial hypothalamus, amygdala, and cingulate cortex *[10]*).

Central to Gray's account of the neural mechanisms serving *anxiety* is the concept of a "behavioral inhibition system." This system analyzes environmental events that are innately fearful or novel (and thus potentially dangerous), or that have been learned to predict punishment or nonreward. In response to such events, the system induces increases in arousal and attention, and inhibits ongoing behavior, the cardinal features of anxiety states. The key anatomical element of the behavioral inhibition system is the septo-hippocampal system. Anxiolytic drugs affect the function of the septo-hippocampal system by reducing activity in noradrenergic and serotonergic inputs to the system. Since the monoamine neurons are activated by inputs from largely glutamatergic afferents *(95)*, these synapses are potential targets for glutamatergic antagonists to reduce activity in these systems. Additionally, however, signaling within the hippocampal system is also dependent upon glutamate, and antagonists acting at intrahippocampal circuits can also be expected to degrade hippocampal information processing.

In keeping with these ideas, intrahippocampal injection of the competitive antagonist AP7 increased open-arm exploration in the plus-maze in rats previously exposed to restraint stress *(96)*. It should be noted, however, that similar anxiolytic effects were not seen in unstressed animals.

Despite the clear implications of these notions for a potential anxiolytic effect of glutamate receptor antagonists infused locally into the relevant brain areas, no work appears to have been carried out attempting to induce anxiolytic effects through modulation of activity in raphe or coeruleus neurons by administering glutamate receptor antagonists into these areas.

3. BEHAVIORAL PHARMACOLOGY OF GLUTAMATE

3.1. NMDA Receptor Modulation as Potential Treatment of Anxiety

A potential effectiveness of NMDA antagonists as anxiolytic agents was suggested independently by Stephens *(97)*, and by Bennett *(98)* from their effects in animal models. Since these early findings, evidence has accumulated that agents acting at several sites on the NMDA receptor complex are effective in animal models of anxiety. Thus, competitive NMDA antagonists, high-affinity open-channel blockers, glycine site antagonists, and polyamine site antagonists have all been reported to exhibit anxiolytic activity in both punishment and nonpunishment models of anxiety in rodents. The most consistent effects have been observed with competitive NMDA antagonists, though until recently, glycine and polyamine site antagonists had received little research attention. Although the majority of these studies were performed in rodents, a few experiments have examined the anxiolytic effects of NMDA modulation in primates (e.g., *see* ref. *99*). This earlier work has been extensively reviewed and will not be dealt with here. It is important to note, however, that whereas at least competitive antagonists appear to exert consistent effects in standard animal tests predictive of anxiolytic activity, all the antagonists are also active in tests predictive of side effects such as sedation, muscle relaxation, and cognitive dysfunction leading to memory impairments. For this reason, emphasis in the majority of recent studies has been on tests of glycine site antagonists, which have been suggested to have fewer problematic side effects than high-affinity open-channel blockers or competitive antagonists.

Nevertheless, results with glycine site ligands have been mixed, regarding both this anxiolytic activity and lack of side effects. For example, 1-aminocarboxycyclopropane (ACPC), a partial agonist at strychnine-insensitive glycine sites, was inactive in the elevated plus-maze model in rats *(100)*, though it did exhibit anxiolytic activity in the Vogel conflict model in rats *(101)*. In contrast, positive findings were obtained for the racemate and for the active isomer of HA-966 [(±)HA-966 and (+)HA-966, respectively], each of which produced modest anxiolytic effects in the elevated plus-maze *(100,102)*. When tested at sufficiently high doses, D-cycloserine also gave rise to anxiolytic-like effects in elevated plus-maze and conflict models in rats *(100,103)*. The anticonflict effect of D-cycloserine was blocked by coadministration of NMDA, but not glycine, suggesting that the effect may not have been mediated through glycine receptor sites *(103)*. At lower doses, D-cycloserine was not active in the elevated plus-maze, but it did block the anxiolytic activity of ethanol in this procedure *(102)*. In contrast to the positive findings with D-cycloserine, negative findings were reported for several glycine site antagonists, including ACEA 1011, ACEA 1021, MRZ 2/570, MRZ 2/571, and MRZ 2/576, and the glycine prodrug milacemide when tested in conflict models in rats *(100,104)*. The MRZ-type glycine-B full antagonists were also not active in the elevated plus-maze in rats *(100)*.

Another compound, MDL 105,519, has been reported to produce decreases in separation-induced vocalizations in rat pups *(105)*, suggesting anxiolytic potential. These effects, however, were accompanied by muscle relaxant activity, suggesting that the compound was not anxioselective. Another compound, L-701,324, produced dose-dependent anxiolytic effects in the elevated plus-maze in rats and mice without changes in overall activity *(100,106,107)*, but the magnitude of the effect was slightly less than that of diazepam *(108)*. In mice, the anxiolytic effect of L-701,324 in the elevated plus-maze was reversed by administration of glycine *(107)*, consistent with its proposed glycine site of action. In rats tested in the Vogel conflict model, the effects of L-701,324 were less positive: in one study, it produced a modest anticonflict effect *(108)*; in another study, it did not produce an anxiolytic effect *(100)*.

Further, there is no relationship between intrinsic activity at strychnine-insensitive glycine receptors (as measured by a patch-clamp technique) and efficacy in an anxiolytic procedure *(100)*. Although such attempts at correlation of potencies ignore the contribution that pharmacokinetic factors may make to the in vivo efficacy of drugs, they may suggest that the anxiolytic effects of these drugs may not be mediated through interaction with the population of glycine-B receptors measured in this study.

As with other subclasses of NMDA antagonists, the inconsistent nature of the anxiolytic effects of glycine site-selective modulators across procedures and labs contrasts sharply with the robust and reliable effects of benzodiazepines. At least two explanations of this contrast are possible: (1) these models were developed to detect benzodiazepine effects and may not be as sensitive for detection of anxiolytic effects of nonbenzodiazepines or (2) the anxiolytic effects of NMDA antagonists may not be as robust as those of the benzodiazepines.

3.1.1. Where in the Brain Do NMDA Antagonists Exert Their "Anxiolytic" Effects?

A number of recent studies have used central, site-directed injection of NMDA antagonists in an effort to determine the brain area(s) in which the anxiolytic effects of these drugs are mediated. Brain areas that have received attention in recent research are the hippocampus, the amygdala, periaqueductal gray, and the ventral tegmental area. The anxiolytic effects of the glycine site partial agonist ACPC produced anticonflict effects when injected ip and intrahippocampally whereas the competitive NMDA antagonist CGP 37,849 was active in the conflict test only when injected ip *(101)*. Curiously, the anxiolytic effects of both of these compounds was blocked by pretreatment with the benzodiazepine antagonist, flumazenil. Why blockade of the benzodiazepine-binding site of $GABA_A$ receptors should influence the action of NMDA antagonists is unclear, but there may be an interaction of glutamate and GABA systems in mediation of the anxiolytic effects of these NMDA antagonists *(109)*. Similarly, intrahippocampal injection of the competitive antagonist AP7 showed no anxiolytic effect in the elevated plus-maze in nonstressed rats; however, in stressed rats, intrahippocampal injection of AP7 was anxiolytic *(110)*. These results suggest that site selectivity within the NMDA receptor complex, as well as stress, affect neural mediation of the anxiolytic effects of NMDA antagonists in the hippocampus. The periaqueductal gray also appears to be important in mediation of the anxiolytic effects of some NMDA antagonists. In previous studies, Guimarães and colleagues *(90,91)* showed that injections of NMDA antagonists into the periaqueductal gray produced anxiolytic effects in the elevated plus-maze. In their more recent study,

they report that injection of a nonselective glutamate antagonist, glutamic acid diethylester, that blocks both NMDA and AMPA/kainate receptors, also has anxiolytic effects in this model *(91)*. Similarly, injection of the glycine antagonist 7-chlorokynurenic into the dorsal periaqueductal gray blocked the anxiogenic effects of penetylenetetrazol in the elevated plus-maze *(94)*. Another glycine-site antagonist/partial agonist, R(+)HA-966, blocked the acquisition and expression of conditioned fear-induced immobility when injected into the ventral tegmental area, but not when injected into the mesoprefrontal area *(111)*. In addition, the extinction of conditioned fear was blocked by an intra-amygdala injection of the competitive NMDA antagonist, AP5 *(112)* whereas intra-amygdala injection of MK-801 did not block acquisition of an anxiogenic effect caused by exposure to a stressor *(46)*. In summary, then, the anxiolytic effects of NMDA antagonists may be mediated in different brain areas depending on the site within the receptor complex at which the specific compound acts. Further, the results of brain site injection studies suggest the possibility of differential distribution of heterogeneous NMDA receptor subunits comprising the binding sites.

As suggested above, stress may modulate the anxiolytic effects of NMDA antagonists. A related and developing area of interest is the evaluation of anxiolytic effects of NMDA antagonists in compromised animals. In a study examining the anticonvulsant effects of NMDA antagonists, Löscher and his colleagues have shown that the effects of competitive and phencyclidine (PCP)-like antagonists on motor behavior are similar in amygdala-kindled rats whereas the effects of these compounds differ in uncompromised rats *(113)*. These results suggest that there may be some fundamental differences in the brains of epileptic rats that change their response to NMDA antagonists. Since anxiety disorders may also involve temporary or permanent changes in brain function *(114)*, it is possible that the effects of NMDA-based anxiolytic agents may also differ in anxious vs nonanxious rats. Several recent studies have investigated this possibility by examining the anxiolytic effects of NMDA antagonists in animals that had been exposed to a stressor or that were undergoing ethanol withdrawal. Adamec and colleagues have developed a preclinical model that they suggest to have features of PTSD, in which long-lasting anxiogenic-like effects in an elevated plus-maze are engendered in rodents following a single exposure to a cat *(115)*. More recently, they have shown that MK-801 and the competitive NMDA antagonists, AP7 and CPP, block the acquisition of this anxiety-like response to a stressor, but have no effect on expression of the response if administered soon after predator exposure *(116)*. When administered a short time before testing in the elevated plus-maze, however, MK-801 (but not the competitive NMDA antagonists) still maintained an anxiolytic effect in these stressed rats. Similarly, intrahippocampal injection of AP7 produced anxiolytic effects in the elevated plus-maze in rats exposed to restraint stress, but not in nonstressed rats *(96)*. Anxiolytic effects in the elevated plus-maze were also observed following systemic injection of AP7 or CGP 37,849 (another competitive NMDA antagonist) in rats stressed by withdrawal from ethanol following induction of dependence *(117)*. Interestingly, MK-801 was only marginally effective and HA-966 was ineffective in attenuation of the anxiogenic effects of ethanol withdrawal, suggesting that the source or cause of "anxiety" is important in determination of anxiolytic efficacy of site-selective NMDA antagonists. Further, the results of the few studies in this area suggest that NMDA antagonists may be differentially effective in the treatment of different types of anxiety disorders or conditions (e.g., generalized anxiety vs PTSD vs ethanol withdrawal).

A final study that should be mentioned used a traditional method of evaluating anxiolysis (i.e., elevated plus-maze), but effected NMDA receptor modulation via a novel method *(118)*. In this study, phosphodiester antisense oligodeoxynucleotide administration was used to reduce synthesis of the NMDA-NR1 subunit. Mice treated with antisense spent more time in the open arms of an elevated plus-maze whereas mice treated with vehicle or with the corresponding sense nucleotide did not show this anxiolytic effect. These results suggest that the NMDA-R1 subunit may be important in mediation of the anxiolytic effects of NMDA antagonists, though changes in trafficking of other subunits following disruption of NR1 should also be considered.

3.2. *Non-NMDA Receptor Modulation as Potential Treatment of Anxiety*

Evidence for the usefulness of non-NMDA receptor antagonists for the treatment of anxiety disorders is considerably weaker than that for NMDA receptor antagonists. To a great extent this reflects the poor availability of drugs that have selective actions at AMPA and kainate receptors and that show good brain penetration and useful pharmacokinetic properties in rodents. Additionally, AMPA receptors are so universally involved in fast transmission throughout the CNS that only a narrow window is available at which selective anxiolytic effects of antagonists might be observed without concurrent disruption of behavior through their sedative and muscle relaxant actions. Nevertheless, positive effects of AMPA antagonists have been described in animal models predictive of anxiolytic action in the clinic.

NBQX is a quinoxalinedione derivative that has little affinity for NMDA receptor sites, but that acts as a mixed AMPA/kainate receptor antagonist. In the four-plate test in mice, NBQX enhanced punished activity at a dose of 0.033 mmol/kg, but higher doses could not be effectively tested since they depressed locomotor activity *(119)*. An agonist at kainate receptors containing the GluR5 subunit, ATPA, had clear anxiogenic-like effects in this test, decreasing punished locomotor activity at a dose (0.002 mmol/kg) that had no effect on spontaneous locomotor activity in unpunished mice. These observations suggest that kainate receptors may be involved in signaling information regarding punishment, consistent with the role for amygdala kainate receptors in anxiety postulated by Li et al. *(41)*. Alternatively, NBQX may have exerted its effect through AMPA receptors. A similar problem of interpretation of the relative roles of AMPA and kainate receptors in mediating anxiolytic effects is provided by LY326325. This mixed AMPA/kainate antagonist induced a dose-dependent increase in a punished drinking test, without concomitant effects on unpunished drinking *(106)*. These effects occurred over a dose range (2.5–5mg/kg, ip) that did not influence locomotor activity. In the plus-maze assay, however, LY326325 (0.5–5 mg/kg) did not alter the percentage of entries into the open arms, though one dose (1 mg/kg) gave rise to a small, though significant increase in the time spent on the open arm. These observations stand in contrast to a previous report from the same group *(120)* in which LY326325 induced a dose-dependent *decrease* in time spent in the open arms, as well as the percentage entries into the open arms. In this study NBQX also caused a dose-dependent reduction in the time spent in the open arms. The authors conclude that AMPA receptor antagonists may give rise to anxiogenic-like behavior in the plus-maze, but the lack of consistency across test situations and the susceptibility of the plus-maze as a model of anxiety to interference from locomotor effects of drugs *(121)* cast doubt on this interpretation. NBQX has also been reported to possess

only limited ability to antagonize the discriminative stimulus provided by the $GABA_A$ channel blocker, pentylenetetazole *(122)*, which has been argued to be based on the anxiogenic properties of pentylenetetrazole *(123)*.

In an extensive study of three quinoxalinedione competitive antagonists of AMPA/kainate receptors (CNQX, DNQX, and NBQX) and a noncompetitive AMPA receptor antagonist (GYKI 52466) in the Vogel test of punished drinking, none of these drugs, tested up to dose ranges that reduced exploratory activity in the rat, were found to increase punished drinking, allowing the authors to conclude that AMPA/kainate receptors probably are not directly involved in the control of rat emotional behavior *(124)*. However, administration of the agonist, S-AMPA, intracerebroventricularly at a dose of 2 μg/5 μL, significantly enhanced the ability of electric shock to suppress drinking in thirsty rats. Interpretation of this observation in terms of an anxiogenic effect of the agonist is complicated, however, by observations that the same dose decreased activity, and even gave rise to "prodromal" symptoms of epileptic activity in some animals. Lastly, given the theoretical importance of behavioral inhibition in the action of anxiolytic drugs *(10)*, it is of interest that NBQX at a very low dose (10–1000 ng/rat) increased premature responding in a two-lever choice reaction time task, without altering response speed or accuracy *(125)*. Nevertheless, in another model of behavioral inhibition, differential reinforcement of low response rates, Stephens and Cole *(126)* found no effects of NBQX.

The ability of AMPA/kainate antagonists to exert anxiolytic-like effects in animal models is thus unreliable. This is surprising given the inevitable importance of these receptors in mediating neurotransmission in CNS circuits involved in processing emotional information, and the quite specific role for glutamatergic fast transmission envisaged in neuronal circuitry accounts of conditioned fear and anxiety outlined in Subheading 2. It seems likely that failure to find anxiolytic-like actions may be accounted for by the nonselective behavioral effects of these drugs, so that behavioral disruption masks their anxiolytic-like effects in many behavioral assays. A possible way of avoiding such nonspecific effects is to administer the drug centrally into areas of the brain accredited with a specific role in anxiety. Few attempts have been made at this kind of experiment, possibly because of the low solubility of the quinoxalinedione compounds at physiological pH values. However, bilateral infusions of CNQX (0.5 μg) into amygdala-impaired performance of a previously acquired passive-avoidance task, as well as decreasing reactivity to footshock, blocking footshock-induced decreases in locomotor activity, and increasing open-arm activity in the plus-maze, to a similar extent to midazolam *(127)*. These observations are consistent with an anxiolytic action of CNQX, though it should be noted that this drug possesses significant affinity for the glycine-B site of NMDA receptors, at which it acts as an antagonist *(128)*. Because specific glycine-B receptor antagonists also possess anxiolytic-like properties *(106)*; *see* previous discussion), and other behavioral effects of CNQX are attributable to an action at this site *(129)*, it is possible that the anxiolytic effects *(127)* are also mediated by CNQX's action at NMDA receptors.

4. METABOTROPIC GLUTAMATE RECEPTORS

In addition to its effects at ionotropic receptors, glutamate acts at a family of G protein-coupled metabotropic receptors *(130)*, classified into three subgroups (*see also* Part II). To date eight metabotropic receptors and multiple splice variants have been cloned group I receptors (mGluR1 and mGluR5) increase phospholipase C activity and phosphoinositol

hydrolysis, are located postsynaptically, and modulate ion channel activity. In contrast, group II receptors (mGluR2 and mGluR3) and group III receptors (mGluR4, 6, 7 and 8) inhibit adenylyl cyclase activity and, with the exception of GluR6, are located presynaptically where they regulate release of glutamate and other transmitters (refs. *131*, and *132)*; but *see* ref. *133* for discussion of postsynaptic distribution of group II receptors). In principle, such receptors may act to facilitate GABAergic or inhibit glutamatergic mechanisms, and might for that reason be expected to possess anxiolytic properties. There is increasing evidence that compounds acting at metabotropic receptors possess anxiolytic-like properties in animal models.

4.1. Group I Metabotropic Receptors (mGluR1 and mGluR5)

Systemic administration of the mGluR5 antagonist, MPEP, gives rise to anxiolytic-like effects in a number of spontaneous models including social interaction, elevated plus-maze, shock-probe, and marble-burying tests, and conditioned models such as the Geller–Seifert conflict test, Vogel punished drinking procedure, and four-plate test *(134–137)*. However, in a parametric comparison with the standard benzodiazepine anxiolytic, diazepam, MPEP was not as effective in increasing punished responding in a modified Geller–Seifter conflict test *(137)*. These anxiolytic-like effects of MPEP may be mediated by mGluR5 receptors in hippocampus since administration of (S)-4-carboxy-3-hydroxyphenylglycine (S-4C3HPG), a mixed group I antagonist and group II agonist, *(138)*, or of the more selective group I competitive antagonist (S)-4CPG and noncompetitive antagonist, CPCCOEt *(139),* into this region, gives rise to anxiolytic-like effects. group I antagonists blocked memory consolidation of contextual conditioning *(140)*, which is hippocampus-dependent, and fear conditioning leads to a transient upregulation of mGluR5 receptors in hippocampus *(141)*.

However, there is also accumulating evidence that mGluR5 receptors in the amygdala may play a role in fear conditioning *(142)* because MPEP blocked the expression of fear-potentiated startle when a discrete light cue, previously paired with shock, was used as the fear stimulus. Such potentiation by discrete cues is thought to be processed by amygdala mechanisms *(6)*. Bilateral infusion of MPEP into the lateral amygdala prevented the acquisition of conditioned fear assessed as fear-potentiated startle, but had no effect when administered immediately after training (to assess consolidation), or immediately before the test (to assess effects on expression of conditioned fear) *(143)*. These behavioral effects were paralleled in studies of LTP, in which MPEP blocked induction, but had no effects when administered following induction of LTP. Interestingly, it has been known for some time that administration of a mGluR agonist, trans-1-amino-cyclopentane-1,3-dicarboxylate, into amygdala facilitates potentiates auditory startle *(144)*.

Thus, mGluR5 receptors in the lateral amygdala appear to play a role in the early stages of synaptic plasticity underlying fear conditioning, but apparently do not contribute to expression of that conditioned fear. Clearly, these processes cannot be involved in putative anxiolytic effects of mGluR5 antagonists.

Perhaps these effects of group I antagonist reflect their ability to prevent glutamate-induced excitation through group I receptors *(131)*.

4.2. Group II/III Metabotropic Receptors

Administration of LY354740, an agonist of mGlurRII receptors, has been reported to possess anxiolytic-like effects in a range of standard tests using both spontaneous and

conditioned behaviors *(145,146)*. These effects may represent an action of LY354740 at hippocampal receptors, because administration of both this compound and another group II agonist, L-CCG-I, into the CA1 region of dorsal hippocampus of rats, increased punished licking in the Vogel test *(139)*. Nevertheless, Moore and colleagues *(147)* found no ability of LY354740 to increase punished responding in a conflict test, at doses that reduced responding during the nonpunished component; the same doses increased responding during a time-out component, reduced the number of reinforcers obtained on a DRL schedule, and shifted responding on an FI60-sec schedule toward the early part of the interval. These results are more consistent with effects of LY354740 on rates of responding, enhancing low rates while decreasing high rates, than with specific effects on punished behavior.

Group II receptors in the amygdala have also been implicated in fear responses and fear conditioning. LY354740 infused into the BLA disrupted the ability of a tone, previously conditioned to footshock, to potentiate a startle response *(148)*, an effect that could be antagonized with the group II antagonist, LY341495.

The ability of agonists at group II receptors to induce anxiolytic-like effects may reflect their ability to reduce glutamate release in several brain areas via activation of presynaptic receptors *(149)*, though they also act to hyperpolarize basolateral amygdala neurons *(150)*, and play a role in long-term depression of synaptic transmission in amygdala circuits *(151–153)*.

5. CLINICAL EVIDENCE

The slow progress in the clinical development of glutamate antagonists means that there is little evidence available from patients that can be used to test the predictions that antagonists should have clinically effective anxiolytic properties. Nevertheless, there are limited relevant data available from the use of ketamine during anaesthesia for surgery or for epidural catheter placements, where anxiety may be significant. Intravenous administration of 5 mg of ketamine given 5–10 min before epidural catheter placement significantly decreased anxiety as assessed using a visual analogue scale *(154)*, or when given orally to children, at a dose of 12.5 mg/kg prior to oral surgery *(155)*. A recent pilot study with PTSD patients also suggests that the glycine site partial agonist agonist, D-cycloserine, may improve anxiety, avoidance behavior, and numbing *(156)*.

6. CONCLUSIONS

Glutamatergic systems play essential roles in the signaling of emotions and in learning about environmental cues informing about threatening situations. In keeping with this functional role for glutamate, animal experimental evidence suggests a potential utility of both NMDA and non-NMDA receptor antagonists for the treatment of forms of anxiety. Despite the clear evidence from behavioral neuroscience of a potential utility of such compounds, little evidence of relevance is available from the clinic. It is less clear whether such treatments will have advantages over current therapies.

ACKNOWLEDGMENTS

Preparation of this chapter was supported by UK Medical Research Council grant G9806260 and Biotechnology and Biological Sciences Research Council grant S14554.

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VII Attention Deficit Hyperactivity Disorder

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Dopamine and Glutamate in Attention Deficit Hyperactivity Disorder

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1. INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a complex condition, thought to have multiple subtypes lurking within a broad, behaviorally defined phenotype, making it difficult to identify specific biological causes of this syndrome. However, the evidence from studies conducted over the past decade suggests that dopamine (DA) plays a prominent role in the etiology and treatment of ADHD. Here we will start with consensus views that have emerged about ADHD at the behavioral, biological, and genetic levels of analysis. Then, we will summarize the evidence that links DA to ADHD.

2. CONSENSUS VIEWS

2.1. Clinical Symptoms

ADHD is considered to be the most prevalent psychiatric condition of childhood. Variants of this condition have been described in the literature for more than 100 yr, dating back to Still *(1)* in 1902. The most accepted modern definitions are provided in the *Diagnostic and Statistical Manual,* 4th ed. (DSM-IV) *(2)* published in 1994 by the American Psychiatric Association and the International Classification of Diseases, 10th ed. (ICD-10) *(3)* manual published in 1993 by the World Health Organization. Both manuals include a list of 18 behavioral items (*see* Table 1) in three domains (nine Inattention, six Hyperactivity, and three Impulsivity items). The Hyperactivity and Impulsivity domains were merged into one in DSM-IV, and for the two domains of Inattention and Hyperactivity/Impulsivity, a separate cutoff criterion was specified as the presence of at least of six of nine items within a domain. At an anatomical level, the names of these domains implicate brain regions (e.g., basal ganglia, where DA receptors are dense) that are involved in the control of attention and activity.

The mere presence of the behaviors listed in Table 1 is not sufficient to be considered psychopathology *(2,3)*. The overall pattern must be developmentally inappropriate, manifested pervasively across settings (i.e., at home and at school) and time (i.e., have onset before the age of 7 and be chronic), and produce significant functional impairment. The

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Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

Table 1
Domains and Symptoms of ADHD

	Nine Hyperactivity/Impulsivity symptoms	
Nine Inattention symptoms	Six Hyperactivity symptoms	Three Impulsivity symptoms
Fails to give close attention to details	Fidgets with hands or feet or squirms	Blurts out answers
Difficulty sustaining attention	Can't remain seated when required	Difficulty waiting turn
Does not seem to listen	Runs about or climbs when inappropriate	Interrupts or intrudes
Does not follow through (fails to finish)	Difficulty playing quietly	
Difficulty organizing tasks	Always "on the go" or "driven by a motor"	
Avoids tasks requiring sustained effort	Talks excessively	
Loses things		
Distracted by extraneous stimuli		
Forgetful		

presence and impairment must not be because of other conditions, which often co-occur with ADHD. Epidemiological studies reviewed by Swanson et al. *(4)* suggest that in the school-age population the prevalence of a broad phenotype defined in DSM-IV is around 6–10% but is much lower (around 1–3%) for a narrow phenotype defined in ICD-10, which does not allow diagnosis in the presence of comorbid depression or anxiety and does not allow diagnosis of subtypes when just one domain is manifested.

2.2. *Cognitive Processes*

The identification of a specific cognitive deficit unique to ADHD has been elusive. Some investigators *(5)* have suggested this is owing to heterogeneity resulting from overlap with other conditions, such as other disruptive externalizing conditions (conduct disorder and oppositional defiant disorder in DSM-IV, which describe children who "won't" rather than "can't" pay attention to the rules of society) or internalizing conditions (anxiety and mood disorders in DSM-IV, which may have different manifestations in children than in adults and produce disorganized behavior). Others (e.g., *see* ref. *6*) have pointed out the terms "inhibition" and "impulsivity" were included in DSM-IV criteria for many other disorders in addition to ADHD (e.g., bipolar disorder, obsessive compulsive disorder, borderline personality disorder, antisocial personality disorder, and others), which may increase the diagnostic overlap owing to similarities in the defining clinical characteristics of multiple disorders. Other investigators have suggested that the core deficit of the clinical syndrome is not cognitive. For example, Barkley *(7)* proposed that a single core deficit (the lack of behavioral inhibition) produced the range and variety of deficits and concluded that there was ". . . no attention deficit in ADHD," and Sonuga-Barke et al. *(8)* proposed that some symptoms of ADHD reflect a motivational difference or "economic" decision to avoid (or minimize the impact of) long periods of delay, which are considered aversive to children with ADHD.

Regardless of the underlying processes, ADHD children clearly have abnormal performance on several standard neuropsychological tasks, especially those that measure frontal lobe or "executive" functions, as discussed by Pennington and Ozonoff *(9)* in a review. On some of these tasks, the primary measure of performance is based on accuracy, and the time to perform the task is secondary. Two examples are the Tower of Hanoi test, which requires planning and rearranging colored rings on a stick to match a pattern, and the Matching Familiar Figures test, which requires inspection of a sample of complex figures that are all similar and comparison to a standard to identify the one that is an exact match. Under the typical conditions of these tasks, children with ADHD tend to make decisions to halt planning or inspecting prematurely, and as a consequence have a performance deficit characterized by premature (fast) responses and lower-than-normal accuracy. This fast-inaccurate pattern of performance has been used as an operational definition of an "impulsive" style of responding (e.g., *see* refs. *10* and *11*). On other tasks, the primary measure of performance is based on the time taken to respond to each of a series of stimuli. Some examples are go-no go tasks, such as the Continuous Performance Task *(12,13)*, which requires detecting the occurrence of infrequent events; the Logan Stop Task *(14)*, which requires motor inhibition of an initiated response; and the Posner Visual–Spatial Cued Detection Task *(15)*, that requires a simple reaction to a cued or uncued stimulus. When these tasks are administered under standard conditions, children with ADHD typically have lower than normal accuracy, but they also have slower instead of faster than normal response times *(16)*. This slow-inaccurate pattern of performance does not meet the operational definition of "impulsivity," and instead suggests an inefficient style or an impaired ability to process information *(13,17)*.

To organize the many neuropsychological tests and in an effort to select the best type for identifying cognitive deficits of ADHD children, in prior discussions of this topic *(18–20)* we were guided by years of work by Posner and his colleagues, summarized in Posner and Raichle *(21),* who proposed three component processes of attention: alerting (suppression of background neural noise by inhibiting ongoing or irrelevant activity or mental effort to establish a state of vigilance), orienting (mobilization of specific neural resources by facilitating some specialized process and inhibiting others), and executive control (coordination of multiple specialized neural processes by detecting targets, starting and stopping mental operations, and ordering multiple responses to direct behavior toward a goal). Posner and Raichle *(21)* linked each of these component processes of attention to a primary cortical brain region and a primary neurotransmitter: for alerting, right frontal and norepinephrine (NE); for orienting, bilateral parietal and acetylcholine; for executive Control, anterior cingulate and DA.

2.3. *Brain Anatomy and Function*

Over the past decade, brain-imaging methods have been applied to investigate differences in brain anatomy and function in children with ADHD. Some of the early studies addressed function. Lou et al. *(22)* used single-photon emission computed tomography (SPECT) to measure blood flow in ADHD and non-ADHD children, and in the ADHD group documented reduced blood flow to frontal lobes and basal ganglia, but increased blood flow to occipital lobes. Zametkin et al. *(23)* used positron emission tomography (PET) to measure glucose metabolism in adults with ADHD, who compared to non-ADHD

adults had lower metabolism in frontal lobes when performing an auditory attention task. Vaidya et al. *(24)* and Rubia et al. *(25)* used functional magnetic resonance imaging (fMRI) to measure blood flow in ADHD children, who compared to non-ADHD children had lower blood flow to frontal lobes and basal ganglia. Thus, the findings of these functional imaging studies converged and suggest underactivity of specific brain regions where DA receptors are dense.

Many groups have used anatomical magnetic resonance imaging (MRI) to compare groups of children with ADHD to non-ADHD control groups. Neuroanatomical differences have been replicated in multiple studies (e.g., refs. *26–37*), and this literature has been discussed in detail by several investigators, most notably by Castellanos *(38)*, Swanson et al. *(4)*, Tannock *(39)*, and Castellanos and Tannock *(40)*, in addition to a review by Swanson and Castellanos *(41)* that was included as part of the ADHD Consensus Conference *(42)* held by the National Institute of Health (NIH) in 1999. Therefore, only a brief summary will be presented here (*see* Table 2). The multiple investigators targeted different brain regions, including those regions associated with executive functions (regions in the frontal cortex) and the control of attention and movement (regions in the basal ganglia and cerebellum). In all of these studies, the differences associated with ADHD were manifested as smaller-than-normal (about 10%) size. Statistically, the average mean difference in standard deviation units (effect size) was about 0.5 (*see* Table 2) for these candidate brain regions where DA receptors are dense.

Two recent studies have used innovative new methods for refining these estimates of neuroanatomical differences between groups of ADHD and non-ADHD children. Castellanos et al. *(43)* used automated techniques to evaluate whole-brain and gross-brain regions, as well as some specific regions targeted in prior studies, and used longitudinal, repeated measures designed to investigate the developmental course of brain anatomy in large samples of ADHD and non-ADHD children. The automated analyses and longitudinal follow-up suggest that the estimated overall brain size was approx 10% smaller for the ADHD group than for the non-ADHD group. In some brain regions the differences (i.e., smaller caudate regions in the ADHD group) were present during early childhood but were no longer evident in adolescence, and in other brain regions (i.e., the frontal lobes) differences previously reported were not detected in this study. Sowell et al. *(44)* evaluated the surface anatomy in a large sample of ADHD and non-ADHD children, and documented in the ADHD group smaller prefrontal brain regions. With these improved methods of analysis, compared to prior reports the differences were more precisely localized to more inferior aspects of prefrontal regions and were represented bilaterally instead of predominately in the right hemisphere.

2.4. Integration

In 1990 *(18)*, 1998 *(19)*, and 2002 *(20)*, our group used the Posner and Raichle *(21)* neuroanatomical network theory of attention as a framework to organize these replicated neural differences associated with ADHD. We initiated a levels-of-analysis *(45)* approach to relate neuroanatomical differences to clinical abnormalities (manifested as symptoms of ADHD) and cognitive deficits (manifested as inefficiencies in neural networks). As shown in Table 3, we separated the nine Inattention symptoms into three subgroups aligned with the cognitive components of attention (alerting for sustained

Table 2
Summary of Neuroanatomical Studies in the 1990s

Team and diagnosis	Sample size, age and clinical contrast group	Estimate of effect size[e] and % reduction[f] by brain region			
		Corpus callosum	Basal ganglia	Frontal lobes	Cerebellar vermis
U Georgia[a]	$n = 7–11$				
DSM–III	11.1 yr	0.51	0.88	0.69	—
ADHD, all but 2 HKD	learning disabilities	10.9%	19.0%	3.6%	—
Harvard U[b]	$n = 15$				
DSM–IIIR	12.4 yr	0.80	0.72	0.82	—
ADHD, all HKD	response to stimulants	12.2%	11.4%	12.7%	—
NIMH[c]	$n = 18–57$				
DSM–IIIR	12.0 yr	0.53	0.40	0.64	0.80
ADHD, all but 2 HKD	none	11.2%	5.4%	9.6%	11.1%
Johns Hopkins[d]	$n = 10–13$				
DSM–IIIR	11.3 yr	0.44	0.7	—	0.79
ADHD, all HKD	Tourette syndrome	5.7%	11.8%	—	12.3%

[a]ref. *34* (anterior width of frontal lobes on single slice; es = 0.69); ref. *35* (5 areas of cc; es = 0.51); ref. *33* (left caudate head; es = 0.88).

[b]ref. *37* (7 areas of cc; overall es = 0.80); ref. *30* (volumetric MRI; l caudate es = 0.81; r caudate es = 0.63; ant.sup. es = 0.74;.ant.-sup. wm es = 0.81; ant.-inf. es = 0.89).

[c]ref. *31* (area of cc; overall es = 0.44); ref. *26* (volume of basal ganglia; l caudate es = 0.56; r caudate es = 0.34; l globus pallidus es = 1.1; r globus pallidus es = 0.80); ref. 36 (area of cerebellar vermis lobules VIII-X es = 0.79).

[d]ref. *32* (7 areas of cc; rostrum es = 0.62; rostral body es = 0.81; total es = 0.15); ref. *28* (volume of basal ganglia; l caudate es = 0.29; r caudate es = 0.54); ref. *29* (volumetric MRI; total area cc es = 0.06; l caudate es = 0.20; r caudate es = 0.52; l globus pallidus es = 0.25; r globus pallidus es = 0.60; ant. frontal es = 0.64); ref. 27 (volume of cerebellar vermis lobules VIII-X es = 0.79).

[e]effect size = (ADHD mean – Control mean)/(standard deviation of control group).

[f]% reduction = (Control mean – ADHD mean)/Control mean.

DSM-IIIR, *Diagnostic and Statistical Manual*, 3rd ed.; ADHD, attention deficit hyperactivity disorder; HKD, hyper kinetic disorder.

attention, orienting for selective attention, and executive control for memory and planning); the three Impulsivity symptoms into a subgroup aligned with executive control for self-regulation; and the six Hyperactivity symptoms into two subgroups aligned with executing of fine motor control and gross motor control. In these prior summaries we used the concept of parallel segregated circuits proposed by Alexander et al. *(46)* and utilized by Castellanos *(38)*, Castellanos and Tannock *(40)*, and Swanson et al. *(4)*. These circuits form DA-sensitive loops that link midbrain, basal ganglia, thalamic, and cortical brain regions and thereby modulate attention (the mesolimbic DA pathway) and movement (the nigrostriatal DA pathway) *(47)*.

Table 3
Alignment of Symptoms Domains, Cognitive Processes, and Neural Networks

Symptom domain	Cognitive process	Neural network
Inattentive–Alerting	Sustained attention	Alerting
– difficulty sustaining attention	– vigilance level/decrement	– cortical: right frontal
– fails to finish	– persistence	– pons:locus coruleus
– avoids sustained effort	– performance	– thalamic
Inattentive–Orienting	Selective attention	Orienting
– distracted by stimuli	– visual cueing	– cortical: parietal
– does not seem to listen	– auditory cueing	– midbrain
– fails to give close attention	– visual search	– thalamic: pulvinary
Inattentive–Memory	Memory/Planning	Executive Control
– has difficulty organizing	– planning	– cortical: prefrontal
– loses things	– memory for objects	– striatal: basal ganglia
– is forgetful	– memory for time	– thalamic
Impulsivity	Cognitive regulation	Executive Control
– blurts out answers	– conflict resolution	– cortical: anterior cingulate
– interrupts or intrudes	– behavioral inhibition	– striatal: nucleus acumbens
– can't wait	– delay aversion	– thalamic
Hyperactivity–Fine motor	Motor/Vocal control	Fine Motor Control
– fidgets	– fine motor control	– cortical: left frontal
– can't play quietly	– non-verbal control	– striatal
– talks excessively	– verbal	– cerebellar: vermis
Hyperactivity–Gross motor	Activation level	Gross Motor Control
– leaves seat	– gross motor control	– cortical: right frontal
– runs about and climbs	– novelty seeking	– striatal: caudate
– always on the go	– arousal level	– cerebellar

3. PHARMACOLOGICAL TREATMENT

3.1. The Primary Pharmacological Treatment

The primary pharmacological treatment of ADHD is with stimulant drugs, amphetamine (AMP), and methylphenidate (MPH). At relatively high concentrations these drugs are known to be indirect DA receptor agonists because they elevate extracellular DA levels and thereby increase the probability of receptor activation and signal transmission. Both of these stimulant drugs interact with all three monoamine (DA, NE, and serotonin) transporters. However, whereas AMP like MPH blocks dopamine transporter (DAT) and the reuptake of DA, AMP also serves as a monoamine transporter substrate that effectively increases intracellular and nonexocytotic release of DA *(48)*.

The clinical effectiveness of stimulants in the treatment of ADHD has been documented by thousands of studies and hundreds of reviews, and an historical account was provided by Swanson et al. *(49,50)*, a "review of review" commissioned after a Congressional Notice of Inquiry *(51)* about ADHD when the Individuals with Disabilities Education Act (IDEA) was debated and passed by the US Congress in 1990. The IDEA provided for the first time official recognition of ADHD as an educational disability (under the IDEA category titled "Other Health Impaired"), and the regulations that

implemented the IDEA *(52)* provided the rules for obtaining special services in public schools specifically for ADHD.

The "review of reviews" *(49,50)* summarized the empirical bases for the use of stimulant drugs to treat children with ADHD. Relatively low oral doses (e.g., 5–20 mg) of the immediate-release (IR) formulations of MPH and AMP produce dramatic reductions in symptoms (decreased activity and increased attention), as well as some associated features of ADHD (aggression and defiance). These pharmacodynamic effects follow closely the pharmacokinetic properties of MPH and AMP *(50)*, which differ somewhat for the two drugs, with a longer time to reach maximum serum concentration (Tmax) and for serum concentrations to drop by 50% (T1/2) for AMP than MPH. But, in general both drugs have rapid onset, achieving maximum efficacy within 1–2 h after acute doses (near Tmax), and rapid offset, with efficacy dissipating about 2–4 h after Tmax (proportional to T1/2), when another dose is required to re-establish clinical efficacy. The optimal doses of MPH for the treatment of children with ADHD range from 5 to 20 mg administered two (BID) to three (TID) times a day. This sixfold difference (i.e., from 10 mg/d to 60 mg/d) makes titration essential to select the best dose for each child.

For children in school, BID or TID dosing requires an administration of a controlled (Schedule II) drug at school, which is inconvenient, costly, and stigmatizing. This created a need for effective sustained-release (SR) formulations of the stimulants that would maintain efficacy across the day with once-a-day administration at home. The first-generation SR formulations (i.e., Ritalin-SR and Dexedrine Spansules) were developed and approved in the 1980s and used a zero-order (flat) drug pattern as a target, but these SR formulations were never well-accepted in clinical practice owing to the perception of decreased efficacy compared to multiple daily doses of IR formulations. First-order (ascending) delivery patterns were proposed in the late 1990s based on the discovery by Swanson et al. *(53)* of acute tolerance to clinical doses of MPH, which later was also suggested for clinical doses of AMP by Greenhill et al. *(54)*. Based on this new knowledge, osmotic-release and polymer-coated beaded drug delivery systems were applied by pharmaceutical companies to develop second-generation of SR formulations of MPH (Concerta, Metadate CD, and Ritalin LA) and AMP (Adderall XR). These new formulations are characterized by smooth-rising (ascending) or double-pulse drug delivery patterns, and when administered once a day, Concerta *(55)*, Metadate CD *(56)*, Ritalin LA *(57)*, and Adderall XR *(58)* all proved to be as effective as multiple daily doses of the old IR formulations. After approval by the US Food and Drug Administration (FDA), the second-generation SR formulations were rapidly accepted by clinicians and now are prescribed in the majority of cases for the treatment of ADHD in the United States.

3.2. Multimodality Treatment and Long-Term Outcome

The NIH Consensus Conference on ADHD *(42)* reviewed evidence from multiple follow-up studies that clearly document poor prognosis, and concluded that ADHD is a serious condition that demands recognition and deserves treatment. The Consensus Conference *(42)* also reviewed evidence that documented the short-term efficacy of two modalities of treatment (pharmacological with stimulant medications and psychosocial with behavior modification), but also noted the lack of information on the effects of long-term treatment on long-term outcome.

In the early 1990s, the National Institute of Mental Health (NIMH) funded the Multimodality Treatment study of ADHD (MTA) *(59)* to address the efficacy of long-term intervention with established pharmacological (stimulant medication) and psychosocial (behavior modification) treatments. A large sample (n = 579) of 7- to 9-yr-old children with ADHD-Combined Type was recruited, and from 144 to 146 cases were randomized to each of four treatments defined by systematic, protocol-based interventions with medication management (MedMgt) alone, behavior modification (Beh) alone, or the combination (Comb), or to a community comparison (CC) condition with treatment sought and provided in a variety of community settings of the six-site study. In the initial 14-mo treatment phase of this randomized clinical trial, both modalities appeared to be effective *(59)*, but the pharmacological modality was more effective than the psychosocial modality (MedMgt > Beh) and the multimodality treatment was not significantly more effective than medication alone (Comb ~ MedMgt) for the reduction of ADHD symptoms. Secondary analyses *(60)* revealed a small multimodality superiority at the end of treatment, based on a higher rate of clinical success (defined by an "excellent response") in the Comb (68%) than the MedMgt (56%) conditions, which were both greater than the success rates for Beh (34%) and the CC (26%) conditions. In addition to clear efficacy of stimulant medication, some stimulant-related side effects on growth were noted *(61)*, resulting from suppression of height gain (about 1 cm/yr) and weight gain (about 2 kg/yr). The next phase of the MTA was a 10-mo follow-up, which revealed continued efficacy of the initial assignment to a treatment with the MTA medication algorithm *(62)* but with a reduction in magnitude of about 50% *(61)*, as well as continued height suppression at about the same magnitude during the initial follow-up (i.e., an additional reduction of about 1 cm/yr in expected gain in height). Subsequent follow-up of the MTA sample is now in progress.

4. INVOLVEMENT OF DOPAMINE

4.1. Early Biochemical Studies

Many theories about the pathophysiology of ADHD have been based on the notion of catecholamine dysfunction, but the emphasis on either deficits or excesses of DA and NE has shifted frequently. For example, Kornetsky *(63)* proposed the hypothesis of overactive DA and NE systems, whereas Wender *(64)* proposed the opposite. Coyle and Snyder *(65)* emphasized the involvement of NE over DA, but Wender *(64)* proposed the opposite. Solanto *(66)* proposed a DA excess hypothesis, whereas Levy *(67)* proposed the opposite. Pliskza et al. *(68)* proposed deficits in NE, but Swanson et al. *(18)* proposed the opposite.

Early studies attempted to test various catecholamine hypotheses by contrasting ADHD and control groups on measures of DA and NE (and their metabolites HVA and MHPG) in blood, urine, and cerebral spinal fluid (CSF). However, a clear picture did not emerge, perhaps because of uncertainty about the relationships of these peripheral measures of catecholamines and their metabolites to central (brain) levels. Mikkelsen et al. *(69)* found no difference in plasma NE between ADHD and control groups, but did report that the intrasubject variation in plasma NE was greater for the ADHD than the control group. Although most studies failed to document group differences on these measures *(70)*, Shekim et al. *(71)* did consistently report lower urinary NE metabolite (MHPG) levels in ADHD groups than in control groups, which paradoxically were

reduced further in ADHD children treated with stimulant medication. The initial studies of the DA metabolite HVA in CSF in ADHD and control groups of children did not consistently show between-group differences *(72–74)*, but later studies by Castellanos et al. *(75,76)* did report lower levels in ADHD than in control groups.

The widespread clinical success of stimulant medications as a specific treatment for ADHD suggests that this pharmacological treatment might correct an underlying deficit *(64)*. Many studies have been conducted to evaluate this hypothesis. An early and influential study that directed this work was by Coyle and Snyder *(65)*, who suggested D-AMP had effects 10 times greater than L-AMP on NE uptake in synaptosome preparations from rat cerebral cortex, whereas these isomers had equal effects on DA uptake in synaptosome preparations from the rat corpus striatum. Bradley's *(77)* initial clinical reports of the uses of stimulant medication in the 1930s documented the efficacy of racemic (D,L) AMP, but the later report *(78)* of use of the D-isomer in the 1940s showed that D-AMP had about the same clinical efficacy at almost half the dose of D,L-AMP. Because clinical response seemed to rely on the D-isomer, the conclusion was that the underlying deficit in ADHD children was primarily related to NE.

However, subsequent studies using synaptosome preparations *(79–81)* reported the opposite effect (i.e., a similar effect on NE but a greater effect of D-AMP than L-AMP on DA uptake), perhaps because Coyle and Snyder *(65)* used animals that previously had received large amounts of reserpine. And, preclinical studies of regional responses to the isomers in rats *(82)* revealed a greater increase in glucose metabolism in the caudate nucleus and globus pallidus after D-AMP than after L-AMP, which provided additional evidence that D-AMP preferentially affected DA neurocircuitry.

MPH was introduced in the 1960s and then rapidly became the stimulant drug of choice of the treatment of ADHD. Swanson et al. *(49,50)* provided a written historical account of when and why there was a shift from AMP to MPH. Over the next several decades, most research was on the available formulation of MPH for clinical use, the 50:50 racemic mixture of the D-threo-MPH and L-threo-MPH isomers (Ritalin®). Recently, a formulation of the pure D-threo-MPH isomer (Focalin®) was approved for clinical use *(83)*. When administered orally, apparently the L-threo-MPH isomer is rapidly metabolized in the stomach and intestine, so little enters the blood stream. Thus, the PK profiles of serum concentrations do not differ for matched doses of D-threo-MPH (i.e., Focalin at half the dose of Ritalin), nor does clinical efficacy *(83)*, except for a slight and unexplained longer duration of action for the pure formulation of D-threo-MPH.

4.2. PET Studies of Effects of MPH in the Human Brain

Studies of animals suggested the primary mechanism of action of MPH was to block the reuptake of DA, but direct evidence from studies of humans was not provided until the 1990s, when PET studies of adults were used to document its primary site of action. A series of PET studies conducted at Brookhaven National Laboratory documented the properties of MPH in the human brain. Ding et al. *(84)* used PET imaging to document the site of binding of the two isomers of MPH (D- and L- threo-MPH). They used intravenous (iv) administration of the formulation used clinically (the 50:50 racemic mixture), and showed nonspecific binding of the L-isomer in all brain regions but specific binding of D-isomer to the DAT in the basal ganglia. Volkow et al. *(85)* used PET to compare iv MPH to another stimulant drug (cocaine) that was a known potent blocker of DAT, and

demonstrated that the dose of MPH required to block 50% (0.075 mg/kg) was even lower than for cocaine (0.13 mg/kg). Intravenous doses of both MPH and cocaine produced maximum concentration in brain very rapidly (within 10 min) and serial PET scans revealed that the brain PK half-life of MPH was relatively long for MPH (90 min) compared to cocaine (10 min). The initial reinforcing effect (the perception of euphoria) occurred for both drugs, but acute tolerance to the reinforcing effects of iv MPH occurred so that although DAT blockade remained high over time, the perception of "high" dissipated rapidly.

Volkow et al. *(86)* evaluated the effects of oral MPH on DAT blockade and documented a dose-response effect *(*5 mg ~ 12%, 10 mg ~ 40%, 20 mg ~ 54%, 40 mg ~ 72% and 60 mg ~ 74% DAT blockade) and a high correlation (~0.8) of DAT blockade with serum concentration of MPH at Tmax. Even though clinically relevant oral doses of MPH (e.g., 20 and 40 mg per administration, or about 0.5 mg/kg in adult subjects) produced DAT blockade that exceeded the threshold expected to produce reinforcing effects (euphoria), acute tolerance emerged during the slow onset of oral MPH and apparently mitigated the reinforcing effects. Volkow et al. *(87)* measured changes in DA with PET using [^{11}C]raclopride, a DA D2 receptor radioligand that competes with endogenous DA for occupancy of the DA D2 receptors. This technique requires two PET scans—one after placebo and one after the drug tested (in this case MPH)—and the difference in [^{11}C]raclopride is used to assess relative changes in extracellular DA induced by the drug. Volkow et al. *(88,89)* showed that oral doses of MPH increased extracellular DA in brain, owing to accumulation of spontaneously released DA and MPH-induced DAT blockade. Recently, Neto et al. *(90)* used this PET technique and also showed increases in DA after oral does of MPH in adolescents with ADHD.

The program of research at Brookhaven National Laboratory (*see* refs. 84–89) provided evidence to account for the similarities and differences in a drug with widespread clinical use (MPH) and a drug with widespread abuse (cocaine), despite a very similar site of action. Volkow and Swanson *(91)* summarized this work and identified four factors that distinguish the clinical use and the illegal abuse of stimulants:

1. Dose, because there is a threshold for MPH-induced DA increases to be perceived as reinforcing and to produce therapeutic effects.
2. Pharmacokinetics, because the reinforcing effects of MPH are associated with rapid changes in serum concentrations and presumably fast DA increases (as achieved by iv injection or insufflation), whereas the therapeutic effects are associated with slowly ascending serum concentrations and presumably smoothly rising DA levels (as achieved orally).
3. Individual differences, because sensitivity to MPH varies across individuals and sets a threshold for blood and brain levels required for reinforcing ("high," drug-liking) and for therapeutic (symptom reduction) effects.
4. Context, because the effects of MPH are modulated by different settings in abuse (rituals of self-administration that may have powerful conditioning and expectation effects) and in clinical use (external demands set by others that require low activity and focused attention).

5. DA GENES AND ADHD

5.1. Candidate Dopamine Genes and ADHD

Family *(92)*, adoption *(93)*, and twin *(94)* studies all suggest that ADHD has a strong genetic component, but these statistical genetic studies cannot identify specific genes that might be involved. Molecular genetic studies have been conducted to evaluate specific

genes. The first molecular genetic studies were based on the DA theories of ADHD *(64,67)* and the DA sites of action of drugs used to treat ADHD *(84–89)*, which led to DA genes as candidate genes for ADHD. In the first two molecular genetic studies of DA genes in humans, the *DAT* gene *(95)* and the DA receptor type 4 (*DRD4*) gene *(96)* were investigated. Both of these genes have polymorphisms based on a variable number of tandem repeats (VNTRs). Strings of base pairs (bp) define a sequence of nucleotides (called a "motif") that is repeated (R) a different number of times in different individuals. For example, the *DAT* gene *(97)* has a 40-bp VNTR in the 3′ untranslated (noncoding) region, and in Caucasian populations the primary allelic variants are the 9R allele ($p \sim 0.25$) and 10R allele ($p \sim 0.75$). The *DRD4* gene *(96)* has a 48-bp VNTR in exon 3 (a coding resion), with the primarily variants defined by 2R, 4R, and 7R alleles. This polymorphism produces structural differences across individuals in the receptor's putative third intracellular loop, an important region of the receptor's protein that couples it to G protein effectors pre- and postsynaptically. In humans the allele frequencies of the *DRD4* gene vary across ethnic groups, but in Caucasians the expected allele frequencies are about 0.10 for the 2R, 0.67 for the 4R, 0.12 for the 7R, and 0.11 for other alleles.

Cook et al. *(97)* investigated parent-to-child transmission rates of the DAT alleles, and reported an increased prevalence *(85)* and transmission *(60)* of the most prevalent 10R-repeat allele in a sample of 119 ADHD children. LaHoste et al. *(98)* observed a higher than expected frequency of the 7R allele *(28)* in a clinical group of ADHD cases, and Swanson et al. *(99)* replicated this finding and extended it by showing linkage disequilibrium in proband–parent triads. Typically, initial positive findings of candidate gene studies are not replicated *(100)*. However, many of the subsequent studies of association of ADHD with the *DRD4* and *DAT* genes replicated the initial findings *(101,102)*. This consistency was described by Collier et al. *(103)* as ". . . a major achievement in psychiatric genetics: an association finding which has been observed in an overwhelming majority of attempts at replication."

5.2. Studies of Functional Differences Associated With DRD4 Genotype

The *DAT* 40-bp VNTR is not in a coding region of the gene so most speculations about its functional consequences have focused on it being in linkage disequilibrium with a nearby polymorphism that is in a coding region of some gene that becomes translated into structural differences in proteins that result in functional differences in brain processes and behaviors. The *DRD4* 48 bp VNTR falls within the protein-coding region of the gene so some studies have investigated functional differences in the resultant DRD4 protein. (Of course, even structural differences in the DRD4 receptor could be inconsequential, and this exon 3 polymorphism might merely be in linkage disequilibrium with polymorphisms in other coding regions that produce functional consequences that are important.)

One of the initial hypotheses about the DRD4 VNTR was that the 7R allele coded for a receptor that was subsensitive to endogenous DA and thus might produce functional differences. Swanson et al. *(104)* tested this hypothesis by evaluating ADHD subjects performing neuropsychological tasks designed to place demands on the alerting and executive control attentional networks proposed by Posner and Raichle *(21)*: the Stroop task, the Cued Detection task, and the Stop task. Two ADHD subgroups were formed based on the presence of at least one 7R allele (7-present) or none (7-absent), which were

compared to a control group of non-ADHD children. Both the 7-present subgroup and 7-absent subgroup differed dramatically from the control group on the behavioral Swanson, Nolan, and Pelham ratings of ADHD symptom severity, but on measures of cognitive performance, only the 7-absent subgroup showed a deficit characterized as a pattern of slow and variable responding. The 7-present subgroup did not differ from the control group in terms of speed or accuracy of performance on these neuropsychological tasks selected to impose demands on the executive control network *(21)*.

Recently, Langley et al. *(105)* reported the results of a study of the *DRD4* gene and ADHD that used one of the same neuropsychological tasks (the Stop task) and the same subgroups based on *DRD4* genotype (the 7-present and 7-absent subgroups) as used in the Swanson et al. *(104)* study. The basic findings were the same: the 7-present subgroup had faster response time than the 7-absent subgroup. In fact, the 7-present subgroup appeared to respond faster than a normal control group, and to make more errors, suggesting "impulsivity" according to the operational definition discussed earlier. Two other studies of the *DRD4* gene have reported similar patterns of differences in performance on neuropsychological tests as a function of *DRD4* genotype, although the tests used and the methods to define subgroups based on genotype were quite different from those used by Swanson et al. *(104)* and Langley et al. *(105)*. Manor et al. *(106)* used a continuous performance task to evaluate a sample of 178 ADHD children subgrouped by long and short *DRD4* alleles (long = 7R-present, and short = 7R-absent), and reported that the subgroup with the 7R-present genotype made fewer errors of commission and had less variable reaction time than the subgroup with the 7R-absent genotype. Fossella et al. *(107)* use the attentional network task to assess reaction time performance and the efficiency of the three attention networks (altering, orienting, and executive control). In a sample of 200 normal adults, they found that the efficiency of the executive control network was greater in the subgroup of those homozygous for the 7R allele or the 2R allele (i.e., the 4-absent subgroup) than the subgroup defined by the presence of a 4R allele.

5.3. Possible Environmental and Genetic Forms of ADHD

These selected studies of the *DRD4* gene and ADHD suggest that the 7R allele may be associated with some benefits, as well as some deficits in behavior. We have speculated *(104)* that the 7R allele may protect children with behavioral symptoms of ADHD from some of the typical cognitive deficits manifested in information-processing tasks. The pattern of performance deficits in the 7-absent genotype (slow and variable responding) is similar to the pattern manifested by patients with brain damage. This led us to speculate that the children with the 7-absent genotype may have an environmental form of ADHD associated with minimal brain damage (MBD) *(108)* that often occurs during fetal development *(109–111)*, rather than a genetic form of ADHD involving the *DRD4* gene. Thus, the full syndrome with behavioral and cognitive deficits may be a result of MBD, and the genetic form of ADHD associated with the 7R allele of the *DRD4* gene may be a "genocopy" that produces a partial syndrome characterized by behavioral but not cognitive deficits. In addition to information-processing benefits, cognitive style (impulsivity) *(10,11,105)*, and personality style (novelty seeking) *(112)* may be associated with the 7R allele. These characteristics may be beneficial in some settings but detrimental in other settings, such as modern school settings that require children to remain still and quiet for extended periods of time.

5.4. Evolutionary Significance of the 7R Allele of the **DRD4** *Gene*

In the framework of evolutionary biology, some investigators have speculated that a genetic form of ADHD associated with the 7R allele may be owing to an "environmental mismatch" *(113,114)* associated with some functional consequences of this allele that clash with some of the demands of modern society but were beneficial to ancestors in the distant past. For example, Chen et al. *(115)* evaluated the migration history of different ethnic groups, and showed that migration distance was highly correlated with the prevalence of the 7R allele in these groups. They proposed that the 7R allele may have played a role in the gene flow "out of Africa" *(116)*, the crossing the Bering Strait during the last ice age *(117)*, and the spread of the human population into the New World.

5.5. Positive Selection of the 7R Allele

Speculation about positive selection of behaviors usually resort to Kipling-like "just so" stories, so restrictions must be imposed to counter the almost unlimited possibilities *(118)*. We have proposed that evidence of positive selection at the nucleotide level should be used as a very stringent restriction *(119)*. The speculations about evolutionary significance of the behaviors associated with the 7R allele have received support from our recent analysis of genetic variation in the *DRD4* gene at the nucleotide level. The 48-bp VNTR in exon 3 of the *DRD4* is an "imperfect repeat." Many "motifs" of the 48-bp building block exist. For example, Lichter et al. *(120)* identified 19 motifs, which were labeled with Greek letters. They noted that the first motif (A CCC GCG CCC CGC CTC CCC CAG GAC CCC TGC GGC CCC GAC TGT GCG CC) and the fourth motif (A CCC GCG CCC **GG**C CTC CCC C**C**G GAC CCC TGC GGC **T**CC **A**AC TGT GC**T** CC) typically occur as the first and last 48-bp sequences for all alleles regardless of the number of repeats, but the internal motifs vary considerably. In the program of research at UC–Irvine, Ding et al. *(121)* resequenced the DRD4 exon 3 polymorphism in 600 chromosomes from a worldwide sample of DNA, which is a sample size selected to identify almost all alleles that appear in the population at a frequency greater than 0.01. Ding et al. *(121)* observed all of the motifs reported by Lichter et al. *(120)* and a few more, and the total of 35 different "building blocks" of the exon 3 VNTR outnumber the letters in Greek alphabet, so numbers were proposed as labels. The labels were used to specify the nucleotide sequence of an allele by a string of 48-bp motifs. For example, the 4R allele consists of $48 \times 4 = 192$ nucleotides, and the typical variant can be specified as 4R:1-2-3-4; the 7R allele consists of $48 \times 7 = 333$ nucleotides, and the typical variant can be specified as 7R:1-2-6-5-2-5-4; and the 2R allele consists of $48 \times 2 = 96$ nucleotides, and the typical variant can be specified as 2R:1–4.

The nucleotide sequence organization of these 600 alleles analyzed by Ding et al. *(121)* clearly indicated that the 4R allele was the ancestral variant and that other variants (2R–6R allele) could have arisen from the simple process of recombination (e.g., two 4R alleles recombining asymmetrically to produce a 2R allele (2R:1–4) and a 6R allele *(6R:1-2-3-2-3-4)*. However, this simple recombination process could not explain the derivation of the 7R allele from the 4R allele. Instead, what distinguished the typical 7R allele from the presumed 4R ancestral allele were unique motifs labeled 5 (A CCC GCG CCC **G**GC CTC CCC CAG GAC CCC TGC GGC CCC GAC TGT GCG CC) and 6 (A **G**CC GCG CCC **G**GC CTC CCC C**C**G GAC CCC TGC GGC CCC GAC TGT GCG CC). The unusual nucleotide sequence would require several events to occur (e.g., multiple recombination, gene conversions, and single nucleotide mutations) to arise from the 4R

allele. Ding et al. *(121)* speculated that the initial formation of the 7R allele was owing to such a rare combination of events and then this unique nucleotide variation rose to a high level in the human population because of positive Darwinian selection. Statistical tests of intra-allelic variability, diversity in African and non-African alleles, and linkage disequilibrium were performed and presented that support this hypothesis over competing hypotheses, such as "bottleneck" effects.

We have used this example to make recommendations for the application of adaptationism *(119)*. Rather than starting with traits and speculating whether selective forces drove evolution in past environments, we propose starting with a candidate gene associated with a trait and testing first for patterns of selection at the DNA level. This can provide limitations on the number of traits to be evaluated subsequently by investigators. If evidence of positive selection at the nucleotide level were required, and the functional significance of such changes were pursued, this would limit the number of "just so" stories so prevalent in evolutionary psychology *(118)*.

Grady et al. *(122)* extended this approach by sequencing DNA from 132 ADHD probands. In addition to replicating our prior findings of increased percentages of 7R allele *(98,99)* and motif variation at the nucleotide level *(121)*, this study of ADHD DNA documented an increased presence of "rare variants"—12 alleles were observed that were not identified in the initial sample of 600. Of these 12, inspection of their nucleotide sequences indicated that 10 arose from recombinations with a 7R allele. This suggests that rare variants of the exon 3 VNTR may be overrepresented in the ADHD population, and that this locus is a "hot spot" for recombination events.

These studies conducted at UC–Irvine *(98,99,104,121,122)* suggest that if the *DRD4* polymorphism plays a causative role in ADHD, it is likely that studies relying on the usual gel-based genotypes (which allow visualization of the length polymorphism defined by the number of repeats of the 48-bp VNTR) will not be sufficient to capture important features of the DRD4 genotype. For example, the longer length may be more likely to harbor an abnormal 48-bp sequence, and it (rather than VNTR length) may have an effect on behavior and play a role in the manifestation of the ADHD phenotype.

5.6. Other Genes and ADHD

Several investigators have extended the candidate gene approach to the investigation of other genes. For example, Fossella et al. *(107)* evaluated the *DRD2*, *DRD3*, and *DRD5* receptor genes, as well as for genes related to the enzymes MAO and COMT. As recommended by Crowe *(99)*, until multiple replications emerge for these candidate genes, a discussion of the significance of the isolated positive findings seems premature.

In addition to candidate gene studies, two genome scans have been reported. Fisher et al. *(123)* evaluated 126 affected sibling pairs and 404 markers and Bakker et al. *(124)* evaluated 164 affected sibling pairs and 402 markers. Neither genome scan revealed a strong signal from a specific location on the human genome to direct the search for a specific gene. The largest but statistically nonsignificant signals in the Fisher et al. *(123)* study were for chromosomal locations 5p, 10q, 12q, and 16p, and for the Bakker et al. *(124)* study the largest nonsignificant signals were from nonoverlapping chromosomal locations (15q, 7p, and 9q). Of course, the reliance on "weak, nonsignificant" signals is very likely to identify chromosomal regions that are owing to chance variation in allele sharing on the group of siblings investigated. Neither genome scan identified the chromosome locations

of the *DAT* or *DRD4* genes. However, in the population the risk alleles for these candidate genes are high ($p \sim 0.15$ for the 7R allele of *DRD4* and $p \sim 0.7$ for the 10R allele of *DAT*). This restricts the maximum value of relative risk (the ratio of the allele probability in the "disease" population over the allele probability in the nondisease population). Risch and Merikangas *(125)* pointed out that for genes with low relative risk and high allele probability of the risk allele, very large sample sizes would be required to detect a signal (e.g., 65,000 sibling pairs for a gene such as the *DRD4*). The failure of these initial genome scans to detect a strong signal does not discount the possibility of genes with high values of attributable risk (the percentage of the disease population with the risk allele, which is about 50% for the *DRD4* 7R allele), of multiple genes that combine to confer risk for ADHD, or of genes with effects that depend on interactions with environmental factors. Recent reviews of the genome scan approach *(125,126)* suggest that much larger sample sizes (thousands rather than hundreds of affected sibling pairs) and many more markers (hundred of thousands rather than hundreds) will be required to detect genes involved in complex conditions, such as ADHD.

6. SPECULATIONS ABOUT INVOLVEMENT OF GLUTAMATE IN ADHD

6.1. The Role of Glutamate in MBD

There is little information on the involvement of glutamate in the manifestation and treatment of ADHD. However, a few preclinical and clinical studies have addressed this issue and suggest that glutamate may be involved in MBD *(22,108,110,111)*. Lou *(109)* developed a theory based on the "excitotoxic" hypothesis of Benveniste et al. *(127)* and suggested that repeated bouts of prenatal anoxia and hypoxia produced damage to germinal cells owing to increased glutamate, which might restrict the population of late-developing neurons. This hypothesis is similar to that of Altman *(110)* and Amsel *(111)*, and suggests that glutamate-induced damage early in prenatal development would be manifested as a size difference in postnatal development. This provides an intriguing neuroanatomical basis for MDB, a concept that was rejected so completely in the report by Bax and McKeith *(108)*, of the conclusions of a 1962 international study group of pediatric neurologists that was convened to address this issue and to combat the widespread acceptance and use of the term MBD in clinical practice.

6.2. The Role of Glutamate in Seizures

Rubinstein et al. *(128)* evaluated the role of DA in the initiation of motor activity and the integration of goal-directed behaviors wildtype and D4 receptor-deficient (knockout) mice, with a focus on the ascending DA pathways from the VTA that innervates the frontal cortex. Absence of the D4 receptor in mutant mice or pharmacological blockade of the D4 receptor in wildtype mice increased excitability in the frontal cortex where D4 receptors are densely expressed. The mutant mice also had increased susceptibility to seizures produced by a convulsant drug (bicuculline). Glutamate immunolabeling of frontal cortex pyramidal neurons was lower in D4-deficient than wildtype mice. Behavioral, electrophysiological, immunocytochemical, and ultrastructural evidence suggested that cortical activity is elevated in mice without D4 receptors. This supported the hypothesis that D4 receptors function as an inhibitory modulator of glutamate activity in the frontal cortex. According to this model, the *DRD4* allele that codes for subsensitive D4 receptors (i.e., the 7R allele) may increase the seizure threshold. One of the early studies of

pathophysiology of ADHD by Laufer and Denhoff *(129)* used the photometrazol test with an ADHD (or "hyperkinetic") and control group of children and documented lower seizure threshold in the clinical group.

6.3. Use of Magnetic Resonance Spectroscopy to Investigate the Role of Glutamate in ADHD

Proton magnetic resonance spectroscopy (MRS) yields information regarding markers of neurons (*N*-acetylaspartate [NAA]), myelin, and other cellular membranes (Choline), and energy metabolism (intracellular creatine and phosphocreatine [Cre]) within a well-defined and limited region of interest. Only a few research groups have published MRS findings in children with ADHD, focusing on the striatum *(130–133)* and the right frontal lobe *(134)*. These groups have utilized magnets with 1.5 Tesla field strengths (T), which render peaks with insufficient spectral resolution to distinguish between glutamate (Glu), glutamine (Gln), and γ-amino butyric acid (GABA), so they report a Glx (Glu + Gln + GABA) resonance.

Interesting findings have been reported by Carrey and colleagues *(130–132)*, such as changes in striatal Glx/Cre ratios as a result of stimulant medication use in children with ADHD. In these initial studies, only small samples have been evaluated so far. A more accurate identification and quantification of each metabolite would significantly contribute to the understanding of the neurochemical basis of ADHD and response to stimulant medication. Work is currently in progress to use magnets with 4T because greater field strengths reliably render distinguishable peaks specific for Glu, Gln, and GABA. Work is now in progress by Carrey and colleagues (personal communication) to develop 4T pulse sequences of MRS to significantly improve spectral resolution of metabolites of interest in studies of children with ADHD.

A MRS study in normal adults reported a strong positive correlation between performance on the Stroop color-word test and (NAA) in the right anterior cingulate cortex *(135)*. This effect was lateralized to the right hemisphere and was robust enough to accurately predict group membership (high interference vs low interference on the Stroop test). Based on this, Juranek and colleagues *(136)* have initiated a program of research to use this technique in studies with ADHD subjects to determine how NAA in the right anterior cingulate cortex is related to cognitive performance on the Stroop test in this clinical population.

7. CONCLUSIONS

We have used a levels-of-analysis approach for over a decade as a framework to organize and relate the consensus views about the behavioral, psychological, and neural abnormalities associated with ADHD. The behavioral areas of clinical manifestation of ADHD (abnormalities in attention and activity), the psychological areas of cognitive deficits (abnormalities in executive functions), and the neural areas of anatomy and genetics (smaller brain regions and statistical association with the *DAT* and *DRD4* genes) all implicate DA as factor in the etiology and treatment of ADHD.

Research on brain imaging provides the strongest evidence for specific involvement of DA in ADHD. Multiple studies of brain anatomy using MRI have documented that groups of ADHD children have smaller brain volumes in regions where DA receptors are dense. Multiple studies of brain function have documented that the primary mechanism of action

of stimulant drugs is DAT blockade in the striatum, which produces an increase in brain levels of DA when administered at clinical doses. Thus, evidence from a variety types of brain imaging studies converge and support the long-standing hypothesis that the clinical symptoms and cognitive deficits associated with ADHD may be the result of a DA deficit, which can be temporarily corrected by the pharmacokinetic and pharmacodynamic effects of low oral doses of stimulant drugs.

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VIII Addiction

14

Dopamine–Glutamate Interactions in Reward-Related Incentive Learning

Richard J. Beninger and Todor V. Gerdjikov

1. INTRODUCTION

Extensive evidence implicates the neurotransmitter dopamine (DA) in reward-related incentive learning (for reviews, *see* refs. *1–13*). DA projections to the nucleus accumbens (NAc; refs. *14–17*), striatum *(18)*, amygdala *(19)*, and medial prefrontal cortex (mPFC; ref. *20*) have been shown to be involved. In recent years, researchers have begun to focus on the neurochemical mechanisms underlying the role of DA in learning and significant advances have been made *(21–23)*. Many data suggest that DA afferents interact with glutamatergic (Glu) afferents common to the same cell when reward-related learning occurs *(see* ref. *22)*. Results further suggest that a number of signaling molecules activated by Glu and DA synaptic transmission interact to bring about short-term and long-term alterations that mediate the neurochemical and structural changes that form the basis of reward-related incentive learning *(see* ref. *22)*. In this chapter, we will review some of the studies examining the role of DA and especially Glu neurotransmission in reward-related learning. This will be followed by a discussion of evidence that provides a basis for understanding the DA–Glu interactions and the signaling pathways that mediate the effects of reward on behavior. Finally, the role of Glu in reward-related learning will be considered from the point of view of this evidence.

2. GLUTAMATE AND REWARD-RELATED LEARNING

In the following subheadings, the role of Glu in reward will be reviewed. Each section will begin with a brief discussion of the role of DA in the phenomenon under consideration. There have been many studies of DA manipulations and no attempt will be made to exhaustively review those studies. Instead, representative studies will be presented to provide a background of the role of DA against which the Glu results can be viewed.

2.1. Glutamate and Appetitive Learning: Acquisition and Expression of Conditioned Approach Responses

When a neutral stimulus is paired with a rewarding stimulus such as food, animals begin to make approach responses to the neutral stimulus *(24)*. This type of learning can

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

be seen in the increase in approach responses to the food tray in operant chambers after food has been delivered there on a number of occasions. DA receptor antagonists decrease this type of learning. For example, the D2-like DA receptor antagonist pimozide impaired the acquisition of conditioned approach responses to a food magazine signaled by a light *(25)*. In a number of related studies, conditioned approach responses of animals that had received conditioning prior to drug treatment also were impaired by pimozide or the related D2-like DA receptor antagonists metoclopramide and haloperidol but not thioridazine *(26–28)*. Dickinson et al. *(29)* paired food with an auditory conditioned stimulus presented to rats pretreated with pimozide or the mixed D1- and D2-like DA receptor-antagonists α-flupenthixol and then presented the auditory stimulus while the animals were lever pressing in a drug-free state. They found that the auditory stimulus increased responding in control rats but not in those that had been treated with a DA receptor-antagonist during conditioning; this suggested that drug treatments had blocked acquisition by the auditory stimulus of the ability to energize responding. A. Phillips et al. *(26)* showed that the maximal impairment produced by pimozide or haloperidol was not immediate but had a gradual onset with repeated presentations of the conditioned appetitive stimulus over test trials; this observation suggested that, although DA was necessary for the maintenance of responding to conditioned stimuli, once they were established, these conditioned stimuli may have been temporarily resistant to the effects of DA receptor blockade. Thus, studies using systemic drug administration implicate DA in the acquisition and long-term maintenance of conditioned approach responses to conditioned appetitive stimuli but, once established, this conditioning may be transiently resistant to the effects of DA receptor antagonists.

DA in the NAc, especially the core region, has been shown to play a role in the acquisition and the expression of conditioned approach responses. Thus, NAc core injections of the D1-like DA receptor antagonist SCH 23390 *(30)*, α-flupenthixol *(31),* or NAc DA depletions with 6-hydroxydopamine impaired both acquisition and expression of conditioned approach responses *(32)*. The latter study found milder effects on expression vs acquisition suggesting again that once conditioning had taken place, it may have been transiently resistant to the effects of decreased DA neurotransmission. In one study, the mPFC was implicated: Baldwin et al. *(33)* reported that mPFC infusions of SCH 23390 impaired acquisition and expression of conditioned approach; however, higher doses were required to impair expression. Like studies using systemic administration of DA receptor antagonists, those using intra-NAc or -mPFC injections showed that DA plays a critical role in acquisition and expression of conditioned approach responses but that expression is somewhat resistant to the drug effects.

Glu *N*-methyl-D-aspartate (NMDA) receptors have been implicated in conditioned approach. In all of the studies reviewed in this paragraph, NMDA receptor antagonists were found to impair acquisition but not expression of conditioned approach responses. Impairments were seen following chronic intracerebroventricular (icv; ref. *34*), unilateral or bilateral basolateral amygdala (BLA; refs. *35* and *36*) or NAc core (but not shell; refs. *31, 36,* and *37*) or bilateral mPFC infusions of the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (AP5; ref. *36*). Injections of AP5 into the dorsal or ventral subiculum were without effect *(36)*. Di Ciano et al. *(31)* studied discriminated approach to a lever that was extended to signal the delivery of a food pellet; they showed that intra-NAc core injections of the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid

(AMPA)/kainate receptor antagonist (3S,4aR,6R,8aR)-6-[2-(1(2)H-tetrazole-5-yl)-ethyl]-1,2,3,4,4a,5,6,7,8,8a-decahydroiso-quinoline-3-carboxylic acid (LY293558) did not block acquisition of approach and contact responses but impaired discrimination so that approach and contact responses were seen to levers that signaled either food or no food. Results implicate NMDA receptors in the NAc core, BLA, and mPFC in the acquisition but not expression of conditioned approach responses. AMPA/kainate receptors in the NAc core may play a role in discrimination learning.

Synergistic effects of DA D1-like and Glu NMDA receptors have been found in the NAc core and mPFC. Thus, coinjections of subthreshold doses of SCH 23390 plus AP5 blocked acquisition but not expression of conditioned approach responses *(30,33)*. Results suggest that both DA D1-like receptors and Glu NMDA receptors in NAc core and mPFC participate in the learning that underlies the acquisition of conditioned approach responses.

2.2. *Glutamate and Appetitive Learning: Lever Pressing Tasks*

2.2.1. Glutamate and the Acquisition of Lever Pressing for Food

One form of appetitive instrumental learning is the acquisition of lever pressing for food in rats. In this paradigm, food-deprived rats learn that food is available from a food hopper in an operant testing chamber outfitted with a lever. Once rats have been fed in this situation, they become more active and often sniff, bite at, and manipulate environmental stimuli associated with food. Occasionally this activity leads to a downward deflection of the lever that is programmed to deliver a food pellet to the food hopper. Rats tend to return to environmental stimuli that were encountered just before the presentation of a rewarding stimulus and this tendency leads the rats to further manipulate the lever and to attain further rewards. This process brings about instrumental learning evidenced by the rat's repeated lever pressing.

It is well-known that DA is necessary for this form of learning. Thus, treatment with the DA D2-like receptor blocking agent pimozide dose-dependently attenuated the acquisition of lever pressing for food *(25,38)*. Tombaugh et al. *(25)* found that animals trained to lever press and then given pimozide showed little effect of the drug in two 22.5-min sessions. This result makes it difficult to attribute the failure of rats to learn to lever press while treated with pimozide to motor effects of the drug; clearly, previously trained rats, when treated with pimozide, can lever press. Thus, the DA receptor antagonist blocked acquisition learning of appetitive instrumental conditioning.

It has also been shown that DA in the NAc and the mPFC is important for the acquisition of appetitive instrumental conditioning. Rats were given daily 15-min sessions in an operant chamber outfitted with two levers, one of which produced a food pellet when depressed. Normal rats generally learned to lever press in the third session and rates increased over subsequent sessions. Smith-Roe and Kelley *(30)* showed that intra-NAc injections of the DA D1-like receptor antagonist SCH 23390 impaired acquisition of lever pressing in this paradigm. The dose of SCH 23390 that impaired acquisition of lever pressing also decreased locomotor activity and increased the average duration of feeding bouts in a 15-min test. However, it did not affect total amount eaten. The authors argued that SCH 23390 did not alter motivation to eat but could not rule out the possibility that the effect of injection of this drug into NAc on lever press acquisition was related to its motor effects.

In a further study from Kelley's laboratory, SCH 23390 injected bilaterally into the mPFC dose-dependently decreased appetitively motivated lever press acquisition *(33)*. In this study, locomotor activity and feeding were not affected by doses of SCH 23390 that impaired lever press acquisition. Results implicated NAc- and mPFC-DA in the acquisition of lever pressing for food.

Kelley and coworkers have extensively evaluated the role of NMDA receptors in this paradigm. They compared the effects of the NMDA receptor antagonist AP5 injected into the NAc core or shell. Injections into both regions impaired lever press acquisition but AP5 was more potent in the core than in the shell. AP5 did not significantly alter locomotor activity or feeding at doses that impaired operant response learning *(37)*. Results demonstrate the requirement of NMDA receptors in NAc for the early stages of learning.

Baldwin et al. *(36)*, in another study from A. Kelley's lab, investigated the effects of bilateral AP5 injections into NAc core, BLA, dorsal subiculum, ventral subiculum, or mPFC on lever press acquisition. Learning was impaired by injections into the NAc core, replicating the effects of Kelley et al. *(37)*. Learning was also impaired by injections into the BLA or mPFC but not into the dorsal or ventral subiculum. BLA or mPFC injections of AP5 did not significantly affect locomotor activity or feeding. Results implicate not only NMDA receptors in the core of NAc but also those in BLA and mPFC in instrumental response learning.

Some studies have used systemic injections of Glu agents. One evaluated the effects of glutamic acid diethyl ester (GDEE) on lever press acquisition; GDEE is a nonselective excitatory amino acid (EAA) receptor antagonist. Consistent with the central injection studies reviewed above, Freed and Wyatt *(39)* found a dose-dependent impairment. The noncompetitive NMDA receptor antagonist ketamine similarly impaired lever press acquisition in a dose-dependent manner; in related experiments, open field performance was unaffected *(40)*. Some studies required rhesus monkeys to learn a new sequence of pressing four levers for food each day and showed that treatment with the noncompetitive NMDA receptor antagonists dizocilpine (aka MK-801) impaired performance *(41)*. Clissold et al. *(42)* showed that the competitive NMDA receptor antagonist (±)-3-(2-carboxypiperazin-4-yl)propyl-l-phosphonic acid [(±)-CPP] or the noncompetitive NMDA receptor antagonists dizocilpine impaired acquisition of a repeated discrimination lever pressing task; phencyclidine (PCP) was without effect. These studies support a role for NMDA receptors in the acquisition of lever pressing for food.

Mélan et al. *(43)* found that BALD/c mice that had been partially trained to lever press for food showed a spontaneous improvement of performance 24 h later. If they were injected with an NMDA receptor antagonist immediately after the acquisition session, they did not show this effect. Thus, γ-L-glutamyl-L-aspartate (γ-LGLA) or (±)-CPP dose-dependently eliminated the spontaneous improvement effect. In a related study, posttraining icv injections of AP5 eliminated the performance improvement effect in mice *(44)*. Results implicated NMDA receptors in acquisition of lever pressing and were consistent with the findings from A. Kelley's lab that injections of AP5 into the NAc core, BLA, or mPFC impaired learning.

The role of metabotropic glutamate receptors (mGluRs) has also been investigated. Mathis and Ungerer *(45)* found that posttraining icv injections of the group I and II mGluR antagonist α-methyl-4-carboxyphenylglycine (MCPG) dose-dependently blocked

the spontaneous improvement effect observed 24 h after training in mice. Coadministration of either the group I and II mGluR agonist (1S,3R)-1-aminocyclopentane -1,3-dicarboxylic acid (ACPD), the group I mGluR agonist (R,S)3,5-dihydroxy-phenylglycine (DHPG), or the group II mGluR agonist (1S,2S,5R,6S)- 2-aminobicyclo [3.1.0]hexane-2,6-dicarboxylate monohydrate (LY354740) reversed the impairments produced by MCPG (for a review of these studies and a discussion of the role of NMDA and mGluRs in consolidation of memory, *see* Ungerer et al. ref. *46*). The group I category of mGluRs includes mGluR5 *(47)*; mGluR5 null mutant mice or wildtype mice treated with the mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pypiridine (MPEP) were not impaired in acquiring a lever press response for food *(48)*. Results suggest that group I and II mGluRs are involved in the acquisition of lever pressing for food but that the group I category receptor mGluR5 is not involved; that leaves mGluR1(the remaining member of the groupI category) and mGluR2 and 3 (members of group II) as candidates for a role in the learning that underlies acquisition of lever pressing for food.

Smith-Roe and Kelley *(30)* and Baldwin et al. *(33)* identified doses of SCH 23390 and AP5 that were ineffective on their own when injected into the NAc core or mPFC during acquisition training. Coadministration of these doses together produced a significant impairment of lever press acquisition without affecting food intake or locomotion. Results suggest that coactivation of DA D1-like receptors and Glu NMDA receptors in NAc and mPFC plays a critical role in appetitive instrumental learning.

2.2.2. Glutamate and the Expression of Lever Pressing for Food

The effects of DA or Glu receptor antagonists on instrumental behavior can be tested during acquisition, as reviewed in the previous subheading, or during expression following acquisition when the behavior is well established. Numerous studies have shown that lever pressing for food is initially resistant to the effects of DA receptor antagonists; however, with continued testing under the influence of these agents, established responding gradually declines, showing a pattern that resembles that seen during extinction (when food reward no longer is presented following lever press responses). In one of the earliest such studies, Wise et al. *(49)* trained rats to lever press for food. On subsequent days, well-trained rats were injected with pimozide prior to testing. On the first day, drug-treated rats responded almost as frequently during a 45-min session as they had during training. With repeated testing while under the influence of pimozide on subsequent days responding declined. Related studies showed that when intrasession response rates were evaluated over time, they also declined in an extinction-like manner *(50–52)*. It has also been shown that systemic injections of SCH 23390 produce a day-to-day decline in responding for food reward *(53)*. Results suggested that DA acting at both D1- and D2-like receptors is critical for the maintenance or expression of responding in well-trained animals but when drug treatments commence, there is an initial period during which responding is resistant to their effects.

Some studies have sought to identify DA terminal regions that may be critical for the expression of operant responding for food. Beninger and Ranaldi *(18)* and Beninger et al. *(54)*, for example, showed that bilateral injections of the DA receptor antagonist α-flupenthixol into the dorsal caudate nucleus, but not into the NAc, central nucleus of the amygdala, or sensorimotor cortex, produced a within-session decline in responding resembling that seen in extinction. Injections of the inactive isomer *trans*-flupenthixol were without significant effect. G. Phillips et al. *(55)* showed similar effects with dorsal

caudate injections of the DA D2-like receptor antagonist sulpiride. Results suggest that the expression of lever pressing for food in previously trained rats depends on intact neurotransmission at both D1- and D2-like DA receptors and that the caudate nucleus may be the brain region critical for this effect.

In her studies of the effects of intracranial injections of AP5 on the acquisition of lever pressing for food and nose poking into the feeder, Kelley and colleagues used the following protocol (all sessions were 15 min in duration): four sessions preceded by intracranial injections; five sessions with no injections; one session preceded by intracranial injections. The final session provided information about the effects of treatments on expression of the lever pressing response once it was established. They found that an intra-NAc core or shell dose of AP5 that impaired acquisition of responding for food had no significant effect on response expression in trained rats tested in one 15-min session *(36,37)*. Similar results were found with BLA and mPFC injections of AP5 *(36)*. Thus, trained operant responses seemed to be resistant to the effects of centrally injected NMDA receptor antagonists when responding was assessed for one session.

Freed and Wyatt *(39)*, who showed that systemic GDEE impaired acquisition of lever pressing for food (*see* Subheading 2.2.1.), also tested the effects of this nonspecific EAA receptor antagonist on the expression of learned lever pressing 2 d and approx 2 wk after acquisition training. GDEE had no significant effect on the performance of the learned operant.

Systemic dizocilpine failed to affect established lever pressing for food *(56,57)*. In the same report, Shoaib et al. *(56)* found that (+)-3-amino-1-hydroxy-pyrrolid-2-one [(+)-HA966], a partial agonist at the glycine site that acts as a functional antagonist of the NMDA receptor complex, produced a decrease. When dizocilpine was infused via sucutaneous minipumps during performance of a well-trained operant lever press reinforced according to a fixed ratio (FR) 30 schedule, a dose-dependent decrease in responding was observed; this effect showed tolerance with repeated testing for 10 d *(58)*. Poling et al. *(59)* and Hudzik and Slifer *(60)* similarly showed a dose-dependent decrease in FR responding by dizocilpine or the NMDA receptor antagonists PCP or (+)-*N*-allylnormetazocine (NANM) with acute systemic dosing. Hudzik and Slifer *(60)* assessed FR 10 responding in the context of a complex multiple schedule with one of the components being a differential reinforcement of low response rates (DRL) 10-s. They found that doses of the NMDA receptor antagonists that produced decreases in FR 10 responding produced increases in DRL 10-s responding. Increases in DRL 10- or 15-s responding similarly were reported following PCP, dizocilpine, NANM, or (±)-CPP but not the noncompetitive NMDA antagonist ifenprodil *(59,61,62)*. These observations made it difficult to attribute the effects of the NMDA receptor antagonists on FR responding to possible motor impairments. In a related study, Genovese and Lu *(63)* showed that dizocilpine dose-dependently decreased FR 20 rates and increased fixed interval (FI) 2-min rates on a multiple schedule. They also assessed repeated testing under the influence of the drug and observed tolerance, in agreement with the findings of Wessinger *(58)*. Other studies evaluated rates and maxima on progressive ratio schedules in rhesus monkeys; both were dose-dependently decreased by dizocilpine *(41)*. Thus, some results showed that systemic doses of most NMDA receptor antagonists dose-dependently decreased continuously reinforced or FR responding and increased DRL or FI responding; others showed no effect of systemic dizocilpine.

The results from many systemic administration studies showing that Glu receptor antagonists decrease the expression of lever pressing for food are not in agreement with those from central-injection studies showing no effect. However, a number of differences between the two sets of studies should be noted. One relates to the doses tested. Thus, the central-injection studies identified a dose of AP5 that impaired lever press acquisition and then used that dose in the test of expression. By contrast, with one exception, the systemic administration studies did not evaluate acquisition. Thus, it is possible that the doses that were observed to affect established operant responding were higher than those that would have affected acquisition. The one exception is the study by Freed and Wyatt *(39)* and when these researchers tested the effects of an acquisition-impairing dose of GDEE on expression, they observed no effect.

Pierce et al. *(57)* evaluated the effects of the AMPA/kainate receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) on established operant responding for food. They observed a significant decrease. Results suggest that AMPA/kainate receptors may be involved in the expression of lever pressing for food.

In their study showing that subthreshold doses of AP5 and SCH 23390 when given together into NAc synergized to produce an impairment of acquisition, Smith-Roe and Kelley *(30)* found that the same treatment also caused a significant reduction in established responding; however, responding was still substantially above the level seen in untrained rats, showing some resistance to the effects of the drug treatment. In their related study examining the effects of mPFC co-injections of subthreshold doses of AP5 and SCH 23390, Baldwin et al. *(33)* found no effect of the coinjections on well-trained lever pressing.

A recent study by Hauber et al. *(64)*, although using a different behavioral paradigm, might be relevant here. They trained rats in a reaction time (RT) task. The rats were presented with a signal to press a lever and then waited (200–800 ms) for an additional signal to release the lever; if they released the lever within a critical amount of time (100–1000 ms from signal onset) they received reward. There were two reward magnitudes, one and five pellets that were indicated in advance by the first stimulus. The authors found that rats released the lever more quickly on trials where they were going to receive five pellets than on trials where they were going to receive one pellet; the difference, termed RT gain, was about 45 ms. Bilateral intra-NAc injections of the D2-like receptor antagonist haloperidol were without effect on RT gain. AP5 significantly reduced RT gain but did not affect number of trials taken to complete the test session. They argued that their results showing that NAc injections of AP5 were without effect on trials to complete the test session were in good agreement with those of Kelley et al. *(37)* showing that, once training had taken place, NAc injections of AP5 did not affect performance. The authors concluded that NMDA but not DA D2-like receptors in NAc of well-trained rats are importantly involved in guiding the speed of instrumental responding under the control of a predictive stimulus that signals upcoming reward magnitude.

The reaction time task of Hauber et al. *(64)* may assess an aspect of complex behavioral control that is added to the control of operant responding by environmental stimuli. Notwithstanding their observations, it appears from the work of A. Kelley's group that Glu NMDA receptors are necessary for acquisition but not expression of lever pressing for food. Related studies implicate mGluRs in lever press learning. Studies with DA receptor antagonists similarly have shown that DA is necessary for acquisition but not

expression. However, those studies have also shown that well-trained animals repeatedly tested under the influence of a DA receptor antagonist gradually decrease responding, showing an extinction-like decline. Well-trained animals repeatedly tested after systemic treatment with an NMDA receptor antagonist did not show an extinction-like decline. It remains to be determined whether repeated tests of well-trained animals treated with intra-NAc, BLA, or mPFC injections of AP5 will show a gradual decline in responding. Results of studies that have looked at both acquisition and expression appear to show that NMDA receptors are necessary for acquisition but not expression. AMPA/kainate receptors, on the other hand, were necessary for expression. This is a general finding from many studies as will be further seen in the following subheadings.

2.2.3. Glutamate and the Expression of Lever Pressing for Brain Stimulation Reward

Since the discovery by Olds and Milner *(65)* that animals could be trained to perform an operant response that produced electrical stimulation of certain brain regions, there has been extensive interest in identifying the neuroanatomical and neurochemical substrates that mediate this effect. It was thought that identification of the critical substrates would point to the brain circuits that mediated the effects of reward on behavior. Extensive research led to the conclusion that DA neurons were important for brain stimulation reward (BSR) *(1)* and these results contributed to the now widely held view that DA neurons play a critical role in reward-related incentive learning *(3)*. For example, Mogenson et al. *(66)* implanted rats with stimulating electrodes in the ventral tegmental area (VTA) and bilateral cannulae into the NAc. Once self-stimulation rates were stable, they injected the DA D2-like receptor antagonist spiroperidol into the NAc either ipsilaterally or contralaterally to the stimulating electrode. They found that ipsilateral but not contralateral injections caused a reduction in rates of responding for BSR. The observation that contralateral injections were without effect eliminated the possibility that the drug was producing its effect by impairing the rats' ability to respond. Results supported the conclusion that VTA BSR depended on stimulation of DA receptors in the NAc.

Some studies have looked at the role of Glu in BSR. Herberg and Rose *(67)* microinjected Glu agents into the VTA to observe effects on BSR produced by electrodes placed rostral to the VTA in the medial forebrain bundle (MFB). AP5 was without effect. Less specific Glu receptor antagonists acting on NMDA and non-NMDA receptors produced a decrease in responding. Thus, the broad-spectrum EAA antagonists *cis*-2,3-piperidine dicarboxylate (cPDA), γ-D-glutamylaminomethyl sulphonic acid (GAMS), or p-chlorobenzoyl-2,3-piperazine dicarboxylic acid (pCB PxDA) reduced responding. Control injections of these agents into the VTA contralateral to the side of the electrode were without effect ruling out the possibility that reductions in response rates were related to nonspecific motor effects of the broad-spectrum Glu receptor antagonists. With electrodes in the ventral pallidum, Panagis and Kastellakis *(68)* found no effect of VTA injections of NMDA or AMPA on BSR thresholds. The results of Herberg and Rose *(67)* implicate non-NMDA Glu receptors in VTA in the expression of MFB BSR and those of Panagis and Kastellakis *(68)* implicate neither NMDA nor AMPA Glu receptors in the VTA in the expression of ventral pallidal BSR.

The effects of intra-VTA injections are generally consistent with results from studies investigating the effects of systemic injections of Glu agents. It was found that neither of the noncompetitive NMDA receptor antagonists dizocilpine nor ketamine impaired BSR

produced by electrical stimulation of the midlateral hypothalamus; in fact, they enhanced responding at low doses (refs. *69–71*). In a related study, Ranaldi et al. *(72)* found that dizolcipine failed to affect thresholds for lateral hypothalamic BSR; however, dizocilpine augmented the threshold-reducing effects of cocaine. Besides antagonizing NMDA receptor function, dizocilpine and ketamine have been shown to activate A10 DA neurons *(73)* or to increase the metabolism of DA in several brain areas *(74,75)*; these DA effects of dizocilpine and ketamine may account for their ability to augment BSR reward. M. Olds *(71)* tested this hypothesis by evaluating the effects of the DA receptor antagonists SCH 23390 or halpoeridol on the BSR-enhancing effects of dizocilpine; both DA agents blocked the effect. Thus, NMDA antagonists fail to impair well-established lever press responding for BSR, suggesting that NMDA receptors are not involved in the expression of responding. The agents tested also produced an increase in DA neurotransmission; this increase can augment BSR reward.

Recall that Herberg and Rose *(70)* found no impairment of BSR with dizocilpine or ketamine. On the other hand, the broad-spectrum EAA receptor antagonist kynurenic acid suppressed responding maintained by BSR, elevated threshold current intensity for producing BSR, and blocked the enhancement of BSR responding produced by morphine *(70,76)*. Herberg and Rose *(67)* concluded that, ". . . the NMDA receptor is unlikely to play an essential role in maintaining self-stimulation in the *fully trained* rat" (italics added). Coupled with the findings reviewed in Subheadings 2.2.1 and 2.2.2 on acquisition and expression of operant responding for food, results point to a role for NMDA receptors in the establishment but not the early expression of reward-related learning with non-NMDA AMPA and kainate receptors perhaps being important for expression.

2.2.4. Glutamate and the Acquisition of Lever Pressing to Self-Administer Drugs

DA has been found to play a critical role in the acquisition of self-administration behavior. Early studies showed that animals learned to perform an operant response when an iv injection of amphetamine *(77)* or cocaine *(78)* was made contingent on that response. Since then, many DA agents have been found to be self-administered including apomorphine *(79)*, the D2-like receptor agonist bromocriptine *(80),* and the D1-like receptor agonists SKF 82958 and SKF 77434 *(81)* and SKF 81297 *(82)* in rats or monkeys. Results suggest that DA receptor stimulation is rewarding.

Carlezon and Wise *(83)* reported that rats would self-administer the NMDA receptor antagonists PCP, dizocilpine, or (±)-CPP directly into the NAc shell or mPFC; injections into the NAc core were not effective. The rewarding effects of these agents were not blocked by coinfusion of the DA D2 receptor antagonist sulpiride. Results suggested that the NMDA receptor-antagonists injected into the NAc shell or mPFC produced reward independent of activation of DA D2 receptors. It is difficult to reconcile these findings with those of A. Kelley and colleagues (*see* Subheading 2.2.1.) showing that blockade of NMDA or DA D1-like receptors in the NAc impaired acquisition of lever pressing for food. Besides the reinforcing stimulus used, some differences between the studies are that Carlezon and Wise *(83)* made multiple response-contingent injections of a smaller volume and a different drug from the one used by A. Kelley and her coworkers; Carleson and Wise *(83)* also tested a D2-, not a D1-like DA receptor antagonist.

Mice were found to learn to choose the correct arm of a Y-maze to receive intra-VTA injections of the competitive NMDA receptor antagonist D(-)-2-amino-7-phosphonoheptanoic acid (AP7) or the AMPA/kainate receptor antagonist DNQX *(84)*. This response

was dependent on DA D2 receptors as pretreatment with sulpiride in well-trained animals led to rapid extinction of the response. These results are consistent with the critical role of DA in reward-related learning. They suggest that normally, Glu afferents to the VTA, acting on either NMDA or AMPA receptors, inhibit DA cell firing, probably through a γ-aminobutyric acid (GABA) interneuron; Glu receptor blockade decreases this GABAergic inhibition, increasing the activity of DA neurons and producing reward. It is difficult to reconcile these findings with the report of Herberg and Rose *(67)* that broad-spectrum EAA receptor antagonists injected into the VTA decreased MFB BSR. Perhaps the use of more selective drugs in the more recent experiments allows for identification of the regionally specific function of different ionotropic Glu receptor subtypes.

The acquisition of cocaine self-administration was impaired by dizocipline. Schenk et al. *(85,86)* observed that rats treated with dizocilpine pressed both the active and inactive lever over training days. When dizocilpine was discontinued, acquisition proceeded in a manner similar to that observed in naïve rats showing that the experience with cocaine during training with dizocilpine did not lead to learning about the cocaine-producing lever.

In summary, acquisition studies show that animals will learn operant responses rewarded by intra-NAc shell, PFC, or VTA injections of NMDA receptor antagonists. Rewarding effects of these agents in the NAc shell or PFC were not blocked by local injection of the DA D2-like receptor antagonist sulpiride but VTA reward was blocked by systemic sulpiride. The latter finding is consistent with a role for VTA DA neurons in reward. Systemic injections of dizocilpine impaired acquisition of self-administration for cocaine. This finding is consistent with observations of impaired lever press acquisition for food in animals treated with NMDA receptor antagonists (Subheading 2.2.1). We know of no reports of acquisition of self-administration for intravenous injections of Glu receptor antagonists.

1.2.5. Glutamate and the Expression of Lever Pressing to Self-Administer Drugs

There are two sorts of experiments in this category. The first involves the use of a substitution procedure. Animals are initially trained to self-administer a stimulant drug, such as cocaine, and then other drugs are substituted for the cocaine to see if they will maintain responding. Because the lever press response is already well trained before the drug substitution takes place, these experiments are classified as tests of expression rather than acquisition. The second type of experiment involved assessing the effects of agents on ongoing responding for a self-administered drug. DA receptor-blocking drugs reduce the rewarding effects of self-administered stimulants. Both D1-like *(87)* and D2-like receptor blockers were effective *(88)*. In recent years it has been discovered that the rewarding effects of many substances including amphetamine, cocaine, heroin, morphine, nicotine, alcohol, and cannabinoids depend on intact function of the VTA-NAc DA system (for a review; *see* ref. *13*).

The NMDA receptor antagonists dizocilpine or PCP were self-administered in a dose-dependent manner by monkeys using the substitution procedure *(89–91)*. If monkeys were trained to self-administer cocaine and then switched directly to dizocilpine, they did not self-administer the substituted drug. When they were trained to self-administer cocaine and then switched to the noncompetitive NMDA receptor antagonist PCP, they self-administered PCP and then when dizocilpine was substituted, self-administration was seen. Thus, in well-trained monkeys, self-administration of NMDA receptor antagonists is seen under some circumstances. As the drugs were administered systemically, it

is not possible to determine where they were acting in the brain. The results of acquisition of self-administration studies using central injections (Subheading 2.2.4) provide some clues.

In other studies using the substitution procedure, mGluR5 null mutant mice failed to self-administer cocaine when it was substituted for food *(48)*. In the same study, wildtype mice treated with the mGluR5 antagonist MPEP showed reduced self-administration of cocaine. Results implicated mGluR5 in cocaine reward.

Turning to experiments involving assessment of the effects of agents on ongoing responding for a self-administered drug, injections of the NMDA receptor agonist 1-aminocyclobutane-*cis*-1,3-dicarboxylic acid (*cis*-ACDA) or AMPA into NAc decreased responding for self-administered cocaine, indicative of increased reward. This study failed to observe any effect of dizocilpine, AP5, or the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) injected into NAc *(92)*. However, studies from Koob's lab with well-trained rats showed that injections of AP5 into the NAc produced an increase in cocaine (but not heroin) self-administration, indicative of a decrease in cocaine reward *(93)*. Related studies showed that NAc injections of AP5 decreased ethanol reward *(94)*. When AP5 or CNQX was injected into the BLA, they failed to affect cocaine reward *(95)*. Results indicate the involvement of NMDA and AMPA or kainate receptors in NAc in cocaine and ethanol but not heroin reward.

Systemic administration of Glu NMDA receptor antagonists increased reward produced by cocaine. In Subheading 2.2.3 on the effects of Glu drugs on the expression of BSR, it was noted that dizocilpine or ketamine appeared to enhance reward *(70,72)*. Similar observations have been made in self-administration studies. Thus, Ranaldi et al. *(96)* showed that systemic injections of dizocilpine increased cocaine reward. Using a self-administration procedure, these authors assessed the maximum FR (breaking point) that a particular concentration of cocaine would support and found that the maximum increased with cocaine concentration. In previous studies, Roberts et al. *(97)* had shown that a DA receptor antagonist decreased breaking points. Ranaldi et al. *(96)* showed that dizocilpine increased breaking points. These results suggest that systemic administration of an NMDA receptor antagonist augmented reward produced by cocaine. Dizocilpine or (+)-HA966 decreased rates of cocaine self-administration in rats *(56,57)*. As increases in dose of cocaine per infusion also led to a decrease in rate, results support the findings of Ranaldi et al. *(96)* suggesting that systemically administered NMDA receptor antagonists augment cocaine reward. On the other hand, Schenk et al. *(85,86)* showed that dizocilpine failed to shift the dose–response curve for cocaine self-administration but it impaired discriminated responding on the cocaine-producing lever, leading to increased responding on the inactive lever.

The findings that the NMDA receptor antagonist dizocilpine augmented cocaine self-administration in most studies can be interpreted in the same manner as the findings reviewed in Subheading 2.2.3 showing a similar effect of this agent on established responding for BSR. Thus, the finding that NMDA receptor antagonists failed to decrease cocaine reward suggests that the expression of responding for cocaine does not require NMDA receptors. The finding that dizocilpine increases cocaine reward is consistent with the ability of this agent to increase activity in VTA-NAc DA neurons *(73)* and its ability to increase the metabolism of DA in several brain areas *(74,75)*.

The AMPA/kainate receptor-antagonist DNQX was found to decrease lever pressing for self-administered cocaine and the same dose decreased lever pressing for food

(*see* Subheading 2.2.2.). Pierce et al. *(57)* also had found that dizocilpine decreased responding for cocaine but had no effect on responding for food. The finding that DNQX similarly decreased responding for both types of reinforcer led Pierce et al. *(57)* to suggest that the effect of DNQX could be attributed to nonspecific motor changes. Alternatively, it could be that NMDA receptors are involved in the expression of lever press responding for cocaine but not food, whereas AMPA or kainate receptors are involved in the expression of both.

These studies can be summarized as follows. Glu NMDA receptor antagonists were self-administered when they were substituted for cocaine. Consistent with these observations, reward produced by self-administered cocaine was augmented by injection of the NMDA receptor antagonists dizocilpine or ketamine in some studies. However, conflicting results showed that systemic dizocilpine failed to shift the self-administered cocaine dose–response curve. Central injection studies showed that intra-NAc injections of an NMDA agonist or AMPA itself increased cocaine reward and AP5 decreased cocaine or ethanol but not heroin reward. These findings might suggest that the apparent rewarding effects of Glu NMDA receptor antagonists or the augmentation of cocaine reward reported following systemically administered NMDA receptor antagonists is not the result of an action of the antagonists in NAc. However, acquisition studies (Subheading 2.2.4.) showing reward based on intra-NAc infusions of NMDA receptor antagonists seem to contradict this conclusion. The finding that intra-NAc AP5 decreased cocaine or ethanol reward is not consistent with the observations of A. Kelley and her colleagues showing that AP5 failed to block expression of responding for food. Perhaps the use of different reinforcers in the studies from Koob's lab (cocaine) vs those from Kelley's lab (food) accounts for the difference. This hypothesis is supported by the findings of Pierce et al. *(57)* showing that dizocilpine decreased responding for cocaine but not for food. Finally, there is evidence implicating AMPA, kainate, and mGluR5 receptors in the expression of lever pressing for stimulant self-administration.

2.2.6. Glutamate and the Resumption (Relapse) of Lever Pressing to Self-Administer Drugs

Animals that have been trained to self-administer a drug (e.g., cocaine) eventually will cease to respond if the drug is no longer available, showing extinction. The presentation of environmental stimuli that have previously been paired with the rewarding drug or exposure to the drug stimulus itself can produce resumption (relapse) of operant responding for the rewarding drug *(98)*. Injection of DA directly into the NAc reinstated responding on a manipulandum that previously produced cocaine self-administration and this effect was blocked by NAc injection of the DA receptor-blocker fluphenazine *(99)*. Injections of SCH 23390 but not the D2-like DA receptor antagonist raclopride into the BLA blocked the ability of drug-paired stimuli to reinstate responding for cocaine *(95)*. Results implicated NAc and BLA DA in resumption of lever pressing for stimulant self-administration.

Response reinstatement produced by intra-NAc injections of DA was blocked by coinjection of the DA receptor antagonist fluphenazine or the AMPA Glu receptor antagonist CNQX; the NMDA receptor antagonist (±)-CPP was ineffective. The same was found for reinstatement produced by systemic cocaine except that NAc fluphenazine did not block the effect. This result suggested that response reinstatement produced by systemic cocaine did not depend on the action of cocaine on DA neurons in NAc *(99)*. These researchers also showed that NAc injections of the NMDA receptor-agonist *cis*-ACDA

or AMPA itself reinstated responding; the effect of AMPA was blocked by CNQX but not by fluphenazine *(92,99)*.

In rats with excitotoxic lesions of the BLA, cocaine-associated cues were ineffective in the reinstatement of extinguished lever pressing previously rewarded with cocaine but now rewarded only with a conditioned stimulus that had been paired with cocaine *(100)*. See et al. *(95)* examined the effects of AP5 or CNQX injected into the BLA in the reinstatement paradigm and observed no effect. Results implicate the BLA but not ionotropic Glu receptors in cue-induced response reinstatement.

Dizocilpine dose-dependently reinstated responding for cocaine in rats after an extinction period of 1–3 wk *(101,102)*. Dizocilpine is known to activate DA neurons and to increase regional DA concentrations *(73,75)* and treatments that increase DA are known to effectively reinstate responding for cocaine *(98)*. Thus, the observation that the NMDA receptor antagonist dizocilpine reinstates cocaine responding is consistent with many previous findings. Furthermore, these results suggest that NMDA receptor stimulation is not necessary for reinstatement. This conclusion is supported by the results of Bespalov et al. *(102)*. They showed that the NMDA receptor antagonists 3-(2-carboxypiperazin-4-yl)-1-propenyl-phosphonic acid (D-CPPene) or memantine had no effect on the reinstatement of responding produced by cocaine.

In summary, systemic cocaine, intra-NAc DA, or exposure to drug-paired cues reinstated previously extinguished responding for cocaine self-administration. The effects of NAc DA or drug-paired cues were blocked by DA or AMPA receptor antagonism but not by systemic or intra-NAc NMDA receptor antagonism. Intra-NAc NMDA or AMPA agonists produced response reinstatement; dizocilpine also produced reinstatement but its effect was probably mediated by DA. The resumption of responding for stimulant self-administration elicited by drug-paired cues was blocked by excitotoxic lesions of the BLA but not by NMDA or AMPA antagonists in BLA.

2.2.7. Glutamate and the Acquisition of Lever Pressing for Conditioned Reward

A stimulus that is repeatedly paired with a primary rewarding stimulus (e.g., food), acquires the ability to act as a reinforcing or rewarding stimulus in its own right; such a stimulus is termed a conditioned reward. Animals will learn an operant response, such as lever pressing when a conditioned rewarding stimulus is made contingent on that response. Many studies have shown that treatment with agents such as amphetamine that augment DA neurotransmission specifically enhance responding for conditioned reward and DA receptor antagonists block learning with conditioned reward. Similar enhancing effects have been reported following intra-NAc injections of amphetamine, DA, and a number of DA agonists (for a review, *see* ref. *22*).

The enhancement of responding for conditioned reward produced by intra-NAc injections of amphetamine was blocked by coinjection of AP5 or CNQX *(35,103)*. CNQX, when injected alone, also decreased the conditioned reward effect itself *(35)*. Coinjection of the Glu receptor agonists NMDA, AMPA, or quisqualate impaired the amphetamine-produced enhancement of responding for conditioned reward. NMDA alone also decreased responding on the conditioned reward lever *(35)*. Results implicate NAc Glu acting at NMDA, AMPA or kainate receptors in the effects of amphetamine on responding for conditioned reward. They suggest that there is an optimal level of receptor stimulation for the acquisition of responding; either increases to levels above or decreases to levels below that putative optimum impair learning.

BLA or ventral subicular lesions decreased responding for conditioned reward *(104–106)*. BLA lesions did not affect the ability to discriminate *(104)*. Whereas BLA lesions did not block the enhancement of responding produced by NAc amphetamine, ventral subiculur lesions did *(105)*. Microinjection of CNQX into BLA or ventral subiculum decreased responding for conditioned reward, but injections of CNQX into BLA also increased responding on a second manipulandum that produced no programmed consequences *(107)*. These results implicate BLA AMPA/kainate receptors in the control of behavior by discriminative stimuli and ventral subiculur AMPA/kainate receptors in reward efficacy.

2.3. Glutamate and Appetitive Learning: Place-Conditioning Tasks

The previous sections focused on the acquisition and expression of lever press responses rewarded by several different types of stimuli (i.e., food, BSR, psychomotor stimulants, or conditioned rewards). It is also possible to assess the rewarding qualities of stimuli by pairing them with a particular environment or place and then assessing the animals' response to that place. These types of studies can use the conditioned place preference paradigm, where the animal chooses between alternatives with differential conditioning histories, or the conditioned activity paradigm, where animals' locomotor activity is observed in an environment previously paired with a rewarding stimulus. Both types of paradigms have been used to assess the role of DA and Glu in reward-related learning.

2.3.1. Glutamate and the Acquisition of a Conditioned Place Preference

The conditioned place prefences (CPP) procedure usually involves the pairing of one chamber of a two- or three-chambered apparatus with a rewarding stimulus (e.g., cocaine or amphetamine). One or more features such as floor texture, wall design, or odor, as well as their location in space, normally distinguish the chambers. In the test, animals are given access to all chambers and the amount of time spent in each is measured. If an animal spends more time in the environment previously paired with the rewarding stimulus, a CPP is said to have occurred.

Many data implicate DA in CPP learning. Thus, DA agonists produce a CPP that is blocked by cotreatment during conditioning with DA receptor antagonists *(108)*. The rewarding effects of a number of agents including opiates also appear to depend on DA *(109)*. Central-injection studies have shown the NAc to be an important region for producing CPP effects *(110,111)*. CPP has been seen with both D1-like receptor agonists *(112)* and D2-like receptor agonists *(113)*. For a thorough review, *see* Tzchentke *(114)*.

The role of Glu in the acquisition of CPP can be considered in studies that evaluate the possible rewarding properties of Glu agents themselves or in studies that evaluate the effects of Glu agents on reward produced by other agents.

2.3.1.1. Acquisition of Conditioned Place Preference With Glutamatergic Agents

The competitive NMDA receptor antagonist DL-(E)-2-amino-4-methyl-5-phosphono-3-pentonoic acid (CPG 37849) produced a CPP. Excitotoxic lesions of the infralimbic, prelimbic, or anterior cingulate regions of the mPFC blocked CPP produced by CPG 37849. A CPP based on systemic cocaine or morphine but not amphetamine was also blocked by lesions to one of these subregions of the mPFC *(115)*. These results implicated these regions of the mPFC in reward produced by the NMDA receptor antagonist CPG 37849.

PCP produced a dose-dependent conditioned place *aversion* in a number of studies *(116–119)*. This effect was blocked by the DA D1-like receptor antagonist SCH 23390 *(118)* and by the serotonin 5-HT3 receptor-antagonists ICS 205-930 or MDL 72222, implicating these monoamines in this action of PCP.

Dizocilpine produced a CPP in many studies *(120–126)*; the one exception, Tzschentke and Schmidt *(127)* tested only one dose. CPP has been reported with CPG 37849, as noted above, and its (R)-enantiomer CPG 40166 *(115,122,125,127)* and with the Glu release inhibitor riluzole *(128)*. 1-Aminocyclopropanecarboxylic acid (ACPC), a partial agonist at the strychnine-insensitive glycine site, that acts as a functional NMDA receptor-antagonist, failed to produce a CPP *(125,129)*. The low-affinity noncompetitive NMDA receptor antagonist memantine did not produce a CPP *(130)*.

We know of only one study that has evaluated the role of AMPA receptors in the acquisition of CPP with Glu agents. The selective AMPA receptor antagonist 1-(4-aminopheyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466) did not produce a CPP *(131)*.

The nonspecific Glu receptor antagonist kynurenic acid did not produce a CPP *(76)*. Similarly, inhibitors of the enzyme *N*-acetylated-α-linked-acidic dipeptidase (NAALADase), which hydrolyzes the dipeptide *N*-acetyl-aspartyl-glutamate (NAAG) to n-acetylaspartate and Glu, failed to produce a CPP. Thus, no CPP effect was seen with 2-(phosphonomethyl)pentanedioic acid (2-PMPA) or GPI 5693 *(132)*.

Papp et al. *(125)* suggested that DA may mediate the CPPs produced by dizocilpine, CPG 37849, and CPG 40166. These agents increase activity of DA neurons of the VTA-NAc system *(73–75)* and Papp et al. *(125)* cite preliminary results showing that the DA receptor antagonist haloperidol blocked the CPP produced by dizocilpine or the CPG compounds. They point out that glycine receptor antagonists like ACPC, a functional NMDA receptor antagonist that failed to produce a CPP, do not modulate the spontaneous activity of DA neurons. Thus, DA may mediate the CPPs produced by dizocilpine, CPG 37849, and CPG 40166. As these agents also block NMDA receptors, results suggest that the acquisition of a CPP to these agents can take place when NMDA receptors are blocked.

2.3.1.2. Effects of Glutamatergic Agents on Acquisition of CPP With Other Agents

The CPP produced by methamphetamine in mice was blocked by dizocipline *(133)* but a similar experiment in rats with amphetamine showed no effect *(121)*. However, in rats the Glu release inhibitor riluzole blocked the CPP produced by amphetamine *(128)* as did ACPC, a functional NMDA receptor antagonist *(129)*. NAc injections of the AMPA/kainate receptor antagonist DNQX blocked the CPP produced by amphetamine in rats *(134)* but systemic injections of the selective AMPA receptor antagonist 2,3-dihydro-6-nitro-7-sulphamoyl-benzo(f)quinoxaline (NBQX) did not *(135)*. Taken together, results suggest that NMDA and possibly NAc kainate but not AMPA receptors are critical for the acquisition of a CPP with amphetamine.

Acquisition of a cocaine-produced CPP in mice or rats was blocked by the noncompetitive NMDA receptor antagonist dizocilpine *(136,137)* and in rats by the partial agonist at the strychnine-insensitive glycine site ACPC, a drug that acts as a functional NMDA receptor antagonist *(129)*. Cocaine CPP was also blocked by NAc injections of the AMPA/kainate receptor antagonist DNQX *(138)* but not by intracerebroventricular injections

of DNQX *(136)*. The NAALADase inhibitors 2-PMPA and GPI 5693 blocked acquisition of a CPP to cocaine *(132)*. Results implicate NMDA and possibly AMPA and kainate receptors in the acquisition of a CPP with cocaine.

The CPP produced by morphine also was sensitive to manipulations of Glu function. Dizocilpine blocked the acquisition of a morphine CPP in mice *(124)* and rats *(127,131)* as did CGP 37849 in rats *(127)*. Morphine CPP was also blocked by the low-affinity noncompetitive NMDA receptor antagonist memantine *(130)* and by ACPC *(129)*. The Glu release inhibitor riluzole or the nonspecific Glu receptor antagonist kynurenic acid blocked the acquisition of a morphine CPP *(76,128)*. The acquisition of a morphine CPP was not blocked by NAc injections of the AMPA/kainate receptor antagonist DNQX *(134)*. Results implicate NMDA receptors in the acquisition of a CPP with morphine.

Agents that reduce Glu neurotransmission also blocked the acquisition of a CPP to other rewarding drugs. Thus, ACPC, a partial agonist at the strychnine-insensitive glycine site that acts as a functional NMDA receptor antagonist, blocked the CPP produced by nicotine, nomifensine, or diazepam *(129)*. These results further implicate NMDA receptors in the acquisition of CPP to rewarding drugs.

Food produces a CPP *(139)*. Acquisition of a food-based CPP was not blocked by memantine *(130)* or by ACPC *(129)*. ACPC did not affect CPP based on sucrose, social interaction, or novelty *(129)*. Results suggest that NMDA receptors may not be necessary for the establishment of a CPP based on a number of natural rewards.

With the exception of natural rewards, results from studies investigating the role of Glu in the acquisition of CPP implicate NMDA receptors. Thus, NMDA receptor antagonists blocked CPP based on amphetamine, cocaine, morphine, and several other rewarding drugs. The effects of AMPA/kainate receptor antagonists were less consistent.

2.3.2. Glutamate and the Expression of CPP

Once conditioning to one side of a CPP apparatus has taken place, the effects of various treatments on the expression of a CPP can be assessed. It has generally been found that conditioned responses that require DA for their acquisition are transiently resistant to DA receptor antagonists during the expression phase (*see* Subheading 2.2.2.). For example, CPP based on cocaine was not blocked by SCH 23390 or sulpiride in the test phase *(136)*.

The expression of a CPP based on amphetamine was blocked by NAc injections of the AMPA/kainate receptor antagonist DNQX *(134)* or by systemic injections of the similarly acting compound CNQX *(135)*. Surprisingly, systemic injections of the selective AMPA receptor antagonist NBQX did not block expression *(140)* but the similarly selective compound GYKI 52466 did *(131)*. L-701,324, an antagonist at the strychnine-insensitive glycine site, blocked expression of CPP based on amphetamine. These observations led Mead and Stephens *(135)* to argue that the effects of CNQX could be attributed to its action at the glycine site of the NMDA receptor. The observation of Papp et al. *(129)* that ACPC, a partial agonist at the strychnine-insensitive glycine site, blocked the expression of a CPP based on amphetamine is consistent with this suggestion. Bespalov *(141)* showed that the NMDA receptor antagonist (±)-CPP blocked expression of a CPP based on amphetamine. Results implicate NMDA receptors and possibly AMPA/kainate receptors in the expression of a CPP based on amphetamine.

NAc or icv injections of DNQX blocked expression of a CPP based on cocaine *(136,138)*. Dizocilpine had no effect *(136)* but ACPC produced a block *(129)*. The

NAALADase inhibitors 2-PMPA and GPI 5693 blocked expression of a CPP based on cocaine *(132)*. Results implicate AMPA/kainate receptors and possibly NMDA receptors in the expression of a CPP based on cocaine.

The expression of a CPP based on morphine was blocked by NAc injections of DNQX *(134)*. Intra-NAc or VTA injections of the NMDA receptor antagonist 2R,4R,5S-2-amino-4,5-(1.2-cyclohexyl)-7-phospoonoheptanoic acid (NPC 17742) also blocked the effect *(142)*. Systemic injections of dizocilpine, memantine, NPC 17742, GYKI 52466, or kynurenic acid blocked the expression of morphine CPP *(76,130,131,142,143)* but ACPC was without effect *(129)*. Results implicate both NMDA and AMPA/kainate receptors in the expression of a CPP based on morphine.

The expression of a CPP based on food was not affected by memantine, ACPC, 2-PMPA, or GPI 5693 *(129,130,132,143)*. Memantine blocked the expression of a CPP based on sexual interaction *(143)*. ACPC did not block the expression of a CPP based on diazepam or nicotine but did block the CPP based on nomifensine *(129)*. Results suggest that the expression of CPP based on food may be independent of Glu NMDA receptors.

2.3.3. Glutamate and the Acquisition of Conditioned Activity

When injections of a psychostimulant drug are repeatedly paired with a particular environment, that environment will acquire the ability to elicit enhanced locomotor responses in the future when the animal is placed there in a drug-free state. This effect has been observed following drug-environment pairings with amphetamine *(144)* or cocaine *(145)*. D1- or D2-like DA receptor agonists *(146,147)* also produced conditioned activity. D1- but not D2-like DA receptor antagonists blocked the establishment of conditioned activity based on amphetamine or cocaine *(147–150)*. Thus, both D1- and D2-like receptors appear to play a role in the establishment of conditioned activity.

Several studies have investigated the effects of Glu receptor antagonists on the establishment of conditioned activity. Thus, coinjections of the NMDA receptor antagonist dizocilpine with amphetamine or cocaine during conditioning blocked the conditioned activity effect; the doses of dizocilpine that blocked the effect did not affect unconditioned locomotion stimulated by amphetamine or cocaine ruling out a nonspecific motor effect *(150–152)*. Intracerebroventricular injections of the NMDA receptor antagonist (±)-CPP blocked acquisition of conditioned activity based on cocaine *(153)*. Icv injections of the AMPA/kainate receptor antagonist DNQX during conditioning sessions also resulted in a loss of the conditioned activity effect *(150)*. Results implicate NMDA, AMPA, and kainate receptors in the acquisition of conditioned activity.

2.3.4. Glutamate and the Expression of Conditioned Activity

Expression is tested after pairing sessions have taken place. When the minimal effective dose for blocking acquisition of the effect was identified and used in the test, it was found that the D2-like DA receptor antagonist pimozide failed to block expression of conditioned activity based on amphetamine or cocaine *(154,155)*. Similar results have been reported for haloperidol *(156)*. SCH 23390 decreased activity levels in amphetamine-conditioned and unpaired groups when given in the test, but it failed to block the expression of conditioned activity based on cocaine *(150)*. When considered in conjunction with the effects of DA receptor antagonists reviewed in Subheading 2.3.4, results suggest that DA acting at both receptor families plays a more important role in the establishment than expression of conditioned activity.

NAc injections of the NMDA Glu receptor-antagonist (±)-CPP dose-dependently blocked expression of conditioned activity based on systemic amphetamine or morphine; similar injections into the dorsal striatum were without effect *(157)*. Systemic injections similarly decreased expression of conditioned activity based on systemic cocaine. Thus, dizocilpine, memantine, D-CPPene, the glycine site antagonist 5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione (ACEA-1021), or the polyamine site antagonist eloprodil decreased the expression of conditioned activity; memantine, ACEA-1021, and eloprodil did this at doses that did not affect spontaneous activity *(158)*. In another study, dizocilpine or icv (±)-CPP failed to block expression of conditioned activity based on cocaine *(150,153)*. Results were conflicting. Some implicated NMDA Glu receptors, possibly in NAc, in the expression of conditioned activity and some results were negative.

In mice conditioned with amphetamine, the AMPA receptor antagonist NBQX blocked expression of the conditioned activity effect at a dose that did not affect spontaneous locomotor activity *(140)*. DNQX similarly blocked the expression of conditioned activity based on cocaine conditioning *(150)*. In rats conditioned with cocaine, the AMPA receptor antagonist GYK 52466 blocked expression of the conditioned activity effect *(159)*. This latter study used intracerebral microdialysis of NAc to show that conditioned stimuli associated with cocaine increased Glu release. Results implicate AMPA and kainate, along with NMDA receptors in the expression of conditioned activity.

3. MECHANISMS OF LEARNING

In recent years, the molecular mechanisms of learning have been extensively studied. This work is exemplified by investigations of the sea slug *Aplysia (160)*. However, numerous other species have been studied. Thus, somewhat similar mechanisms have been identified in *Caenorhabditis elegans (161), Drosophila (162)*, honey bee *(163)*, chick *(164),* and rat *(160,165,166)*. There appears to be a high level of conservation of the mechanisms for producing learning and memory across phylogeny. This apparent conservation extends to the putative mechanism underlying learning produced by rewarding stimuli.

3.1. Role of Glutamate and Dopamine in the Striatum and NAc

Beninger *(3)* first proposed a synaptically based mechanism for reward-related learning. He proposed a heterosynaptic facilitation model involving the ability of DA afferents, acting at D1-like receptors, to modulate coterminating cholinergic afferents on medium spiny straital neurons; at the time there was good evidence for such an interaction between DA and cholinergic synapses but little evidence for a DA–Glu interaction. In the following decade, evidence for a DA–Glu interaction accumulated, leading Wickens *(21,167,168)* and his coworkers *(6,169)* to propose that DA-mediated reward-related learning occurred as a result of the modulation by DA of Glu synapses made by cortical afferents on the spines of medium spiny striatal neurons. There is now extensive electrophysiological and neurochemical evidence supporting this model *(170–174)*.

The model fits into the following context (cf. ref. *175*). Most of the neurons in the striatum and the NAc are of the medium spiny type. These neurons use GABA as their principal neurotransmitter and are the principal projection neurons of the striatum *(176)*. The spines of these neurons receive Glu inputs from cortical neurons and DA inputs from mesencephalic neurons *(177)*. Among other things, cortical inputs carry information

about the perception of stimuli in the environment and the output neurons of the striatum and NAc influence motor action. DA, by modifying the strength of Glu synapses in the striatum and NAc, would be able to change the behavioral impact of associated environmental stimuli that activate those synapses. One important feature of the model is that DA, although released at multiple synapses when a rewarding stimulus is encountered, would only act to strengthen Glu synapses that were recently active when stimuli associated with reward were present; that is, at Glu synapses that are in a state of readiness *(178)*. As outlined in the next Subheading, the molecular events underlying DA-mediated learning are beginning to yield to the efforts of researchers.

3.2. Role of Signaling Molecules in Reward-Related Learning

Space does not permit an extensive review of the molecular signaling cascades that are thought to mediate the modulating influence of DA on Glu synapses and the reader is referred to several recent reviews *(22,23,179,180)*. The series of events might include the following: When environmental stimuli are encountered, a subset of allo- and neocortical cells is activated and their corresponding synapses in the striatum and NAc release Glu. This event leads to stimulation of NMDA receptors and an increase in calcium concentrations ($[Ca^{2+}]$) in the dendritic spines that receive these synapses. Wickens *(21)* proposed that this event might represent the state of readiness proposed by Miller *(178)*. Increased spine $[Ca^{2+}]$ leads to activation of enzymes including Ca^{2+}-dependent protein kinase (PKC) and Ca^{2+}- and calmodulin-dependent protein kinases (CaMKs); these enzymes phosphorylate a variety of proteins including, for example, AMPA receptors, altering their open time, and are known to be necessary for some of the molecular signals produced by stimulation of D1-like DA receptors *(181,182)*. In the absence of a DA input in close temporal contiguity with the Glu input that establishes this state of readiness, the enzyme protein phosphatase I (PP I) will dephosphorylate recently phosphorylated proteins and undo the putative state of readiness.

When reward occurs and DA is released, stimulation of D1-like receptors will lead to activation of adenylyl cyclase and stimulation of cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA). PKA phosphorylates DA- and cAMP-regulated phosphoprotein (DARPP-32), which, in turn, inhibits PP I. As a result, the newly phophorylated proteins can endure within the synaptic spine; for example, recently phosphorylated AMPA receptors would remain phosphorylated. In addition, activation of PKA leads to activation of cAMP-response-element-binding-protein (CREB), a transcription factor involved in gene expression. CREB activation requires not only PKA but also stimulation of NMDA receptors and increases in $[Ca^{2+}]$ *(181,182)*; this makes it an excellent candidate for mediating temporally contiguous activation of DA and Glu receptors on synaptic spines of medium spiny neurons.

These are only a few of the many molecular events that occur upon stimulation of DA or Glu receptors. Another class of enzymes is the mitogen-activated protein kinase (MAPK) family. These include extracellular signal-regulated kinase (ERK), which has been implicated in learning and memory *(183)*; ERK mediates the ability of PKC to phosphorylate CREB. DA directly activates the p38 MAPK in a PKA-dependent manner and it activates the transcription factors CREB and Elk-1 *(184)*. c-Jun-*N*-terminal kinase (JNK) is another MAPK that phosphorylates activating transcription factor 2 (ATF-2), a CREB family member *(185)*. The ability of amphetamine to activate MAPKs has been

found to depend on mGluRs *(186)*. These kinases also might play a role in reward-related learning. Relevant studies are reviewed in the next section.

3.3. Pka and Reward-Related Learning

3.3.1. PKA and the Acquisition of Approach Responses

Appetitive conditioning is dependent on a corticostriatal circuit involving the BLA *(136)* and PKA manipulations in the BLA affect the acquisition of approach behavior. Jentsch et al. *(187)* infused cholera toxin (CTX), the PKA inhibitor adenosine 3′,5′-cyclic phosphorothioate-Rp (Rp-cAMPS), or the PKA activator adenosine 3′,5′-cyclic phosphorothioate-Sp (Sp-cAMPS) into the BLA and assessed acquisition of approach responses (nose pokes into a food tray) to a conditioned stimulus signaling food. G proteins of the G_s family are positively coupled to the cAMP-PKA pathway. CTX, binding to G_s, prolongs the activation of G_s proteins effectively upregulating the cAMP-PKA pathway. BLA CTX increased approaches to the food tray during the conditioned stimulus. The same was true for lower doses of Sp-cAMPS infused either immediately before or after the training sessions, but a higher dose of Sp-cAMPS decreased food tray approach. Pretraining BLA infusions of Rp-cAMPS decreased approach responses. Baldwin et al. *(33)* also analyzed nose pokes into the food tray in the context of a lever pressing task. Consistent with Jentsch et al. *(187)*, they found that mPFC Rp-cAMPS impaired the acquisition of nose pokes. In an identical task, Baldwin et al. *(188)* found that NAc infusions of the broad serine/threonine kinase inhibitor H7, Rp-cAMPS, or Sp-cAMPS impaired acquisition of approach responses.

Results with agents that reduce PKA activity in the NAc, BLA, or mPFC were consistent in showing impairment in the acquisition of approach responding during an appetitive conditioned stimulus. Agents that augmented PKA activity in the BLA augmented learning at low doses but impaired it at higher doses and these agents in NAc impaired learning. Results implicate NAc, BLA, and mPFC PKA in the acquisition of approach responses during conditioned stimulus presentation.

3.3.2. PKA and the Acquisition of Lever Pressing for Food

PKA may be necessary for the acquisition of lever pressing for food. Baldwin et al. *(188)* trained rats to lever press for food over 10 d on an FR 1 schedule. Drug manipulations were introduced on days 1–4 to study the role of PKA in acquisition. Immediate posttraining NAc infusion of H7 or immediate pretraining infusion of the PKA inhibitor Rp-cAMPS dose-dependently impaired acquisition of lever pressing for food. Smaller impairments were produced by infusion of Rp-cAMPS immediately or 1 hr after training sessions. Acquisition also was impaired by NAc infusion of the PKA activator Sp-cAMPS. This finding suggested that reward-related learning occurred at an optimal window of activation for PKA and that either lower or higher levels of activation resulted in impairment. A. Kelley's group also showed that infusion of Rp-cAMPS into the mPFC 5 min before training impaired learning *(188)*.

In summary, inhibition of PKA in NAc or mPFC impaired the acquisition of lever press responding for food. Stimulation of PKA in NAc also impaired acquisition.

3.3.3. PKA and the Expression of Lever Pressing for Food

To assess the role of PKA in the expression of lever pressing for food, Baldwin et al. *(188)* injected rats on test day 10 of training for lever pressing for food with Rp- or

Sp-cAMPS into NAc. Neither drug impaired the expression of lever pressing for food. Results reported by Self et al. *(189)* appear to agree with the these findings. In a study on self-administration of cocaine, Self et al. *(189)* tested a food reward group in which rats had been trained to lever press for food pellets on an FR 1/time-out 2-min schedule. Neither Rp- nor Sp-cAMPS, injected into NAc 30 min before testing, impaired lever pressing for food on the FR 1 schedule. However, Rp-cAMPS decreased responding during time-out periods and on an inactive lever. Neither of these studies tested the effects of repeated NAc treatments with a PKA inhibitor on the expression of lever pressing for food. Results showed that established responding was resistant to PKA inhibition in NAc on the first day of drug testing.

3.3.4. PKA and the Expression of Lever Pressing for Stimulant Self-Administration

Self et al. *(190)* found that inhibition of NAc G proteins G_i and G_o with pertussis toxin (PTX) produced long-lasting (up to a month) changes in iv self-administration of cocaine or heroin. PTX produced a rightward shift in the dose–response curve for both drugs. This effect was consistent with reducing the self-administered dose of the drug, causing the animal to compensate by increasing drug intake. Thus G_i/G_o proteins may be necessary for the rewarding effects of cocaine and heroin. G_i/G_o proteins are negatively coupled to the cAMP-PKA pathway, suggesting that an upregulation of PKA may have resulted in decreased reward and hence higher responding.

Self et al. *(189)* directly studied the role of PKA in cocaine self-administration. When multiple doses of cocaine were tested, the PKA inhibitor Rp-cAMPs produced a leftward shift in the dose–response curve, consistent with an *enhancement* of reward. The opposite was found for Sp-cAMPS, suggesting that increased activation of PKA decreased reward. In addition, Rp- but not Sp-cAMPs induced relapse in cocaine seeking when injected into the NAc and enhanced cocaine-induced relapse of cocaine seeking. The effects of PKA inhibition and PKA activation resembled the effects of respectively increasing and decreasing the unit dose of cocaine per injection; this observation suggested that the levels of PKA activation varied negatively with the rewarding properties of cocaine! The finding that PKA inhibition failed to block established responding for stimulant self-administration is consistent with the finding that PKA inhibition failed to affect established lever press responding for food (Subheading 3.2.3.) but the apparent increase in reward with PKA inhibition appears to be inconsistent. The difference may be explained by the nature of the self-administration paradigm. Animals were trained to self-administer cocaine in daily sessions over a period of 10 d before drug testing. As it has been shown that this may result in long-term adaptations at the cellular level (e.g., ref. *180*), it is possible that the functional role of PKA was affected by these changes.

3.3.5. PKA and the Acquisition of Lever Pressing for Conditioned Reward

Kelley and Hollahan *(191)* paired a compound light/click stimulus with food over several days. They then injected rats with NAc CTX and evaluated the acquisition of lever pressing for the compound stimulus alone. Responding was markedly enhanced by infusion of CTX into the NAc but not into the dorsal striatum. Results suggest that enhanced coupling of G_s proteins to receptors in NAc and subsequent increased activation of PKA, which occurs when G_s-coupled receptors are stimulated by DA acting at D1-like receptors, increases the acquisition of lever press responding for conditioned reward.

3.3.6. PKA and the Acquisition of a CPP

CPP is produced by NAc injections of amphetamine during pairing sessions *(110,111)*. Beninger et al. *(192)* found that coinjections of amphetamine plus the PKA inhibitor Rp-cAMPS produced a dose-dependent blockade of the CPP effect. Rp-cAMPS or the PKA activator Sp-cAMPS alone failed to affect time spent on the paired side. Coinjections of a subthreshold dose of amphetamine plus Sp-cAMPS also failed to affect side preference. On the other hand, coinjection of a dose of amphetamine that produced a CPP on its own plus the PKA activator Sp-cAMPS during conditioning led to a loss of the CPP effect.

Cocaine CPP may also be mediated by PKA. Icv infusions of the nonselective protein kinase inhibitor H7 impaired systemic cocaine-induced CPP when infused immediately before or after each conditioning session. The PKA inhibitor H89 when given immediately after each conditioning session also impaired the cocaine CPP *(193)*.

Results with Rp-cAMPS suggested that PKA activation consequent to injections of amphetamine into NAc was necessary for the establishment of a CPP and those with icv H7 or H89 similarly suggested that PKA activation may be necessary for the acquisition of a CPP produced by cocaine. The finding that CPP acquisition based on NAc amphetamine was impaired by activation of PKA was consistent with the similar findings that acquisition of approach responses to an appetitive conditioned stimulus (Subheading 3.3.1.) or lever pressing for food (Subheading 3.3.2.) were impaired by Sp-cAMPS injected into NAc.

3.3.7. PKA and the Expression of a CPP

One study reported on the effects of H7 injected icv during testing following conditioning with systemic cocaine. There was no effect on the expression of cocaine CPP *(193)*. Although H7 is a nonspecific serine/threonine kinase inhibitor, this result is consistent with the finding that the expression of lever pressing for food (Subheading 3.3.3.) or stimulant self-administration (Subheading 3.3.4.) was not blocked by PKA inhibition.

3.3.8. PKA and the Acquisition or Expression of Conditioned Activity

Conditioned activity resulting from pairing NAc amphetamine administration with the test environment was blocked dose-dependently by coinfusion of Rp-cAMPs *(194)*. NAc infusions of Rp-cAMPs enhanced unconditioned amphetamine-induced locomotion on conditioning days showing a dissociation of the role of PKA in locomotor activity vs learning. Results of a related study showed that NAc PKA inhibition on the test day not only failed to block the expression of conditioned activity, it enhanced the effect (Beninger et al., in prep.). Results support previous findings implicating PKA in the acquisition but not the expression of reward-related learning.

3.4. PKC and Reward-Related Learning

As mentioned in Subheading 2.2.1., Ungerer and associates evaluate the effects of treatments on the acquisition of lever pressing for food by injecting drugs after training and evaluating their effect on the spontaneous improvement normally seen 24 h later. Using this approach, Stemmelin et al. *(195)* showed that mice injected icv with the PKC inhibitor GF 109203X failed to show spontaneous improvement. Results supported a role for PKC in the acquisition of lever pressing for food.

A few recent studies have implicated PKC in the acquisition of a CPP based on amphetamine, cocaine, or morphine. Intra-NAc co-infusions of the PKC inhibitor NPC 15437 before each conditioning session impaired CPP produced by NAc injections of amphetamine *(196)*. Similarly, icv injection of the PKC inhibitor chelerythine immediately after but not before pairing sessions impaired CPP produced by systemic cocaine *(193)*. No CPP was observed following injection of the PKC inhibitors NPC 15437 alone into NAc *(196)* or calphostin C alone icv *(197)*.

The opioid morhpine also has the ability to elicit a robust CPP and this effect requires intact DA transmission *(198)*. Narita et al. *(197)* found that icv infusion of the PKC inhibitor calphostin C impaired place preference produced by morphine. These authors also tested mutant mice lacking the PKCγ gene. These mice did not show morphine-produced CPP suggesting that the PKCγ isoform mediates the rewarding effects of morphine.

Paradigms other than CPP have also been investigated. One recent study implicated the PKCγ isoform in associative learning for drug-related cues *(199)*. These authors paired a conditioned stimulus with cocaine injection in a self-administration procedure. Subsequent presentation of the conditioned stimulus alone resulted in upregulation of PKCγ expression in NAc core and BLA, suggesting that PKCγ may play a role in expression of learning in this paradigm.

In summary, PKC inhibition in NAc blocked CPP produced by NAc amphetamine and icv PKC inhibition blocked CPP produced by cocaine or morphine. Morphine-produced CPP was also absent in mutant mice lacking the PKCγ gene. PKCγ levels were increased in the NAc core and BLA following presentation of reward-related cues. Results implicate PKC in reward-related learning.

3.5. MAPK and Reward-Related Learning

The MAPKs include three subfamilies: ERK, p38, and JNK. Some recent work has implicated MAPKs in reward-related learning. None of the ERK inhibitor PD98059, the p38 inhibitor SB23580, or the JNK inhibitor SP600125 injected alone into NAc produced a CPP *(200)*. However, MAPKs may mediate CPP produced by cocaine, amphetamine, or morphine.

Systemic administration of the ERK inhibitor SL 327 impaired cocaine-induced CPP and cocaine-stimulated locomotion in mice *(201)*. Our lab has recently performed experiments testing the effects of all three MAPK subtypes on NAc amphetamine-produced CPP. We found that the ERK inhibitor PD98059 and the p38 inhibitor SB23580 but not the JNK inhibitor SP600125 dose-dependently impaired amphetamine-produced CPP when injected into NAc 10 min before NAc amphetamine on conditioning days *(200)*. Unlike Valjent et al. *(201)*, we did not observe a decrease in amphetamine-produced locomotion during conditioning sessions. This finding showed a dissociation between the ability of NAc amphetamine to produce an increase in activity and its ability to produce a CPP.

The ERK MAPK subfamily includes ERK1 and ERK2. Most behavioral work has involved manipulations that did not discriminate between these two kinases. One recent study suggests that this approach may be an oversimplification. Mazzucchelli et al. *(202)* found that ERK1 knockout mice showed enhanced striatal ERK2 activation after an ip injection of the D1-agonist SKF 38393. Moreover, in contrast to the studies described above the ERK1 mutants showed an enhanced systemic morphine CPP. Clearly, more research is needed to better understand the separate contributions of ERK1 and ERK2 to reward-related learning.

4. CONSIDERATION OF THE EVIDENCE FOR A ROLE FOR GLUTAMATE IN REWARD-RELATED LEARNING FROM THE POINT OF VIEW OF THE MECHANISM OF DA–GLU INTERACTIONS IN LEARNING

If the acquisition of reward-related learning involves a DA–Glu interaction like that described in Subheading 3, it would be expected that NMDA receptor antagonists would impair this acquisition. Furthermore, if reward-related learning is mediated in part by a change in AMPA or kainate receptors, it might be expected that AMPA/kainate receptor antagonists would block expression of conditioned responses. As corollaries to these hypotheses, it might further be expected that NMDA receptor antagonists would have less of an effect on the expression of conditioned responses and that AMPA/kainate receptor antagonists would have less of an effect on the acquisition of conditioning. What do the data show?

Results from the data reviewed in Subheading 2 are summarized in Table 1. It is clear that NMDA receptor antagonists impaired acquisition of reward-related learning. An effect was observed in every case where data were available with one exception. The exception was that an NMDA receptor antagonist did not block acquisition of a CPP based on food; however, only two compounds have been tested and more data are needed. Results revealed that NMDA receptor antagonists impaired acquisition of responding in conditioned approach, lever press and place conditioning tasks based on a number of rewarding agents. Although limited data are available for specific brain regions, where regional specificity was examined, the NAc core, BLA, and PFC were implicated with a small number of studies implicating the NAc shell and VTA. It appears that NMDA receptors are necessary for the acquisition of reward-related learning.

Table 1 also reveals that AMPA/kainate receptors appear to be necessary for the expression of reward-related learning. With the exception of lever pressing to self-administer cocaine, where CNQX injected into the NAc or BLA had no effect, decreases in the expression of reward-related learning were observed in every case. Thus, the expression of lever pressing for food or BSR, place conditioning based on amphetamine, cocaine, or morphine, or conditioned activity based on either cocaine or amphetamine was decreased by an AMPA/kainate receptor antagonist. The NAc has been implicated. These results suggest that AMPA/kainate receptors are necessary for the expression of reward-related learning.

It was suggested that a corollary to the observation that NMDA receptors are necessary for the acquisition of reward-related learning is the hypothesis that they may not be necessary for the expression of this type of learning. As can be seen in Table 1, this was often observed. The expression of conditioned approach, lever pressing for food, BSR, or cocaine, or CPP or conditioned activity based on cocaine was not blocked by NMDA receptor antagonists in some studies. However, conflicting results (no effect or a decrease) were found in a number of cases and only a decrease was reported in others. It is interesting to note that the only column in Table 1 where conflicting data are reported within a particular paradigm and brain region is the one for expression of reward-related learning following treatment with an NMDA receptor antagonist. As discussed in Subheading 2.2.2., one explanation for the conflicting data relates to dose. In studies of the effects of DA receptor antagonists on established responding for reward, some studies have reported little initial effect whereas others have found a decrease. However, if care is taken to identify the minimum effective dose needed to block acquisition and then that

Table 1
Summary of the Effects of NMDA and AMPA/Kainate Glutamate Receptor Antagonists Injected Systemically or Regionally on the Acquisition and Expression of Responding for Reward in a Number of Paradigms

		NMDA antagonist		AMPA/kainate antag	
Paradigm	Region	Acqisition	Expression	Acquisition	Expression
Conditioned approach	Systemic/icv	Decrease	No effect	—	—
	NAc core	Decrease	No effect	No effect	—
	BLA	Decrease	No effect	—	—
	PFC	Decrease	No effect	—	—
Lever press: food	Systemic/icv	Decrease	No effect/Dec	—	Decrease
	NAc core	Decrease	No effect	—	—
	NAc shell	Decrease	No effect	—	—
	BLA	Decrease	No effect	—	—
	PFC	Decrease	No effect	—	—
Lever press: BSR	Systemic/icv	—	No effect[a]	No effect	—
	VTA	—	—	No effect	Decrease
Lever press: cocaine	Systemic/icv	Decrease	No effect[a]/Dec	—	Decrease
	NAc	—	No effect/Dec	—	No effect
	BLA	—	No effect	—	No effect
CPP: amphetamine	Systemic/icv	Decrease	Decrease	No effect[b]	Decrease
	NAc	Decrease	—	Decrease	Decrease
CPP: cocaine	Systemic/icv	Decrease	No effect/Dec	No effect	Decrease
	NAc	—	—	Decrease	Decrease
CPP: morphine	Systemic/icv	Decrease	Decrease	—	—
	NAc	—	Decrease	No effect	Decrease
CPP: food	Systemic/icv	No effect	No effect	—	—
Conditioned act to cocaine or amphetamine	Systemic/icv	Decrease	No effect/Dec	Decrease	Decrease
	NAc	—	Decrease	—	—

[a]Increase observed probably due to increased DA neurotransmission (*see* Subheading 2.2.3).
[b]AMPA-specific antagonist NBQX.
—indicates no data available.
Antag, antagonist; BLA, basolateral amygdala; BSR, brain stimulation reward; CPP, conditioned place preference; Dec, decrease; icv, intracerebroventricularly; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.

dose is tested on the expression of previously conditioned responding, minimal effects are seen. An example of this approach can be found in the elegant studies from A. Kelley's lab. These studies repeatedly have shown that doses of DA receptor antagonists

that block acquisition of responding for food reward have little or no effect on expression. It is well-known that higher doses of DA receptor antagonists produce decreases in motor activity or catalepsy so the observation that they also decrease conditioned responding based on reward is not surprising. By analogy to the observations from studies using DA receptor antagonists, perhaps the studies that have found that NMDA receptor antagonists impair the expression of reward-related learning have used higher doses than those necessary to block acquisition. These considerations and the results summarized in Table 1 suggest the tentative conclusion that NMDA receptors play a less important role in the expression than in the acquisition of reward-related learning. However, it is possible to disrupt the expression of conditioned responses with NMDA receptor antagonists. Further studies are needed to evaluate the hypothesis that this latter effect occurs at higher doses.

A second corollary to the finding that AMPA/kainate receptors are necessary for the expression of reward-related learning is the suggestion that they are less important for acquisition. Although fewer studies have been done in this category, examination of Table 1 reveals that when the effects of AMPA/kainate receptor antagonists were evaluated on the acquisition of reward-related learning, often no effect was seen. Thus, the acquisition of conditioned approach, lever pressing for BSR, and amphetamine, cocaine, or morphine CPP was unaffected by an AMPA/kainate receptor antagonist. On the other hand, a decrease in acquisition of amphetamine or cocaine CPP was reported following NAc DNQX and icv administration of this agent blocked acquisition of conditioned activity based on cocaine. Overall, results favor the conclusion that AMPA/kainate receptors are less important for acquisition than for expression of reward-related learning, but there are sufficient contradictory findings to keep this conclusion tentative until further studies are carried out.

Researchers studying other aspects of cognition also have concluded that NMDA Glu receptors appear to play a differential role in acquisition vs expression. For example, in a recent-memory task on the radial maze, it was found that the noncompetitive NMDA receptor antagonist dizocilpine impaired acquisition; when rats were pretrained on the maze and then tested with dizocilpine, no effect was observed *(203)*. These results are consistent with those reviewed here for reward-related learning (Table 1) and suggest that the acquisition but not the expression of recent memory may similarly depend on NMDA Glu receptors.

The effects of manipulations of Glu neurotransmission have been evaluated extensively in tests of spatial learning and fear conditioning. A review of these studies is beyond the scope of the present paper. Riedel et al. *(204)* have recently reviewed this material.

5. CONCLUSIONS

Although there are conflicting results, when the data reviewed in Subheading 1 are taken together (Table 1) they appear to provide support for the DA–Glu interaction model of reward-related learning presented in Subheading 3. Subheadings 3.3.–3.5. reviewed the relatively small number of studies that have evaluated the contribution of signaling molecules that are affected by DA and/or Glu to reward-related learning. Results supported a role for signaling pathways in reward-related learning and indirectly supported the DA–Glu interaction model. It will remain the task of future studies to

continue to explore the contribution of Glu receptor subtypes, their interaction with DA, and the contribution of signaling molecules in specific brain regions to reward-related learning. Continued success at this enterprise will move us closer to the identification of more effective treatments for a range of human disorders, many reviewed in this volume, that have been linked to DA–Glu interactions in the brain.

ACKNOWLEDGMENTS

Dedicated to Shan and Carmen. We wish to thank Dr. M. C. Olmstead for her helpful comments on an earlier draft of this manuscript. Funded by a grant from the Natural Sciences and Engineering Council of Canada to Dr. Richard J. Beninger.

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15

Sensitization and Relapse

Dopamine–Glutamate Interactions

David A. Baker and Peter W. Kalivas

1. INTRODUCTION

A consequence of repeated use of cocaine or other drugs of abuse is a transition in drug consumption from a casual and recreational style of use to a more compulsive and excessive pattern. Acute administration of psychomotor stimulants is associated with numerous effects that can contribute to casual drug consumption, including feelings of euphoria and increased energy, which can contribute to repeated recreational consumption. However, chronic administration of psychomotor stimulants results in the emergence of persistent craving and paranoia that contributes to compulsive drug-taking behavior *(1,2)*. The transition in drug use from casual to compulsive likely occurs as a result of drug-induced plasticity in brain functioning that is pathogenic for the relapse of drug-taking behavior *(3,4)*. Accordingly, identification of drug-induced plasticity that underlies the emergence and maintenance of compulsive drug-taking behavior is considered to be critical to the development of effective pharmacotherapies. Toward this end, the circuitry underlying compulsive drug-taking behavior must be delineated in order to narrow the search for drug-induced plasticity. Next, plasticity in brain functioning occurring following drug administration must be identified. Finally, the relevance of plasticity to drug-seeking behavior should be determined because plasticity in brain functioning could contribute, oppose, or be unrelated to compulsive drug-seeking behavior.

This chapter will summarize some of the progress toward identifying circuitry implicated in addiction-related behaviors, drug-induced plasticity within this circuitry, and the relevance of plasticity using animal models of addiction. Toward this end, the chapter will focus on findings obtained using cocaine in the behavioral sensitization and reinstatement paradigms, owing in part to the sheer volume of extant data with all drugs of abuse. Behavioral sensitization was chosen because it has been the most widely used paradigm to unearth behaviorally relevant drug-induced plasticity. Findings obtained using the reinstatement model are reviewed because this paradigm is one of the most valid animal models of relapse. In addition, both paradigms have been critical in identifying the circuitry underlying addictive-related behaviors.

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

2. ANIMAL PARADIGMS

2.2. *Overview of Reinstatement*

Drug addiction is characterized by a high relapse rate, which can be precipitated in human addicts by a drug prime, exposure to drug-paired stimuli, or stressful events *(5–8)*. These stimuli appear to have the capacity to elevate incentive motivation for cocaine, thereby increasing the likelihood of drug-seeking behavior. Although incentive motivation cannot be directly modeled, it can be inferred by assessing drug-seeking behavior in animals. In the reinstatement paradigm, rats are trained to self-administer a drug, such as cocaine, by performing an operant response (e.g., pressing a lever) for an infusion of drug. After adequate training and drug exposure, subjects typically undergo repeated extinction training in which an operant response either results in an infusion of saline instead of cocaine or has no scheduled consequences. Reinstatement of drug seeking can then be produced by the very stimuli that induce relapse in humans addicts, including a drug prime or exposure to drug-paired stimuli, or stress *(9–16)*. Thus, the reinstatement paradigm represents one of the most valid animal models of relapse of drug-seeking behavior, yet the difficulty and lower throughput relative to other animal paradigms, including behavioral sensitization, have limited the use of this paradigm.

The mechanisms underlying reinstatement differ from those that underlie the reinforcing properties of the drug. For instance, the rewarding properties of cocaine are dependent on dopamine (DA) neurotransmission, particularly within the nucleus accumbens *(17–21)*. In support, D1-like DA agonists are self-administered by rodents and nonhuman primates indicating that, similar to cocaine, D1-like agonists produce reinforcement *(22–25)*. In contrast, systemic administration of a DA agonist *blocks* cocaine-induced reinstatement in rodents or nonhuman primates *(24,26)*. The capacity of the reinstatement paradigm to distinguish between the reinforcing properties of cocaine, which are thought to underlie self-administration behavior, from the incentive motivational properties of the drug, which are thought to contribute to reinstatement behavior, is a critical feature of the reinstatement paradigm because the latter is thought to be critical for drug craving in human addicts *(27,28)*.

2.2. *Overview of Behavioral Sensitization*

Behavioral sensitization is defined as a progressive, enduring enhancement of drug-induced behavioral activation following repeated drug administration *(29–32)*. Studies examining the biological basis of behavioral sensitization typically distinguish between drug-induced plasticity that underlie the induction and expression of behavioral sensitization *(31)*. The *induction* of behavioral sensitization result from drug-induced plasticity occurring in response to acute pharmacological properties of the drug, as well as compensatory plasticity. Although typically short in duration, plasticity associated with induction may be necessary for the expression of sensitization. The *expression* of behavioral sensitization involves long-term plasticity that persist for months or even years following drug discontinuation *(33,34)*. Interestingly, the mechanisms underlying behavioral sensitization can be distinguished from those mediating the acute locomotor response to cocaine, which is dependent on DA neurotransmission *(35–38)*. This implies that sensitization is not merely an amplification of the acute locomotor response, but instead involves drug-induced plasticity resulting in abnormal activity of the circuitry.

The relevance of behavioral sensitization to addiction has been questioned, in part owing to the lack of face validity. However, findings obtained using behavioral sensitization demonstrate a close concordance to those obtained using other models of addiction, such as the reinstatement paradigm *(33)*, and in imaging studies in human addicts *(7,39–41)*. Moreover, behavioral sensitization, similar to addiction-related behaviors, occurs as a consequence of drug-induced plasticity within the motive circuit, and exhibits a similar temporal profile as the emergence of compulsive drug use and paranoia in human addicts *(42,43)*. Thus, sensitization may involve similar processes that underlie compulsive drug use. In addition, the ease of operation and high throughput capacity make it a productive and easily implemented behavioral screen regardless of discipline.

3. ANATOMY OF ADDICTION

Efforts to identify the neural basis of drug addiction have focused on the contributions of the mesoaccumbens DA system *(31,44,45)*, which originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens. This was in part because of the identification of monoamine transporters as the molecular site of action for cocaine and other psychomotor stimulants. Cocaine and methylphenidate bind to monoamine transporters preventing reuptake of extracellular DA norepinephrine, and serotonin *(46)*. Amphetamines serve as false substrates at monoamine transporters resulting in the release of cytosolic stores of these neurotransmitters *(47)*. Systemic administration of DA antagonists blocks the locomotor and reinforcing properties of cocaine, indicating that the acute behavioral effects of cocaine arise largely from binding to DA transporters *(37,48,49)*. Conversely, DA transmission does not appear to be necessary for behavioral sensitization because DA antagonists do not prevent the induction of cocaine behavioral sensitization *(50–53)*. Further, D1-*agonists* block cocaine-induced reinstatement *(24)*. As opposed to DA, glutamate transmission is critical for cocaine behavioral sensitization and reinstatement *(32,33)*. Taken together, these data demonstrate the importance of examining the contribution of the entire circuit of which the mesolimbic DA system is embedded to behavioral sensitization and reinstatement, notably the contribution of cortical and allocortical glutamatergic afferents to the mesolimbic projection.

3.1. Motive Circuit

The circuitry outlined in Fig. 1 has been termed the motive circuit and is key in translating incoming stimuli into a behavioral response *(54,55)*. A central component of the motive circuit is the mesoaccumbens system. Although primarily a dopaminergic projection, as much as 30% of the this pathway contains γ-aminobutyric acid (GABA) *(56)*. A second critical pathway originating in the VTA is the mesoprefrontal pathway, which sends dopamine projections to the prefrontal cortex (PFC). Akin to the mesoaccumbens pathway, almost 40% of the neurons in the mesoprefrontal pathway are GABAergic *(57)*. In addition to the PFC, the nucleus accumbens also receives glutamatergic afferents originating in the hippocampus, and basolateral amygdala *(58–62)*. The cells projecting from the nucleus accumbens are GABAergic medium spiny neurons that terminate in the ventral pallidum and ventral mesencephalon *(63,64)*.

DA and glutamate neurotransmission interact throughout the motive circuit. For instance, pyramidal cells in the PFC and medium spiny neurons in the nucleus accumbens are innervated by dopaminergic and glutamatergic projections. In fact, DA and

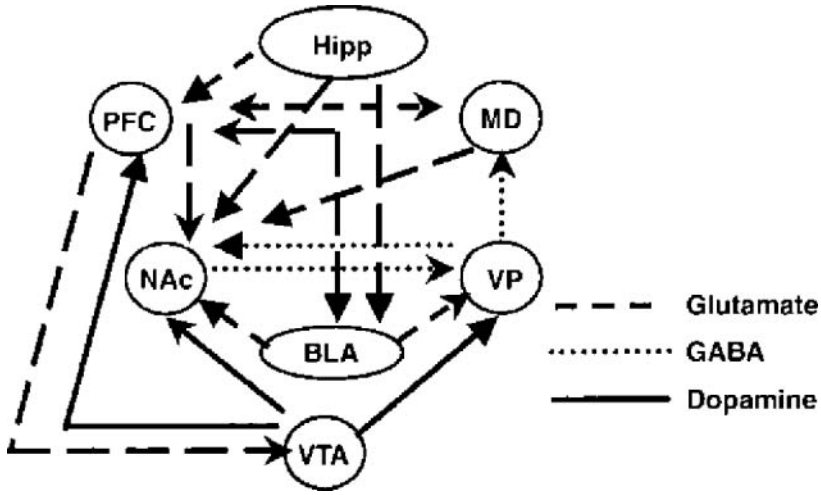

Fig. 1. Schematic illustrating a portion of the anatomical connections between regions typically included in the motive circuit: nucleus accumbens, mediodorsal thalamus, prefrontal cortex, basolateral amygdala, ventral pallidum, ventral tegmental area, and hippocampal formation. GABA, γ-aminobutyric acid; Hipp, hippocampal formation; PFC, prefrontal cortex; NAc, nucleus accumbens; BLA, basolateral amygdala; VTA, ventral tagmental area; VP, ventral pallidium; MD, mediodorsal thalamus.

glutamate afferents within the nucleus accumbens directly synapse onto the neck and head of the spine, respectively *(65,66)*. A similar orientation has been observed in the PFC where DA tends to synapses on more proximal regions of the pyramidal cell dendrite than glutamatergic afferents *(67,68)*. Within the VTA, cortical glutamatergic afferents synapse onto both GABAergic and dopaminergic neurons projecting to the nucleus accumbens and PFC *(69)*.

3.2. Neuronal Activity in Motive Circuit

A number of laboratories have utilized electrophysiological techniques in order to characterize the flow of information through the motive circuit. Collectively, these studies indicate that excitatory afferents to medium spiny neurons from the hippocampus and amygdala gate the activity of glutamatergic afferents from the PFC *(70)*. Medium spiny neurons in the nucleus accumbens exhibit bistable states characterized by cycling through a depolarized phase in which the cell is more excitable (up state) and a hyperpolarized nonfiring phase (down state) in which the cell is unlikely to fire *(71–73)*. Hippocampal glutamatergic afferents appear to regulate the transition to the depolarized phase because stimulation of fimbria–fornix produces a long-lasting duration of the up state *(72)*. Stimulation of basolateral amygdala also contributes to the transition to the depolarized phase, however, stimulation of this region produces a brief transition to the up state *(74)*. Conversely, PFC glutamatergic afferents to the accumbens do not alter the frequency of up or down states, but instead can produce action potentials in medium spiny neurons that are in the up state *(72)*. Stimulation of either *N*-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methylisoxazole-4-proionate (AMPA) receptors in the accumbens produces excitation in medium spiny neurons, however, AMPA but not NMDA receptor antagonists block glutamate-induced excitation of medium spiny neurons *(75)*.

DA neurotransmission in the accumbens also modulates the biphasic states observed in medium spiny neurons. D1-like receptors in the nucleus accumbens promote the function of NMDA receptors *(76,77)*, however, this occurs only when the neuron is in the up state owing to the voltage-dependent Mg^{2+} block of NMDA receptors. In contrast, D2-like receptors in the nucleus accumbens inhibit AMPA-mediated effects, but only while the cell is in the down state *(70)*. The compound effect of dopamine transmission is thought to increase the signal-to-noise ratio in the nucleus accumbens by hyperpolarizing the cell in the absence of sufficient glutamatergic input, while maintaining a depolarized state in the presence of depolarizing input. This dopaminergic filter on the transit of information is consistent with behavioral studies that have concluded that dopamine serves to "gate" information through the nucleus accumbens *(78)*.

3.3. Motive Circuit and Behavioral Sensitization

Separate mechanisms underlie the induction and expression of behavioral sensitization. The VTA is of particular importance to the induction of behavioral sensitization. Infusion of cocaine or amphetamine into the VTA produces behavioral sensitization to a systemic injection of cocaine or amphetamine *(79–81)*. Glutamatergic, but not dopaminergic, neurotransmission in the VTA is necessary for the induction of cocaine behavioral sensitization. In support, blockade of NMDA glutamate receptors in the VTA prevents the induction of cocaine behavioral sensitization *(82)*, whereas negative findings are obtained with systemic or intra-VTA infusion of DA receptor antagonists *(50–53)*. The prominent role for glutamate in cocaine behavioral sensitization is further indicated by the observations that regions within the motive circuit that send glutamatergic projections to the nucleus accumbens also contribute to the induction of behavioral sensitization. Thus, the PFC, amygdala, or hippocampus have all been shown to be necessary structures for the induction of sensitization produced by systemic administration of cocaine *(82,83)* or amphetamine *(84,85)* although *see* refs. *84* and *86*.

Unlike induction, the nucleus accumbens is the critical structure for the expression of cocaine sensitization. In support, infusion of amphetamine or cocaine into the nucleus accumbens results in a sensitized behavioral response in rats previously treated with systemic or intra-VTA infusion of amphetamine or cocaine *(87–89)*. Within the nucleus accumbens, the expression of cocaine behavioral sensitization is dependent on both glutamatergic and dopaminergic neurotransmission *(90–92)*. Collectively, these studies indicate that repeated cocaine produces plasticity in the VTA that underlies the induction of behavioral sensitization, which then results in plasticity in the nucleus accumbens that are necessary for the expression of behavioral sensitization.

3.4. Motive Circuit and Reinstatement

Reinstatement of extinguished cocaine-seeking behavior in rats can be induced by a variety of stimuli that induce relapse in human addicts, including a drug prime or exposure to a drug-paired cue or environment. The extant data indicate that separate but overlapping circuitry underlie drug or cue reinstatement. Exposure to cocaine-paired stimuli elicits an increase in Fos protein expression in the basolateral amygdala, anterior cingulate, hippocampal formation, and nucleus accumbens *(93,94)*, indicating increased neuronal activation in these regions. Conversely, a cocaine-priming injection increased Fos protein expression in the VTA and DA terminal regions, such as the central nucleus of

the amygdala, dorsal striatum, and the nucleus accumbens *(93,94)*. Interestingly, a cocaine-primed increase in Fos protein expression was also obtained in the anterior cingulate, but the increase was evident only in cocaine-withdrawn rats *(93)*. A strikingly similar profile of activation is observed in imaging studies conducted in human cocaine addicts. Presentation of drug-associated images or paraphernalia increases self-reports of craving and neuronal activation in the striatum, amygdala, anterior cingulate, and prefrontal cortex *(7,39–41)*. Likewise, cocaine increased activity in the nucleus accumbens, hippocampus, cingulate, and hippocampal formation *(95)*.

A variety of studies have established a causal role for the regions identified in the studies above and further support the notion that separate but overlapping circuitry modulate reinstatement produced by cocaine or cocaine-paired stimuli. Specifically, lesion or transient inactivation of the dorsal PFC nucleus accumbens core, ventral pallidum, and VTA blocks cocaine-primed reinstatement, whereas inactivation of the basolateral or central nucleus of the amygdala do not *(96,97)*. Within the nucleus accumbens core, cocaine-induced reinstatement is dependent upon glutamatergic, but not dopaminergic transmission *(97–99)*. The later observation is surprising since dopamine infusion into the nucleus accumbens is sufficient to reinstate cocaine-seeking behavior. However, DA-induced reinstatement may arise from diffusion to the nucleus accumbens shell because D1 DA receptor blockade in the shell blocks cocaine reinstatement *(99)*. Thus, the extant data reveal that a cortical-striatal-pallidal circuit underlies cocaine-primed reinstatement of cocaine-seeking behavior. Lesion or temporary inactivation of the basolateral amygdala, PFC or dorsal hippocampus blocks reinstatement produced by exposure to drug-paired stimuli *(13,100,101)*. Within the basolateral amygdala, cue-induced reinstatement is dependent on dopaminergic, but not glutamatergic neurotransmission *(102)*. The role of the nucleus accumbens in cue-induced reinstatement remains equivocal since inactivation of this structure was without effect *(96)*, yet blockade of AMPA receptors in this region prevented cue-induced reinstatement *(103)*.

In summary, the extant data support the view that separate but overlapping circuitry underlie reinstatement of cocaine-seeking behavior produced by a drug challenge or exposure to drug-paired stimuli. Specifically, cocaine-primed reinstatement involves the VTA, PFC, nucleus accumbens core, and ventral pallidum. As detailed in the description of the motive circuit, anatomical connections between these regions have been delineated with the ventral tegmental area sending dopaminergic and GABAergic projections to the PFC. The PFC then sends glutamatergic projections to the nucleus accumbens core, which in turn sends GABAergic projections to the ventral pallidum. Interestingly, blocking DA in the PFC or glutamate in the nucleus accumbens core can prevent cocaine-induced reinstatement. Reinstatement produced by drug-paired cues may involve a similar pathway (PFC-nucleus accumbens-ventral pallidum), but it likely gains access to the pathway via the basolateral amygdala (discrete cues) or the hippocampus (contextual associations), both of which project to the PFC and nucleus accumbens.

4. DRUG-INDUCED PLASTICITY WITHIN THE MOTIVE CIRCUIT

An influential zeitgeist in the addiction field is that drug-induced plasticity in brain functioning, which can range from changes in a single protein to a permutation in the activity of an entire neuronal circuit *(34,104–107)*, underlies the transition in drug use from casual to compulsive *(3)*. Repeated cocaine administration produces a variety of

adaptations throughout the motive circuit that are commonly categorized on a temporal basis. Thus drug-induced plasticity may emerge during the course of drug administration or during withdrawal *(32–34)*. Plasticity in the former category are often transient, but likely contribute to persistent drug-seeking behavior by serving as a necessary conduit for more permanent drug-induced plasticity or may remerge and contribute to craving following exposure to stimuli capable of producing relapse (e.g., a drug prime).

4.1. Cocaine-Induced Plasticity

The extant data indicate that drug-induced plasticity render the circuitry underlying cocaine-seeking behavior sensitized. As described above, a variety of studies indicate that the PFC and nucleus accumbens are necessary for cocaine-primed reinstatement and the expression of behavioral sensitization. These structures, in addition to the basolateral amygdala, have also been implicated in cue-primed cocaine-seeking behavior. Enhanced mesolimbic DA activity is evident as increased DA release in the nucleus accumbens *(108)* and amygdala *(109)* following a cocaine challenge and a transient increase in the firing rate of mesoaccumbens DA cells *(34,110)*. Cocaine-induced presynaptic alterations mediate the enhanced releasability of DA in the nucleus accumbens and include a transient decrease in D2 autoreceptor inhibitory feedback *(89,111,112)* and an enduring increase in calcium signaling through calmodulin-dependent protein kinase II (CaMKII) *(113)*. Interestingly, medium spiny neurons in the nucleus accumbens are more sensitive to the inhibitory effects of DA following repeated cocaine administration *(104,107)*. This effect is mediated by enhanced activity of D1-receptors, which is not because of an increase in the number of receptors *(34)*, but instead may involve an upregulation in the activity of the cyclic adenosine monophosphate (cAMP) *(114)*. In support, D1-receptor stimulation is coupled to increased cAMP activity, which results in increased levels of cAMP-dependent protein kinase (PKA). The elevation in PKA levels may result in increased phosphorylation of voltage-dependent sodium channels by PKA *(115–117)*, which would decrease voltage-sensitive sodium currents and reduce the excitability of accumbal neurons. Further, this cascade is capable of inducing stable isoforms of the transcription factor ΔFosB *(106)*. Interestingly, these changes in the nucleus accumbens may arise as a function of cocaine on the glial-derived neurotrophic factor (GDNF) in the VTA. Nestler and colleagues have provided evidence that mice exhibit a reduction in GDNF signaling in the VTA during short-term cocaine withdrawal *(118)*. The reduction of GDNF in the VTA appears to be necessary for the sensitized D1/PKA/ΔFosB pathway in the NAc because infusion of GDNF into the VTA blocked cocaine-induced elevations in PKA activity and ΔFosB in the nucleus accumbens *(118)*. Collectively, these data illustrate how drug-induced plasticity in the VTA, which is necessary for the induction of sensitization, can lead to plasticity in the nucleus accumbens, which is necessary for the expression of sensitization.

Evidence of enhanced activity of corticofugal glutamate systems following repeated cocaine treatment includes a sensitized cocaine-induced increase in extracellular glutamate in the VTA and the nucleus accumbens *(90,119,120)*, and increased responsiveness of AMPA receptors in the VTA and nucleus accumbens *(90,121)*. Interestingly, elevated levels of glutamate in the nucleus accumbens may arise from a decrease in cocaine-induced dopamine release in the PFC *(122)*, which would lead to a reduction in inhibitory dopaminergic tone. Alternatively, the cocaine-induced sensitized glutamate

response may arise from adaptations in presynaptic release mechanisms. In support, repeated cocaine produces a long-lasting decrease in basal extracellular levels of glutamate *(90,123,124)*, and thereby contributes to a reduction in tonic stimulation to group 2/3 metabotropic glutamate receptors that function as autoreceptors *(125–127)*.

4.2. Relevance of Drug-Induced Plasticity

A need to examine the impact of drug-induced plasticity on behavior is evident, in part, by the observation that drug plasticity can perpetuate or oppose the emergence of compulsive drug-seeking behavior. For instance, repeated administration of cocaine results in an increase in mRNA levels of the transcription factor NAC-1 in the nucleus accumbens *(128)*, and this appears to oppose rather than result in the emergence of addictive-related behaviors because viral-mediated overexpression of this protein blocked the induction of cocaine sensitization *(129)*. Likewise, cocaine-induced elevations in the neuronal protein kinase Cdk5 may be compensatory in nature because Cdk5 inhibitors infused into the nucleus accumbens enhanced behavioral sensitivity to cocaine *(130)*.

Despite the intuitive and popular nature of the hypothesis that drug-induced plasticity in brain functioning underlies the transition in drug use from casual to compulsive use, there have been relatively few studies supporting this claim. An exception involves the increased activity of D1 receptor/PKA/ΔFosB pathway in the nucleus accumbens. Several studies indicate that manipulations that reverse or block the development of D1-receptor supersensitivity inhibit the expression of behavioral sensitization *(131–133)*, indicating that D1-receptor supersensitivity is necessary for drug-induced behavioral plasticity. Further, there is evidence indicating that upregulation of the cAMP pathway can result in behavioral sensitization *(134)* and increase reward-related behaviors *(135)*. However, a reduction in cAMP activity was also found to enhance the rewarding properties of cocaine *(136)*. Likewise, the role of ΔFosB levels appears equivocal with reports of enhanced sensitivity to the rewarding properties of cocaine in FosB knockout mice *(137)* and mice overexpressing ΔFosB in the striatum *(138)*. These data seem to indicate that the role of cAMP and/or ΔFosB is complicated so that any deviation from normal may alter behavior *(139)*. Consistent with this interpretation, either stimulation or blockade of DA D1-like receptors produces a similar change in cocaine-induced reinstatement in nonhuman primates *(26)*. Adaptations in glutamatergic neurotransmission also seem necessary for behavioral plasticity. Specifically, cocaine-induced sensitized glutamate response is necessary for the expression of sensitization and cocaine-primed reinstatement *(97,124)*. Collectively, these studies demonstrate the need to examine the impact of plasticity on animal models of addiction-related behaviors such as behavioral sensitization or drug-seeking behavior in the reinstatement paradigm.

5. SUMMARY

The emergence of compulsive drug use likely occurs as a result of drug-induced plasticity in brain functioning that is pathogenic for the relapse of drug-taking behavior. Regions within the motive circuit are critical for the expression of behavioral sensitization and cocaine-primed reinstatement in rats, as well as drug- and cue-induced craving in human addicts. Drug-induced plasticity within this circuitry results in altered activity such that the mesolimbic DA systems projecting to the nucleus accumbens and amygdala and corticofugal glutamatergic systems projection to the nucleus accumbens and VTA

tend to be overactive. Further, a subset of drug-induced plasticity has been shown to be necessary for the expression of behavioral sensitization or cocaine-primed reinstatement. Accordingly, pharmacotherapies that alter or reverse these adaptations may prove to be effective in the treatment of cocaine addiction in humans.

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16

Glutamatergic Neurotransmission in Sensitization

Thomas M. Tzschentke

1. INTRODUCTION

Behavioral sensitization, the intensification of a drug-induced behavior upon repeated drug administration, is a complex phenomenon. As outlined in the preceding chapters, the mesocorticolimbic dopamine system plays a central role in the various stages of initiation, development, expression, and long-term maintenance of sensitization. Although dopamine is likely to be one of the major transmitters involved, other transmitters act to modulate these processes in various and complex ways. Transmitter systems that have been shown to modulate sensitization or that undergo adaptations during sensitization include, for example, serotonin *(1,2)*, γ-aminobutyric acid (GABA) *(3)*, noradrenaline *(4)*, or acetylcholine *(5,6)*.

Glutamate has also been demonstrated to be importantly involved in sensitization. There are several reviews that give a good and comprehensive overview of the literature pertaining to the role of glutamate in sensitization *(7–16)*. Because there is such a broad coverage of this issue in the literature, this chapter focuses on some of the most recent important findings and on some phenomena and mechanisms where glutamate appears to play a particularly crucial role.

After several years of extensive behavioral work on the role of glutamate and its receptors in sensitization, the focus in recent years has clearly shifted to the cellular and molecular level, and the field continues to be very productive on that level. A crucial challenge for the future will be to integrate behavioral and cellular/molecular data into an encompassing model of sensitization. This will, however, be an exceedingly difficult task, since sensitization is not a unitary phenomenon. Both, context-independent, nonassociative and context-dependent, associative mechanisms contribute to the development and expression of sensitization, and both components are likely mediated, at least in part, by distinct brain areas and neurochemical mechanisms *(17–19)*. Also, sensitization is not an effect produced by drug exposure *per se*. The way of drug administration can have a high impact on the kind of behavioral change that is produced by the drug. Whereas intermittent (usually daily) bolus injections of drugs of abuse commonly produce behavioral sensitization, chronic administration (e.g., via osmotic mini-pumps or implanted pellets) of the same daily total drug dose can produce tolerance to the drug. Furthermore, although sensitization produced by different drugs is likely to be dependent to a large

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

degree on similar basic mechanisms (which is mirrored in the wide extent of cross-sensitization among different drugs; e.g. refs. *20–22*, but *see* ref. *23*), there also appear to be a number of differences in the mechanisms involved in the development of sensitization produced by different drugs. Notably, even for the behaviorally closely related psychostimulants cocaine and amphetamine, a number of differences have been described with respect to alterations in the glutamatergic and other transmitter systems induced by these drugs with repeated administration (cf. ref. *8*).

Although some of these differences have been characterized, it is not yet entirely clear of what relevance these differences are and whether they contribute to the development of distinct forms of sensitization that might have distinct characteristics with respect to speed of development, persistance, or cross-sensitization to other drugs or stimuli, such as stress, or whether they are just distinct epiphenomena of otherwise common underlying mechanisms.

2. THE RELATION BETWEEN SENSITIZATION AND ADDICTION

The high scientific interest in the phenomenon of sensitization is to a large extent because of the assumption that sensitization processes may be importantly involved in the development of addiction, and that stable changes that can be seen in sensitization may also contribute to the long-term maintenance of adaptations on the behavioral and neurobiological level that maintain a propensity to relapse even after long drug-free periods. Thus, the hope is that by understanding sensitization one might also make progress in understanding addiction. Because glutamate antagonists have been shown to interfere with sensitization in various ways this has spurred hopes that such drugs may also be useful in the treatment of addiction in humans.

The view that sensitization is associated with addiction and that glutamate may be importantly involved in several instances is supported be the following findings:

1. Sensitization is a long-lasting phenomenon; that is, its underlying functional alterations are very stable. In the rat, sensitized locomotor activity can persist for several months after the end of drug administration *(24)*.
2. The degree of sensitization appears to predict the vulnerability for relapse; that is, the stronger an individual is sensitized, the more easily it relapses *(25,26)*.
3. A sensitized individual will more readily acquire drug self-administration than a nonsensitized individual *(27)*. This phenomemon may be owing to a process of reward sensitization *(28)* or incentive sensitization *(29)*.

Relating to each of these points, there have been findings that glutamate antagonists can interfere with these phenomena:

1. Under certain conditions, glutamate antagonists can block both the cellular and behavioral manifestations of development and expression of sensitization *(30)*. Moreover, there appears to be some evidence that under certain conditions glutamate antagonists can reverse a pre-existing sensitization *(31)*.
2. Glutamate antagonists might be able to reduce craving and the propensity to relapse *(32,33)*, although it should be noted that certain classes of glutamate antagonists may also produce relapse themselves *(34)*.
3. Glutamate antagonists can delay the acquisition of drug self-administration *(35)*.

The fundamental problem with a glutamate antagonist-based approach toward addiction therapy is that glutamate is an almost ubiquious transmitter in the central nervous system (CNS). The actions of antagonists are thus not limited to the areas and mechanism

involved in addition but can produce a number of very undesirable side effects that make them not useful as a medication in many cases. The hope here clearly lies in the development of well-tolerated low-affinity *N*-methyl-D-aspartate (NMDA) channel blockers and in subtype-selective compounds targeting the NR2B subunit, the glycinB-binding site at the NMDA receptor complex, or drugs targeting certain subtypes of metabotropic glutamate receptors (in particular mGluR5) (*see* refs. *36* and *37*).

3. ANATOMICAL BASIS FOR THE ROLE OF GLUTAMATE IN SENSITZATION

Extensive research over the years has shown that the initiation of sensitization, that is, the adaptations that transform an organism from a nonsensitized to a sensitized state, is likely to be mediated, at least to some extent, by different mechanisms and in different brain areas than the expression of sensitization (i.e., the generation of a sensitized response to a drug challenge in a sensitized organism that can occur even a long time after the initial sensitizing drug treatment). In simplified terms, initiation of sensitization is mediated primarily at the level of the ventral tegmental area (VTA), whereas the expression of sensitization is mediated primarily at the level of the nucleus accumbens (NAS) *(19)*. Thus, the core element of the processes involved in sensitization appears to be the mesolimbic dopamine system, projecting from the VTA to the NAS. Of course, this is an oversimplification, since other transmitters and brain structures are also importantly involved in various aspects of sensitization. There are substantial glutamatergic projections to both VTA and NAS, originating from limbic cortical structures, such as medial prefrontal cortex (PFC), amygdala, and possibly hippocampus (*see* refs. *15* and *38*). A large body of evidence shows that the PFC functionally interacts with NAS and VTA by virtue of its glutamatergic efferent projections. Whereas the connections between PFC and NAS are rather straightforward in that there is a direct projection from the PFC to the NAS that terminates on and excites spiny neurons (GABAergic projection neurons of the NAS) *(39,40)*, the anatomical and functional relationship between PFC and VTA is more complex. A direct (glutamatergic) projection from the PFC to the midbrain has been shown repeatedly *(39,41)*, and it has long been argued that prefrontal afferents to the VTA directly activate VTA dopaminergic neurons *(42,43)* and that this dopaminergic activation is mediated via the action of glutamate in the VTA *(44,45)*. On the other hand, it has also been shown that the majority of the glutamatergic terminals of prefrontal origin in the VTA does not terminate on dopaminergic cells *(46)*, and more recent evidence suggests that the PFC input to the VTA is highly specific and not compatible with a direct excitation of mesoaccumbens dopamine neurons by PFC input. Carr and Sesack *(47)* showed that PFC projections to the VTA make contact only with those dopaminergic cells that project back to the PFC, but avoid mesoaccumbal dopamine neurons. On the other hand, mesoaccumbal neurons that do receive input from the PFC were found to be exclusively GABAergic. Thus, excitatory prefrontal input to the VTA selectively targets dopaminergic mesocortical and GABAergic mesoaccumbal neurons. This suggests that the activating effects of the PFC on mesoaccumbal dopamine neurons and on dopamine release in the NAS cannot be mediated by a direct projection from the PFC to the VTA. Rather, the PFC may project to and stimulate other brain structures that in turn project back to and activate mesoaccumbal dopamine neurons in the VTA. One area that has been implicated in such a mechanism is the pedunculopontine tegmental nucleus *(48–50)*.

Thus, there is a multitude of levels of interactions between the mesolimbic dopamine system and the glutamatergic system. As will be outlined below, this anatomical and functional complexity is mirrored in the complexity of adaptations that develop in these systems with repeated drug administration.

4. SENSITIZATION AND NEUROADAPTATIONS IN THE GLUTAMATERGIC SYSTEM

As mentioned in the Subheading 1, sensitization is not a unitary phenomenon. In many cases, the sensitized behavioral response that can be observed in animals after repeated drug treatment is composed of a nonassociative, unconditioned and an associative, conditioned component *(17,51)*. Different neurobiological mechanisms are likely to be involved in these two components of sensitization. Furthermore, the relative contribution of these two components to the observed sensitization may be variable. It appears that for sensitization to some drugs the conditioned component is more important than for sensitization to other drugs. The contribution of associative factors to sensitization also crucially depends on the design of the behavioral experiment. Treating animals repeatedly with a drug in the same test environment clearly favors the strengthening of the conditioned component of sensitization, whereas treating animals in a similar manner (with respect to dosing and intertreatment interval) in the home cage and then testing them in a different, distinct test environment reveals the unconditioned component of sensitization. Yet, this statement is also somewhat of an oversimplification. The injection procedure and the drug stimulus itself can serve as a conditioning context that cannot be circumvented even when treatment and test environment are different (for elegant studies on this issue, *see* the work of Robinson and colleagues, e.g., refs. *52–54*).

Although nonassociative mechanisms probably provide the basis for many of the enduring adaptations in the CNS that develop during sensitization, they cannot account for the specificity of drug-associated cues to provoke relapse. Relapse, in many cases, involves drug-associated stimuli that "reactivate" the urge to take drug in humans and experimental animals *(55,56)*. There is growing interest in the idea that context-dependent sensitization may be a special form of habit learning. Habit learning refers to the learning of a consistent relationship between a stimulus and a certain behavioral response, and in the context of addiction there is some evidence that through excessive habit learning the controlled intake of drugs, which initially is a flexible, voluntary, and evaluative behavior, develops into an automated, involuntary stimulus–response habit *(57–59)*. This process can also be conceptualized in terms of sensitization.

Changes that occur during the induction phase of sensitization include: a subsensitivity of D2 autoreceptors in the somatodendritic region of dopamine neurons in the VTA *(60)*; an enhanced dopamine-stimulated glutamate release from prefrontal cortical afferents (via D1 heteroreceptors) that could be the basis of the observed increase in drug-induced release of glutamate in the VTA during sensitization *(61)*; and an increased excitability of dopamine neurons in the VTA that is probably owing to increased excitatory currents through α-amino-3-hydroxy-5-methylcoxazole-4-propionate (AMPA) receptors *(62)*, which in turn could be related to an NMDA receptor-dependent long-term potentiation of AMPA receptor-mediated currents that can be found even after a single adminstration of cocaine *(63)*. All these changes lead to a hightened responsitivity and activity of dopamine neurons and contribute to the shift from the nonsensitized to the

sensitized state. They can be observed only during the drug treatment period and/or shortly afterward and disappear within a short period of time. On the other hand, there are adaptations that are responsible for the long-term maintenance of sensitization. Accordingly, these changes develop only toward the end of or after the drug treatment phase and are very stable in nature and persist for weeks or months (possibly years, or even permanently). These changes include: a subsensitivity of NAS spiny neurons to the excitatory effects of glutamate *(64)*, which might be related to long-term depression at excitatory synapses that has been observed between PFC afferents and spiny neurons in the NAS following a sensitizing treatment with cocaine *(65)*; an increased releasability of glutamate in the NAS *(66,67)*; and an increased inhibition of NAS spiny neurons by dopamine via D1 receptors *(68)*.

There is a large number of other changes in the CNS relating to the glutamatergic system that occur in the course of sensitization. A number of representative studies are summarized in Table 1. Although in some cases the relevance of a particular adaptation has been demonstrated experimentally, in many other cases the actual contribution to and relevance for sensitization is not clear. It is also important to keep in mind that most of these changes have been found following administration of only one particular drug (either cocaine or amphetamine in most cases), and it has not been determined to date whether similar changes would occur with other sensitizing drugs as well. Thus, it is not known to what degree these adaptations are universal phenomena of sensitization or to what degree they are drug-specific.

Behavioral pharmacological approaches have been widely used to characterize the role of the glutamatergic system in sensitization. It is known for some time that several forms of learning and behavioral plasticity depend on glutamatergic mechanisms, which are in large part mediated by the NMDA receptor (e.g., ref. *69*). Karler et al. *(70)* were the first to report that sensitization, too, can be blocked by the non-competitive NMDA receptor antagonist dizocilpine (MK-801), and in the meantime, a considerable number of studies have shown that interference with glutamatergic neurotransmission at NMDA receptors can disrupt the development, maintenance and/or expression of sensitization *(10,11)*. Similar findings have been reported for the development, maintenance, and expression of tolerance, another form of neural and behavioral plasticity *(11,71)*. From these findings the view has emerged that glutamate antagonists, and in particular, NMDA receptor antagonists, block or interfere with behavioral plasticity, and over the years this view has become widely accepted.

However, there is also evidence that the picture might not be as simple as this. The importance of glutamatergic neurotransmission for neural and behavioral plasticity notwithstanding, several findings indicate that there may be cases where strong modification of behavior can occur in the presence of NMDA receptor blockade. In particular, studies using dizocilpine have generated results that do not fit well within the conceptual framework outlined above. A state-dependency explanation was put forward by Carlezon, Wise, and colleagues *(72,73)* for their findings on the effects of dizocilpine on bromocriptine-induced sensitization. They found a very strong day-to-day increase in locomotion during repeated treatment with bromocriptine + dizocilpine but an absence of sensitization when the (clearly sensitized) animals were subsequently challenged with bromocriptine alone. According to this explanation, the coadministration of dizocilpine has made the sensitization state-dependent; that is, sensitization can be expressed only in

Table 1
Alterations Within the Glutamatergic System Induced by Repeated Sensitizing Drug Treatment

Observed effect	Structure	Observed at time point	Shown for (drug)	Reference
Increased mGluR2/3 levels, associated with reduced GTP-binding and reduced agonist-induced inhibition of glutamate releases	NAS, PFC	21 d of withdrawal	Cocaine (7 d)	*91*
Increased excitatory response of dopaminergic neurons to stimulation with AMPA (but not NMDA)	VTA	3 d, but not 10–14 d of withdrawal	Amphetamine (5 d)	*92*
Increased release of glutamate by AMPA (but not NMDA)	VTA	3 and 10–14 d of withdrawal	Amphetamine (5 d)	*92*
Increased nerve terminal levels of glutamate	VTA	17 d of withdrawal	Cocaine (7 d)	*93*
Increased drug-induced release of glutamate	striatum, NAS	4 d of withdrawal	Cocaine (7 d)	*94*
Increased expression of mGluR1	striatum, NAS	3 h, but not 7–28 d of withdrawal	Amphetamine (5 d)	*95*
Reduced expression of mGluR5	striatum, NAS	3 h and 7, 14, and 28 d of withdrawal	Amphetamine (5 d)	*95*
Increased mRNA and protein level for GluR5 receptor	dorsal PFC	21 d of withdrawal	Cocaine (7 d)	*96*
Increased GABAergic inhibition (via GABA-B receptors) of extracellular glutamate levels	VTA	3 d, but not 10–14 d of withdrawal	Amphetamine (5 d)	*97*
Increased drug-induced release of glutamate	ventral pallidum	10–14 d of withdrawal	Amphetamine (14 d)	*98*
No change in GluR1-4 levels	VTA, substantia nigra	3 and 14 d of withdrawal	Amphetamine (5 d)	*99*
No change in GluR1 receptor and mRNA levels	VTA, substantia nigra	16–18 h and 24 h of withdrawal	Amphetamine (5 d); cocaine (7 d)	
No change in GluR1-4 levels	VTA	30 min of withdrawal	Amphetamine (3 or 10 d)	*100*
No change in NMDA receptor density and affinity	striatum, NAS	14 d of withdrawal	Cocaine (5 d)	*101*

The coverage of literature in this table is not comprehensive. It intends to give a representative overview of some of the more recent findings that are not covered by previous reviews. The selected references demonstrate the heterogeneity of experimental designs and findings, illustrating the difficulty of building a general and consistent model of the role of glutamate in sensitization based on the available data.

GTP, guanosine triphosphate; NAS, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; NMDA, *N*-methyl-D-aspartate; GABA, γ-aminobutyric acid.

the same state under which it developed. If sensitization develops while the animals are treated with drug + dizocilpine (which very clearly was the case), then this sensitization can be expressed in the presence of dizocilpine but not or only to a much lesser degree when the animals are challenged with the drug (in this case bromocriptine) alone.

It has been argued *(10)* that such effects may be owing to the fact that a rather unusual sensitizing drug was used. Bromocriptine has some particular pharmacological properties and the sensitization it induces appears to be entirely context-dependent *(74),* which might render this form of sensitization particularly vulnerable to state-dependency effects. Although this is an intuitive assumption for a drug like bromocriptine, in the meantime very similar effects have been demonstrated for amphetamine-, morphine-, and nicotine-induced sensitization *(12,75–78)*. Thus it seems to be very unlikely that state-dependency phenomena are restricted to bromocriptine-induced sensitization, but rather they seem to have a more general applicability in the context of sensitization.

On the other hand, there have been reports that drugs other than dizocilpine can genuinely block the development of sensitization. For example, Li and Wolf *(79)* found that coadministration of the competitive NMDA receptor antagonist CGS19755 prevented the day-to-day increase in psychomotor activation during the amphetamine sensitization induction phase. Likewise, animals pretreated with CGS19755 + amphetamine did not show a sensitized response to a subsequent amphetamine challenge. In another study, coadministration of the AMPA receptor antagonist LY293558 resulted not only in the absence of a sensitized response in cotreated animals to a morphine challenge but also in a prevention of the day-to-day increase in locomotion during the sensitizing treatment schedule *(80)*. Results like these cannot be explained in terms of state-dependency and suggest that the behavioral plasticity induced by repeated amphetamine administration was truly blocked by these drugs. Unfortunately, studies like these are still an exception with respect to the experimental design. Only a few laboratories test animals on a daily basis during the sensitizing phase or run an additional challenge test with drug plus glutamate antagonist. This, however, would be necessary to obtain the additional information required to interpret the data appropriately. Of course, even with this experimental procedure not all questions relating to the mechanisms underlying glutamate receptor antagonist effects on behavioral sensitization can be addressed and answered, but at least it can enable the investigator to discard (or accept) one possible alternative interpretation.

Table 2 summarizes a number of recent behavioral pharmacological studies that exemplify the heterogeneity of both, the experimental designs and findings, illustrating the difficulty of building a general and consistent model of the role of glutamate in sensitization based on the available data.

5. CONCLUDING REMARKS AND FUTURE DIRECTIONS

Sensitization involves adaptations at the behavioral and neuronal level. Whereas some of these adaptations are transient, others persist over prolonged periods of time. This persistance, along with other factors, makes sensitization an interesting potential model for at least some aspects of drug addiction. It is clear that neuroadaptations within the glutamatergic system contribute importantly to the initiation and maintenance of sensitization. One open question is which of these adaptations are a cause and which are an effect of sensitization. Another question is to what extent these adaptations gain relevance via

Table 2
Behavioral Studies Showing an Involvement of Glutamatergic Mechanisms in Sensitization

Sensitizing drug (mg/kg)	Glutamate antagonist (mg/kg)	Treatment schedule	Effect obtained	Reference	Remarks
Amphetamine (1)	LY379268 (1)	Five injections of amphetamine, one every 2–3 d, challenge 2 wk later with amph. + LY	LY379268 blocks the expression of sens.	*106*	SD as possible explanation for observed effect
Cocaine (20)	2-PMPA (50–100)	Five daily injections of cocaine ± 2-PMPA, challenge with cocaine 3 d later	2-PMPA blocks the development of sens.	*107*	SD as possible explanation for observed effect
Cocaine (20)	Riluzole (2.5–20)	Five daily injections of cocaine ± riluzole	Riluzole has no effect on induction and expression of sens.	*108*	
Morphine (3)	LY293558 (0.1–3)	Five injections every other day of morphine ± LY293558; challenge with morphine 2 d later, and with morphine + LY293558 4 d later	LY293558 blocks the development, but not the expression of sens.	*80*	
Cocaine (15)	Dizocilpine (0.1and 0.3); CGS 19755 (5 and 10); memantine (4)	10 d of daily cocaine, on days 14–20 daily injections of a DA agonist in combination with a glutamate antagonist; 3 d or 2 wk later cocaine challenge	Combinations of cocaine + dizocilpine, quinpirole + dizocilpine,quinpirole + CGS 19755, and pergolide + memantine reverse an established cocaine sens.	*31*	
Amphetamine (1)	AP-5 (1 and 5 nmol/side; local infusion)	Four injections, one every 3 d, of amphetamine ± AP-5;challenge with amphetamine 2 wk later	AP-5 infused into the VTA but not into the NAS, blocks the development of sens.	*109*	
Ethanol (2 g/kg)	dizocilpine (0.25 mg/kg)	21 d of daily ethanol ± dizocilpine; challenge with ethanol 4 d later	dizocilpine blocks the development of sens.	*110*	SD as possible explanation for observed effect

Amphetamine (14) and apomorphine (40)	CPP (8–20);dizocilpine (0.03–0.25)	One (context-dependent) or three (context-independent) daily injections of amphetamine or apomorphine ± CPP or dizocilpine; challenge 2 d later with amphetamine or apomorphine ± dizocilpine	CPP and dizocilpine block induction of context-dependent, but not of context-independent sens.	*111*	
Haloperidol (0.25 and 0.5)	Dizocilpine (0.16)	Seven daily injections of haloperidol ± dizocilpine; challenge 1 d later with haloperidol	Dizocilpine does not block the development of sens. of catalepsy; lack of sens. during challenge likely owing to SD effects	*112*	SD as possible explanation
Haloperidol (0.25 and 0.5)	D-CPPene (5 and 10); eliprodil *(30)*; Ro 25-6981 *(15)*	Seven daily injections of haloperidol ± NMDA antagonist; challenge with haloperidol ± NMDA antagonist on several days afterwards	NMDA antagonists do not block the development of sens. of catalepsy; lack of sens. During challenge likely owing to SD effects	*113*	SD as possible explanation

The coverage of literature in this table is not comprehensive. It intends to give a representative overview of some of the more recent findings that are not covered by previous reviews. The selected references demonstrate the heterogeneity of experimental designs and findings, illustrating the difficulty of building a general and consistent model of the role of glutamate in sensitization based on the available data. There are a number of further studies that used very diverse experimental protocols that could not be accommodated in the table (e.g., refs. *102–105*).

Dizocilpine (MK-801): noncompetitive, high-affinity NMDA receptor channel blocker; memantine: noncompetitive, low-affinity NMDA receptor channel blocker; CPP, D-CPPene, AP-5, CGS 19755: competitive NMDA receptor antagonists; eliprodil, Ro 25-6981: NR2B subtype-preferring NMDA receptor antagonists; riluzole: glutamate release inhibitor; 2-PMPA: NAALADase inhibitor; LY379268: antagonist at metabotropic group II receptors. Sens, sensitization; SD, state-dependency; NMDA, *N*-methyl-D-aspartate; PMPA, 9-(2-phosphonylmethoxypropyi) adenine; CPP, 3,3-(2 carboxypiperazine-4-yl)-propyl-1-phosphonate; NAS, nucleus accumbens.

interaction with other transmitter systems, most notably the dopaminergic system, or to what extent they are the primary cause of sensitization, independently of other systems.

A very crucial issue will be to determine whether sensitization processes do indeed contribute to the development of human addiction. For obvious ethical reasons, human studies on this subject have been extremely rare so far since it necessarily involves the repeated administration of drugs of abuse to healthy volunteers. In the few existing studies *(81–87),* no attempt has been made to test the influence of glutamatergic drugs on sensitization effects. There is some evidence from human drug discrimination studies that the NMDA receptor antagonist memantine may enhance, rather than attenuate, the subjective effects of cocaine and may possess possess some stimulant-like properties itself *(88,89)*. On the other hand, memantine may attenuate the expression of opiate physical dependence *(90)*, and acamprosate reduces the likelyhood of relapse in withdrawn alcoholics *(33)* (although it should be mentioned here that acamprosate is only a weak NMDA receptor antagonist and also acts on other transmitter systems, so that the relevance of its NMDA antagonistic properties for the observed therapeutic effect is not clear).

Thus, although there is good evidence from the animal literature, neither the involvement of sensitization in human drug addiction nor the involvement of glutamate in these processes in man has been established to date with any certainty.

It is likely that many more adaptive processes will be identified with the development of more sophisticated experimental methods and with the shift of focus from the behavioral level to the cellular and, in particular, molecular level. The big challenge for future research will be to fit the novel findings into a coherent picture of how repeated drug adminstration can produce significant and long-lasting alterations at the neuronal, and ultimatitely, at the behavioral level. The studies reviewed in this chapter represent first steps toward this goal, but they also show that it will be an exceedingly difficult task to piece together a host of heterogenous and often conflicting findings and to apply them to the human situation.

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17

Glutamatergic Mechanisms of Drug Relapse

Withdrawal and Conditioning Factors

Anton Y. Bespalov and Robert L. Balster

1. WITHDRAWAL SYNDROME AND CUE REACTIVITY: TWO FACTORS CONTROLLING THE RECURRENT PATTERNS OF DRUG SEEKING AND TAKING

Drug abuse is best viewed as a chronic relapsing disorder *(1)*. Two factors are usually said to be important for controlling the recurrent patterns of drug seeking and taking—the somatic/behavioral distress associated with discontinued drug administration and exposure to the drug-associated environment. The relative contribution of these two factors is likely to be different for various classes of drugs of abuse, primarily because some classes (e.g., opiates and depressants) have a more intense and aversive withdrawal syndrome than others (e.g., stimulants and cannabinoids).

When translated into the terms of the experimental analysis of behavior, drug taking can be said to rely on a combination of positive and negative reinforcing effects of drug administration. The positive reinforcing effects probably play a greater role in the initiation of drug use and the establishment of dependence; the negative reinforcing effects are probably more important in maintaining regular patterns of self-administration as users attempt to avoid or escape from the aversive conditions associated with the withdrawal syndrome. Because they contribute to the maintenance of regular patterns of drug use, physical dependence and the associated withdrawal syndromes are often the first targets of substance abuse treatment through the use of various pharmacotherapies for detoxification. In addition, understanding the basis for drug withdrawal states offers unique opportunities to look into the basic mechanisms of adaptive changes associated with repetitive drug exposures. Indeed, repeated experiences of the withdrawal state may come to enhance the incentive motivational value of the drug *(2)*, and play a role in the hedonic dysregulation that is an important feature of addictive disorders *(3)*. In addition, discontinued drug administration leads to protracted withdrawal states that are more difficult to manage and may or may not have the same mechanisms as the acute withdrawal syndrome. Protracted withdrawal affects the chances of the individual both to relapse and to respond to treatment.

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

Although the development of physiological dependence has been known since the beginning of the last century to play an important role in the substance abuse, we now know that it often does not have a primary role. It has long been known from experimental studies that drug self-administration in experimental subjects occurs readily in subjects that are not dependent *(4)*. In addition, there are many patterns of abusive repetitive drug taking behavior that do not lead to clinically significant physiological dependence, as acknowledged by such diagnostic tools as the *Diagnostic and Statistical Manual of Mental Disorders* (4th ed.), which allows the diagnosis of abuse with or without physiological dependence. Patients who have been detoxified and no longer exhibit withdrawal states are still prone to relapse, even after many months or years of abstinence. The important phenomenon of relapse is poorly understood, but conditioning factors are believed to play a very important role. These include the production of conditioned responses by environments associated with drug taking occasions or drug withdrawal that lead to the resumption of drug taking possibly by instituting cravings or other motivational elements of the relapse process. This conceptualization is well supported by laboratory research over the last few decades. Classical animal studies conducted in 1960s and 1970s indicated that associative learning was implicated in the mechanisms of various drug-related phenomena, such as tolerance and dependence *(5–7)*. In human experimental subjects, drug-associated conditioned stimuli were shown to arouse neural states that mimic features produced by the drugs and to elicit drug-related effects including the production of drug-like alterations in motor activity, subjective effects, reinstatement of drug seeking and drug taking behaviors, and so on *(8–13)*. Exposure to the drug-associated cues results in both intense craving and/or withdrawal-like symptoms *(14,15)*. Indeed, it is the ability of drug-associated cues to produce craving and withdrawal-like effects that serves as a bridge between studies of physical dependence and conditioning, the two major topics of this review. Looking for common neural substrates in the brain for these phenomena can facilitate our ability to link these phenomena, both conceptually and experimentally.

Drugs of abuse belong to diverse pharmacological groups and target many receptors with vast representation in the brain. Despite that, there is one neurotransmitter system that is uniquely positioned to be involved in the adaptive changes associated with repeated exposures to drugs of abuse. This is the glutamatergic system *(16)*. Over the last several years, many review articles and monographs have summarized various aspects of the glutamate involvement in drug tolerance *(17)*, dependence *(18)*, and sensitization (*19; see also* Chapter 15), as well as the potential for this research to yield novel pharmacotherapies for substance abuse treatment *(20,21)*. Because of space constraints, the sections that follow cannot provide a complete account of the work published to date. Instead, the goal of this review is to demonstrate the importance of glutamatergic neurotransmission in the phenomena associated with drug seeking and taking and to analyze critically the effects of glutamatergic manipulations on drug withdrawal and reactivity to drug cues.

2. GLUTAMATE AND DRUG WITHDRAWAL

In 1991, reports began to appear showing that *N*-methyl-D-aspartae (NMDA) antagonists could markedly attenuate the development and expression of morphine dependence *(22,23)*. Since then, these findings have been confirmed and extended in a number of studies with various types of glutamate receptor antagonists and drugs of abuse in several

species of laboratory subjects. Evidence for glutamatergic involvement in drug withdrawal comes from various types of studies, including research showing facilitated glutamatergic neurotransmission during the expression of drug withdrawal, attenuation of withdrawal signs and symptoms by glutamatergic antagonists, and modulation of the effects of withdrawal-associated cues. These areas of research will be briefly reviewed.

2.1. Facilitated Glutamatergic Transmission in Drug-Withdrawn Subjects

Because of the "excitatory" nature of most drug withdrawal syndromes, it seemed logical to expect that glutamatergic transmission would be hyperactive in withdrawal episodes. The initial evidence linking the expression of drug withdrawal syndromes with hyperactive glutamatergic neurotransmission came from studies showing enhanced glutamate release in several brain areas and the spinal cord *(24–27)*. The increase in glutamate release may turn out to be secondary to some other event and it is unlikely that a single area will be found responsible for the entire withdrawal syndrome. Nevertheless, it is worth noting that withdrawal-induced facilitation of glutamate release is often seen in the locus coeruleus *(24)*, the disputed "locus of drug withdrawal" *(28)*, where the blockade of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors and stimulation of mGlu group II receptors may attenuate both withdrawal-induced activation of the neurons and overt withdrawal signs *(29)*.

Overall, at least for opiate drugs, it appears that expression of withdrawal correlates with the increase in glutamate release (e.g., ref. *27*). However, facilitating glutamate uptake with MS-153 had no effect on the expression of the morphine withdrawal syndrome, although this drug did affect the development of both tolerance and dependence *(30)*. Nor is morphine withdrawal affected by the inhibitor of *N*-acetylated-α-linked-acidic dipeptidase (NAALADase), an enzyme catalyzing the cleavage of glutamate from *N*-acetyl-aspartyl-glutamate *(31)*. Thus, enhanced glutamate release may not necessarily be the triggering cause of the expression of the opiate withdrawal syndrome.

Alternatively, glutamatergic adaptations may involve changes in neurotransmitter uptake, receptor number, affinity, or other functional quality but experimental evidence on this is rather scarce. Though some controversy exists, it is generally found that withdrawal from opiates, benzodiazepines, and barbiturates is associated with an increased ligand binding to NMDA and AMPA receptors *(32–34; see*, however, ref. *35*). Such increases in binding are in accord with the reported alterations in the expression of various subunits of glutamate receptors, although the precise pattern of subunit expression changes varies across abused drugs *(33,36–38)*. Increased glutamate binding and enhanced expression of glutamate receptor subunits are also found in ethanol-withdrawn subjects *(39–41)*. However, these data should be treated with caution because ethanol acts as a glutamate receptor antagonist *(42)* and this is a likely reason for causing upregulation of glutamate receptor systems.

Experiments with direct stimulation of glutamate receptors in drug-withdrawn subjects have yielded mixed results. Facilitated emergence of the withdrawal symptoms has been reported after central administration of glutamate *(43)* but systemic administration of various glutamate receptor agonists had little or no effect *(44)*. Meanwhile, there is some very limited evidence indicating enhanced withdrawal intensity in animals exposed to repeated administration of glutamate (NMDA) receptor antagonists, which was thought to result in the upregulation of this receptor system *(45,46)*. Studies with overex-

pression of glutamate receptor subtypes or subunits are probably necessary to obtain more conclusive information on the functional role of increased number or affinity of glutamate receptors in the expression of a drug withdrawal syndrome. In summary, although evidence for glutamatergic hyperactivity would fit well with the reported ability of glutamate receptor antagonists to attenuate drug withdrawal symptoms, it is not yet proven that the expression of drug withdrawal effects is causally related to the hyperactivation of glutamatergic systems.

2.2. *Negative Modulation of Glutamatergic Transmission Impairs Expression of Drug Withdrawal Syndromes*

If glutamatergic systems are hyperactive during drug withdrawal, it could easily be predicted that antagonists at glutamate receptors would attenuate dependence development and/or the expression of withdrawal signs and symptoms. However, to prevent the development of drug dependence, glutamate receptor antagonists need to be coadministered with the dependence-inducing agent. Many such studies have been reported (Table 1). These findings complement similar studies showing that glutamate antagonists attenuate the development of tolerance and sensitization (reviewed in refs. *17* and *19*.) Indeed, a combination product containing morphine and the weak NMDA antagonist dextromethorphan has been under development as an analgesic in part because of the lower likelihood of opiate tolerance development *(47)*. However, from a practical viewpoint in treatment of drug abuse, medications to prevent the development of tolerance and dependence would be of limited usefulness. Thus, most research has been on the effects of these antagonists on the expression of withdrawal effects.

Table 1 lists various glutamate receptor antagonists and other negative modulators of glutamatergic neurotransmission that have been tested in animals withdrawn from repeated morphine administration; morphine serves as a prototypic dependence-inducing agent in the majority of studies.

The overwhelming majority of studies in rodents revealed suppressed expression of the morphine withdrawal syndrome (*see also* ref. *18*); however, in the only studies done in monkeys, phencyclidine (PCP) and dextrorphan failed to selectively suppress morphine withdrawal signs using a standard single-dose suppression model utilized for identifying potential treatments for opiate dependence *(48,49)*. A study in genetically modified animals lacking components of AMPA receptors is generally supportive of the idea that glutamate is critically involved in the development/expression of drug dependence *(50)*. However, to the best of our knowledge, there are no studies yet that evaluated the effects of inducible mutations. Thus, any results that were described to date can be attributed to the effects of genetic modifications on the development of drug dependence and, therefore, are difficult to evaluate within the lines of present discussion.

As with opiates, it appears that glutamate receptor antagonists inhibit the expression of withdrawal syndrome in animals dependent on ethanol *(51,52)*, barbituates *(53,54)*, benzodiazepines *(55–57)*, and nicotine *(58)*. Despite this almost unanimous agreement on the effects of negative modulators of glutamatergic transmission in drug-withdrawn animals, one could still argue that this evidence is not fully conclusive.

First, as exemplified by the studies that measured withdrawal-induced jumping as the dependent variable, these experiments typically include no control groups to prove that the effects of glutamatergic manipulations are selectively related to withdrawal-induced

Table 1
Inhibition of the Morphine Withdrawal Syndrome in Rodents by Glutamatergic Agents[a,b]

Drug type	Drugs and representative references
NMDA receptor channel blockers	MK-801 *(23)*; ketamine *(131)*; dextromethorphan *(131)*; memantine *(72)*; MRZ 2/579 *(132)*
Competitive NMDA receptor antagonists	LY 235959 *(133)*; LY 274614 *(23)*; D-CPPene *(59)*; CGS 19755 *(134)*; CGP 39551 *(135)*
NMDA/Glycine site antagonists and partial agonists	5,7-DCKA *(136)*; ACEA-1021 *(59)*; MRZ 2/576 *(137)*; MRZ 2/570; L-701,324 *(132)*; felbamate *(138)*; (±)HA-966 *(138)*; *D-cycloserine (138)*, (+)HA-966 *(134)*
AMPA/kainate receptor antagonists	DNQX, nonselective *(136)*; LY 293558 *(139)*; LY 300168 *(140)*; *LY382884*, GluR5 selective *(140)*
Metabotropic receptor ligands	(1S,3R)-ACPD, nonselective agonist *(141)*; DCG-IV, group II agonist *(141)*; LY 354740, group II agonist *(142), MCPG*, nonselective antagonists *(143)*; (*S*)-4C-PG, group I antagoist *(143)*; *MCCG*, group II antagonist *(143)*; *MAP4*, group III antagonist *(143)*
Miscellaneous	Eliprodil, polyamine site NMDA receptor antagonist *(59)*; NMDA-R1 antisense oligonucleotide *(144); 2-PMPA*, NAALADase inhibitor *(31); MS-153*, GLT-1 activator *(30); ibogaine (113,145)*, lamotrigine *(146), riluzole* (147)*, acamprosate (148)*

[a]The only study in monkeys was with PCP and showed negative results *(48,49)*.

[b]Italics indicate the agents that have little or no effect, increase the severity of withdrawal, or produce inconsistent pattern.

NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; PMPA, 9-(2-phosphonylmethoxypropyl) adenine; NAALADase, *N*-acetylated-α-linked-acidic dipeptidase.

jumping. In other words, withdrawal-induced jumping could be inhibited by the agents that produce nonspecific impairments in the "jumping" behavior. There is no obvious way to solve this problem because the withdrawal symptoms (e.g., jumping, teeth chattering, abdominal gasps, etc.) are likely to be absent or expressed at near-zero rates in normal subjects that are not drug-dependent. It may appear that, in some cases, withdrawal-like phenomena can be studied in nondependent subjects. For example, drug withdrawal may result in seizures (e.g., ethanol) or in aversive-like state (e.g., benzodiazepines), both of which can be modeled in drug-naive animals with the use of proconvulsant or anxiogenic agents, respectively. Unfortunately, this approach also has limitations because, as suggested above, glutamate receptor antagonists may have effects of their own that would make the interpretation of the results difficult (e.g., anticonvulsant and anxiolytic properties for the given examples).

Second, it is often observed that the doses needed to block the expression of drug withdrawal are rather high and significant behavioral toxicities can be observed at these dose levels. For instance, NMDA receptor antagonists were reported to attenuate discriminative stimulus effects of naloxone in morphine-dependent rats (Fig. 1; ref. *59*). However, these effects were most pronounced with the high-affinity channel blocker, MK-801, which possesses significant PCP-like effects at the effective dose levels. Mean-

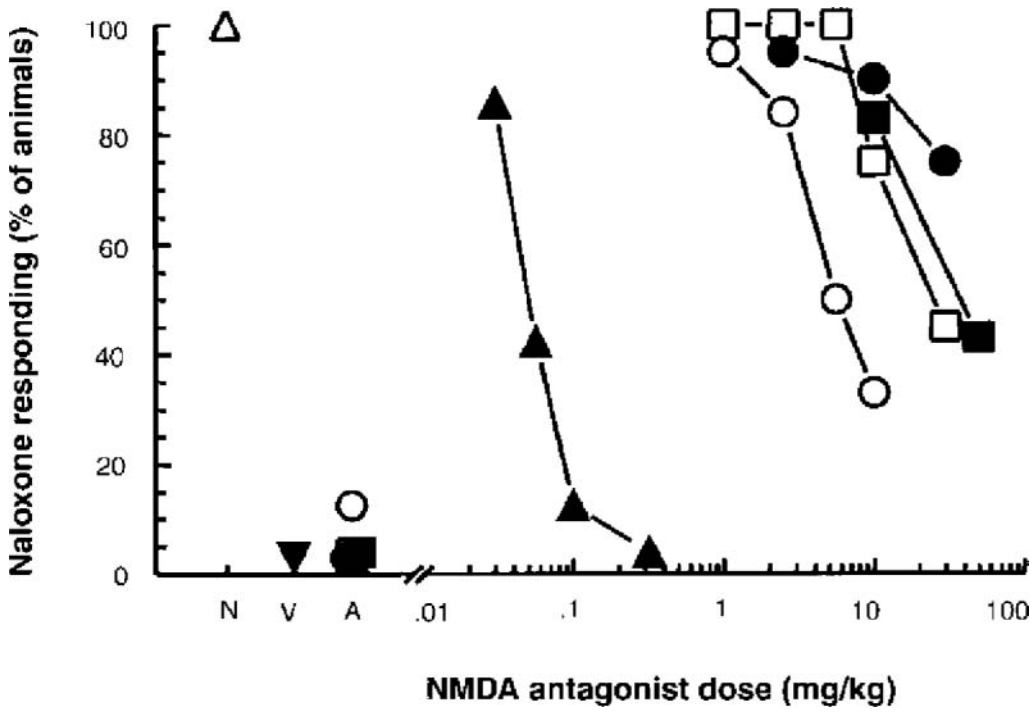

Fig. 1. *N*-methyl-D-aspartate (NMDA) receptor antagonism produces partial attenuation of discriminative stimulus effects of opiate withdrawal. Data are presented as percentage of the naloxone-appropriate responding following the administration of the training dose of naloxone (0.1 mg/kg, sc) in combination with ip dizocilpine (▲), memantine (□), D-CPPene (○), eliprodil (●), or ACEA-1021 (■) in morphine-dependent rats. Points above "V" and "N" represent the results of saline and naloxone test sessions, respectively. Points above "A" represent the results of the tests with the combinations of sc saline and the highest doses of NMDA receptor antagonists. Each point is based on observations made in seven to eight rats, except for 0.3 mg/kg of dizocilpine where only one rat was tested. (Reproduced with permission from ref. *59.*)

while, antagonists and doses that are less likely to produce PCP-like effects had little or no effects on naloxone's discriminative stimulus effects. Similarly, in monkeys, significant observable impairment occurs with NMDA channel blockers before any suppression of opiate withdrawal signs is obtained *(48,49)*.

Third, suppression of drug withdrawal is not complete and some symptoms persist in animals treated with glutamate receptor antagonists (e.g., refs. *59* and *60*). Such evidence may argue against the view that glutamate (in a single brain area) plays a causal role in triggering the expression of a full withdrawal syndrome.

Fourth, as illustrated by studies with ethanol, suppression of withdrawal symptoms may be explained by the ability of certain glutamate receptor antagonists to substitute for ethanol producing cross-dependence (animals: ref. *61*; humans: ref. *62*). These data are usually explained by glutamate receptor antagonist properties of ethanol. However, it should be noted that glutamate receptor antagonists (more specifically, those acting at NMDA receptors) also substitute for barbiturates with less well established NMDA antagonist properties *(63)*. Furthermore, such potentially confounding interactions are not limited to sedative-hypnotics as some NMDA receptor antagonists are also found to

produce synergistic effects with psychostimulant drugs *(64)*. Taken together, these data indicate that there are alternative mechanisms through which glutamate receptor antagonists may produce drug-like effects that would facilitate false-positive results in drug withdrawal experiments.

Fifth, NMDA antagonist drugs can themselves produce dependence. Studies in monkeys *(65)* and rats *(66)* have shown that PCP can produce significant physical dependence when administered repeatedly. Although it is possible to view the expression of withdrawal from NMDA antagonists as a rebound hyperactivity of glutamatergic systems, it is clear that dependence can develop while NMDA receptors are chronically blocked. Little is known about dependence on other types of glutamate antagonists, so it is not clear whether this is true of glutamatergic systems in general.

2.3. Protracted and Drug Cue-Induced Withdrawal Symptoms

Most, if not all, of the signs and symptoms that were recorded in the drug withdrawal studies described above are rather short-lived and disappear soon after the drug administration is suspended or when drug withdrawal is precipitated by an injection of a pharmacological antagonist. Some withdrawal symptoms may be apparent after the first 24 h and become manifest when the somatic and autonomic signs fade. For example, facilitation of aggressive behavior is observed following the cessation of repeated morphine injections and is maximally expressed at 48 h postmorphine. Acute administration of NMDA receptor antagonists (low-affinity channel blockers, memantine, and MRZ 2/579) significantly attenuates this withdrawal-facilitated aggression at dose levels that do not impair spontaneous motor activity or aggressive behavior in nondependent subjects *(67)*. However, one could question the validity of withdrawal-facilitated aggressive behavior as a sign of protracted withdrawal because it is known to fade over a period of 3–4 d, which is approximately the same speed with which other behavioral changes disappear (e.g., withdrawal-induced elevations in intracranial self-stimulation thresholds, ref. *68*, or ultrasonic vocalizations, ref. *69*.)

One class of dependence-related phenomena to survive significant periods of abstinence are those elicited by withdrawal-associated cues. This area of research has not received much attention and few experimental models have been developed that permit the analysis of withdrawal cue-induced behaviors. One of these models involves repeated pairings of the precipitated withdrawal state with specific environments. Subsequent tests in the drug-free state reveal an aversion to the withdrawal-associated environment, even when the test occurs 14 d after the last injection of the dependence-inducing agent *(70)*. Both development and expression of such conditioned place aversions are blocked by NMDA receptor channel blockers and competitive antagonists (systemic administration), as well as an AMPA receptor antagonist injected into the central amygdala *(70–73)*. Although still limited, these data suggest that NMDA receptor blockade negatively affects withdrawal cue-induced place aversions. However, this evidence needs to be extended to other classes of glutamate antagonists.

2.4. Glutamate and Repeated Withdrawal Experiences

So far we have seen that glutamatergic systems can be overactivated during drug withdrawal and negative modulation of this hyperactivation can reduce the expression of withdrawal symptoms. Nevertheless, for the reasons discussed above, it is still not clear whether this evidence *per se* may have practical significance. As pointed out, glutamate

receptor antagonists are capable of producing nonspecific reductions in the expression of drug withdrawal syndrome. Second, facilitated glutamatergic neurotransmission may serve a purpose different from that involved in triggering the expression of drug withdrawal.

Another way to conceptualize these relationships is to hypothesize that glutamatergic hyperactivity acts to facilitate withdrawal-related learning. Beginning with the work of Wikler *(74)*, it has been well established that classical conditioning plays a key role in appearance of drug abstinence signs and symptoms and that re-exposure to environments where drug taking and drug withdrawal had occurred in the past is a major factor in relapse *(75)*. Such learning may be seen in the laboratory in the form of environmental cues eliciting withdrawal-like effects *(5)*, conditioned place aversions *(71,72)* or as a progressive enhancement in the severity of withdrawal syndrome with repeated withdrawal experiences *(51,56,76)*. In both latter cases, glutamate seems to be critically involved, and the effectiveness of glutamate receptor antagonism may be to blunt this associative process. The role of glutamatergic systems in learning the drug withdrawal-related information serves as a link to its potentially broader role in drug-conditioned responses, a topic that will be discussed next.

3. GLUTAMATE AND DRUG-CONDITIONED RESPONSES

3.1. Brain Areas Involved in Drug Cue Reactivity

Glutamate is heavily represented in the projections to the brain areas that are thought to be involved in various aspects of drug cue reactivity. For instance, the ventral striatum, a critical structure for response-reinforcement learning, receives extensive glutamatergic projections from hippocampus, amygdala, thalamus, and several cortical areas *(77)*. In agreement with the role of glutamate in memory, glutamatergic pathways are found to be important for establishing various forms of stimulus–response and response–reinforcement learning including those forms that involve classical conditioning mechanisms *(78)*. Direct evidence for the involvement of glutamate in drug cue responses is provided by studies where drug cue presentations increased extracellular glutamate concentration in relevant brain areas, such as ventral striatum *(79)* or where lesions of areas sending efferent glutamatergic projections to mesolimbic targets produced impairment of specific forms of responding to drug-associated cues *(78)*. Of note, electrical stimulation of the glutamatergic projections to the ventral striatum may mimic some of the effects of drug cue exposures, such as reinstatement of extinguished drug self-administration behavior *(80)*. Direct administration of glutamate receptor agonists, such as AMPA produces similar effects *(81)*.

Exposures to drug cues activate many brain areas and there seems to be at least some degree of specialization in that different types of information are processed through pathways that overlap partially or do not overlap at all. For instance, it is generally accepted that contextual stimuli are processed in hippocampus *(82)*, which sends contextual stimuli-related information to several brain areas including ventral striatum via glutamatergic pathways *(83)*. The amygdala seems to play a critical role in responding to various types of discrete stimuli. More specifically, the central nucleus of the amygdala was found to be important for conditioned approach behavior and Pavlovian-to-instrumental transfer, whereas basolateral amygdala reportedly mediates the effects of conditioned reinforcers. Cingulate-striatal pathway may mediate "directional" properties of (discrete) conditioned stimuli. This subject received an extensive treatment in a recent review by Robbins and

Everitt *(78)*. Quite remarkably, the amygdala and anterior cingulate cortex are the areas that are reliably activated in drug abusers presented with drug-associated, presumably discrete, cues *(9)*.

It should be noted that glutamate and its interactions with dopamine play a key role in appetitive learning and drug reinforcement beyond just their involvement in drug cue reactivity. In the presence of glutamate, dopamine may turn from mainly inhibitory into a facilitatory neurotransmitter *(84)*. Conversely, dopamine may enhance glutamate-mediated excitation *(85)*. These interactions may present a mechanism that facilitates the selective storage of relevant information. Experimental data strongly suggest the functional importance of dopamine–glutamate interactions. For instance, coincident activation of glutamate and dopamine receptors is required for successful learning in appetitive learning tasks *(86)*.

Thus, glutamate is vastly represented in brain reward areas and is involved in the processing of stimuli associated with various reinforcers, not limited to drugs of abuse. In subsequent sections, we discuss experimental evidence on the ability of various negative modulators of glutamatergic neurotransmission to alter responding to drug-conditioned cues. Design of such experiments typically permits test drugs to be administered either during the conditioning phase or just prior to the final expression session. Although inhibition of glutamatergic neurotransmission retards the development of drug-conditioned behaviors, and this is well in line with the role of glutamate in learning and memory, mechanisms of such effects are difficult to interpret (e.g., these effects may involve direct interactions with the unconditioned effects of the drugs) and have questionable practical significance; therefore, these studies are not discussed. For the same reasons, until appropriate studies are done in animals with inducible mutations in glutamatergic systems, discussion of the results generated using genetically engineered animals is complicated by the fact that these manipulations may not have dissociable effects on development and expression of drug-conditioned responses.

3.2. Behaviors Elicited by Drug-Associated Stimuli

Presentation of the conditioned stimuli associated with drug administration elicits a variety of effects, some of them overtly resembling the effects of drugs themselves. For instance, drug-associated cues are often found to enhance motor activity in laboratory subjects. When administered prior to the test session, various glutamate receptor antagonists were reported to inhibit the expression of this drug-conditioned motor activity *(79,87)*. It should be remembered that some glutamate receptor antagonist, such as MK-801 and memantin, can stimulate motor activity themselves and, therefore, their effects on drug-conditioned activity are difficult to interpret. Several other NMDA receptor antagonists including glycine and polyamine site antagonists attenuate drug (cocaine)-conditioned motor activity at doses that have no effects of their own *(87)*. Similarly, systemic administration of the AMPA receptor antagonist at doses that are without effect on spontaneous activity attenuated the expression of cocaine-conditioned activity *(79)*.

3.3. Drug-Conditioned Place Preference

It was initially found that nonselective glutamate receptor antagonists such as kynurenic acid inhibited both the development and expression of morphine conditioned place preference *(88)*. Subsequent studies extended this finding with various types of glutamatergic modulators and abused drugs (Table 2). Though most evidence linking

Table 2
Inhibition of the Expression of the Drug-Conditioned Place Preference and Aversion by Glutamatergic Agents[a]

Glutamatergic agent	Conditioning drug	References
NMDA receptor antagonists		
– channel blockers	Morphine (↓), cocaine (↓0), amphetamine (0)	*72,149–152*
– competitive antagonists	Morphine (↓), amphetamine (↓), pentilenetetrazol (↓)	*70,94*
– glycine site antagonists	Morphine (↓), cocaine (0), amphetamine (↓)	*132,153*
– glycine site partial agonists	Morphine (↓), diazepam (↓), nicotine(↓), amphetamine (0), cocaine (0), nomifensine (0), naloxone (0), picrotoxin (0)	*7,151*
AMPA receptor antagonists	Morphine (↓), cocaine (↓), amphetamine (↓0)	*149,150,154*
Metabotropic mGluR5 antagonists	Morphine (↓)	*155*
NAALADase inhibitors	Morphine (↓), cocaine (↓)	*31,156*

[a]↓, inhibition; 0, no effect.
NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; NAALADase, *N*-acetylated-α-linked-acidic dipeptidase.

glutamate with the expression of drug-conditioned place preferences was obtained using systemic administration of glutamatergic agents, local blockade of glutamate receptors in ventral striatum and ventral tegmental area produces similar effects in morphine-conditioned animals *(89)*. Enhanced cocaine-conditioned place preference in animals with overexpression of the GluR1 subunit in the ventral tegmental area *(90)* is well in line with the inhibitory effects of glutamate receptor antagonists *(91,92)* and glutamate receptor subunit gene knockout (NR2B; ref. *93)* on the development of drug-conditioned place preferences. However, data obtained in genetically modified animals are difficult to interpret because both development and expression of place preferences could have been targeted by these manipulations (*see also* Subheading 3.1.).

Thus, negative modulation of glutamatergic neurotransmission attenuates the expression of drug-conditioned place preferences. It has been argued that these effects do not result from general motivational or learning deficits. For instance, studies report no effect on place preferences conditioned with nondrug reinforcers *(7,72)*, although some glutamate receptor antagonists may still inhibit the expression of drug-conditioned place aversions *(94)*. Also, it is worth noting that, with the exception of PCP-like NMDA receptor antagonists, which are known to affect various forms of discriminative behaviors *(13,95,96)*, glutamate receptor antagonists are not likely to impair discrimination between drug- and vehicle-conditioned environments and thereby produce false positive results.

3.4. Responding With Conditioned Reinforcement

Similarly to the drug exposure itself, classically conditioned cues are also known to reinstate previously extinguished drug seeking and taking behavior *(11)*. Response-contingent

drug-paired stimulus presentations are also important in maintaining lengthy sequences of drug self-administration *(97)*. One of the experimental designs commonly used to investigate the role of glutamatergic systems in conditioned reinforcement based on drug reinforcers involves response-contingent presentations of drug-associated cues after an extinction period during which drug cues are not presented. A critical issue in all such studies is whether administration of glutamatergic antagonists selectively alters the reinforcing properties of the drug-associated stimuli. Nonselective effects on both cue-maintained and drug-maintained responding are difficult to interpret. Administration of NMDA receptor antagonists produced mixed effects on cue-induced reinstatement of cocaine seeking *(98)*. In this study, the competitive antagonist, D-CPPene, but not the channel blocker, memantine, reduced the cue-induced responding on a lever that previously resulted in cocaine deliveries. Local injections of either the NMDA receptor antagonist AP-5 or nonselective AMPA receptor antagonist CNQX into the basolateral amygdala also were without appreciable effects on cue-induced reinstatement of cocaine self-administration *(99)*.

When injected into the nucleus accumbens core of rats trained to lever-press for cocaine under a second-order schedule of reinforcement, the AMPA receptor antagonist LY293558 produced rather nonselective effects, attenuating responding during both an initial cocaine-free period and after the first cocaine infusion was delivered; the NMDA receptor antagonist AP-5 again had no effect *(100)*. A recent study obtained data that systemic administration of CNQX selectively attenuated cocaine-seeking behavior during the drug-free period controlled at least in part by cocaine-associated cues *(101)*. However, a close inspection of the reported cumulative records may indicate that, at least in some subjects, CNQX was also affecting responding after the first cocaine infusion was delivered (Fig. 2).

Thus, it appears that glutamate receptor antagonists (particularly those acting at NMDA receptors) are less likely to produce selective impairment of drug cue-induced behaviors when discrete stimuli are used for conditioning. Indeed, most self-administration studies use discrete stimuli and negative or mixed results are obtained in the experiments with glutamate receptor antagonists. In contrast, both conditioned place preferences and conditioned motor activity are observed when exposing animals to contextual stimuli and glutamate receptor antagonists are usually found to attenuate the expression of these phenomena. Unfortunately, there have been few attempts so far to directly compare the effects of glutamatergic manipulations on behaviors controlled by discrete vs contextual stimuli. In one such study, as shown in Fig. 3, effects of these stimulus types may be successfully dissociated using glutamate (NMDA) receptor blockade.

3.5. Assessing Cue Reactivity in Protracted Withdrawal

Most animal drug cue reactivity studies are conducted shortly after the drug exposure is terminated in subjects that have a limited history of drug exposure and are not dependent on the drug. Furthermore, commenting on one of the most popular reinstatement models of relapse behavior, Everitt and Wolf *(102)* wrote that "although an effective and fruitful model of relapse, extinction of drug self-administration is not a means by which human addicts achieve abstinence, which is more likely to arise through an active decision to abstain or through forced abstinence."

Meanwhile, it was recently shown that responding maintained by conditioned reinforcement may become increased with prolonged abstinence *(103,104)* and this is well in line with the clinical experience indicating that the drug cue reactivity does not

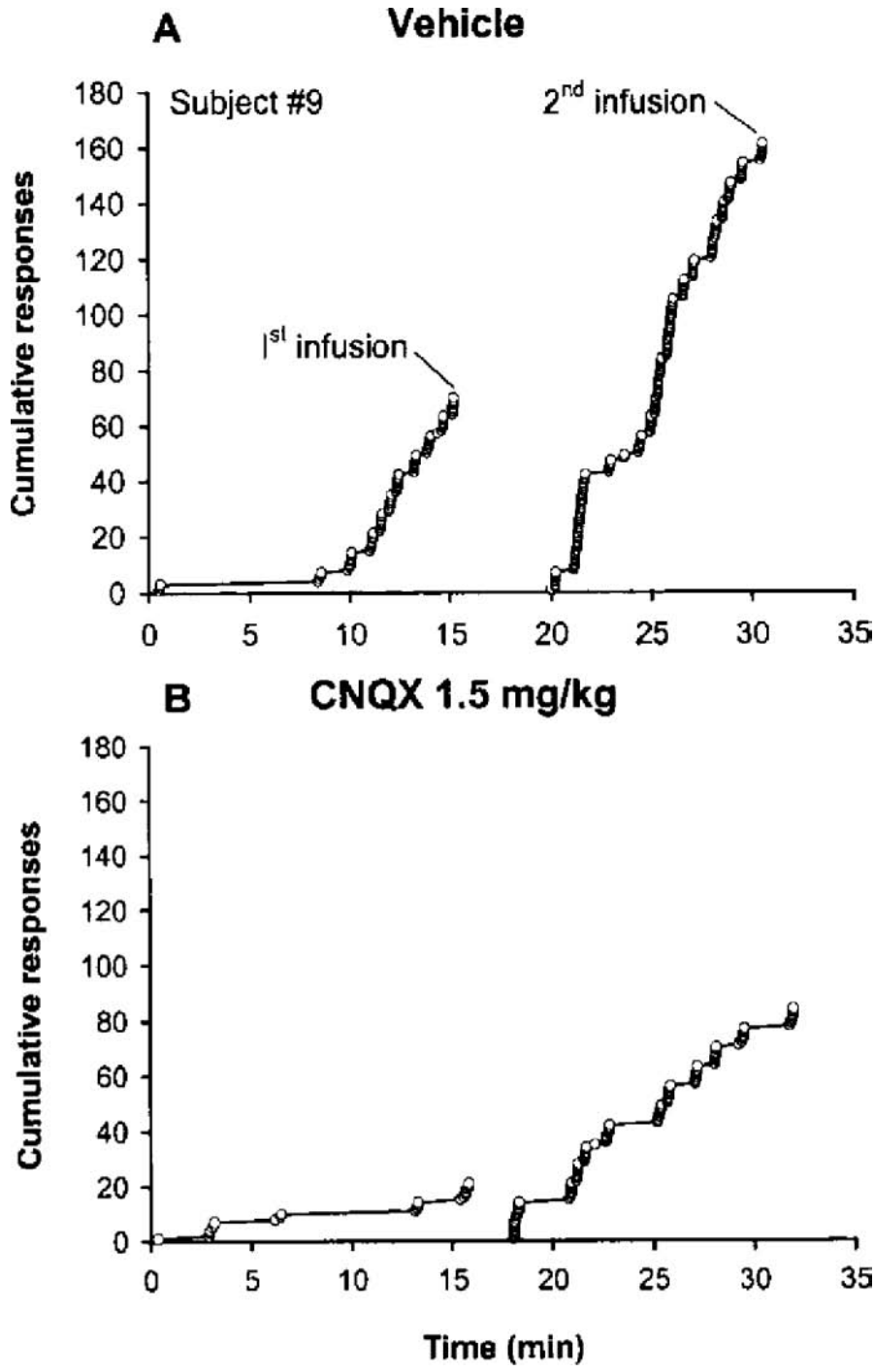

Fig. 2. Pattern of responding by a single rat during the first two intervals under the fixed interval 15-min (fixed ratio 7:S) second-order schedule of intravenous cocaine reinforcement (0.50 mg/infusion). The cumulative number of responses is plotted against the elapsed time from the start of the session. Each open circle indicates a response on the active lever. **(A)** responding after the vehicle injection. **(B)** responding in the same rat pretreated with 1.5 mg/kg of CNQX (ip, 20 min presession injection time). (Reproduced with permission from ref. *101.)*

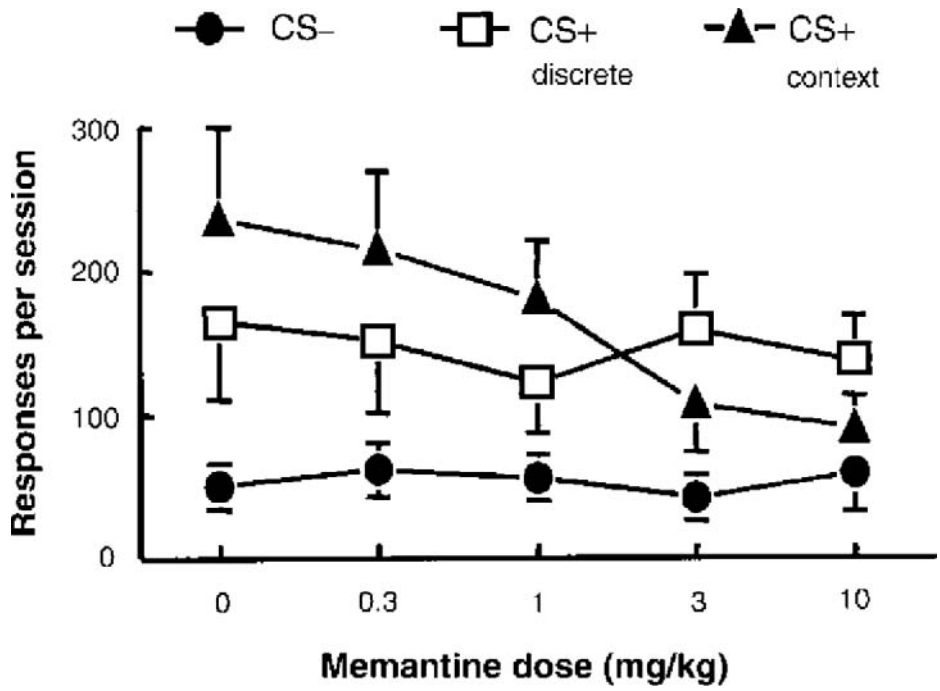

Fig. 3. Effects of memantine on conditioned facilitation of intracranial self-stimulation behavior. Rats with bipolar electrodes implanted unilaterally into the ventral tegmental area were trained to lever press for response contingent electrical stimulation (continuous reinforcement). After preliminary lever-press training, two types of daily sessions were held on 10 consecutive days: type T+, during which current intensity was set at the threshold level (high-intensity stimulation), and type ST–, during which current was set at the subthreshold level (low-intensity stimulation). In separate groups of animals, these two session types were further differentiated by: (a) the specific contextual stimuli presented continuously during the entire session, or (b) compound stimuli consisting of discrete visual (stimulus lights above the lever briefly going off at each lever press) and auditory (buzzer clicks) signals. On d 11, memantine or its vehicle were injected 30 min prior to the 15-min test phase, during which each lever-press response was accompanied by ST stimulation. During the tests, rats were presented with no stimuli (CS–), contextual stimuli, or discrete stimuli (contingent on each response). Combination of the ST current intensities and stimuli previously associated with the threshold stimulation resulted in the significantly elevated response rates compared to the performance under the subthreshold current without visual stimuli (data point above "0"). Memantine reduced the response rates in a dose-dependent manner only for the group of rats that were exposed to the contextual stimuli. Other training and testing procedures were essentially similar to that in an earlier study, which did not explicitly separate discrete and contextual stimuli *(157)*. $n = 6–10$.

decline soon after abstinence is achieved *(75)*. This phenomenon, labeled as "incubation of drug craving," seems to be remarkably similar to what is known as a "deprivation effect" *(105)*. The deprivation effect was originally described in alcohol-drinking rats as an increase in the level of free-choice consumption of alcohol following a period of forced abstinence. The deprivation effect can be observed in several species of laboratory animals (as well as in humans) exposed to long-term, free-choice access to various drugs of abuse and nondrug, reinforcers such as saccharin *(106)*. The deprivation effect

procedure features several key characteristics relevant for modeling drug addiction: (a) drug consumption is increased with repeated deprivation (forced abstinence) episodes *(105)*, (b) expression of the deprivation effect is context-dependent, and (c) extinguishing the cue reactivity may prevent the development/expression of the deprivation effect *(106)*.

Apart from the above-mentioned studies by Grimm and colleagues *(103,104)*, there is very limited evidence on the drug cue reactivity in subjects that were exposed to long-term forced abstinence with no explicit extinction training during the withdrawal periods. Accordingly, there have been hardly any studies that assessed the effects of glutamatergic manipulations on the drug cue reactivity during an extended abstinence phase. Nevertheless, it is worth noting that NMDA receptor channel blockers, memantine, and MRZ 2/579 (neramexane), as well as acamprosate, potently inhibit the alcohol deprivation effect *(107–109)*. These results indicate that glutamate may be involved in the reinstatement of drug seeking and taking in animals that are withdrawn from long-term access to the drug. It is yet to be studied whether glutamate receptor blockade would also affect drug cue reactivity in subjects exposed to prolonged, forced abstinence.

4. DEVELOPING GLUTAMATERGIC STRATEGIES OF DRUG RELAPSE PREVENTION

4.1. General Rationale

As reviewed above, experimental evidence strongly suggests that glutamatergic drugs may affect various behaviors and phenomena associated with the relapse to drug seeking and drug taking. For this reason, there has been considerable interest in the possibility that various types of glutamate antagonists may be developed for as pharmacotherapies for drug abuse treatment *(21)*.

Although this may be a good strategy, it important to understand exactly what behavioral mechanisms such prospective medications might target. To do this, a more refined understanding is needed of how glutamate receptors are involved in drug abuse-related phenomena. For example, we are not convinced that glutamate antagonists will have direct benefits for treatment of the acute withdrawal syndrome, except possibly for alcohol where NMDA receptor antagonists may directly substitute. Although glutamate release is often enhanced in drug-withdrawn subjects and negative modulation of glutamatergic transmission affects the expression of drug withdrawal syndrome in various species of laboratory animals, these findings alone are not very conclusive. In the first place, these phenomena have been seen primarily in rodent subjects; monkey studies so far have produced no evidence for selective attenuation of opiate withdrawal, at least not by NMDA receptor channel blockers. But, what is most important is that the expression of drug withdrawal has yet to be causally related to the hyperactivation of glutamatergic systems. Instead, we suggested that enhanced glutamatergic neurotransmission serves to facilitate the acquisition and storage of information relevant to the drug withdrawal state. Acute drug withdrawal effects are thought to contribute significantly to the maintenance of regular patterns of drug use. With repeated withdrawal experiences, the subjective effects of the withdrawal state may come to act as a discriminative stimulus for drug seeking and taking behavior or it may enhance the incentive value of the drug itself. Moreover,

repeated drug withdrawals were shown to result in progressive enhancement in the expression of withdrawal symptoms, and this sensitization was prevented by glutamate receptor antagonists. Thus, it is hypothesized that the withdrawal-induced glutamate release contributes to this progressive sensitization.

Acute drug withdrawal is described as a highly aversive state and protracted withdrawal is commonly associated with negative affect and craving. Negative affect, including cue-induced negative mood, is thought to contribute to relapse *(110)*. Meanwhile, drugs acting at various subtypes of glutamate receptors (e.g., NMDA, metabotropic) possess significant anxiolytic (*see* Chapter 12) and antidepressant (*see* Chapter 10) activity. Such properties make glutamatergic agents especially warranted in the clinical drug dependence trials.

Highly orchestrated interactions between glutamate and dopamine in the brain reward circuits are seen as the primary mechanisms for attributing increasing motivational valence to the environmental and other stimuli associated with repeated drug exposures. Cue-induced reactivity is one of the major factors in initiating drug seeking behavior, and glutamate is critically involved in the development and expression of drug-conditioned behaviors.

Overall, there is a large body of evidence indicating that negative modulators of glutamatergic neurotransmission reduce the severity of acute withdrawal syndrome and attenuate responding to cues previously associated with the drugs. This evidence is likely to result in a successful treatment strategy. Clinical research data on these matters will be discussed next.

4.2. Preliminary Human Data

Although the first studies implicating glutamate in the mechanisms of drug dependence were conducted in the late 1980s to early 1990s *(22,23,111)*, clinical application of this knowledge is still delayed. To date, there have been only a handful of studies that assessed the effects of glutamate receptor antagonists on drug dependence and other addictive behaviors in humans. Antiaddiction medication is rarely a primary goal for pharmaceutical companies when developing glutamatergic agents. This is why there were no drug dependence studies with newly developed, selective glutamatergic agents with more favorable side-effect profiles than such prototypic antagonists as NMDA receptor channel blockers. In addition, drug dependence studies have also been conducted with medications that have been on the market for a number of years and for which glutamatergic activity seems important for their clinical effectiveness but is not the only mechanism of their action. This point is best exemplified by acamprosate, a drug that features NMDA receptor antagonist properties among many other receptor mechanisms and that is currently used in Europe and several other countries for alcohol dependence treatment (*see* ref. *112* for review). Similarly, NMDA receptor antagonist properties are attributed to ibogaine, which is claimed to have antiaddiction effects in humans *(113)*; however, this drug too has actions at many other receptors and it is unlikely that most of its central nervous system effects are related to its NMDA antagonist properties *(114)*. Other available agents for clinical studies include compounds that act as NMDA receptor channel blockers, such as ketamine, memantine, and dextromethorphan. Although some of these drugs have abuse potential of their own (ketamine), they have proven to be useful clinical tools as briefly reviewed later.

4.2.1. Opiate Dependence

Initial studies with dextromethorphan *(115)* revealed little or no effect of this drug on the expression of opiate withdrawal (spontaneous withdrawal procedure in inpatients stabilized on morphine before the dextromethorphan administration). Similar negative conclusions were reached in two more recent studies, one of which was conducted in opiate-dependent patients stabilized on methadone *(116)* and another one in nondependent subjects using acute morphine-induced dependence procedure *(117)*. These negative results are in agreement with the results of studies of single-dose suppression in morphine-dependent rhesus monkeys reviewed above *(48,49)*. Meanwhile, several detoxification studies with significantly higher doses of dextromethorphan (360–375 mg/d) suggested that this agent facilitated the dissipation of withdrawal signs and symptoms and reduction in heroin craving *(118,119)*. However, any further interpretation of these findings was obstructed by lack of placebo control, use of adjunct medications (e.g., diazepam), and/or small sample sizes.

Results with memantine appear more positive. A recent placebo-controlled study with memantine reported attenuated expression of opiate dependence in eight heroin-dependent inpatient volunteers who were maintained on morphine prior to being subjected to naloxone-recipitated withdrawal *(120)*. Memantine significantly reduced the intensity of the withdrawal syndrome as measured by objective rating scales (Clinical Institute for Narcotic Withdrawal Scale and Objective Opioid Withdrawal Scale) and this effect was evident for at least 54 h after the drug administration. Although it appeared that the drug was well tolerated, the tested dose (60 mg) is quite high as it significantly exceeds the dose recommended for clinical use by the manufacturer. At this dose level, memantine is likely to produce some side effects typical for NMDA channel blockade such as hallucinations, confusion, and dizziness.

Furthermore, memantine was found to reduce heroin craving in addicts that were detoxified for 7–10 d prior to the memantine treatment phase *(121)*. In this single-blind placebo-controlled study, memantine was administered at lower dose levels (10–30 mg/d) for three consecutive weeks and was found to reduce heroin craving and withdrawal-related anhedonia. In the placebo group ($n = 22$), 36% of patients left the hospital against medical advice and relapsed to heroin use during the 3-wk study period. In contrast, in the memantine group ($n = 2l$), all but one subject remained heroin-free through the end of the study. As memantine appeared to be both safe and effective, these data provide a rationale for further studies on the use of memantine in heroin dependence treatment.

4.2.2. Ethanol Dependence

A large number of clinical trials focused on the effects of acamprosate in alcohol addicts *(122)*. Overall, there was a moderate but fairly consistent reduction in the rates of relapse to alcohol abuse. These studies did not focus on the ethanol withdrawal; nor was it reported whether responding to ethanol-associated cues was affected (hence, discussion of these studies is not fully within the scope of this review). With the mechanism of action of acamprosate still unclear, it is hard to judge whether its NMDA receptor antagonistic properties make a determinative contribution to these positive effects in alcoholics. Clinical trials with agents that have better defined mechanisms of action are currently in progress (e.g., clinical trial with neramexane as cited in ref. *123*).

4.2.3. Psychostimulant Dependence

Compared with opiate and alcohol dependence, clinical trials with glutamatergic agents in psychostimulant drug abusers are less supported by experimental evidence that would suggest that these agents block psychostimulant withdrawal or reduce responding to psychostimulant-related cues.

Meanwhile, it is known that psychostimulant withdrawal is characterized by marked decrease in extracellular dopamine concentration in ventral striatum and, in amphetamine- but not cocaine-treated animals, this deficit is restored by the NMDA receptor channel blocker MK-801 *(124)*. MK-801, as well as most other NMDA receptor channel blockers, indirectly stimulates the dopaminergic system and, therefore, it is not surprising that these agents may be effective when the dopaminergic system is hypoactive. Indeed, there is some evidence suggesting that amantadine may be effective in cocaine-dependent patients with severe withdrawal symptoms *(125)*. Amantadine is well established as a dopaminergic agonist, but it is not clear that it has NMDA antagonist properties in vivo *(126)*. These beneficial effects are likely to be limited to the early phase of withdrawal syndrome and there was found no effect of amantadine on cue reactivity in cocaine-dependent subjects *(127)*.

5. CONCLUSIONS AND PERSPECTIVES

Experimental studies provided a very straightforward rationale for developing and testing glutamatergic therapies for drug dependence. It is firmly established that glutamatergic neurotransmission is facilitated in drug-withdrawn subjects and data suggest that glutamate receptor antagonists may block at least some of the drug withdrawal signs and symptoms. However, clinical studies have yielded mixed results and the utility of negative glutamatergic modulators for managing unconditioned drug withdrawal remains obscure.

Reconsidering the role of glutamate in drug withdrawal, we propose that enhanced glutamate release during the drug withdrawal serves to facilitate storage of memories associated with the withdrawal experiences. Thus, the rationale for applying glutamatergic modulators during the drug withdrawal may be not to block the expression of the withdrawal syndrome but rather to prevent acquisition and expression of the withdrawal-related information that plays an important role in protracted withdrawal and relapse. Some correlates of such withdrawal-related learning are well known and were shown to be sensitive to blockade of glutamatergic transmission (e.g., conditioned place aversion, progressive enhancement in the severity with repeated withdrawal experiences). It follows then that beneficial effects glutamatergic inhibitors in drug abuse treatment may not be evident upon a single or short-term administration.

Interference with the drug cue reactivity represents another alternative whereby glutamatergic agents may affect the behavior of drug-withdrawn subjects. Although awaiting definitive clinical evidence, one could note several features of cue reactivity in drug abusers that may be critical for both analyzing the role of glutamate and developing a successful medication.

Common logic suggests that the final result of cue reactivity treatment should be extinguished responding to drug cues. Extinction is new learning and agents that attenuate reinforcement-related learning may not be effective in facilitating extinction. Knowing the role of glutamate in the acquisition of new memories *(16)*, it will not be surprising if negative modulators of glutamatergic transmission are found to attenuate extinction. On the other hand, context dependence of the extinction can limit the effectiveness of clinical cue

extinction treatment programs *(128,129)*,which, therefore, may be significantly improved by the therapeutic agents that selectively interfere with acquisition of context-related information. Experimental evidence suggests that different neuroanatomical pathways may subserve responding to discrete vs contextual cues and at least some types of glutamate receptor antagonists may preferentially reduce responding to contextual cues. Thus, it is important to assess the contribution of various types of glutamatergic receptors, subunits, and release/uptake mechanisms to processing of different types of drug-related information.

Furthermore, extinction of the drug cue reactivity is not complete if the cue exposure programs do not include drug onset cues *(130)*. Thus, analysis of glutamate involvement in drug cue reactivity should take into account the possibility of direct pharmacological interferences between glutamatergic agents and drugs of abuse.

Finally, glutamate is vastly represented in brain reward areas and is involved in the processing of responding to stimuli associated with various drug and nondrug reinforcers. There have been relatively few studies that directly compared the effects of glutamatergic manipulations on behaviors conditioned with drug vs nondrug reinforcers and, therefore, it cannot be fully predicted whether glutamatergic therapeutics would selectively target responding to drug-related stimuli and spare behaviors maintained by nondrug responding. It should be noted that there is little concrete scientific basis yet for explaining how associative learning and unlearning about drug-related phenomena would have different neural bases, and hence selective medication targets, compared with other forms of learning and memory. However, one could ask the question: Is such selectivity a necessary condition for developing effective and safe medications?

For example, behaviors maintained by nondrug reinforcers may also become excessive and become addictive (eating, gambling, shopping). Stimuli associated with nondrug reinforcers may contribute to the acquisition, maintenance, progression, and recurrence of the nondrug addiction just as they contribute to drug addiction. Therefore, effects of glutamatergic manipulations on non-drug-conditioned behaviors is not necessarily a negative finding. Instead, it may indicate that glutamatergic systems subserve general mechanisms common for drug and nondrug addictions.

Furthermore, although rarely the focus of experimental studies, drug cue reactivity is a not an acute phenomenon, and successful clinical treatment programs are expected to counteract cumulative effects of repetitive exposures to drug-associated cues in their patients. Future studies will need to establish and validate procedures assessing drug cue reactivity as a function of withdrawal (abstinence) duration and evaluate the role of glutamate in the interaction between cue reactivity and withdrawal factors.

ACKNOWLEDGMENTS

Supported by the NIH Fogarty International Research Collaboration Award #TW00714 and NIDA grant DA-01442. The authors would like to acknowledge the many individuals and groups whose research has contributed to this body of work, but who could not be cited because of the lack of space.

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IX Neurodegeneration

18
Dopamine and Neurodegeneration

Gabriele Gille and Peter Riederer

1. INTRODUCTION

Dopamine (DA) is the prevalent catecholaminergic neurotransmitter in the brain and dopaminergic neurons are mainly located in the substantia nigra (SN), the ventral tegmental area (VTA), and arcuate nuclei. They constitute at least three important dopaminergic pathways: the nigro–striatal, the mesocortical–mesolimbic, and the tubero–infundibular system, which are responsible for locomotor movement, motivation and reward, and secretion of pituitary gland hormones, respectively *(1,2)*. The dysfunction of these systems can lead to neurological, psychological and endocrinological diseases like, e.g., Parkinson's disease (PD), attention deficit hyperactivity disorder (ADHD), and prolactinoma *(3)*. There is increasing evidence that DA, besides acting as a neurotransmitter, can become neurotoxic at high concentrations or in an oxidative environment. Especially in idiopathic PD, even though the SN is not the only affected region in the brain, the main pathological hallmark is the progressive degeneration of the neuromelanin-containing dopaminergic neurons in the SN pars compacta (SNpc) causing the cardinal symptoms of tremor, rigidity, and bradykinesia. It is believed that the interplay between the high DA content and a local pro-oxidative environment in the SN mutually promote the increase of oxidative stress and thus the degeneration of dopaminergic neurons.

Principally, DA can exert its neurotoxic effects via oxidative and nonoxidative mechanisms: enzymatic and nonenzymatic oxidation of DA yield redox-active compounds like reactive oxygen species (ROS) and DA-quinones/semiquinones. Condensation reactions of DA with carbonyl groups of aldehydes and α-ketoacids generate tetrahydroisoquinolines (TIQs), which are converted into toxic cations by *N*-methylation. Moreover, DA can be neurotoxic *per se* without involving oxidative stress.

Possible mechanisms of neurotoxicity and molecular events underlying cell death induced by DA will be discussed in this chapter.

2. OXIDATION REACTIONS OF DA

In the striatum the average DA concentration amounts to 65 μ*M* and in dopaminergic nerve endings a concentration as high as 50 μ*M* is reached, even though most of the DA will be stored in synaptic vesicles *(4)*. When pathological conditions lead to an elevated tunover or release of DA from the vesicles, but also during normal aging, DA-driven

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

production of ROS (H_2O_2, $O_2^{\bullet -}$, $^{\bullet}OH$) and toxic quinone species are accumulated and finally may account for neurodegeneration of the dopaminergic system.

2.1. Enzymatic Oxidation Reactions of DA

The oxidative breakdown of DA can occur via two routes, enzymatically and nonenzymatically including a process called auto-oxidation. The predominant form of enzymatic oxidative metabolism of DA in the brain is via monoamine oxidase (MAO), which is located in the outer mitochondrial membrane. MAO catalyzes the oxidative deamination of DA to Dopaldehyde (DOPAL) with concomitant production of H_2O_2 (Fig. 1). Of the two MAO forms MAO A preferentially, though not exclusively, metabolizes serotonin and noradrenaline MAO A is localized mainly in the locus coeruleus and other catecholaminergic cells of the brain stem, whereas only a light staining for MAO A was detectable in the SN *(5,6)*. DA is preferentially metabolized by MAO B, which is found in the raphe nucleus, certain cell groups of the hypothalamus, and brainstem areas, and in astrocytes *(5–7)*. Also other enzymes found in the brain are capable of oxidizing DA and comprise lipoxygenase *(8)* and xanthine oxygenase *(9)*, which catalyze the oxidation of DA in the presence of hydrogen peroxide, as well as tyrosinase and prostaglandin H synthase. During all these enzymatic reactions DA is oxidized to the highly reactive DA quinone (DAQ) (Fig. 2). It still is not clear to what extent these enzymes contribute to the oxidation of DA in the human brain. However, for prostaglandin H synthase it was demonstrated that the activity in the SN of PD brains was significantly higher than in matched Alzheimer's disease (AD) and control brains *(10)*. There is a controversial debate over whether tyrosinase exists to a significant extent in the SN. Although no tyrosinase immunoreactivity could be detected in neuromelanin-pigmented neurons *(11)*, tyrosinase mRNA was demonstrated in SN *(12)*.

2.2. Nonenzymatic Oxidation Reactions of DA

The most relevant and best studied DA oxidation products with respect to their neurotoxic action in vivo are DAQ, 6-hydroxydopamine (6-OHDA), neuromelanin (NM), and 5-*S*-cysteinyl DA. Their possible nonenzymatic formation pathways will be presented in this section.

The catechol moiety of DA is a phenolic compound and as thus may undergo oxidation by oxygen at physiological pH *(13)*. Such auto-oxidation of DA yields DAQ (*see* Fig. 2) analogously to the enzyme-catalyzed oxidation reaction by tyrosinase or prostaglandin H synthase. The complex oxygen-driven oxidation reaction also gives rise to the highly reactive semiquinone radical and results in the univalent reduction of oxygen with consequent production of superoxide anion, hydrogen peroxide, and hydroxyl radical *(13,14)*:

$$DA + O_2 \rightarrow SQ^{\bullet} + O_2^{\bullet -} + H^+ \quad (1)$$

$$DA + O_2^{\bullet -} + 2\,H^+ \rightarrow SQ^{\bullet} + H_2O_2 \quad (2)$$

$$\text{transition metal}^{2+} + H_2O_2 \rightarrow \text{intermediate complex} \rightarrow {}^{\bullet}OH + HO^- + \text{transition metal}^{3+} \quad (3)$$

(Note: $SQ^{\bullet}$ stands for semiquinone radical.)

However, the uncatalyzed auto-oxidation reaction of DA is relatively slow at physiological pH, but is accelerated by free-transition metal ions like iron and manganese. Both

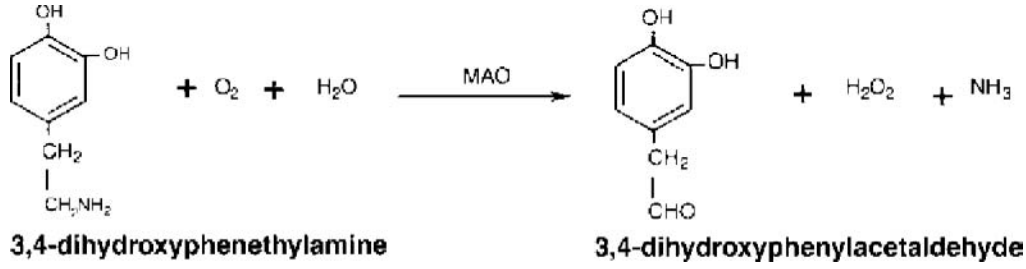


Fig. 1. Oxidative deamination of 3,4-dihydroxyphenethylamine (dopamine) to 3,4-dihydroxyphenylacetaldehyde leading to the generation of H_2O_2.

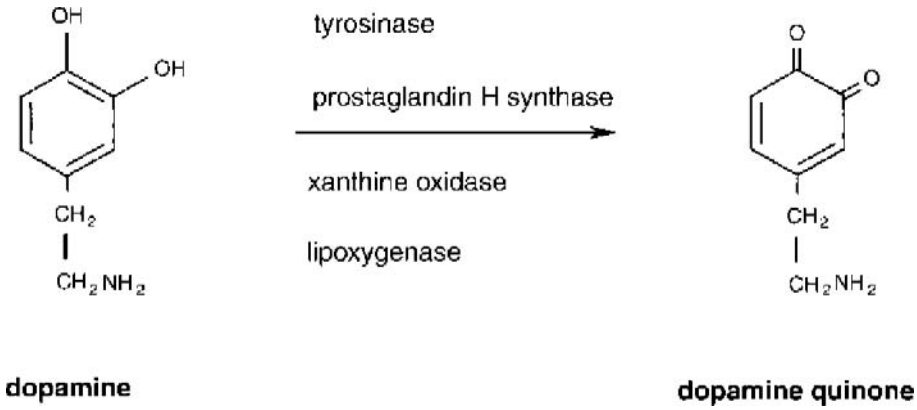
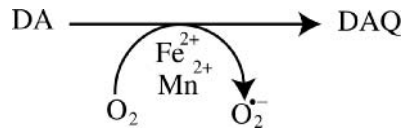


Fig. 2. Enzymatic oxidation of dopamine to dopamine quinone, substrates and (by-)products of the enzymatic reactions are not shown.

metals catalyze the oxidation of DA into DAQ with concomitant production of ROS *(15,16)*:



(4)

This is of special importance in PD, where the iron content was found to be markedly increased in severe cases *(17,18)*. Moreover, it was shown that also $ONOO^-$ and its decomposition product NO_2^- efficiently promote the oxidation of DA to DAQ *(19)*. In PD, glutamate-mediated, secondary excitotoxicity owing to impaired energy metabolism and consequently, elevated $NO^\bullet$ and $ONOO^-$ levels, are believed to contribute to the pathogenetic process *(20,21)*. The formation of 6-nitroDA by nitrogen oxides derived from $NO^\bullet$ was also demonstrated at physiological pH *(22)* even though 6-nitroDA has not yet been identified in vivo *(23)*.

Fig. 3. Chemical structures of (6-OHDA) 6-hydroxydopamine and 6-OHDA quinone.

6-Hydroxylation of DA via nucleophilic attack of water on DAQ results in the formation of the neurotoxin 6-OHDA *(24)* (Fig. 3). However, the uncatalyzed 6-hydroxylation of DA is rather slow, but may be enhanced at physiological pH by the addition of $^{\bullet}$OH-generating systems like the Fenton reagent (Fe^{2+}/H_2O_2) or Fe^{2+}/ascorbate *(25)*. Iron *(26,27)* and manganese *(28)* have also been shown to facilitate the oxidation of DA to 6-OHDA, though the manganese-catalyzed formation of 6-OHDA was later disproved by others *(29)*. An enzymatically accelerated formation of the 6-OHDA quinone was achieved by peroxidase/hydrogen peroxide oxidation of DAQ via nucleophilic addition of the H_2O_2 anion *(29)*. A novel mechanism was proposed for the generation of 6-OHDA quinone (Fig. 3) involving a direct interaction of lipid hydroperoxide, derived from oxidation of polyunsaturated fatty acids, with a DA-Fe^{3+} chelate. The reaction is independent of $^{\bullet}$OH-mediated hydroxylation/oxidation processes *(30)*. 6-OHDA as such is relatively unstable and undergoes rapid auto-oxidation to form the corresponding quinone and H_2O_2 *(31)*:

$$6-\text{OHDA} + O_2 \rightarrow 6-\text{OHDA quinone} + H_2O_2 \quad (5)$$

The auto-oxidation involves the production of $O_2^{\bullet -}$ and the 6-OHDA semiquinone as intermediates:

$$6-\text{OHDA} + O_2 \rightarrow 6-\text{OHDA SQ}^{\bullet} + O_2^{\bullet -} + H^+ \ (\text{slow}) \quad (6)$$

$$6-\text{OHDA SQ}^{\bullet} + O_2 \rightarrow 6-\text{OHDAQ} + O_2^{\bullet -} + H^+ \ (\text{fast}) \quad (7)$$

$$6-\text{OHDA} + O_2^{\bullet -} + 2H^+ \rightarrow 6-\text{OHDA SQ}^{\bullet} + H_2O_2 \ (\text{fast}) \quad (8)$$

Auto-oxidation studies of catecholamines in vitro helped to elucidate the complex oxidation pathways leading to the formation of NM from DA or 6-OHDA *(14,32)*. Figure 4 summarizes the main steps leading to the polymerization of NM. After oxidation to the corresponding quinones, a cyclization to leukoaminochrome and further oxidation to aminochrome takes place. Aminochrome polymerizes to form NM via indole quinone intermediates. The in vivo formation pathway of NM is presumably far more complicated because 5-*S*-cysteinyl derivatives of DA and iron might also be involved, as

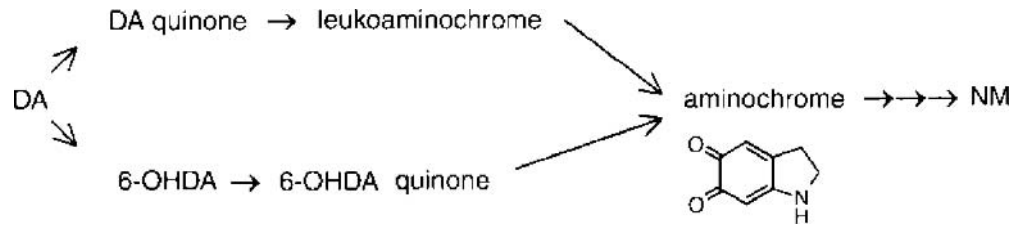


Fig. 4. Schematic presentation of the oxidative formation of neuromelanin from dopamine and 6-hydroxydopamine (adapted from refs. *32* and *224*). 6-OHDA, 6-hydroxydopamine; DA, dopamine; NM, neuromelanin.

reviewed by Zecca *(33)*. Also, an enzymatic synthesis pathway of NM cannot be excluded *(33)*. The chemical structure of human NM is not yet fully characterized either, but recently, it was shown that it contains a proteinaceous component as an integral part of the polymer structure apart from DA-derived components *(34)*.

As mentioned, the DA oxidation product DAQ cyclizes readily and may finally lead to the polymerization of NM (*see* also Fig. 4). However, the nucleophilic addition rate of the sulfhydryl-containing amino acid compounds, glutathione (GSH), and cysteine are three orders of magnitude greater than the intracyclization reaction when measured in vitro at physiological pH *(35)*. Hence, addition of GSH or protein sulfhydryl groups should be an important reaction of DAQ in the central nervous system. The reaction with GSH will yield 5-*S*-glutathionyl DA, which can be degraded by γ-glutamyltranspeptidase and peptidases to 5-*S*-cysteinyl DA *(36)*. Indeed, γ-glutamyltranspeptidase was found to be significantly increased in the SN of PD brains *(37)*. 5-*S*-cysteinyl DA is also directly formed by nucleophilic addition of cysteine (Fig. 5). It has been shown that increasing concentrations of cysteine to DA solutions at physiological pH inhibit and finally block the oxidation pathway of DA to the melanin pigment by scavenging the DAQ to yield 5-*S*-cysteinyl DA *(38)*. These authors put forward a hypothesis according to which the massive, irreversible loss of GSH in the parkinsonian brain without corresponding increase in oxidized glutathione (GSSG) or alterations in GSH peroxidase, GSSG reductase, or GSH transferase activities indicate an elevated reaction of GSH with DAQ ending up in the increased formation of 5-*S*-cysteinyl DA *(38)*. This would lead to decreased NM synthesis and depigmentation of dopaminergic neurons. 5-*S*-cysteinyl DA oxidizes more rapidly than DA and, in the presence of free cysteine, gives toxic 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid (DHBT-1) and other DHBT-cysteinyl conjugates *(39)*. This reaction sequence can result in an increased rate of DA oxidation *(38)*. Iron salts and manganese potentiate the oxidation of DA in the presence of cysteine with concomitant production of 5-*S*-cysteinyl DA and DHBT *(40)*.

3. TOXICITY OF DA-DERIVED OXIDATION PRODUCTS

3.1. Theory of Increased DA Turnover

An important, early established theory regarding the potential toxicity of DA for dopaminergic neurons is the theory of increased DA turnover *(41–45)*. Because of the ongoing loss of dopaminergic neurons during the course of PD, the residual neurons

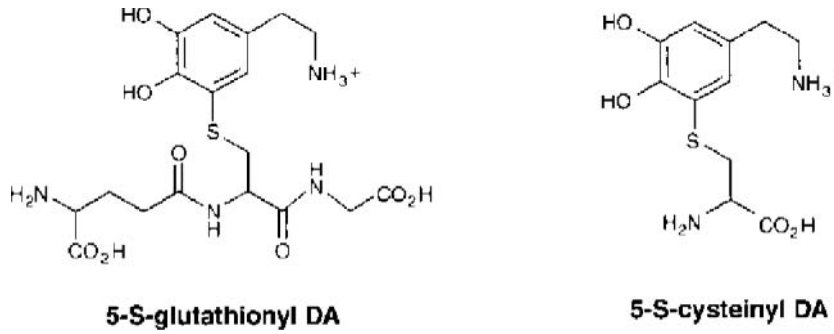


Fig. 5. Chemical structures of 5-*S*-glutathionyl DA and 5-*S*-cysteinyl DA.

might elevate their metabolism of DA as a compensatory mechanism. This theory is supported by the finding that the activity of the remaining tyrosine hydroxylase (TH) molecules, which catalyze the formation of L-DOPA from tyrosine, is increased in the nigro-striatal region of PD brains *(46)*. In this context it is noteworthy that TH was shown to be able to catalyze the generation of •OH probably via Fenton-type reaction of the redox-active nonheme iron in the catalytically active center *(47)* and also other ROS *(48)*. It remains to be established, however, to what extent this process might contribute to the pathogenetic process in PD.

3.1.1. Dopaldehyde

It seems reasonable that increased DA turnover should be associated with increased DA-degrading MAO activity that leads not only to the production of H_2O_2, but also to the aldehyde DOPAL (*see* Subheading 2.1.), which undergoes further dehydrogenation to 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase. As already mentioned, DA is preferentially metabolized by MAO B, which is found in astrocytes. However, MAO A is also able to metabolize DA to a significant extent *(49)*. More than 50 years ago Blaschko *(50)* speculated that MAO aldehyde metabolites would be toxic to the tissues in which they are formed. Indeed, it has been shown that DOPAL is neurotoxic in vitro and in vivo. With rat neostriatal synaptosomal preparations, it was shown that DOPAL enters dopaminergic neurons via the high-affinity DA reuptake system *(51)* and was also identified in the SN of parkinsonian and control brains *(52,53)*. Differentiated PC-12 cells and dopaminergic neurons in primary dopaminergic cultures from ventral mesencephalon of fetal rat embryos selectively exhibited a dose-dependent reduction of neuritic network and viable cells under DOPAL treatment *(10)*. Moreover, the cytotoxicity of DOPAL administered to differentiated PC-12 cells at concentrations close to those identified in normal human SN (2.3 μ*M*) was shown to be much higher than that of DA *(54)*. In PC-12 cells with inhibited mitochondrial respiratory chain activity after treatment with the complex I inhibitor rotenone, DOPAL levels were markedly increased, especially in combination with glucose deprivation, and exerted toxicity *(55)*. Because the DOPAL-metabolizing enzyme aldehyde dehydrogenase is nicotinamide adeninedinucleotide (NAD)-dependent, reduced NAD levels as a consequence of complex I inhibition were thought to be responsible for the accumulation of DOPAL. It was speculated that the observed complex I inhibition in PD and the concomitant rise in

DOPAL concentration might contribute to the specific vulnerability of dopaminergic neurons in PD. When DOPAL was injected into the ventral tegmental area of rats a marked loss of TH immunoreactivity could be detected in the projection areas nucleus accumbens and caudate putamen after 4 d *(56)*. The mechanism of DOPAL toxicity may reside in its instability and reactivity as DOPAL quinone with SH groups of proteins or GSH. Moreover, the aldehyde group can potentially condensate with DA to form the neurotoxic alkaloid TIQ. Recently, it was also demonstrated that DOPAL is able to generate $^{\bullet}OH$ from H_2O_2, eventually through formation of a quinone *(57)*. Because the production of DOPAL takes place at the outer mitochondrial membrane, this might be of relevance for proper mitochondrial function. Interestingly, in isolated, energetically compromised mitochondria physiological concentrations of DOPAL induced the permeability transition pore 1000-fold more effectively than DA *(54)*. However, actively respiring mitochondria proved to be highly resistant to DOPAL, pointing to the importance of mitochondrial integrity to counteract DOPAL toxicity. Thus, the interplay between MAO B activity, which is increased during normal aging *(58,59)* and possibly even more in the SN of PD, impaired mitochondrial function and elevated DA turnover with increased DOPAL production might all contribute to the selective vulnerability of dopaminergic neurons in the SN of PD.

3.1.2. Dopamine Quinone

As described in Chapter 2, the formation of DAQ proceeds via auto-oxidation, enzymatic oxidation, and metal-catalyzed oxidation. Especially in the parkinsonian SN with its pro-oxidative environment owing to, for example, elevated iron content, impaired respiratory chain activity, and increased prostaglandin H synthase activity, the production of highly reactive DAQ might contribute significantly to the degeneration process of dopaminergic neurons.

3.1.2.1. DAQ and Thiol Conjugates

As already mentioned, the electron-deficient nature of DAQ makes it an ideal candidate for nucleophilic addition reactions, thereby potentially inactivating vital proteins in the cell. Because SH groups are the strongest and most ubiquitous nucleophiles in the cell at physiological pH, they are ideal targets for reacting with DAQ *(60,61)*. The predominant source of SH groups is the amino acid cysteine, which is a component of GSH, but also exists free in the cell and as part of physiologically important proteins like, for example, glyceraldehyde-3-phosphate dehydrogenase of glycolysis, with easily-oxidizable accessible SH groups, or ribonuclease inhibitor, rich in cysteine residues *(4)*. Inactivation of protein function because of the reaction between DAQ and SH groups might finally lead to cell death. The covalent bond between DAQ and the SH group will occur preferably at the 5 position of the ring, giving rise to 5-*S*-cysteinyl DA. 5-Cysteinyl derivatives of DOPA and DOPAC are also found in the brain *(62)*. Characteristically, in patients with depigmented SN the ratio of 5-*S*-cysteinyl DA to DA was found to be significantly higher, although only one case was pathologically confirmed as PD in this investigation *(63)*. However, because protein-bound cysteinyl DA is more stable than non-protein-bound forms, it is regarded as a more sensitive index of DA oxidation and a direct measure of cytotoxic events *(64,65)*. Nevertheless, in the guinea pig striatum 5-*S*-cysteinyl DA was found to increase with age pointing to a rising extent of DA auto-oxidation, which could contribute to the loss of dopaminergic neurons in senescence and PD *(61)*.

These findings could be confirmed in the SN of PD brains, where 5-*S*-cysteinyl DA was found to be significantly higher than in control brains *(66)*. However, it should be kept in mind that almost all patients with advanced stages of PD receive L-DOPA therapy, which could contribute to elevated levels of DA thioethers *(67)*. Direct toxic effects of 5-*S*-cysteinyl DA on an immortalized nigral cell line (CSM 14.1) were recently demonstrated by a reduction in cell survival, increase of the apoptosis-indicating enzyme caspase 3 and elevated DNA base oxidation products *(68)*.

As already mentioned, 5-*S*-cysteinyl DA oxidizes more rapidly than DA, giving rise to toxic DHBTs (Subheading 2.2.) that are also easily oxidized *(69)*. Intracerebral injection of DHBT-1 into mouse brains provoked motoric abnormalities and was lethal at higher concentrations *(38)*. In intact rat brain mitochondria DHBT-1 evoked inhibition of complex I respiration *(70)* and in rat brain mitochondrial membranes a time-dependent, irreversible inhibition of complex I activity, which could not be blocked by superoxide dismutase (SOD) or catalase, but could by excess concentrations of GSH *(71)*. DHBT-1 was further oxidized by mitochondrial membranes to *o*-quinone imine intermediates, which presumably bind to catalytically active SH groups at complex I, thus inhibiting its activity. The *o*-quinone imine metabolites are scavenged by excess GSH to form glutathionyl conjugates. These reaction sequences are discussed as contributory factors to complex I inhibition in the SNc in PD, although relatively high concentrations near to the millimolar range were used in these experiments. Similar results have been obtained for the α-ketoglutarate dehydrogenase complex, which is decreased in parkinsonian SN, and pyruvate dehydrogenase complex; both are also inhibited by oxidized DHBT-1 that binds to active site cysteine residues *(72,73)*. Moreover, the cysteine conjugate of the DA metabolite DOPAC, 5-*S*-cysteinyl DOPAC, was shown to exert excitotoxic (via activation of the *N*-methyl-D-aspartate [NMDA] receptor) degeneration of pyramidal neurons in organotypic cultures of hippocampus, which was also attributed to inhibition of the mitochondrial repiratory chain *(74)*. Another DA thioether, *N*-acetyl-*S*-cysteinyl DA, can be formed via the mercapturic acid pathway by measuring cysteine-*S*-conjugate *N*-acetyltransferase (CNAT) activity. CNAT activity was demonstrated to be operative in human SN of control and PD patients *(75)*, but later this result could not be confirmed *(76)*. However, minor amounts of mercapturyl DA were detected in human striatum *(67)*. Subcytotoxic concentrations of 5-*S*-*N*-acetyl-*S*-cysteinyl DA significantly enhanced DA-induced apoptosis in MES 23.5 cells, an immortalized dopaminergic rat mesencephalic/neuroblastoma hybrid cell line *(77)*. This might be of relevance in the pathological condition of PD, with excess extravesicular DA. Similar results were obtained with 5-*S*-homocysteinyl DA and 5-*S*-homocysteinyl DOPAC, but none of the thioethers was neurotoxic when acting alone. On molecular level, 5-*S*-*N*-acetyl-*S*-cysteinyl DA and DA itself produced extensive nicking of supercoiled plasmid DNA when incubated together with Cu^{2+} as metal catalyst *(75)*. Interestingly, *s*-glutathionyl DA and 5-*S*-cysteinyl DA reduced DNA nicking possibly by chelation of the metal ions in solution.

When 5-*S*-cysteinyl DA and 5-*S*-*N*-acetyl-*S*-cysteinyl DA were tested for their interference with DA transport, 5-*S*-cysteinyl DA was shown to reversibly inhibit [^{3}H]DA uptake into rat brain synaptosomes and impede storage of DA in vivo as proved by microdialysis studies in rat striatum whereas 5-*S*-*N*-acetyl-*S*-cysteinyl DA was much less effective in synaptosomal and ineffective in microdialysis studies *(76)*. In contrast, no neurotoxic effect of both DA thioethers could be detected on dopaminergic cells in

primary culture after 48 h incubation as measured by [^{3}H]DA uptake capacity. However, the interference with DA uptake and increase of extracellular DA might well contribute to neurotoxicity in PD, because DA inhibits its own transport by covalently modifying cysteine residues of the DA transporter (DAT) owing to DA oxidation products like DAQs and/or produced ROS as was shown in rat striatal synaptosomes *(78)*. In studies with the human DAT individual cysteine residues were identified to react with DAQ and Cys^{342} was the most relevant of all 13 cysteinyl residues for DAQ-induced inhibition of binding *(79)*. Similar results have been obtained with the glutamate transporter in rat striatal synaptosomes, whose function is likewise affected by DA, DAQ, or ROS *(80)*. It is speculated that increased extracellular glutamate (Glu) concentrations, because of the deleterious effects of DA oxidation products to the Glu transporter, could lead to additional ROS production, promoting further DA oxidation, thus creating a vicious circle in which DA and Glu mutually exacerbate toxicity.

In rat brain mitochondrial-synaptosomal fractions incubation with DA lead to the formation of protein-bound quinones and induced protein crosslinking *(81)*. These events could be prevented by coincubation with GSH, but not ROS scavengers, confirming the direct action of DA and not production of ROS as the main contributory factor to protein crosslinking. The authors speculate on the potential deleterious role for proteins of the respiratory chain or membrane-bound transport proteins.

Tetrahydrobiopterin, an obligatory cofactor for TH, exerts selective toxicity on DA-producing cells via DAQ production, which can be prevented by antioxidants *(82,83)*. Tetrahydrobiopterin is spontaneously released from the cells of its synthesis in proportion to the rate of synthesis, which can be readily upregulated by cellular stress like calcium influx *(84)*. As an unstable molecule it generates H_2O_2 and $O_2^{\bullet -}$ via auto-oxidation in aqueous solution. Both ROS can easily penetrate the plasma membrane and induce the oxidation of DA to reactive DAQ, which leads to the formation of protein-bound quinones and finally to cell death *(83)*. Interestingly, TH itself is inactivated by catechol (DA, DOPA, *N*-acetyl DA) quinones, forming cysteinyl catechols, and converted to a redox-cycling quinoprotein that is able to reduce transition metals like iron and copper, which can participate in the Fenton reaction *(85,86)*. Consequently, under conditions of elevated oxidative stress, remaining TH, the rate-limiting enzyme in DA synthesis, whose activity is tremendously increased according to the lack of DA in PD, can contribute to cell death of dopaminergic neurons.

Another important detoxifying mechanism for DAQ and derived oxidation products thereof, is the conjugation with GSH, because DAQ can catalyze the release of iron from ferritin *(87)*. In PD, depletion of GSH is the earliest known indicator of nigral degeneration and is preceding loss of dopaminergic neurons *(88)*. Thus, its elevated consumption in the early stage of the disease may represent elevated oxidative stress conditions owing to toxic DA metabolites and ROS formation.

3.1.2.2. DAQ/DA and α-Synuclein

In PD, fibrillar α-synuclein is a major component of Lewy bodies *(89–91)*. Recently, it was discovered that DAQ might contribute crucially to α-synuclein-associated toxicity in PD *(92)*. Although α-synuclein is not composed of any SH group containing cysteine, other reactive amino acid residues like tyrosine or lysine possibly react via radical coupling (DAQ to tyrosine) and/or nucleophilic attack (DAQ with lysine) to form DA-α-synuclein covalent adducts *(92)*. These adducts prolong the lifetime of protofibrils that are usually

converted to fibrils deposited in Lewy bodies. There exist hints that actually the protofibrillar form is the toxic compound whereas the fibrils seem to be inert *(93)*. α-Synuclein protofibrils were shown to bind tightly to synthetic vesicles and with high selectivity to brain-derived vesicles *(94,95)*. Although monomeric α-synuclein also bound to the synthetic vesicles, only protofibrillar α-synuclein caused significant permeabilization. It is speculated that this permeabilizing effect is similar to the action of pore-forming protein toxins and could cause degeneration of dopaminergic neurons via increased calcium influx, depolarization of the mitochondrial membrane potential, and leakage of DA from storage vesicles into the cytoplasm *(94)*. Thus, a kind of vicious circle is created in which protofibrils and DA mutually promote their potentially cytotoxic chemical states, especially under conditions of elevated oxidative stress like in the SN of PD. Interestingly, the two autosomal dominant mutations of α-synuclein linked to PD (A53T and A30P) accelerate the formation of protofibrils in vitro and A30P also inhibits the conversion to fibrils *(96)*. The formation of nonfibrillar aggregates, also from proteins not associated with any protein misfolding disease like AD, seems to be a general feature for cytotoxicity of these species as was shown for mouse fibroblasts *(97)*.

Recently, the DA-dependent neurotoxicity of α-synuclein was confirmed with cultured human fetal dopaminergic neurons overexpressed with wildtype or mutant α-synuclein, which underwent apoptosis through the action of endogenous DA *(98)*. α-Synuclein seemed to potentiate DA-dependent generation of ROS, because spin traps and antioxidants were protective and inhibition of TH to block DA synthesis completely abolished apoptosis. However, soluble α-synuclein complexes were sufficient to induce apoptosis and no aggregate formation was necessary. The soluble α-synuclein complex also contained 14-3-3 protein, which counteracts apoptosis by binding and inactivating pro-apoptotic proteins like Bad, for example. Elevated sequestration of 14-3-3 to α-synuclein may reduce its antiapoptotic activity and render the dopaminergic neurons in turn more susceptible to the oxidative metabolism of DA. Interestingly, in cultures of nondopaminergic human cortical neurons wild-type α-synuclein overexpression not only failed to induce apoptosis, but even significantly increased neuronal survival. These results indicate selective vulnerability of dopaminergic neurons to α-synuclein toxicity coupled with the toxic potential of endogenous DA. Notably, elevated levels of soluble α-synuclein complexes were found in the SN of PD patients, but not in the VTA, which contains dopaminergic neurons that are relatively resistant compared to SN *(98)*. Moreover, these neurons express relatively high levels of the vesicular monoamine transporter 2 (VMAT2) (takes up DA into synaptic vesicles) and low levels of the DAT (takes up DA into cytoplasm) *(99)*.

There also exist hints that α-synuclein is an important modulator of synaptic vesicles recycling and that misfolded or dysfunctional α-synuclein owing to oxidative stress or mutations might lead to a reduction in vesicle number and accumulation of cytoplasmic DA again accompanied by increased ROS production *(100,101)*.

In another study it was shown that overexpressed α-synuclein in a dopaminergic cell line (MN9D) significantly reduced TH activity and concomitantly DA synthesis by direct binding to TH or indirect regulation via inhibition of TH-phosphorylating kinase or stimulation of phosphatase, respectively *(102)*. It is speculated that a loss of soluble, TH-regulating α-synuclein, for example, by aggregation, could lead to elevated DA synthesis and correspondingly increased ROS production.

Moreover, exposition of human neuroblastoma cells (BE-M17), overexpressing wild-type or mutated α-synuclein, to iron or to radical-generating Fenton reagent promoted the aggregation of α-synuclein with higher levels in mutant cells *(103)*. Additionally, the combination of iron with DA, but not the treatment with DA alone, also induced formation of α-synuclein aggregates. The aggregation was greater with the combination of oxidants and iron pointing to additive effects of this treatment. These results underline the important role of iron in α-synuclein aggregation. Also, iron was shown to be present in Lewy bodies in the SN *(104)* and overexpression of α-synuclein sensitized cells to iron-mediated toxicity *(103)*. The increased iron content in the SN of PD brains and the presence of DA may thus accelerate the aggregation of α-synuclein and formation of Lewy bodies. Another study also demonstrated that DA incubation alone was able to increase expression of α-synuclein in SH-SY5Y neuroblastoma cells via activation of stress-response kinases *(105)*.

It was also reported recently that α-synuclein binds to the DAT in cultured human progenitor dopaminergic cells and in HEK293 cells contransfected with α-synuclein and DAT *(106)*. This complex formation facilitated membrane clustering of DAT and consequently DA uptake and DA-induced apoptosis in transfected HEK293 cells. It is speculated that α-synuclein and DAT form functional complexes that allow for the effective targeting of DAT proteins to the cell surface, thereby modulating dopaminergic presynaptic function. Thus, mutations or changes in expression levels of α-synuclein in dopaminergic neurons may disturb complex formation between the two proteins and lead to elevated cytosolic DA uptake followed by increased oxidative stress.

3.1.2.3. DAQ/DA AND NM

The biosynthetic pathways of NM, in which DA plays a central role, are roughly outlined in Subheading 2.2. NM contains organic free radicals and large amounts of paramagnetic metals, mainly iron *(107,108)*. It also contains a peptide component of which the amino acid content corresponds to 15% of the neuromelanin weight *(109)*. Interestingly, only residual PD NM isolated from SNpc was found to be mainly composed of highly crosslinked, protease-resistant proteic material identified as α-synuclein, whereas control brains had none *(110)*. NM is located in the perikaryon where it is packed in granules surrounded by membranes *(111)*. The most pigmented areas in the human and primate brain are the SN and the locus coeruleus *(112,113)*, containing dopaminergic and noradrenergic nuclei, respectively. During the last decades there have been many debates on the role of NM under physiological and pathological conditions. It was controversially discussed as a cytotoxicant or cytoprotectant. Based on its stable free-radical content, NM was supposed to serve as an "electron trap," thereby functioning physiologically as an "impulse inhibitor" *(114)*. Thus, NM was considered a "cellular energy repository" similar to a semiconductor, because NM is deposited mainly around the axon hillock of the neuron blocking the electron flow from the cell cytoplasm into the axon cylinder *(114)*. It was also speculated that NM granules might serve to propagate quantum mechanical events in the brain, because of their ability to semiconduct, thus acting as a temporary memory owing to deposited information on the NM molecules. Further considerations on the role of NM as part of bioelectronic mechanisms in the brain are reviewed by Lacy *(115)*.

Definitely more research will be needed to finally elucidate the physiological role of this peculiar brain pigment. Independent of all speculative assumptions concerning its functionality, an important characteristic is its binding capacity of organic molecules and heavy metals, especially iron. A well-known example for the binding of organic molecules is the binding of the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrdine, 1-methyl-4-phenyl-pyridinium (MPP^+) *(116)*, which kills dopaminergic neurons in the SN and leads to PD *(117)*. It is presumed that MPP^+, after entering the dopaminergic neuron, binds to NM, which functions as a depot and continuously releases MPP^+ finally ending up in cell death *(118)*. NM was also shown to bind dopaminergic drugs, implicating a similar depot-like effect as is the case with MPP^+ *(119)*. This might have consequences for the effective dose of the drugs and may end up in toxicity. NM is also hypothesized to protect neurons by binding toxic quinones like dopaminochrome or adrenochrome *(120)*, which covalently bind to DNA and sulphydryl groups in proteins *(10)*. Also, the recently found incorporation of α-synuclein into PD NM *(110)* might be protective against the cytosolic accumulation of excess or potentially oxidized α-synuclein. Moreover, the NM synthesis itself might be a protective mechanism, since it is driven by excess cytosolic catechols, not accumulated in synaptic vesicles *(121)*, which could otherwise produce toxic oxidation products.

Besides having a strong binding capacity for iron, NM also binds zinc, copper, manganese, chromium, cobalt, mercury, lead, and others. Thereby, NM might play a protective role in chelating toxic metals, but once the binding capacity is overwhelmed, it can be converted into a pro-oxidant, which is of special importance concerning the binding of iron *(108)*. Iron is chelated as ferric iron by catecholic groups of the NM polymer *(122)*. Importantly, the iron-storage molecule ferritin is not detectable in dopaminergic neurons of the SN *(123)*, therefore, NM seems to take over the role of an iron-storage system *(124)*. As mentioned before, the total iron content in the SN of severe PD cases is significantly increased and may thus exceed the binding capacity of NM, since the accumulation seems to occur within the NM granules *(125)*. In this context it is noteworthy that in PD the melanized neurons are more vulnerable than the nonmelanized ones *(126,127)*. However, very heavily melanized neurons seem relatively resistant and the most dramatic neuronal loss is seen in the pars α of the SN, which contains a relatively low proportion of heavily to lightly pigmented neurons *(127)*. Although NM binds iron as an Fe^{3+}-complex *(122,125)*, thus preventing it from redox activation and potential cell damage via the Fenton reaction, when the binding capacity is exceeded, the formation of $^{\bullet}OH$ radicals is stimulated as was shown for DA-melanin, a synthetic model for NM *(128)*. Additionally, NM from PD brains is supposed to have a decreased iron-binding capacity, which might potentially lead to elevated free-iron levels within the cells *(129)*.

It was also shown that synthetic DA-melanin was actively phagocytosed into PC-12 cells and induced cell death by apoptosis *(130,131)*. Similar results were obtained with primary dopaminergic cell cultures from mouse mesencephalon, where synthetic DA-melanin also induced cell death of dopaminergic neurons *(132)*. In both culture systems, addition of iron enhanced the toxic effect. However, it has to be kept in mind that whereas synthetic melanins might differ from natural NM with respect to structure, stability, and redox and chelating properties *(133)*, the more PD NM obviously differs from "normal" NM.

Summarized, DA-derived NM seems to be able to play a dual role in dopaminergic neurons; i.e., it can act as a cytoprotectant or cytotoxicant, depending on the environmental

conditions. Its binding of potentially toxic organic molecules and iron might serve as a detoxifying mechanism as long as the binding capacity is not overwhelmed. But especially the increase in iron content in PD SN seems to overload NM which, in PD, has possibly a decreased iron-binding capacity. Thus, NM might confer an increased vulnerability to dopaminergic neurons. In this context it is interesting that in India the prevalence of PD is lower than in Western countries and Indian brains have about 40% less melanized nigral neurons than do UK brains *(134)*, eventually indicating a relation between the degree of melanization and the liability to develop PD. Surely, the meaning of NM is more complex than being a mere waste product of DA oxidation.

3.1.2.4. DAQ AND DNA

Reactive quinone species derived from DA oxidation are also able to form DNA adducts besides protein adducts. The formation of DNA adducts after DNA incubation with DA was demonstrated in peroxidase-containing human leukemia HL-60 cells *(135)*. This process could be further aggravated by H_2O_2 and prevented by ascorbic acid. Less DNA adduct formation was observed with peroxidase-free glioblastoma cell lines. It was speculated that the corresponding quinone or semiquinone radical of DA was responsible for the DNA adduct formation. These activated species were supposed to be formed via peroxidase (HL-60) or nonenzymatically (glioblastoma cells) via trace metals like iron.

Similar results were obtained with isolated DNA incubated with DA in the presence of tyrosinase *(136)*. The DA quinone forming enzyme tyrosinase increased the DA-DNA adduct and antioxidants reduced it significantly. Also, prostaglandin H synthetase, capable of oxidizing DA, led to the accumulation of DA-DNA adducts in isolated human SN *(10)*.

The DNA oxidizing effects of 5-*S*-cysteinyl DA or 5-*S*-*N*-acetyl-*S*-cysteinyl DA in presence of Cu^{2+} were already mentioned (Subheading 3.1.2.2.) *(68,75)*.

The oxidative metabolism of DA by DA oxidizing enzymes or enhanced iron levels leading to DA-DNA adduct formation may contribute to the selective vulnerability of dopaminergic neurons and accelerate their demise.

3.1.3. 6-OHDA

Generally, 6-OHDA is regarded as a classical neurotoxin for catecholaminergic cells, which is commonly used to model the degenerative events underlying the pathogenesis of PD. However, when 6-OHDA was detected in human caudate nucleus *(137)* and in significantly higher concentrations in the urine of L-DOPA-treated PD patients compared to patients not treated with L-DOPA *(138)*, concern about the contribution of endogenously formed 6-OHDA to the pathognesis of PD arose. The role of 6-OHDA in the development of PD is still a matter of debate. Possible formation pathways of 6-OHDA are described in Subheading 2.2. Again, it becomes clear, that under conditions of elevated cytosolic DA and iron, as well as increased oxidative stress the nonenzymatic formation of 6-OHDA might become relevant *(139)*. This hypothesis is supported by the observation that inhibition of DA metabolism by the mixed MAO A and B inhibitor pargyline and the catechol-*O*-methyl transferase (COMT) inhibitor pyrogallol, leads to a substantial amount of 6-OHDA in mouse striatum, which can be further enhanced by additional treatment with the DA-releasing agent methamphetamine *(140)*.

When endogenous 6-OHDA is formed, the potential neurotoxicity of this substance is not only owing to its high instability leading to the formation of ROS via auto-oxidation, but also to its property to be able to release iron from ferritin in the reduced form of Fe^{2+},

which can initiate cell-damaging processes via the Fenton reaction *(139,141)*. This mechanism of toxicity is supported by a study in which rats were treated unilaterally by injecting 6-OHDA intracerebroventricularly *(142)*, because 6-OHDA does not cross the blood–brain barrier *(143)*. It was shown that 6-OHDA caused loss of DA and the decrease of DA metabolites homovanillic acid and DOPAC, but that this loss was attenuated when the iron chelator and radical inhibitor desferrioxamine was injected prior to 6-OHDA treatment *(142)*. Moreover, unilateral infusion of 6-OHDA into the medial forebrain bundle produced degeneration of dopaminergic neurons and an 35% increase in iron content (per mg wet wt.) in the SN ipsilateral to the side of lesion *(144)*. Recently, it was shown that 6-OHDA is also capable of releasing iron from transferrin leading to a ROS-producing redox-cycling of iron by 6-OHDA similarly to the iron released from ferritin *(145)*.

Another important feature of 6-OHDA toxicity is its inhibitory effect on complex I and IV of isolated rat brain mitochondria, which is not mediated by free-radical production and is reversible *(146)*. It was concluded that adenosine triphosphate (ATP) depletion is one decisive mechanism of 6-OHDA-induced neurodegeneration. However, in SH-SY5Y neuroblastoma cells treatment with 6-OHDA did not lead to a decrease of ATP content, ATP/adenosine diphosphate ratio, or NAD^+ content *(147)*. Instead, the antioxidant D-α-tocopherol attenuated cell death, suggesting that ROS production plays a main role for the cytotoxic mechanism of 6-OHDA. Moreover, although 6-OHDA is taken up by the DAT, inhibition of DA transport did not prevent 6-OHDA toxicity *(147)*. Similarly, in PC-12 cells, 6-OHDA induced cell death by a mechanism apparently independent of mitochondrial inhibition and no effect on cytotoxicity was observed when MAO inhibitors were added *(148)*, although 6-OHDA is a substrate for MAO *(149)*. These observations suggest that externally added 6-OHDA does not necessarily need to enter cultivated cells to exert toxicity. Remarkably, when rats were injected unilaterally with 6-OHDA into the SN, a 20% decrease in complex I activity in the SN could be observed, pointing to mitochondrial inhibitory effects of 6-OHDA in vivo *(150)*. Also, 6-OHDA induced a change in Ca^{2+}-evoked swelling and slowed succinate-induced formation of membrane potential in isolated brain mitochondria *(151)*. These effects were attributed to ROS as a consequence of 6-OHDA oxidation, because they were attenuated by the antioxidant enzymes SOD and catalase. The important role of $O_2^{\bullet -}$ in 6-OHDA toxicity for dopaminergic neurons was demonstrated when the survival of primary dopaminergic neurons was significantly enhanced after overexpression of the human SOD gene *(152)*. Similar protective results against 6-OHDA-induced nigrostriatal lesions were obtained with SOD transgenic mice *(153)*. In mice overexpressing human GSH peroxidase, dopaminergic neurons in the SN and DA levels in striatum were protected against loss after intracerebroventricular injection of 6-OHDA, pointing also to the participation of H_2O_2 in 6-OHDA toxicity *(154)*.

Recently, the impact of 6-OHDA on protein degradation pathways was described. When PC-12 cells were incubated with 6-OHDA, protein degradation and levels of free and ubiquitin-conjugated proteins were markedly increased *(155)*. When proteasome activity was inhibited by the specific inhibitor MG132, 6-OHDA toxicity to PC-12 cells was even potentiated, but was attenuated when the antioxidant *N*-acetylcysteine was simultaneously added, again indicating the role of oxidative stress in 6-OHDA toxicity. Interestingly, in PC-12 cultures DA incubation induced inhibition of the proteasome *(156)*. Similarly to the experiments with 6-OHDA, inhibition of proteasome activity by the specific inhibitor lactacystin also potentiated DA toxicity, whereas the simultaneous

application of the antioxidant GSH monoethyl ester and DA attenuated not only DA toxicity, but also DA-induced proteasome impairment *(156)*. Such protective effects could also be achieved by MAO and DA uptake inhibitors. Theses results can likewise be attributed to DA-dependent ROS formation, but DA uptake and metabolism seems to be a prerequisite for proteasome inhibition as a result of DA-induced ROS formation.

When 6-OHDA is generated intracellularly, it can potentially compete with the VMAT2 *(157)*, can cause irreversible modification of the reuptake carrier *(158)*, is a substrate for MAO *(149)*, and inactivates COMT *(159)*, and may thus unfavorably increase cytosolic DA concentrations. Moreover, under L-dopa treatment, when patients are supplied with bolus doses of DA, and when receiving MAO inhibitors, 6-OHDA formation might be favored *(160)*. In this context it is noteworthy, that acute L-DOPA pretreatment potentiated 6-OHDA-induced toxicity as measured by decreases in striatal levels of DA and DA metabolites in mice *(161)*.

Irrespective of the fact, if endogenously formed 6-OHDA levels in the brain of PD patients are high enough to significantly contribute to the pathogenesis of the disease, the substance remains a valuable tool to study on a cellular and molecular level the degenerative processes underlying PD.

4. DA TOXICITY–SIGNAL TRANSDUCTION PATHWAYS AND APOPTOSIS

For years, there has been an extensive ongoing debate about the mechanism of dopaminergic cell death in PD. "Apoptosis or not apoptosis" seems to be the question. Apoptosis is an active, gene-directed, programmed mode of cell death that serves to remove cells that are no longer needed or damaged and thus plays a central role in development and cell homeostasis of metazoans *(162,163)*. This mode of cell death is usually opposed to necrosis. Apoptosis is characterized by membrane blebbing, cytoplasmic and nuclear condensation, fragmentation of chromosomal DNA, loss of mitochondrial membrane potential, translocation of phosphatidylserine to the outer layer of the plasma membrane (phagocytosis signal), and formation of apoptotic bodies *(162,164,165)*. Necrosis, on the other hand, is accompanied by cellular and mitochondrial swelling, dilatation of endoplasmic reticulum, and extensive vacuolation of the cytoplasm, and does not show the aforementioned hallmarks of apoptosis *(166)*. When the necrotic cells lyse and release their cellular content, an inflammatory response in the surrounding cells is often induced, whereas in apoptosis damage to surrounding cells is less severe and usually not followed by inflammatory response *(166)*.

Today, apoptosis and progammed cell death (PCD) are often used synonymously, although in its original sense apoptosis refers to a specific morphologic pattern *(164)*, because PCD can also occur without the typical morphological features of apoptosis as was shown for the intersegmental muscle cells of the moth *Manduca sexta* at the end of metamorphosis, for example, ref. *167*. Moreover, the opposition of apoptosis and necrosis seems to be a too simplified view on cell death. Recently, a classification of four different patterns of cell death ("from apoptosis to necrosis") was presented *(168)*. In this classification classical apoptosis is characterized, besides the morphological features described above, by the typical biochemical marker of caspase activation, especially activation of effector caspase 3. In "apoptosis-like PCD" chromatin condensation is less compact and phagocytosis-recognition molecules are displayed before lysis of the

plasma membrane. Most caspase-independent forms of apoptosis described in the literature (ref. *169* and further references reviewed by Leist, ref. *168*) belong to this class. In "necrosis-like PCD" no distinct chromatin condensation can be observed, but externalisation of phosphatidylserine might occur before lysis. This pathway is usually initiated by specialized caspase-independent pathways. Finally, the other end of cell death mechanisms is represented by necrosis, which is initiated by severe cellular insults like, e.g., ROS. However, the typical feature of necrosis, namely cytoplasmic vacuolation, can sometimes also be found in a special form of PCD called "paraptosis" *(170)*. Although this form of cell death requires gene expression and is thus "programmed" by definition, no other signs of apoptosis including lack of effect of caspase inhibitors can be observed. Interestingly, paraptosis is mediated by an alternative, caspase inhibitor-independent, caspase 9 activity, which is usually a known inducer of apoptosis via cleavage and activation of pro-caspase 3.

All these findings show that some forms of cell death may be accompanied by both apoptotic and necrotic features. But there exist further factors that are decisive for the mode of cell death and contribute to the complexity of this process. The intensity of the insult and the duration of exposition to it play an important role in the decision for apoptotic or necrotic cell death. For example, when cortical cell cultures are treated with an intensive excitotoxic insult (2 m*M* NMDA for 10 min) neurons die by necrosis within 30 min, whereas mild insults (300 μ*M* for 10 min) triggers apoptosis of delayed onset (50% apoptotic nuclei after 18 h) *(171)*. Although it is well known that initial apoptosis can turn into secondary necrosis when adequate phagocytosis is missing, also the opposite sequence can take place; namely cells that survive a necrotic phase can later undergo apoptosis as was shown for cerebellar granule cells treated with excitotoxic glutamate *(172)*. In this system cells that exhibited a rapid loss of mitochondrial membrane potential and energy charge died by necrosis, whereas cells that survived this period recovered energy levels and mitochondrial membrane potential and subsequently died by delayed-onset apoptosis. That intracellular ATP concentration is a decisive factor for the mode of cell death is demonstrated by a switch from apoptosis to necrosis when human T cells are predepleted of ATP before being exposed to apoptotic triggers such as staurosporine or CD95 stimulation *(173)*. In these experiments it was shown that ATP was required for chromatin condensation and DNA degradation, which are late steps in apoptosis. ATP generation by glycolysis turned out to be sufficient for apoptotic cell death. Also other events in apoptosis, like activation of pro-caspase 9 to caspase 9, are ATP-dependent *(174)*. Moreover, it is well known that onset of the mitochondrial permeability transition via the permeability transition pore with concomitant collapse of the mitochondrial membrane potential is an early and irreversible event of the cell death process *(175)*. As a consequence either necrotic or apoptotic cell death can occur depending on the availability of cellular ATP *(174)*. When apoptosis follows there exist two operative ways: a mitochondrion-mediated and a death-receptor-mediated, but mitochondrion-independent pathway, also often referred to as "intrinsic" and "extrinsic" pathway *(176)*. Death receptor (e.g., tumor necrosis factor receptor) mediated apoptosis leads to activation of initiator caspase 8 via a death-inducing signaling complex, whereas in mitochondria-induced apoptosis cytochrome c is released from mitochondria often through activation of pro-apoptotic members of the Bcl-2 family (e.g., *Bax*), which are themselves activated by death signals like ROS and that can rupture the outer mitochondrial membrane or interact with

the voltage-dependent anion channel of the permeability transition pore *(177,178)*. Cytochrome c forms with apoptotic protease-activating factor 1 and pro-caspase 9 the apoptosome complex that finally activates caspase 3 *(179,180)*.

The occurrence of apoptosis as a causative mechanism of degeneration of dopaminergic neurons is discussed quite controversially. Because mitochondria may play a central part in the apoptotic process, it seems attractive to speculate if the demonstrated complex I defect in the SN of PD contributes to apoptosis as the cell death mode of dopaminergic neurons. The precise knowledge of the death mode is important, because it bears intrinsic consequences for new treatment strategies that rely on the identification of specific drug targets and the development of appropriate pharmaceuticals.

The fact that preferentially the dopaminergic neurons in the SN die during the course of PD has supported the assumption that apoptosis might be the reason for the selective loss of this neuronal cell type. Actually, studies carried out on postmortem brain tissue of PD patients have delivered quite contradictory results. With respect to morphologic criteria, several investigators failed to demonstrate apoptotic neurons in the SN of PD patients when using *in situ* terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) of DNA fragments *(165,181,182)*. Instead, TUNEL-positive glial cells and activated microglia in the SN were discovered *(165,181,182)*. Others, however, detected TUNEL-positive neurons in the SN of PD patients *(183)*, and apoptotic dopaminergic neurons based on morphological evaluation like chromatin condensation or shrinkage of cell bodies *(184)*. Concerning biochemical markers of apoptosis, similar contradictory results have been obtained. One study using immunohistochemistry revealed no differences in the expression of apoptosis-related proteins c-Jun, c-Jun/AP1 (ASP), Bcl-2, Bax, and Bcl-x in SN neurons between PD and controls *(165)*. Moreover, no expression of p53 or activated caspase 3 was detected in neurons, but reactive astroglia and microglia showed reactivity for Bcl-2, Bax and, to a lesser extent, for Bcl-x_L and caspase 3. In contrast, others measured increased caspase 1 and 3 activity as well as increased tumor necrosis factor receptor R 1 in the SN of PD patients, although no distinction between neurons and glial cells was made owing to measurements in homogenized tissue *(185)*. Evaluating activated caspase 3 on neuronal cell level, it was found that the proportion of activated caspase 3 positive melanized nigral neurons was significantly higher in the SNpc of PD patients relative to controls *(186)*. Another study of the same group showed that all melanized SNpc neurons of PD brains with activated caspase 3 were also positive for the pro-apoptotic protein Bax and the percentage of *Bax*-positive melanized SNpc neurons containing Lewy bodies was significantly higher than the overall percentage of *Bax*-positive neurons among melanized neurons *(187)*. Moreover, the expression of the anti-apoptotic protein Bcl-x_L was significantly higher in melanized neurons from SNpc of PD patients than in control brains, which is preferably interpreted by the authors as being indicative for a protection of those dopaminergic neurons that express the highest level of *Bcl-x_L* before the onset of the disease *(188)*.

The question if apoptosis is the main mechanism of dopaminergic cell death in PD is still not finally resolved. The quantitative analysis of apoptotic morphology of dopaminergic neurons in PD brains must be evaluated cautiously, because a higher number of apoptotically dying dopaminergic neurons in PD brains compared to controls may be attributable to a higher vulnerability of predamaged dopaminergic neurons to hypoxia secondary to the patient's agonal state *(163)*. There is a general agreement that a

pro-apoptotic environment prevails in the SN of PD patients, which might be generated by oxidative stress because of complex I inhibition, toxic (DA?) metabolites, and iron. Predamaged dopaminergic neurons may thus be more vulnerable to apoptosis. In this context it is interesting to investigate the question to what extent DA and the metabolism thereof might contribute to apoptosis of dopaminergic neurons. This issue has been addressed in numerous cell culture studies. Apoptotic cell death induced by DA was demonstrated in, for example, PC-12 cells *(189–192)*, neuroblastoma SH-SY5Y cells *(193,194)*, striatal neurons *(195–197)*, cerebellar granule cells *(198–200)*, the immortalized neural cell line CSN14.1 *(201)*, an immortalized olfactory neuronal cell line (13.S.1.24) *(202)*, chick embryo sympathetic neurons *(198,203)*, a Neuro-2A cell line originating from mouse neuroblastoma cells *(204)*, a catecholaminergic CATH.a cell line derived from the CNS *(205)*, and a SN/neuroblastoma hybrid cell line (MES 23.5) *(206)*. But also for oligodendrocytes *(207)* and mouse thymocytes *(208)* DA-induced apoptosis was shown. In a rat model DA induced apoptosis after intrastriatal injection *(209,210)*. The production of ROS caused by DA and the subsequent activation of apoptosis-related genes and signal transduction pathways play a central role as apoptosis-inducing factors in these studies. One important effect of DA-induced ROS is DNA damage. All of the above-mentioned systems showed the two important characteristics of apoptosis on the nuclear level: chromatin condensation and DNA fragmentation. In cerebellar granule and leukemia cells an increase in p53 phosphorylation was observed *(199)*, a transcription factor that can promote cell cycle arrest or apoptosis in response to DNA damage and is a transcriptional activator of the *Bax* gene and suppresses the anti-apoptotic protein Bcl-2 *(162,211)*. Moreover, p53 can directly mediate apoptosis via the death receptor and mitochondrial pathway *(212)*. In leukemia cells inactivation of p53 significantly decreased DA toxicity and p53 activation was a prerequisite for DA-induced DNA degradation *(199)*. In SH-SY5Y cells DA was shown to induce a significant rise in the p53 protein level until 12 h after treatment and the anti-apoptotic protein *Bcl-2* remained at normal level up to 6 h, but then decreased *(194)*. Supporting the importance of the level of expression of Bcl-2 for DA-induced apoptosis, PC-12 cells, which do not normally express Bcl-2 protein *(213)* when transfected with the mouse *Bcl-2* cDNA, were highly resistant against DA toxicity *(189)*. Interestingly, extracts of Bcl-2 expressing PC-12 cells inhibited the auto-oxidation of DA and the formation of DA-melanin pointing to an antioxidant role of the protein *(131)*. Overexpression of Bcl-2 in immortalized CSM14.1 neuronal cells similarly suppressed DA-induced apoptosis that was attributed to ROS formation *(201)*. In this study, antioxidative effects of *Bcl-2* were also demonstrated by inhibition of ROS production from DA. Moreover, DA-induced apoptosis is also known to be accompanied by activation of the c-Jun *N*-terminal kinase (JNK) signal transduction pathway. The JNK group of mitogen-activated protein kinases, also designated as stress-activated protein kinases, are intracellularly activated upon exposure of the cells to cytokines, growth factor withdrawal, or other environmental stresses *(214)*. JNK, which is activated by phosphorylation, activates again c-Jun by phosphorylation. Although relatively little is known about the physiological functions of JNK, there is experimental evidence that it is a potent effector of neuronal apoptosis. In the HEK293 cell line and primary rat striatal neurons, DA induces apoptosis and activates the JNK pathway via increased JNK activity, phosphorylation of c-Jun, and subsequent increase of c-Jun protein *(197)*. The authors speculate that stimulation of the JNK pathway after DA treatment leads to

activation of cell cycle gene expression through c-*Jun*-dependent activated protein -1 activity, which drives terminally differentiated neurons into an inappropriate cell cycle that finally results in apoptosis. Because the antioxidants *N*-acetyl cysteine and catalase block the JNK pathway and apoptosis, DA-derived ROS are interpreted as the genuine effectors of these events. Additionally, apoptosis following intrastriatal injection of DA was accompanied by activation of nuclear transciption factor–κB (NF–κB) *(210)*. Upon activation NF–κB is translocated from the cytosol to the nucleus where it induces the transcription of target genes that possibly belong to the family of cell death genes when cells are stressed with ROS. Interestingly, it was shown that in PD the proportion of dopaminergic neurons with immunoreactive NF–κB in their nuclei was more than 70-fold that in control brains *(215)*. Also, the translocation of NF–κB to the nucleus was preceded by a transient production of ROS when apoptosis was induced by C_2-ceramide. Again, *N*-acetyl cysteine prevented ROS production, the subsequent translocation of NF–κB, and consequently cell death, which was interpreted in such a way that cell death was a result of ROS-stimulated NF–κB activation. However, the functions of NF–κB in the nervous system are bidirectional; it can promote or inhibit apoptosis, depending on the specific cell type, and on the inducer of apoptosis *(216)*. Even within the same cell type, activation of NF–κB can have opposite effects. In PC-12 cells it was shown to be essential for DA-induced apoptosis *(191)*, but also to counteract apoptosis signals induced by auto-oxidized DA *(192)*.

In a recent study it was postulated that DA induces apoptosis *per se*, independent of oxidative stress, since increases in caspase 3 activity occurred only when DA auto-oxidation, and thus ROS production, was prevented by ascorbic acid as measured in Neuro-2A cells, a cell line originating from mouse neuroblastoma *(204)*. In contrast, increase of caspase 3 activity induced by L-DOPA was insensitive to ascorbic acid. These interesting findings doubtless warrant further exploration. Another study demonstrating nonoxidative DA toxicity points to a similar direction. Chinese hamster ovary cells, which do not stably express the DAT, but take up L-DOPA, were stably transfected with aromatic acid decarboxylase thus enabling the selective elevation of intracellular DA via aromatic acid decarboxilase *(217)*. Whereas ascorbic acid significantly protected against externally applied DA, no protection by the antioxidant ascorbic acid could be achieved against L-DOPA toxicity, which was interpreted as a nonoxidative mechanism for intracellular DA toxicity, but as an oxidative mechanism of extracellular DA toxicity. Furthermore, intracellular DA generated by L-DOPA treatment induced a rapid and potent activation of NF–κB, which was also relatively insensitive to ascorbic acid, whereas extracellular DA induced a slower and less potent activation inhibitable by ascorbic acid, thus being oxidative in nature, in contrast to the nonoxidative activation by intracellular DA.

Finally, DA is also capable of inducing autophagic cell death as a consequence of intracellularly generated oxidative stress, as was recently demonstrated *(105)*. Autophagy is a third classification of cell death mode, besides apoptosis and necrosis. Autophagy is characterized by autophagic vacuoles (lysosomes) in which the cells degrade cytoplasm and organelles. First, the cytoplasm and organelles are sequestered into double-membrane autophagosomes originating from the endoplasmic reticulum and are then delivered to the lysosome that contains a variety of hydrolases. This energy-dependent process is highly regulated through various kinases, phosphatases, and guanosine triphosphatases *(218)*. Lysosomes and the proteasome represent the two major ways for degradation of

macromolecules in eukaryotic cells, although the proteasome seems to be more selective, but has less degradative capacity *(219,220)*. Further features of autophagy comprise pyknosis of the nucleus, endocytosis, and blebbing of the plasma membrane, and dilated endoplasmic reticulum, mitochondria, and Golgi apparatus *(163)*. DA-derived NM was also found to be sequestered in autophagic vacuoles *(121)*. Importantly, simultaneous autophagic and apoptotic degeneration of dopaminergic neurons was discovered in the SN of PD patients *(184)*. As already mentioned, it has to be kept in mind, however, that all modes of cell death, apoptosis, necrosis, and autophagy, may depend on the severity of the insult the cell has suffered and that mixed forms of cell death, showing features of different kinds of cell death, may occur.

5. CONCLUSION

In summary, DA is able to exert neurotoxic effects via enzymatic or nonenzymatic production of ROS and toxic metabolites as DAQ and its thiol conjugates, DOPAL, 6-OHDA, and formation of NM, which can have both neuroprotective and neurotoxic properties when iron-binding capacity is overwhelmed. The final outcome is predominantly the oxidative modification of vital proteins in the neurons and possibly the induction of apoptosis, necrosis or autophagy and potentially mixed forms of all cell death modes. However, recent interesting findings imply also a nonoxidative form of DA toxicity *(204,217)*, which warrants further exploration in the future.

From all the observed neurotoxic pathways one generally valid conclusion can be drawn: the intracellular compartmentalization of DA and appropriate removal of intercellular DA is of fundamental importance to prevent DA-induced cytotoxicity. But particularly this important prerequisite for "autonomous" neuronal protection might be severely perturbed in idiopathic PD. The lack of sufficient energy supply owing to complex I malfunctioning of the respiratory chain and a pro-oxidative environment will also impair the proper activity of DAT and VMAT2. It is important to note that in PD putamen, the most severely affected region, the ratio of DAT to VMAT2 is higher than in control caudate *(221)*, thus causing a disbalance between cellular DA uptake and intracellular sequestration. The altered transporter protein ratio is also reflected in the significantly reduced expression of DAT mRNA in the SNpc of PD brains from where the synthesized DAT protein is transported to axons and nerve terminal endings *(222)*. Interestingly, the surviving nigral neurons in the PD SN have lower average DAT mRNA levels than in control brains and these lower levels correspond to the least levels expressed by control SN *(99)*.

It seems that inadequate DA sequestering is maybe not the primary causative factor, but plays an integral part of the oxidative stress-generating vicious circle. Moreover, the theory of increased DA turnover for compensating dopaminergic neuronal loss in early PD has recently been confirmed by a new modeling approach to position emission tomography ^{18}F-fluorodopa data in very early stages of PD *(223)*, thus potentially contributing to elevated intracellular stress. Although the development of therapeutics that increase vesicular DA uptake might help to reduce intracellular pro-oxidant conditions, the major task still remains to elucidate the primary causative factors for increased oxidative stress in the SN of PD. Therapeutic strategies aiming at preserving and restoring proper energy metabolism and an array of diverse treatment options to reduce the oxidative stress should help to slow down the progression of dopaminergic cell loss in PD.

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Glutamate and Neurodegeneration

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1. HISTORICAL PERSPECTIVE

Psychiatric diseases have stimulated human interest since ancient times, evoking mixed feelings of fear, compassion, appreciation of originality, and medical impotence. The search for their biological causes and therapeutical remedies has been predominantly inspired either by fantasy, as retracted in the famous masterpiece of Fig. 1, or by religious convictions, similarly to other diseases involving consciousness, such as epilepsy. Following the development of neuroscience, the problems of the mind started to receive attention according to the scientific method and to biological hypothesis, and the discovery of useful psychoactive drugs revolutionized our understanding of the nature of psychiatric diseases. Curiously, at the same time as antipsychotics began to be known as such, another molecule, glutamate, began to be known for both its neurotoxic properties *(1)*, and its potential for being an excitatory neurotransmitter in the central nervous system (CNS) *(2)*. However, in the early 1960s only few molecules, such as acetylcholine, norepinephrine, dopamine, and γ-aminobutyric acid could be definitively considered to play a role as neurotransmitters. Thus, it should not be surprising that glutamate, a widespread amino acid, struggled to establish its role as the most important excitatory neurotransmitter in the brain. Furthermore, after the initial observation of Lucas and Newhouse *(1)*, most of the evidence assigning a neurodegenerative role to glutamate came from studies where glutamate and its analogs were either administered peripherally in high doses or injected directly in the CNS, two experimental conditions that did not suggest a neurodegenerative role for endogenous glutamate. In this respect, it should be noted that the first seminal paper describing neurodegeneration in the brain following peripheral administration of glutamate, could evidence neuronal loss only in the arcuate nucleus and other nuclei of the ventral hypothalamus of neonatal rodents, suggesting an important role of the adult blood–brain barrier in preventing accidental neurodegeneration by alimentary glutamate ingestion *(3)*. Among studies using direct injection of excitatory amino acids (EAAs) in the CNS, an important study by Coyle and Schwarcz in 1976 *(4)* pointed out the usefulness of kainic acid in mimicking, in rats, the striatal gabaergic neurodegeneration observed in Huntington's disease. The specificity of the lesions induced by kainic acid also suggested that EAA receptors could be involved

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

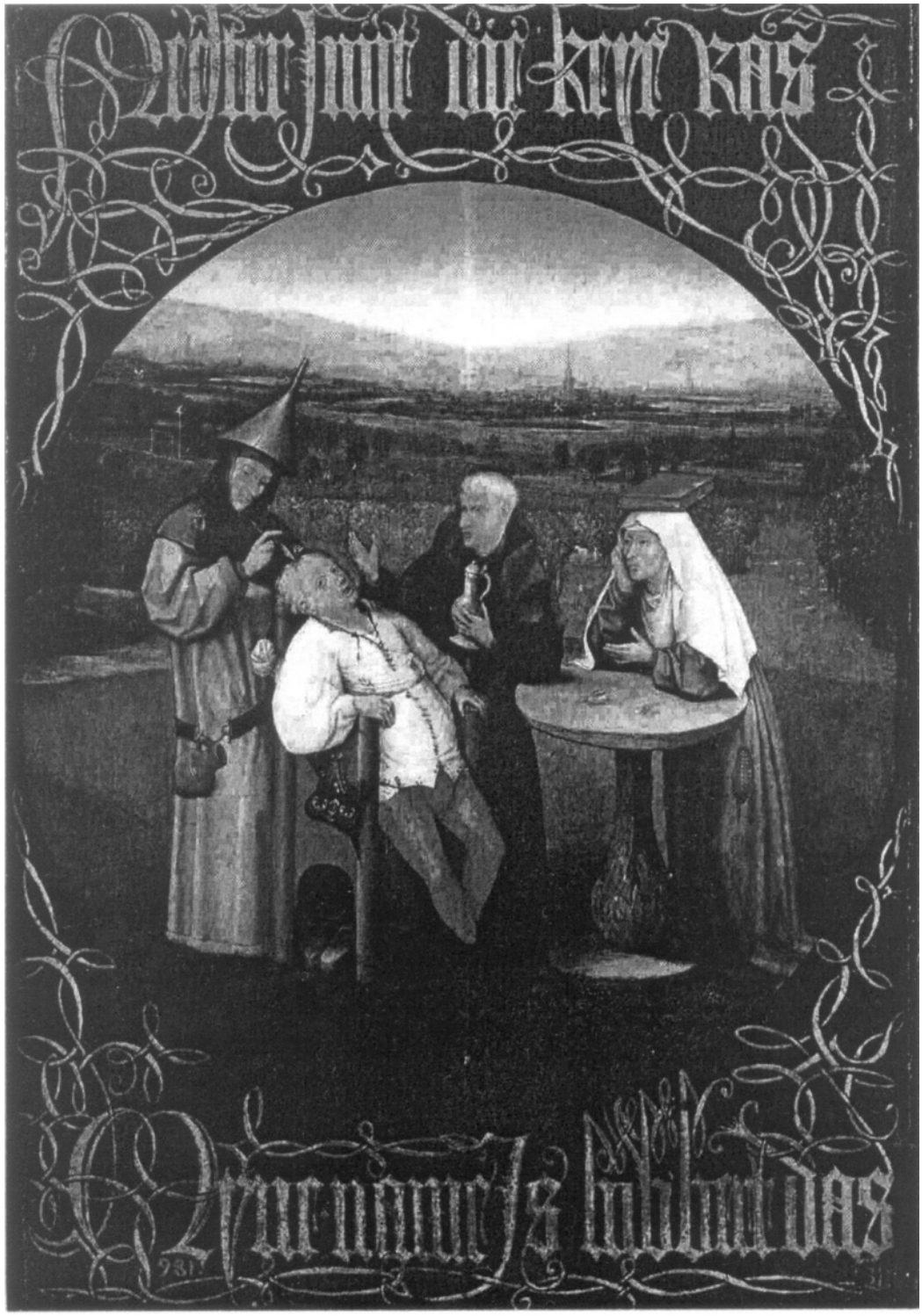

Fig. 1. *Extraction of the Stone of Madness*, 1475–1480, by Hieronymus Bosh, Museo del Prado, Madrid, Spain.

in this human disease of neuropsychological and neuropsychiatric relevance. However, the importance of glutamate as an endogenous potential excitotoxin was difficult to establish at that time owing to both the lack of specific and potent EAA receptors antagonists and a necessarily approximate receptor classification based mainly on electrophysiological and receptor-binding studies, using a variety of glutamate analogs *(5)*. Much important evidence in the 1980s attracted the attention of neuroscientists on glutamate, and linked its presence in the brain to long-term potentiation (LTP) of synaptic response and neurodegenerative diseases. As for the former, several independent investigators were first able to obtain two important evidences: that the induction of LTP in the hippocampus was associated with an increase in glutamate release, and that EAAs were responsible for LTP *(6,7–9)*. Next, the discovery in 1984 of Mg^{2+} voltage-dependent block of the electrophysiological response to *N*-methyl-D-aspartate (NMDA) *(10,11)* was of fundamental importance to begin understanding how endogenous glutamate could elicit LTP, as it lead to a parmenidean classification of the EAA receptors mediating the response to glutamate in NMDA and non-NMDA types *(12)*. It should be noted that non-NMDA receptors at that time included all the ionotropic receptors that were not blocked by Mg^{2+}, such as kainate receptors, as well as the newly pharmacologically identified glutamate receptor coupled to phosphatidylinositol (PI) metabolism *(13,14–16)*. As a result, the number of publications reporting on LTP and glutamate rose from two in the 1970–1980 decade, to 89 in the 1980–1990 decade, indicating the importance of these findings (Fig. 2). Neither less important nor exciting were the findings that prompted to the assignment of a role for endogenous glutamate in some neurodegenerative disorders. In fact, evidence became compelling when several independent research groups confirmed three important findings:

1. A role for synaptic activity and EAA receptors in both hypoxic and hypoglycemic neurons *(17,18)*.
2. A large release of endogenous glutamate during anoxia and hypoglycemia both in vivo and in vitro *(19–21)*.
3. A key role for glial cells in the uptake of glutamate *(22)*.

At this point, knowledge of the voltage-dependent nature of the Mg^{2+} block at the NMDA receptor was crucial to understand how otherwise nontoxic concentrations of glutamate could elicit neurodegeneration when cellular energy was depleted *(23)*. Thus, the conceptual framework for considering brain glutamate as a potential endogenous neurodegenerative agent responsible for some diseases of the CNS was completed *(24–26)*. By the end of 1990 the number of publications relating glutamate and neurotoxicity was 231, as compared to only 32 publications on this subject from 1970 to 1980 (Fig. 2), demonstrating the interest of neuroscientists in this molecule. During the following years the number of publications per year on glutamate neurotoxicity grew ostensibly thanks to other important discoveries, most noticeably the cloning of EAA receptors, and now, when more than 2000 articles have been published on excitotoxicity (Fig. 2), it may be a convenient time to revise some aspects of our knowledge on the the molecular mechanisms leading to excitotoxicity.

2. MOLECULAR STRUCTURE OF GLUTAMATE RECEPTORS

The molecular structure of glutamate receptors was mostly determined between 1989 and 1992 *(27)*. These receptors may be classified at first based on being either ionotropic

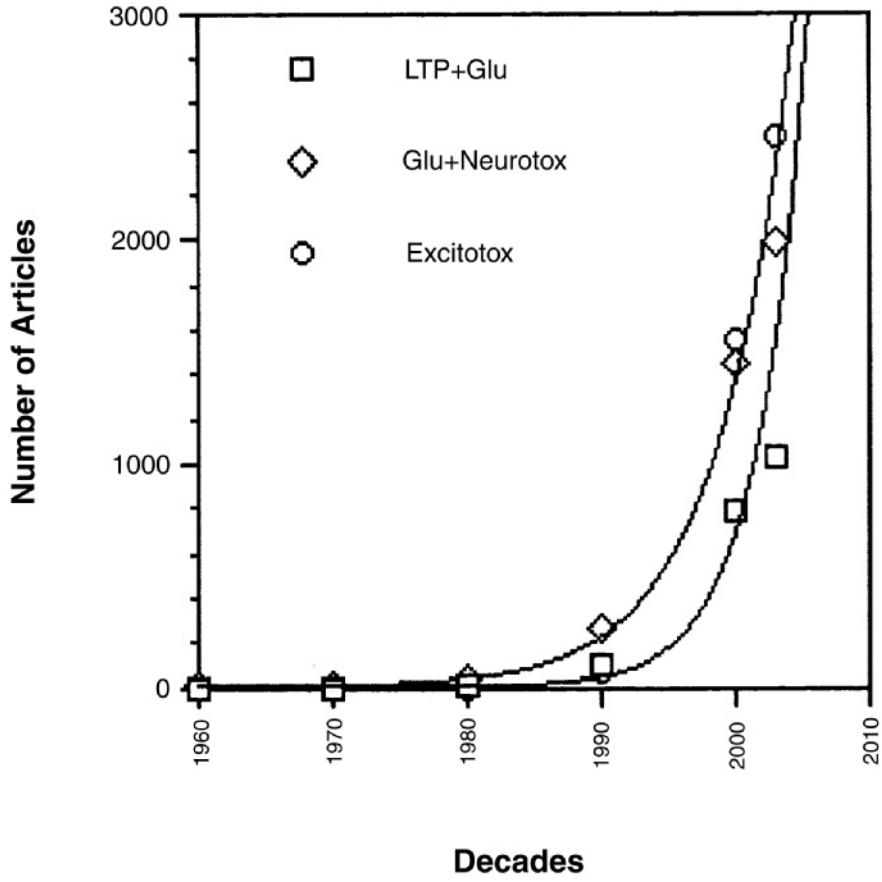

Fig. 2. Increase in the number of publication on the role of glutamate in synaptic plasticity and neurodegeneration from 1960. Data were obtained from the National Library of Medicine at NIH (http://www.ncbi.nlm.nih.gov/PubMed/) using the following key words: neurotoxicity (Neurotox), glutamate (Glu), LTP, excitotoxicity (Excitotox). LTP, long-term potentiation.

or metabotropic receptors. Ionotropic receptors include three molecularly different receptors that are preferentially activated by NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate respectively, whereas metabotropic receptors contain G protein-coupled receptors.

2.1. Ionotropic Receptors

The NMDA receptor is bearing multiple regulatory sites in addition to the glutamate/NMDA-binding site, such as a glycine site where glycine is acting as a coagonist of NMDA for receptor activation, a site for polyamines, a site for Zn^{2+}, and a redox modulatory site. The receptor gates a channel permeable to both Na^{+} and Ca^{2+} that holds inside a Mg^{2+}-binding site where Mg^{2+} binds, upon the activation of the receptor, with a strength directly proportional to the negativity of the intracellular potential *(10,11)*. Furthermore, the channel holds a binding site for drugs such as phencyclidine, which are occluding the channel pore, therefore acting as noncompetitive antagonists *(27,28)*. The

need for the combined presence of glutamate and glycine and a concomitant depolarization in order to achieve receptor activation and ion influx, is the most peculiar feature of the NMDA receptor, and the one that makes it a perfect mediator of LTP of synaptic activity *(29)*. Five subunits from two gene families for the NMDA receptor have been cloned (NR1, NR2A-2D), and their combination according to a conservative tetrameric model containing two NR1 and two NR2 subunits may lead to functionally different NMDA receptors with a specific distribution in the brain *(28,30)*. Two additional subunits named NR3A and NR3B, more distantly related to the others, are encoded by a third gene family. The association of either one of these two subunits with the NR1 subunit has been reported to profoundly change the characteristics of the NMDA receptor, converting it in a type of excitatory glycine receptor *(31)*.

Three gene families are encoding the nine subunits from which ionotropic non-NMDA receptors are assembled, generating at least two receptor types: the AMPA receptor and the kainate receptor (Table 1). AMPA receptors are assembled from four subunits, GluR1 through GluR4, that belong to the same gene family, and generate functional non-NMDA receptor channels with high affinity for AMPA and low affinity for kainate. Multiple subtypes may exist based on subunit combination, providing important functional differences among AMPA receptors located both in different areas and within the same neuron. One of the most eloquent example is perhaps provided by GluR2-lacking AMPA receptors, which are both insensitive to spermine and polyamine spider toxins and more permeable to Ca^{2+} *(32–34)*. Kainate receptors originate from the combination of five subunits that belong to two gene families: one encoding for the subunits GluR5, GluR6, and GluR7, the other encoding for the subunits KA1 and KA2. GluR5-7 generate functional non-NMDA receptor channels with a relatively low affinity for kainate and little or no affinity for AMPA, whereas KA-1 and KA-2 possess high-affinity binding for kainic acid but need to combine with GluR5, GluR6, or GluR7 in order to form functional non-NMDA receptor channels. Interestingly, heteromeric receptors containing KA1 or KA2 subunits possess unexpected properties, such as a low affinity for kainate and some affinity for AMPA *(28,35,36)*.

In addition to the variety of properties provided by subunit combination, the ionotropic glutamate receptors may further increase their physiological diversity through the modification of the molecular structure of their subunits. Thus, two post-transcriptional modifications, *editing* and *alternative splicing,* are operative. Pre-mRNA processing known as *editing* guarantees, for example, the existence of non-NMDA receptors with higher or lower ion permeability as the result of one amino acid exchange in one or more sites of their subunits *(37)*. In particular, a CAG codon for glutamine in the M2 membrane domain of GluR2 can be edited to CIG, leading to the substitution of glutamine by arginine at the Q/R site, and determining low-Ca^{2+} permeability of AMPA receptors containing the edited GluR2 subunit. Similarly, editing of the Q/R site present in the M2 domain of GluR6 reduces the permeability to Ca^{2+} of the kainate receptor although it also increases its permeability to Cl^{-}. Furthermore, the M1 membrane domain of GluR6 can be edited at the I/V and Y/C sites, reversing the effect of the editing of the Q/R site in M2 to arginine. The editing process may also affect other receptor properties, such as desensitization, which can be reduced by the editing of the R/S site in the region between M3 and M4 of GluR2–4 to glycine *(28,38–41)*.

Table 1
Ionotropic Non-NMDA Receptors Classification of Ionotropic Non-NMDA Receptors Based on Subunit Composition and Affinity for the Agonists

Type	Subunits	Subunit relative affinity for the agonist
AMPA	GluR1, GluR2, GluR3, GluR4	QUIS > AMPA > DOM > GLU > KA
Kainate	GluR5[a], GluR6, GluR7[b]	DOM > KA > QUIS > GLU > AMPA
KA-1, KA-2	KA>DOM	

QUIS, quisqualic acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; DOM, domoate; GLU, glutamate; KA, kainate.

[a]Only for GluR5.

[b]For receptors formed by the subunit GluR7, binding studies revealed the following affinity order: DOM>KA>GLU>QUIS, whereas functional studies on homomeric receptors indicated a low affinity for kainate and glutamate and insensitivity to domoate *(35,46)*.

Alternative splicing may increase both NMDA and non-NMDA receptor heterogeneity. This biochemical mechanism of RNA processing allows the insertion or deletion of RNA stretches into the mRNA, determining the presence of EAA receptors with a different molecular weight and with different properties. The NR1 subunit of the NMDA receptor undergoes alternative splicing at three exons, one at the N-terminus and two at the C-terminus, resulting in eight splice variants that are differentially localized in adult and developing animals, according to their physiological role. Alternative splicing-derived properties include affinity for the agonist, Zn^{2+} and polyamine modulation, receptor clustering and sites for posttranslational modifications, and cellular signaling *(28)*. Concerning the AMPA receptor, all four subunits may undergo alternative splicing at two exons located before the M4 membrane domain, thus generating the *flip/flop* variants *(42,43)*. The flop variants desensitize more rapidly than the flip variants and their number increases after animal birth, until they reach the same abundance as the flip variants. Additionally, the AMPA receptor subunits GluR2 and GluR4, and the kainate receptor subunits GluR5-7 may present splice variants at the C-terminus. These variants may be important for the interaction of glutamate receptors with intracellular proteins, and it cannot be excluded that may also affect electrophysiological properties as observed when comparing the two splice variants of homomeric GluR7 receptors *(35,44,45)*.

2.2. *Metabotropic Receptors*

Metabotropic glutamate receptors (mGluR) possess two well-defined structural regions: one consisting of seven transmembrane domains, and the other being a large extracellular domain bearing the agonist binding site. This structure is characteristic of all G protein-coupled receptors, and all mGluR are coupled to G proteins linked to phospholipase C or adenylate cyclase activities. Eight receptor subtypes can be divided in three groups based on their pharmacology, amino acid sequence similarities, and coupling to second messengers *(47,48)*. Thus, group I is formed by mGluR1 and mGluR5, which are coupled to PI hydrolysis, whereas group II and group III are formed by mGluR2-3 and mGluR4 plus mGluR6–8, respectively, all coupled to cAMP synthesis. The pharmacology of mGluRs is not as specific as it would be necessary for the unequivocal identification of the role of each receptor subtype. Thus, whereas a clear pharmacological distinction between group I, group II, and group III receptors can be made, no

specific agonists nor antagonists are available to distinguish between mGluR4, mGluR6, mGluR7, and mGluR8. Furthermore, the only possibility to identify the role of mGluR2 from that of mGluR3 relies on their differential activation by *N*-acetylaspartylglutamate. Only mGluR1 and mGluR5 possess several subtype specific antagonists, allowing for the identification of each one role (for a review, *see* ref. *47*). An interesting characteristic of mGlu receptors, shared by other G protein-coupled receptors, is their capability to form dimers *(49–51)*. This molecular arrangement, which possibly relies on the existence of several points of interaction between the two subunits, may be particularly important for the activation of the mGlu receptors and their physiological function *(52,53)*. Similarly to ionotropic glutamate receptors, mGlu receptors may also be expressed in splice variants. It has been suggested that alternative splicing is developmentally controlled and may allow targeting of mGlu receptors to dendrites and axons, intracellular signal transduction, and synaptic clustering, as well as tissue specificity *(54–57)*.

Besides being determined by permanent structural modifications, such as those already described, the activity of all EAA receptors may also be dynamically modulated within a short time frame, by intracellular signaling. The mechanisms involved will be presented and discussed later on, when relevant to neurodegeneration.

3. ION INFLUX AND NEURODEGENERATION

Glutamate activation of the ionotropic and metabotropic receptors may initiate an extremely complex signaling in the neurons that host these receptors, depending, among other things, on their molecular structure. However, it is possible to define a general scenario where ionotropic glutamate receptors interact synergistically to initiate both physiological and pathological processes. Thus, upon activation, the majority of ionotropic non-NMDA receptors allow only the passage of monovalent ions, and subsequent neuronal excitation may be triggered by a depolarizing Na^+ influx into the neuron. Such depolarization reduces the Mg^{2+} block at the glutamic acid-activated NMDA receptors, allowing the influx of Ca^{2+}, which in turn initiates the intracellular biochemical cascade which, in physiological conditions, may be leading to LTP of synaptic responses, a form of synaptic plasticity that, particularly in the hippocampus, is considered to be the molecular base for explicit learning and memory *(29)*.

However, a variety of both acute and chronic conditions, either endogenous or exogenous to the organism, such as epilepsy, stroke, or exposure to toxins, may eventually torn a physiological excitatory stimulation into a neurotoxic one, possibly leading to neurodegeneration *(58–60)*.

Thus, the whole process leading to neurodegeneration following EAA receptor stimulation, also known as excitotoxicity *(61)*, may begin with an overstimulation of glutamate receptors owing to either an excessive synaptic release of glutamate, or failure of glutamate uptake by glial cells. Both conditions may occur during stroke, because of the rapid decrease in cellular energy levels and progressive neuronal and glial depolarization *(19–22,62)*. The use of selective NMDA receptor antagonists significantly prevents neurodegeneration in most experimental conditions, indicating a pivotal role of this ionotropic receptor in excitotoxicity *(18,23,63–65)*, although in some animal models of ischemia, neuroprotection has been achieved by using ionotropic non-NMDA receptor antagonists *(66)*. Because of these observations, preclinical and clinical investigators have focused their attention on glutamate ionotropic receptors and the signaling that they

generate. Among second messengers, Ca^{2+} in particular has received most of the attention because of the higher Ca^{2+} permeability of NMDA receptors and the idea that Ca^{2+} overload may be responsible for brain damage during ischemia *(24,67–70)*. Initially, the idea that Ca^{2+} could be mediating glutamate neurotoxicity contrasted with the observation that the rapid neuronal swelling that follows exposure to glutamate and precedes neurodegeneration, could be abolished by eliminating either Na^+ or Cl^- *(71,72)*. Later on, substantial but not complete agreement was achieved by defining two different components in the time course of glutamate-induced neurodegeneration: (1) a rapid neuronal swelling, totally dependent upon the presence of Na^+ and Cl^-, and (2) a delayed neurodegenerative process that is independent of Na^+ and Cl^- but requires the influx of Ca^{2+} *(73–77)*. The possibility that Ca^{2+} may also enter the neurons via voltage-gated Ca^{2+} channels (VGCCs) and participate in the delayed component of neurodegeneration by glutamate, was also taken into consideration, and indeed, substantial neuroprotection by L-type VGCC blockers against stimulation of ionotropic non-NMDA receptors has been reported *(78)*. However, several observations argue against a too simplified hypothesis of ionic toxicity. In fact, acute neuronal swelling is elicited only by stimulation of NMDA receptors, whereas stimulation of non-NMDA receptors in the presence of NMDA receptor antagonists produces the opposite effect, that is, a slow, progressive shrinking of the cell bodies that requires long-term exposures and preceeds neurodegeneration *(23,78–81)*. The rapid neuronal swelling associated with the NMDA receptor stimulation and mediated by Na^+ and Cl^-, has been tentatively linked to the Ca^{2+} permeability of its channel, whereas the Ca^{2+} influx via VGCCs has been associated with the delayed-type of toxicity elicited by non-NMDA receptor agonists *(78)*. However, the rise in intracellular Ca^{2+} following ionotropic non-NMDA receptor agonists may be quite rapid, and may result in the formation of Ca^{2+}-dependent second messengers with kinetical parameters that are indistinguishable from those of NMDA receptors, and may even be additive *(80,82,83)*. Furthermore, non-NMDA receptor agonists may promote the rapid release of endogenous NMDA receptor agonists that are capable of producing the characteristic neuronal swelling within the same time frame of exogenously applied NMDA receptor agonists *(80,81,84)*. Both phenomena are strongly dependent on the opening of VGCCs, and may be reproduced by using depolarizing stimuli, such as the voltage-gated Na^+ channels activator veratridine, instead of ionotropic non-NMDA receptor agonists *(85,86)*. Interestingly, both the morphology and the kinetics of neurodegeneration by veratridine are comparable to those of non-NMDA receptor agonists.

Recently, direct evidence has been provided against a prejudicial role for Ca^{2+} entering the neurons via VGCCs during excitatory aminoacid receptor stimulation. In fact, it has been observed that the blockage of these channels may worsen non-NMDA receptor-mediated toxicity, as well as the toxicity by depolarizing stimuli *(81,86,87)*. These observations are in agreement with the idea that Ca^{2+} entering via VGCC may indeed have a trophic role in neuronal development and in activity-dependent cell survival *(88,89)*. This may have been one of the reason why clinical trials involving the use of VGCC antagonists for neuroprotection in ischemia were not successful *(90,91)*.

It should be noted that Ca^{2+} may play an important role in neuroprotection also when entering via the receptor-gated NMDA channel. In fact, as shown in Table 2, the divalent ion chelator ethylene diamine tetraacetic acid (EDTA) is capable of inducing NMDA receptor-dependent neurodegeneration, including neuronal swelling. Different from

Table 2
Extracellular Cation Chelators and NMDA Receptor-Mediated Excitotoxicity[a]

	Treatments							
	None		EDTA 5 m*M*		BAPTA 5 m*M*		Glu 40 μ*M*	
	10′	6 h (%)	10′	6 h (%)	10′	6 h (%)	10′	6 h (%)
None	–	<5	+++	100	–	30	++	81
Mg^{2+} 2m*M*	–	<5	–	30	nd	nd	nd	nd
MK-801 1 μ*M*	–	<5	–	20	–	20	–	<5
EDTA 5 m*M*	+++	100					nd	nd
BAPTA 5 m*M*	–	30					+++	100

[a]Cultures of rat cerebellar neurons were examined under phase contrast microscopy for the presence (+) or the absence (–) of early signs of excitotoxicity, such as swelling and darkening of the cell, 10′ after exposure to the indicated drugs in the growth medium. Then, 6 h later, the percentage of degenerating neurons was calculated after determining the number of neurons that were retaining the vital stain fluorescein diacetate, excluded ethidium bromide, and were morphologically similar to those of the untreated group. nd, not determined. The concentration of endogenous glutamate in the growth medium was less than 9 μ*M*. EDTA, ethylene diamine tetraacitic acid; BAPTA, 1,2-*bis* (2-aminophenoxy) ethane - *N, N, N′, N′*-tetraacetic acid.

ethyleneglycotetraacetic acid (EGTA) or 1,2-*bis* (2-aminophenoxy) ethane-*N, N, N′, N′*-tetraacetic acid, the effect of EDTA does not require exposure to exogenous glutamate, and is antagonized by the addition of extra Mg^{2+}. It is important to note that in the presence of EDTA, the extent of NMDA receptor-mediated neurodegeneration can be superior to that of exogenously applied glutamate in the absence of the divalent anion chelator. Because the concentration of endogenous glutamate in the growth medium does not exceed one-fourth of that of exogenously applied glutamate and is not toxic in the selective absence of Mg^{2+} *(23)*, it is possible that Mg^{2+}-unblocked NMDA receptors may become overactive in the absence of Ca^{2+}. These results are in agreement with both previous reports of an increase in NMDA receptor-mediated neurotoxicity when extracellular Ca^{2+} was removed *(92)* and the notion that excitotoxicity may imply an Na^{+}/Ca^{2+} interplay at discrete synaptic regions *(93)*, physically separated from other microdomains where Ca^{2+} may exert a trophic role. Of course such notion makes obsolete any attempt to prevent excitotoxicity by manipulation of either the extracellular ionic milieu or the ion influxes, and focuses the attention on two topics:

1. The biochemical pathways that are activated by Ca^{2+} and may play a role in neurodegeneration.
2. The molecules that define the microdomain where such potentially lethal reactions may take place.

4. MICRODOMAINS AND SIGNALING

The molecular definition of microdomains began few years ago with the identification of several postsynaptic density (PSD) proteins that control the targeted clustering of EAA receptors. First came the discovery that NMDA receptors may bind to, among others, the PSD-95/SAP90 family of synaptic-associated proteins (SAPs) *(94,95)*. The PSD-95 protein contains three protein–protein interaction motifs named PDZ domains *(96)*, which bind to specific peptide sequences near to the carboxy terminus of interacting proteins. The NR2 and NR1 subunits of the NMDA receptor may bind to two of these

PDZ domains, whereas the third one may bind to other proteins involved in signal transduction, such as neuronal nitric oxide sinthase (nNOS), which possesses a PDZ domain and may interact with PSD-95 through a PDZ–PDZ domain interaction *(97)*. PSD-95 contains also two other domains, an SH3 domain and a guanylate kinase-homology domain, through which it may bind other proteins, such as synGAP, which has been suggested to link NMDA receptors to synaptic Ras signaling *(98–100)*. Furthermore, PSD-95 may associate by a disulphide bridge to chapsyn-110/PSD-93, which has been suggested to bind the GluR6 subunit of kainate receptors, thus providing a continuity between NMDA and kainate receptors *(100)*. Similarly, the AMPA receptor subunits were shown to bind specifically to two out of seven PDZ domains of a glutamate receptor–interacting protein (GRIP) *(44,101)*. The PDZ domains of GRIP allow the binding to an amino acid sequence nearby the carboxy terminus of the interacting protein that is different from that required by PSD-95. Also in the case of GRIP, the five unused PDZ domains may bind other proteins linking AMPA receptors to the cytoskeleton or to signal-transducing enzymes. In particular, the seventh PDZ domain of GRIP binds to GRASP-1, a neuron-specific guanine nucleotide exchange factor (GEF) for the Ras family of the small G proteins *(102)*. Thus, both NMDA and AMPA receptors may possibly be linked to a common biochemical pathway involving some of the members of the Ras family. In fact, the proteins rin and rit are known to bind calmodulin, and may possibly provide a molecular bridge between the Ca^{2+} influx through NMDA receptors, the AMPA receptors, and the Ras-like signaling pathways. Such molecular bridge may be useful, for example, in the dynamic regulation of the presence of AMPA receptors at excitatory synapses, not only in synaptic plasticity *(102,103)* but also in excitotoxicity, where a prominent role of AMPA receptors has been suggested *(90)*. It should be noted that the AMPA receptor has recently been reported to interact with the protein stargazin *(104)*, which was also found to copurify with VGCC of skeletal muscle and brain, and it has been considered to be the γ-subunit of these channels *(105,106)*. The presence of stargazin may be mandatory in functional AMPA receptors, and it may allow the targeting of AMPA receptors at the postsynapse via its binding to PSD-95, although both AMPA receptors and stargazin may also colocalize with presynaptic protein, such as synaptophysin-1 *(104)*. Thus, it is possible that VGCC may get associated to AMPA receptors and NMDA receptors either at the synapse under certain conditions, or in specific neuronal populations. The identification of a family of γ-like subunits, each with both a different homology to stargazin and a distinct expression pattern in the brain, may guarantee the independence of this association. Interestingly, the absence of stargazin in cerebellar neurons from the stargazer mouse did not alter the functioning of VGCC, despite the complete and selective absence of functional AMPA receptors *(104)*. Thus, given the highly sophisticated organization of receptors and ion channels at the synapse, we may still have to wait to achieve further knowledge before we may explain how elevated calcium influxes through the Q/R site-unedited of the GluR2 subunit of AMPA receptors may cause neurological dysfunctions without excitotoxicity, as otherwise predicted (refs. *90,107,108*; *see also* the following subheadings).

4.1. Neuronal Nitric Oxide Synthase

One important biochemical pathway that has been involved in glutamate receptors signaling and neurodegeneration involves neuronal nitric oxide synthase (nNOS). nNOS

is coupled to the NMDA receptor via the same PSD-95 protein that holds the NMDA receptor. Therefore, nNOS may get in the proximities of the AMPA receptor when they are inserted in the synapse for the maintenance of LTP *(103)*. This localization should be important for the calcium-dependent activation of nNOS by Ca^{2+} entering through both the NMDA receptor and the AMPA receptor bearing the Q/R site-unedited GluR2 subunit *(109,110)*. The activation of nNOS via NMDA receptors has been suggested to be potentially harmful for neurons owing to the synthesis of nitric oxide (NO). This free-radical molecule may then react with superoxide and other oxygen radicals, leading to protein nitrosylation and neurodegeneration *(111)*. Accordingly, in mutant mice deficient in nNOS activity, both infarct volume and neurological deficits following cerebral ischemia were lower than in normal mice *(112)*. Furthermore, the suppression of PSD-95 expression reduced both cyclic guonosine monophosphate (cGMP) synthesis and excitotoxicity following NMDA receptor stimulation *(113)* to a similar extent as the uncoupling of NMDA receptors to PSD-95 in ischemic brain damage *(114)*. Interestingly, in both studies the influx of Ca^{2+} following NMDA receptor stimulation was unchanged, suggesting that neurodegeneration may depend exclusively upon the proper activation of nNOS. Although these results disclose an important therapeutic potential to membrane-permeant compounds, which may disrupt the association between NMDA receptors and NOS, it should be noted that neuroprotection may only be partial. In fact, in neuronal cultures where PSD-95 synthesis was impaired, the protection from NMDA toxicity was progressively vanishing as the NMDA concentration was increasing, whereas cGMP synthesis never exceeded 50% of the corresponding NMDA-stimulated value in untreated cultures *(113)*. However, when PSD-95 was just uncoupled from the NMDA receptor, the reduction in both cGMP stimulation and neurodegeneration following NMDA exposure were comparable *(114)*. In vivo, this approach allowed also for a reduction of both total and cortical infarct areas following middle cerebral artery occlusion (MCAO), which was similar or higher than the reduction in NMDA-mediated cGMP stimulation *(114)*. On the other hand, in nNOS-deficient mice, MCAO failed to increase cGMP concentration over brain basal levels, but both brain infarct volume and area were reduced only by approx 40%, resulting in a similar 40% reduction of the neurological deficit score *(112)*. Thus, nNOS-mediated neuronal injury may possibly be limited, and neuroprotection from excitotoxicity may depend more upon the uncoupling of PSD-95 from the NMDA receptor than on the inhibition of nNOS. Part of the problem may be that the toxicity of NO may also change depending on the neuronal population that is considered. In fact, in some experimental systems the inhibition of nNOS did not provide neuroprotection *(115)*. Furthermore, it should also be considered that NO is a short-lived free radical that after NMDA receptor stimulation rapidly activates guanylate cyclase, promoting the transient formation of the second-messenger cGMP. This makes unlikely that NO can be responsible for delayed neurotoxicity processes. However, NO from nNOS activation during reperfusion of ischemic areas, particularly in human stroke *(116)*, may be important, and the extent of neuroprotection during reperfusion may possibly be similar to that of nNOS/cGMP inhibition. On the other hand, cGMP, which appears to be devoid of neurotoxic activity *(117)*, has been recently coupled to the phosphorylation of cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB) and neuronal survival *(118)*, and therefore the inhibition of its synthesis may be prejudicial for a neuroprotective strategy.

4.2. PSD-95

The beneficial effect of PSD-95 uncoupling from the NMDA receptor may actually involve the uncoupling of an unknown pathway, perhaps linked to the activity of metabotropic glutamate receptors. Group 1 mGlu receptors are coupled to the N-terminal EVH1 domain of the Homer protein that contains also an additional C-terminal domain with predicted coiled-coil (CC) structure-mediating homo- and heteromultimerization between Homer proteins. CC-Homers are localized at PSD and appear to provide a bridge between group 1 mGlu and inositol trisphosphate receptors (IP3Rs). In fact, IP3Rs possess a peptide sequence in the cytosol-oriented N-terminus, similar to that allowing mGlu receptor binding at the EVH1 domain of Homer. Furthermore, Homer may bind another postsynaptic protein, Shank, that is part of the NMDA receptor-associated PSD-95 complex, therefore crosslinking mGlu and NMDA receptors *(100,119–122)*. The importance of this link for neurodegeneration is still unclear, as opposite effects of Group 1 mGlu receptor activation on NMDA receptor mediated neurodegeneration have been reported both in vivo and in vitro *(123)*. However, the tight association between mGlu and NMDA receptors may shed new light on the early observation that the block of mGlu receptor-stimulated PI hydrolysis by the noncompetitive receptor antagonist L-2-amino-3-phosphonopropionic acid (L-AP3) leads to neurodegeneration via both NMDA receptor-dependent and -independent mechanisms *(124)*. This study pointed out for the first time that NMDA receptor-dependent neurodegeneration was mediated by endogenous mechanisms linked to mGlu receptors. Other studies have confirmed that group I mGlu receptor antagonists, including L-AP3, may potentiate glutamate toxicity, whereas some agonists may have an opposite effect *(125,126)*. The mechanisms by which group I mGlu are controlling NMDA receptor activity are still unclear, although the tight association between NMDA, mGlu, and IP3Rs suggests an important role for protein kinase C (PKC) and protein phosphatases, such as protein phosphatase 2B (calcineurin) and protein phosphatase 1 and 2A. In this respect, PKC activation has been reported to lower the activity of NMDA receptors containing the NR2C subunit *(127)*, and the NR2C subunit appears to be necessary to allow a reduction of NMDA toxicity by group I mGlu receptor agonists *(123,128)*. It is worth noting that the activity of mGlu receptors may also be under the control of PKC, and it has been suggested that mGlu receptors may reduce NMDA receptor-mediated excitotoxicity only when they are activated both before and during the activation of NMDA receptors *(123)*. Taken together, these observations suggest that the mGlu–NMDA receptor interaction may possibly be leading toward excitotoxicity or excitoprotection depending, among other factors, on the NMDA receptor subunit composition and the timing of receptor activation.

mGlu receptors may also possess a trophic role independent of NMDA receptors. The block of mGlu activity by L-AP3 can induce an NMDA receptor-independent slow but progressive neurodegeneration of cerebellar neurons in culture *(124)*. Such neurodegeneration is associated to a progressive DNA cleavage, an hallmark of apoptosis (Fig. 3). A similar trophic role for group I mGlu receptors has been suggested following in vivo studies in which L-AP3 induced retinal degeneration *(129)*. Furthermore, the activation of group I mGlu receptors may rescue cerebellar neurons from apoptosis when cultured in suboptimal conditions *(123,130)*. In this respect, activation of group I mGlu receptors has been shown to trigger a functional coupling between ryanodine receptors, located on intracellular stores, and L-type VGCC leading to a cyclical facilitation of Ca^{2+} influx

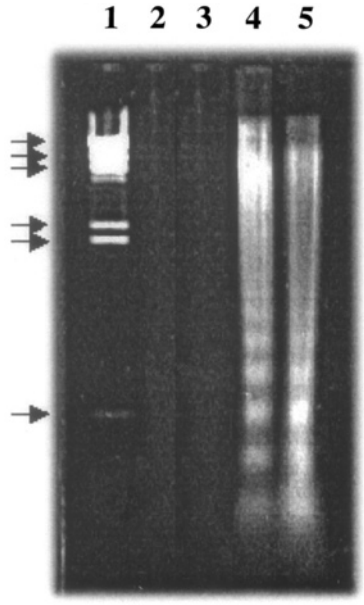

Fig. 3. DNA fragmentation following exposure to L-AP3 of cultured cerebellar neurons. Cultured cerebellar neurons at 15–19 d in culture were exposed to 50 μ*M* L-AP3 in the presence of 1 μ*M* MK-801 in order to avoid the rapid *N*-methyl-D-aspartate receptor-mediated onset of neurodegeneration *(124)*. Exposure to L-AP3 was protracted for 24 h (line 3), 48 h (line 4), or 72 h (line 5). Untreated cultures (line 2) were processed at the same time as cultures treated with L-AP3 for 72 h. Soluble DNA agarose gel electrophoresis reveals a considerable ladder-pattern DNA fragmentation, a hallmark of the apoptotic process, in neurons beginning 48 h after exposure to L-AP3. It should be noted that at this time most of the neurons were still alive and were retaining their gross morphology. No fragmentation can be observed in either untreated neurons (line 2) or neurons treated with L-AP3 for 24 h (line 3). Arrows indicate agarose gel electophoresis of DNA markers of different molecular size (in base pairs: 9416, 6557, 4361, 2322, 564) in line 1. Soluble DNA agarose gel electrophoresis was performed as described *(183)*.

through the latter structure *(122,131)*. The beneficial effect of Ca^{2+} influx trough L-type VGCC for neuronal survival has been known for a long time *(88)*, and therefore we may now have an integrated view of mGlu receptor role on both NMDA receptor-mediated neurotransmission and neuronal trophism.

4.3. Ephrins and Eph Receptors

The control of NMDA receptor activity relies also on other mechanisms that are potentially important for excitotoxicity. One of them is the regulation via the ephrinB2 receptor (EphB) *(132)*. Eph receptors and their ephrin ligands are both membrane-anchored proteins that have initially been shown to be important regulators of axon path-finding and neuronal cell migration, and now are known to have a role in controlling cell–cell interaction in a variety of tissues *(133)*. Eph receptors possess tyrosine kinase activity and can be classified in EphA and EphB based on their activation by ephrinA ligands, a group of proteins attached to the membrane by a glycosyl–PI linkage, and ephrinB ligands, a group of transmembrane proteins, respectively. The binding of ephrinB to EphB is a clear example of bidirectional signaling since it promotes tyrosine

phosphorylation of the cytoplasmic domain of both the receptor and the ligand *(133)*. In the CNS, this unique capability of ephrinBs and EphBs to mediate bidirectional signaling makes them perfect candidates to participate in the control of synaptic plasticity. EphB activation by ephrinB2 appears to induce both NMDA receptor clustering at the postsynaptic sites and potentiation of NMDA receptor activity *(134–137)* . The latter may occur via the phosphorylation of three tyrosine residues in the NR2A/B subunit, leading to an increase in calcium influx through the NMDA receptor *(137)*. It is interesting to note that the phosphorylation of the NMDA receptor is performed by a cytoplasmic Src family kinase that associates to the ephrin B2-activated EphB receptor tyrosine kinase.

4.4. NMDA Receptor Inactivation and Rundown

Based on our present knowledge, the increased activity of the NMDA receptor is not at odds with neuroprotection from excitotoxicity. Activated NMDA receptors undergo two different Ca^{2+}-dependent processes: inactivation and rundown of the channel associated with the NMDA receptor. These two processes occur in a different time frame and involve different mechanisms, but both reduce the open probability of the activated NMDA receptor channel and the risk of excitotoxicity. Ca^{2+} entering through the NMDA receptor channel may bind to nearby calmodulin (CaM), allowing its binding to either the low- or the high-affinity sites located on the COOH-terminus of the NR1 receptor subunit. This results in the rapid inactivation of NMDA receptor channels, and the whole process takes place within hundreds of milliseconds to a few seconds from the activation of the receptor *(138,139)*. The high-affinity site is particularly important for the functional regulation of the receptor, and is located in the 37 amino acid C1 exon cassette which serves as a phosphorylation target for both PKC and protein kinase A (PKA). Furthermore, the C1 cassette contains a motif for PKC and PKA-mediated retrieval/retention of the NR1 subunit from the endoplasmic reticulum, allowing its interaction with the cytoskeleton and clustering at the membrane *(140)*. It is possible that CaM binding and phosphorylation of C1 are mutually exclusive, therefore allocating in C1 the control of both NMDA receptor activity and presence in the membrane. It should also be considered that the inactivation of the NMDA receptor channel depends on its NR2 subunit composition as well, since the presence of NR2C subunits appears to prevent it *(141)*. NMDA receptor channel inactivation has also been related to a Ca^{2+}-CaM-dependent process, which is the activation of calcineurin *(142,143)*. This phosphatase may dephosphorylate several sites on NR2 subunits *(144)*, allowing for a rapid CaM-dependent inactivation of the NMDA receptor channel, independently of the C1 cassette sites on NR1 that participate in CaM binding. Furthermore, Ca^{2+} influx through the NMDA receptor-channel has been shown to induce their rundown within several minutes from the activation of the receptor, by reducing the polymerization of actin filament surrounding the NMDA complex *(145)*. Inactivation and rundown may possibly be particularly important for neuroprotection when a progressive nonexcitotoxic stimulation of the receptor may preceed an excitotoxic stimulation.

5. PRECONDITIONING NEUROPROTECTION

A selective reduction of NMDA receptor-mediated intracellular signaling and excitotoxicity was first observed after a sustained block of glutamate uptake *(146)*, and was confirmed by pre-exposing neurons to low subtoxic concentrations of NMDA *(147)*. The role of Ca^{2+} in this model is also consistent with earlier observations on the worsening of

excitotoxicity in neuronal cultures in the absence of this cation *(92,115)*. Furthermore, the increse in NMDA receptor-mediated excitotoxicity observed after depolarization of dihydropiridine-treated neurons *(86)*, is consistent with a protective role of Ca^{2+} entering through VGCC and participating in the inactivation of the NMDA receptor channel *(148)*. Neuroprotection by preconditioning with subtoxic concentrations of NMDA receptor agonists was shown by Marini and Paul *(147)* to be dependent upon new RNA and protein synthesis. This observation suggests a dynamic turnover of proteins functionally related to NMDA receptor signaling, perhaps one of those we know now controls the activity of the NMDA receptor such as the Src family kinase, CaM, and/or other proteins located downstream from the receptor. Recent studies have demonstrated that nuclear factor κB (NF-κB) activation is necessary for excitotoxicity protection by preconditioning *(149)*. Non-excitotoxic activation of NMDA receptor appears to induce the activation of NF-κB by two pathways, one under the control of the NMDA receptor and leading to the synthesis of new brain-derived neurotrophic factor (BDNF) molecules, the other under the control of the receptor TrkB receptor following its activation by BDNF (Fig. 4). Neuroprotection from excitotoxicity would finally be mediated by both BDNF-TrkB receptor and NF-κB signaling. Both BDNF and NF-κB have been described to have a neuroprotective role in most experimental models (for review, *see* refs. *150–153)*, and are likely to participate in the tolerance to ischemic insults *(154)*. It has been shown that in addition to NF-κB, other transcription factors, such as the CREB are binding to the *BDNF* gene promoter III and are activated by the Ca^{2+} entering through the NMDA receptor *(155,156)*. Interestingly, the simultaneous activation of CREB and NF-κB is required for maximum BDNF synthesis, together with a third transcription factor that represses a negative regulatory region located between the CREB and the BDNF regions of the BDNF promoter III *(156)*.

At this point, a consideration is important: protection from excitotoxicity in preconditioning by NMDA receptor agonists is likely to be the result of the adaptation to potentially toxic stimuli related to synaptic plasticity changes, such as LTP. In fact, the sudden repetitive firing of glutamatergic neurons inducing LTP would possibly lead to excitotoxic neurodegeneration in the absence of mechanisms capable of both, reducing temporarily NMDA receptor activation, and promoting the activation of survival pathways that later may allow the recovery and the potentiation of NMDA receptor activity as required for LTP *(132)*. In this scenario, growth factors, such as BDNF, appear not only to mediate neuroprotection but also to be secreted in a similar fashion as other neuropeptides *(157)* and to be active contributors to synaptic plasticity (for review, *see* refs. *158* and *159*). Thus, not surprisingly, BDNF and glutamatergic transmission have been recently related also to psychiatric disorders *(160–163)* and epilepsy *(164)*. In order to fully elucidate the importance of the discussed molecular mechanisms in neurological and psychiatric disorders, it would be of interest to know whether neuroprotection may develop when synaptic plasticity-related processes, such as LTP, are altered, since the clinical manifestation of plasticity-related processes does not necessarily correlate with neurodegeneration. It has recently been reported that in mutant mice lacking PSD-95, the frequency function of NMDA receptor-dependent LTP and long-term depression (LTD) is shifted to produce strikingly enhanced LTP and impaired learning, without affecting cell density or citoarchitectonic patterns *(165)*. On the other hand, animals in which PSD-95 was uncoupled from the NMDA receptor were more resistant to damage by MCAO, but the reduction of

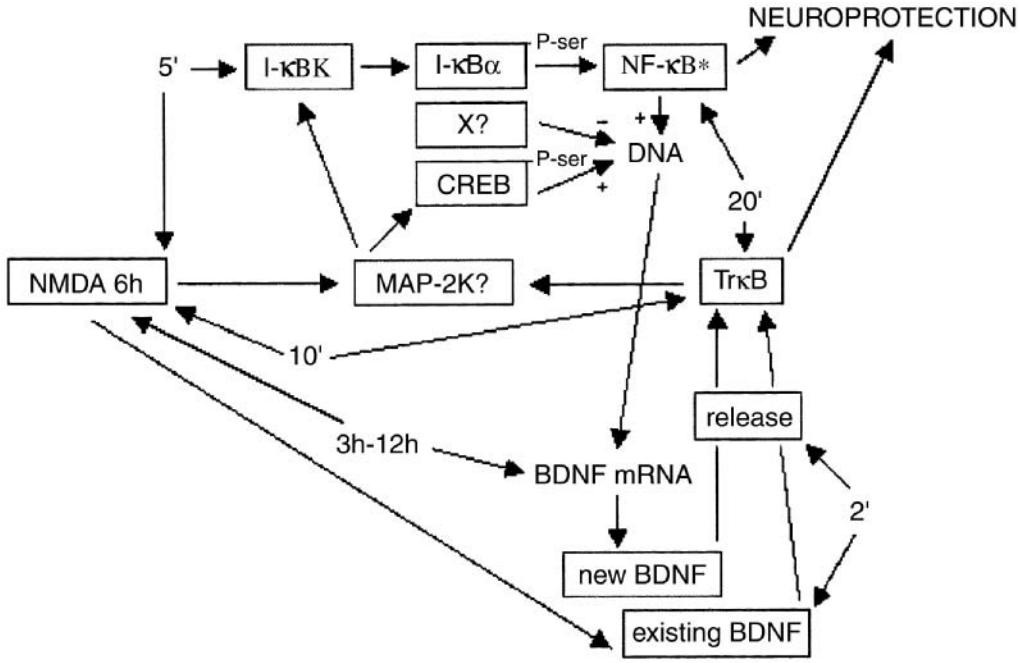

Fig. 4. Time course of the signaling leading to preconditioning neuroprotection via activation of nuclear factor κB. Schematic representation of the chronological sequence of signaling that leads to neuroprotection according to the data of the literature *(149,156)*. The role of mltogen-activated protein-2 kinase is still uncertain. Activation of NF-κB may lead to neuroprotection by promoting the transcription of several genes, one of which appears to be that of brain-derived neurotrophic factor. A significant degree of neuroprotection can be achieved after approx 1 h exposure to *N*-methyl-D-aspartate, whereas maximal neuroprotection will be obtained after 6 h exposure to this drug. BDNF, brain-derived neurotrophic factor; MAP-2k, mltogen-activated protein -2 kinase; NMDA, *N*-methyl-D-aspartate; TrkB, neurotrophin TrkB receptor.

both cortical and total brain infarct following MCAO was superior to the improvement in the neurological score *(114)*. Although no clear explanation for this dichotomy is available at the moment, it could be speculated that preservation of NMDA receptor-mediated Ca^{2+} influx in the experimental models presented may be sufficient to activate the neuroprotective pathways downstream from the receptor, whereas disruption of the protein domain specific for NMDA receptor signal transduction affects synaptic plasticity. Interestingly, Ca^{2+} entering the neurons via VGCC has shown to regulate BDNF gene transcription via a CREB transcription factor *(166–168)*. The stimulation of VGCC was also shown to induce neuroprotection from excitotoxicity, albeit not without the activation of NMDA receptors by endogenous glutamate (ref. *115* and unpublished results). Thus, Ca^{2+} entering the neurons from different structures may activate the pathway leading to BDNF synthesis and neuroprotection. Consistent with this possibility, mice expressing AMPA receptors bearing the Q/R site-unedited GluR-B subunit, which allows Ca^{2+} influx, were reported to present neurological dysfunctions including epilepsy, altered LTP, and dendritic architecture, but no neuronal death *(107)*. Because the activation of Ca^{2+}-permeable AMPA receptor may favor the release of glutamate, it is also likely that NMDA receptor may participate in neuroprotection as previously discussed.

6. EXTRASYNAPTIC RECEPTORS

The role of activated NMDA receptors in guaranteeing neuroprotection is possibly undissociable from their known neurotrophic role in neurons actively participating in a circuit *(169,170)*, and apoptosis occurs when NMDA receptors are blocked *(171–174)*. Furthermore, it has recently been shown that blockade of NMDA receptors may worsen traumatic brain injury *(175,176)* and possibly ischemic brain injury as well *(177)*. Although NMDA receptor activation may be beneficial for neuronal survival, it can definitely mediate excitotoxic neurodegeneration in sudden pathologies such as stroke *(64,90)*, thus originating a puzzling scenario where it has been postulated that two different populations of NMDA receptors, synaptic and extrasynaptic, may be responsible for neuroprotective and excitotoxic effects respectively *(155)*. Extrasynaptic NMDA receptors may represent a relevant amount of total NMDA receptors in the postsynaptic membrane. It has been suggested that extrasynaptic NMDA receptors participate in the dynamic organization of the synaptic NMDA receptor pool, in analogy to what has been observed for the insertion of AMPA receptors *(103,178)*. Extrasynaptic NMDA receptors may not have the same scaffolding, adaptor, cell adhesion, and cytoskeletal proteins of synaptic receptors, nor the same coupling to intracellular signaling *(179)*. They have been shown to oppose the action of synaptic NMDA receptors by triggering CREB shut-off, therefore preventing the synthesis of new BDNF *(155,180)*. Extrasynaptic NMDA receptors may get activated by glutamate spillover, particularly in brain trauma and stroke, and their activity is controlled by Ca^{2+} and tyrosine phosphorylation through mechanisms that are different from those of synaptic receptors *(179)*, thus facilitating the prevalence of their signaling over that of synaptic NMDA receptors. As discussed earlier, the synthesis of new BDNF molecules is likely to have a delayed neuroprotective effect, and therefore its block by activation of extrasynaptic NMDA receptors is expected to weaken the long-term resistance of neurons to repetitive excitotoxic insults. However, the rapid onset of neurodegeneration following an excitotoxic stimulation of the NMDA receptors, has been suggested to be owing to the uncontrolled Ca^{2+} influx through extrasynaptic NMDA receptors, causing the loss of mitochondrial membrane potential *(180,181)* and the rapid decrease in neuronal energy charge. This scenario is likely to occur in experimental models where high concentrations of glutamate are applied acutely to a healthy population of neurons, but it is possible that the activation of extrasynaptic NMDA receptors may not be as relevant for human neurodegenerative disorders unless other energy-limiting conditions are provided. In fact, Ca^{2+} influx via extrasynaptic NMDA receptors may not be potentiated by ephrins *(132)*, and it is expected to face an Mg^{2+} block of the receptor-channel stronger than in synaptic receptors, because of the likely absence of non-NMDA receptors in the proximities. Thus, the influx of Ca^{2+} via extrasynaptic NMDA receptors should depend heavily on the occurrence of a generalized depolarization of the postsynaptic neuron, which should be expected when the synthesis of ATP is already partially compromised. Furthermore, it should be considered that a decline in the energy charge owing to extrasynaptic NMDA receptor-mediated mitochondrial damage may not be sufficient to induce neuronal death *per se* in healthy neurons, as it depends on the number of mitochondria loading an excess of Ca^{2+}. Thus, the extent of neuronal death following the stimulation of extrasynaptic NMDA receptors is likely to depend on the existing energy charge of the neurons in the examined area. In stroke, extrasynaptic NMDA receptors may have a role in the penumbra of the ischemic

area, where there is a decline in energy charge, an important release of glutamate from injured presynaptic neurons of the core, and a reduced glial uptake *(20–22,59,62)*. On the other hand, in the core part of the ischemic brain area, the lack of energy production substrates nullifies the contribution of mitochondria to the energy charge, which is lowering progressively to the point of weakening significantly the Mg^{2+} control of the active synaptic NMDA receptors, leading to an ion influx that reduces further the neuronal energy charge and causes neuronal death *(23,59)*. Among other acute pathologies that may have cellular analogies to the ischemic penumbra, may possibly be head trauma, where the mechanical injury induces both the release of large amounts of glutamate and a variable degree of both anoxia and hypoglycemia.

It should be considered, however, that a chronic stimulation of extrasynaptic NMDA receptors may be relevant to the development of neurodegenerative disorders, as it may produce a progressive impairment in mitochondrial energy production *(26,182)*. In fact, Ca^{2+} loaded mitochondria may increase their generation of superoxide radical anions, causing, among other cellular damages, repetitive permanent damage of mitochondria, and a progressive reduction of neuronal energy charge.

Again, whether extrasynaptic NMDA receptors participate in the onset and/or progression of neurodegenerative disorders remains to be established. Several aspects need to be clarified before we may have a complete view of the acute and chronic excitotoxic process. For example:

1. What links extrasynaptic NMDA receptor-mediated Ca^{2+} influx to mitochondria? Why does Ca^{2+} entering via VGCC or non-NMDA receptors not affect mitochondria?
2. How non-NMDA receptors may induce excitotoxic neurodegeneration?
3. How can we explain the neuroprotective effect of uncoupling the NMDA receptor from PSD-95 in ischemia ?
4. Are extrasynaptic NMDA receptors stimulating cGMP-mediated neurotrophic functions?

7. CONCLUSIONS

The answer to these and many other questions will be very important to understand how the stimulation of glutamate receptors may cross the borderline between physiology and pathology and induce neurodegeneration. It is now clear that neurotoxic reactions, similarly to all reactions in neurons, are occurring somehow in an organized manner, which was unexpected years ago, and this notion will bring us more opportunities for therapeutical treatments. Furthermore, neurodegeneration is likely to be a process taking place only in extreme conditions when the mechanisms that neurons have developed to avoid it have failed. Such mechanisms are being discovered now and may lead us to the conclusion that many neurological, neuropsychological, and neuropsychiatric disorders may not be associated with neurodegeneration until late in life, whereas for most of the lifetime neuronal disfunctioning is more likely to occur, affecting important plasticity processes, such as LTP and LTD. Studying neurodegeneration is changing its philosophical prospective and is now becoming more and more another way of looking at physiology, instead of being a fight against molecular disorder.

ACKNOWLEDGMENTS

This chapter has been supported by grant CICYT REN2001-2959-C04-04 and grant F192BU-C8 from the Brain and Spinal Column Injury Defense Program (United States).

We would like to thank Dr. A. M. Marini and Dr. R.H. Lipsky for helpful discussion, and Dr. A. Torreblanca and Dr. A. García-Rodriguez for sharing their data.

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X Parkinson's Disease

20

Presymptomatic and Symptomatic Stages of Intracerebral Inclusion Body Pathology in Idiopathic Parkinson's Disease

Heiko Braak and Kelly Del Tredici

1. INTRODUCTION

The pathological process that underlies idiopathic Parkinson's disease (IPD) progresses relentlessly and requires years to reach its full extent, provided it is not terminated prematurely by death. The severity of the pathology increases gradually during the course of the disorder *(1–8)*. As such, the lesions develop already, to a mild or moderate degree, even in the nervous system of persons whose clinical protocols fail to note the onset or presence of classical IPD-associated motor symptoms *(9–17)*. Thus, the course of the disease process can be subdivided into presymptomatic and symptomatic phases (Fig. 1A). *(3,5)* Like the tip of an iceberg, it is only the symptomatic, later phase of the larger degenerative process that presently can be detected clinically.

2. IPD-ASSOCIATED INCLUSION BODIES

Assessment of distinctive inclusion bodies that appear as spindle- or thread-like and, in part, arborizing Lewy neurites (LNs) within neuronal processes and as globular or spherical pale bodies and/or Lewy bodies (LBs) in the perikarya of vulnerable nerve cells is a prerequisite for the postmortem diagnosis of both the presymptomatic and symptomatic phases of IPD *(3,18–22)*. Patients who present with a clinical picture of parkinsonism, but whose brain tissue lacks LNs and LBs, should be classified in the heterogenous group of non-IPD motor disorders.

A major element of LNs, pale bodies, and LBs is a misfolded and aggregated form of the protein α-synuclein *(8,23–26)*. The small, 140 amino acid-containing, hydrophilic and natively unfolded molecule exists in many, but by no means all, nerve cells in the human adult nervous system. This means that all of the vulnerable neurons have to be supplied with sufficient amounts of normal α-synuclein in order to become involved in IPD in the first place *(27)*. Usually, the bulk of this protein is located in both synaptic boutons and the axon, for the most part bound to synaptic vesicles or to membranes that are rich in acidic phospholipids *(28)*.

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

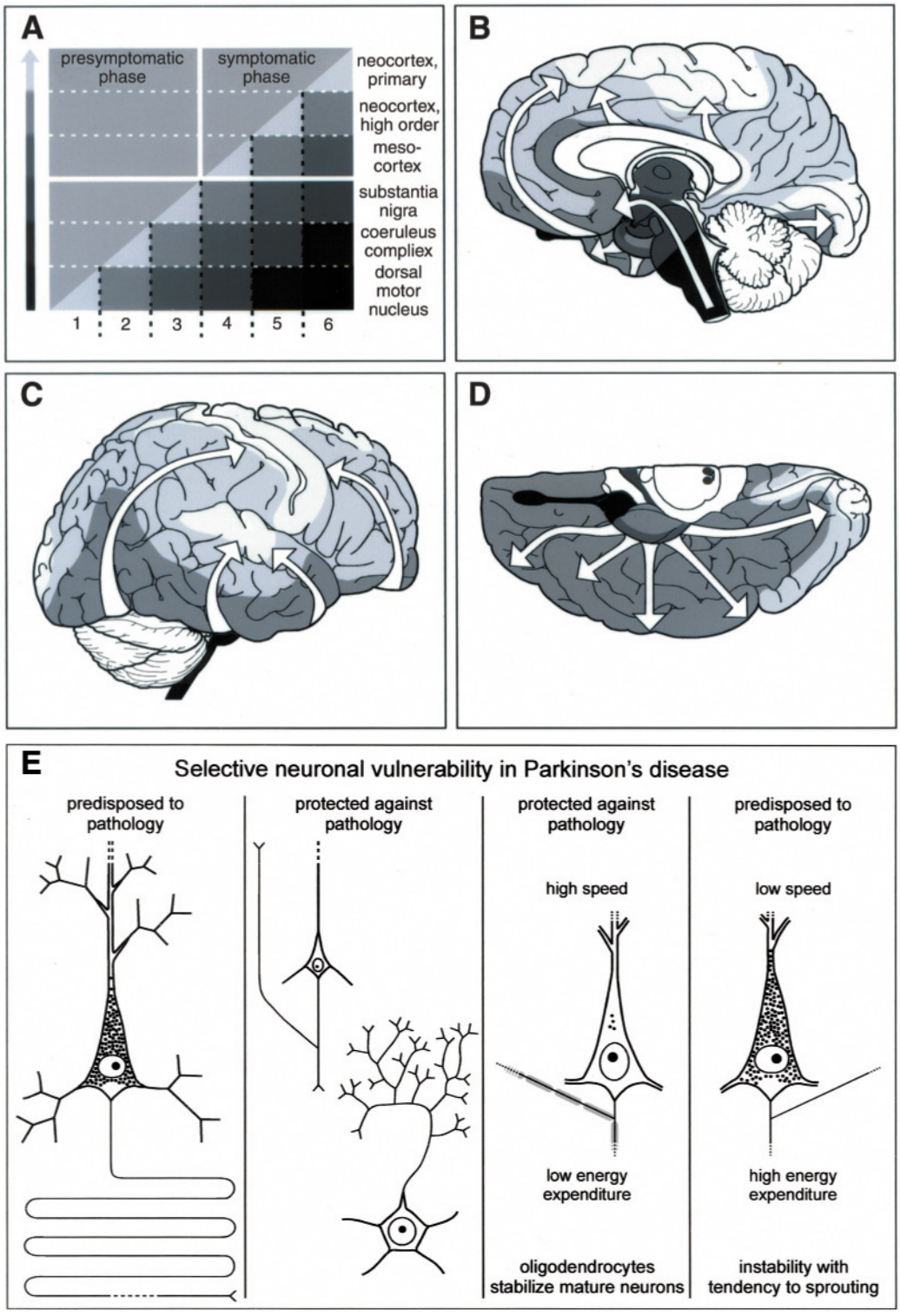
A
presymptomatic phase
symptomatic phase
neocortex, primary
neocortex, high order
meso-cortex
substantia nigra
coeruleus compliex
dorsal motor nucleus
1 2 3 4 5 6
B
C
D
E
Selective neuronal vulnerability in Parkinson's disease
predisposed to pathology
protected against pathology
protected against pathology
high speed
low energy expenditure
oligodendrocytes stabilize mature neurons
predisposed to pathology
low speed
high energy expenditure
instability with tendency to sprouting

Fig. 1.

In a few predisposed neuronal types and under conditions that are still the subject of intensive study, normal α-synuclein molecules lose their binding capacity and take on a β-pleated sheet formation. In this abnormal form, together with other components, such as synphilin-1, phosphorylated neurofilaments, and ubiquitin, a heat shock protein required for the nonlysosomal adenosine triphosphate-dependent breakdown of abnormal proteins, the molecules aggregate and undergo transformation into virtually insoluble LNs and LBs *(29–32)*. It is not clear why the affected neurons are unable to eliminate the misfolded protein in a timely manner by means of ubiquitination and subsequent proteasomal recycling, thereby preventing the aggregation process altogether *(32–35)*.

In the course of IPD, all of the involved neurons develop nonbiodegradeable LNs and LBs. Initial traces of LB material in the neuronal soma generally are observable close to deposits of lipofuscin or neuromelanin granules, which might function as initiation sites for the promotion of oxidative crosslinking of the proteinaceous material *(36,37)*. Despite the presence of the inclusion bodies, neurons may survive for a certain period of time. The mere persistence, however, of such neurons is no proof of their functional integrity, and LB/LN-bearing nerve cells probably cease functioning long before cell death occurs.

IPD can be assigned to the collectivity of degenerative disorders designated as synucleinopathies *(38–45)*. The inclusion bodies in IPD can be readily differentiated from those associated with other synucleinopathies *(46–48)*. Furthermore, the combination of vulnerable neuronal types is peculiar to IPD. It should be emphasized that the IPD-related inclusion bodies are by no means harmless lesions that routinely accompany healthy brain aging *(49,50)*. Instead, LNs/LBs are pathognomonic hallmarks of IPD that facilitate differential neuropathological assessment of the disease process both in symptomatic patients and in individuals whose clinical protocols make no mention of the presence of IPD-associated symptoms *(6,7)*.

3. SELECTIVE NEURONAL VULNERABILITY

Damage to specific subnuclei of the substantia nigra resulting in severe obliteration of their neuromelanin-laden projection neurons has long been considered the most important hallmark of IPD *(51)*. Nevertheless, the nigral damage is always accompanied

Fig. 1. **(A)** Presymptomatic and symptomatic phases of idiopathic Parkinson's disease (IPD). The presymptomatic phase of the disorder is characterized by the appearance of IPD-associated lesions in the brain of asymptomatic persons. Individuals first become symptomatic when the neuropathological threshold is exceeded (approximated by the white vertical line). Increasing density of the shading in areas underneath the diagonal indicates the growing severity of the pathology in vulnerable key regions indicated at the right-hand margin. Arabic numerals mark the stages of the neuropathological process. **(B–D)** Schematic diagrams showing the gradual ascent of the pathological process underlying IPD (white arrows). **(E)** Selective vulnerability and resistance of specific neuronal types to IPD. Projection cells that generate long and thin axons are among the nerve cell types most vulnerable to the pathology, whereas projection cells and local circuit neurons with short axon resist the lesions. Heavy axonal myelination offers the following advantages: high speed of conduction, low energy expenditure, greater stability of the parent neuron. Resistant against IPD-related pathology are long-axoned and sturdily myelinated projection neurons. In contrast, vulnerable neuronal types give off unmyelinated or poorly myelinated and thin axons. Reproduced from ref. *6* with permission from Steinkopff Verlag.

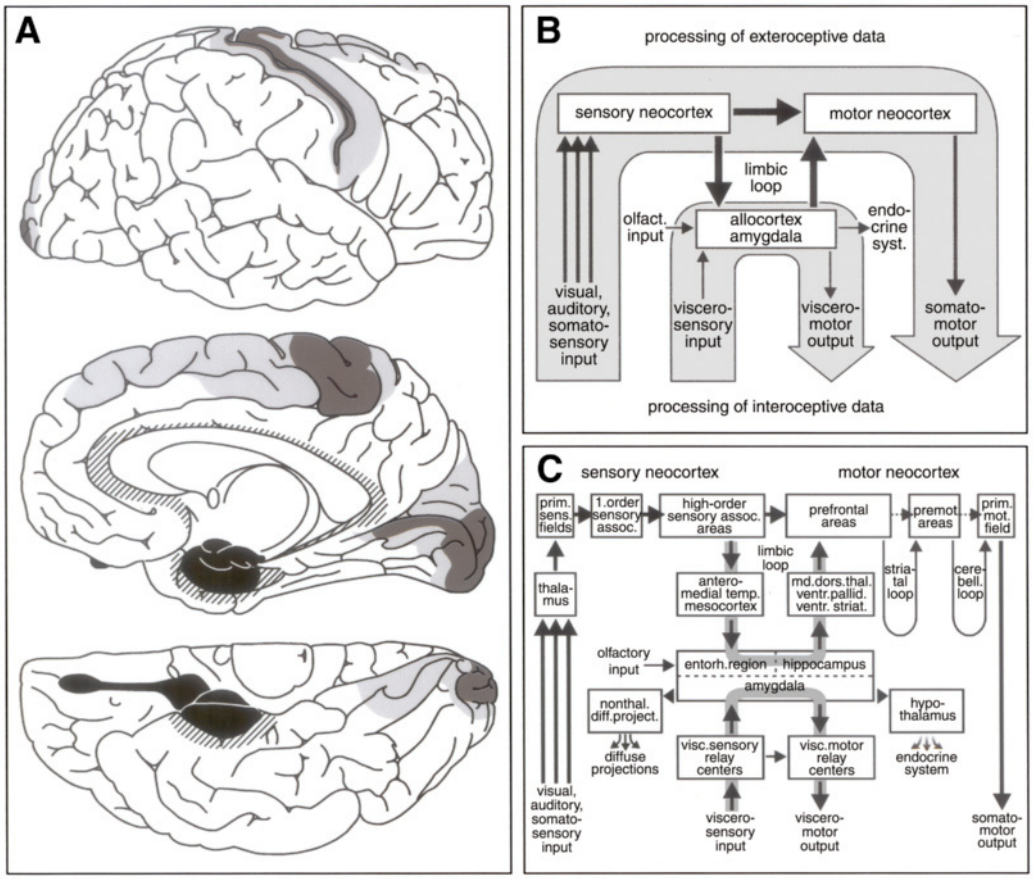

Fig. 2. **(A)** The human cerebral cortex is dominated by its neocortical areas. The allocortex (olfactory bulb and associated areas, as well as entorhinal region, presubiculum, and hippocampal formation—black), as well as the mesocortex (cross-hatching) are small in comparison. The neocortex consists of primary fields (dark gray), premotor fields, and first-order sensory associations areas (light gray), and extended prefrontal and high-order sensory association areas (white). **(B)** The neocortex chiefly processes and controls exteroceptive data. It receives visual, auditory, and somatosensory input and regulates the somatomotor system. The afferent and efferent trunks of the limbic loop interconnect the neocortex with the allocortex. Aside from olfactory input, the allocortex mainly receives and processes interoceptive data, and influences both the endocrine system, as well as centers that regulate visceromotor output. **(C)** Detailed diagram displaying the principal subdivisions of the neocortex and the major components of the limbic loop (entorhinal region, hippocampal formation, amygdala). The afferent trunk of the limbic loop includes the anteromedial temporal mesocortex, a portion of the mesocortex that is exceptionally well developed in the human brain. The efferent trunk chiefly includes the ventral striatum, ventral pallidum, and mediodorsal thalamus, which direct the data toward the prefrontal areas. anteromedial temp. mesocortex, anteromedial temporal mesocortex; entorh. region, entorhinal region; high-order sensory assoc. areas, high-order sensory association areas; md. dors. thal., mediodorsal nucleus of the thalamus; nonthal. diff. project., nonthalamic diffusely projecting nuclei; premot. areas, premotor areas; prim. mot. field, primary motor field; prim. sens. fields, primary sensory fields; ventral pallid., ventral pallidum; ventral striat., ventral striatum; visc.motor relay centers, visceromotor relay centers of the brainstem; visc.sensory relay centers, visceroseonsory relay centers of the brainstem; 1. order sensory assoc., first-order sensory association areas.

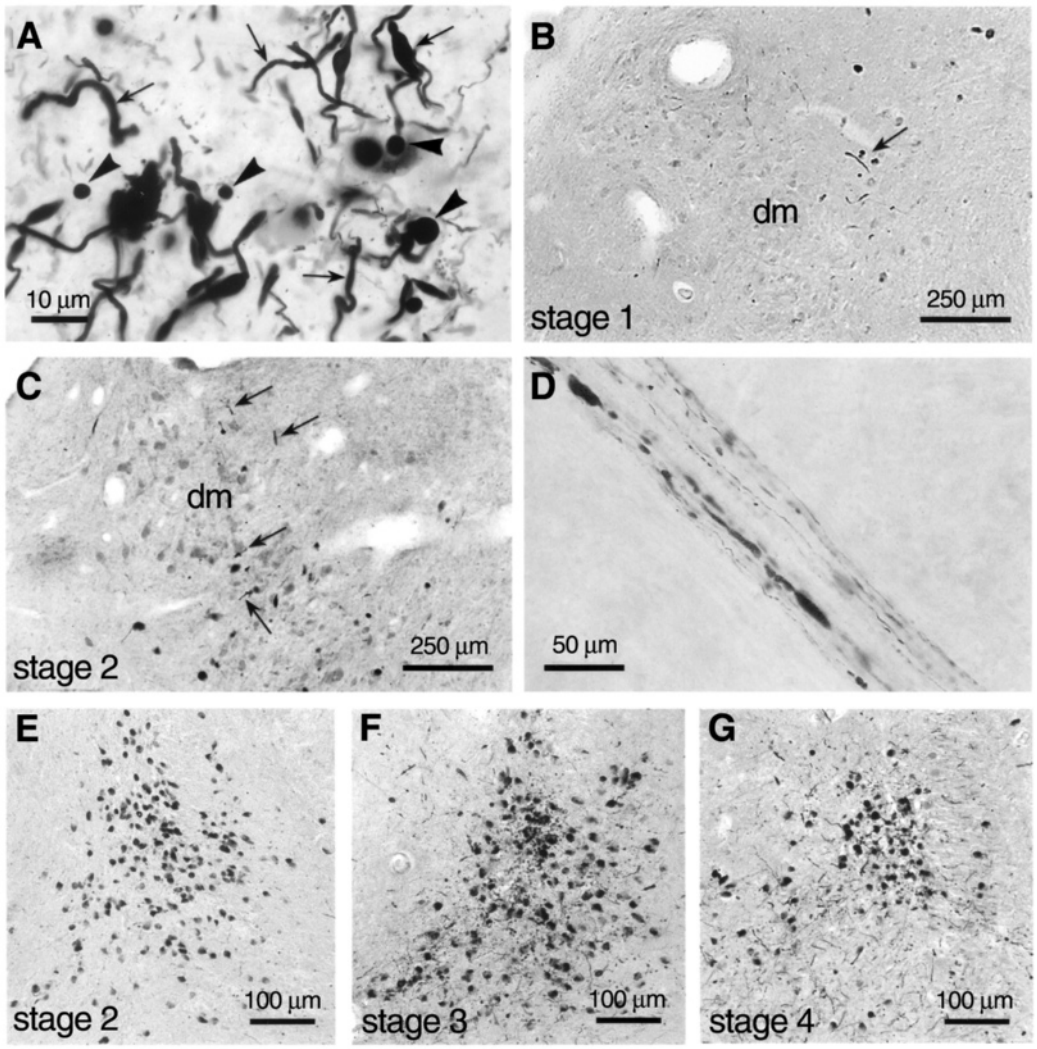

Fig. 3. Idiopathic Parkinson's disease (IPD)-related inclusion body pathology (α-synuclein immunoreactions, 100 µm *[24]*). **(A)** Lewy neurites (LNs) (arrows) and Lewy bodies (arrowheads) in the dorsal visceromotor nucleus of the vagal nerve. **(B)** Dorsal visceromotor nucleus of the vagal nerve (dm). The pathology can commence with a single LN (arrow in B) in stage 1 and increases in severity in stage 2 **(C)**. **(D)** α-synuclein immunoreactive aggregates in preganglionic axons of the vagal nerve en route through the medulla oblongata. **(E–G)** IPD-related pathology in the coeruleus–subcoeruleus complex. The lesions increase in severity, as shown here between stages 2 **(E)** and 4 **(G)**. Note the pronounced loss of melanoneurons in stage 4. Figure 3b reproduced from ref. *6* with permission from Steinkopff Verlag.

by a broad spectrum of extranigral pathology, including that in the olfactory bulb and related olfactory areas, in the dorsal visceromotor nucleus of the vagal nerve and adjacent intermediate reticular zone, in some nuclei of the reticular formation and caudal raphe nuclei, the coeruleus–subcoeruleus complex, tegmental pedunculopontine nucleus, nonthalamic nuclei with diffuse projections, intralaminar and midline nuclei of the thalamus,

amygdala, anterior mesocortex, and second sector of the Ammon's horn. Cases with severe damage usually show lesions extending into the neocortex (Fig. 1B–D) *(5,6,52)*.

IPD displays a pronounced affinity for select nuclear grays and cortical areas. All sensory, somatosensory, or viscerosensory relay centers of the brain remain uninvolved or, for the most part, intact—with the exception of olfactory structures. The disease-related neuronal destruction revolves completely around "motor" areas, particularly the superordinate centers of the somatomotor, visceromotor, and limbic systems.

The neuronal types that are prone to develop the lesions have at least two properties in common. First, all of them can be classified as projection neurons. Among these, only projection cells with axons that are disproportionately long and thin in relation to the size of the cell body demonstrate a pronounced tendency to develop the lesions (Fig. 1E). By comparison, projection cells with short axons, e.g., the small pyramidal cells of neocortical layers II and IV, the granule cells of the fascia dentata, and the neurons of the presubicular parvocellular layer, resist the pathology. To date, no LNs/LBs have been seen in short-axoned local circuit neurons (Fig. 1E).

All of the endangered neuronal types share an additional feature that is required but in itself not wholly sufficient to account for the formation of the protein aggregates: namely, the long and thin-caliber axons are unmyelinated or poorly myelinated *(3,4,6,53)*. The countertest also applies: All nerve cells that are equipped with lengthy and sturdy axons insulated by thick-caliber myelin sheaths are protected against the formation of LNs/LBs (Fig. 1E). In this context, the question arises as to what neuroprotective properties or attributes emanate from a powerfully built myelin sheath. Three advantages come to mind: First, the speed of axonal conduction increases with growing thickness of the myelin sheath. Second, a neuron with a well-myelinated axon requires less energy for the transmission of impulses *(54)*. Third, the parent cell achieves a greater degree of stability and is less susceptible to pathological sprouting owing to interaction of the axon with the oligodendroglial cells that produce and sustain the myelin sheath *(55)*. These three properties are all the more pronounced the earlier an axon begins myelination and the thicker the myelin sheath becomes during the maturation process. Seen against this background, the projection neurons with long, thin-caliber, and poorly myelinated, or even unmyelinated axons that reside in superordinate limbic, visceromotor, and somatomotor regions constitute an identifiable *locus minoris resistentiae* in the construction of the human brain.

All in all, only a small number of the many neuronal types that constitute the human nervous system are prone to develop the abnormal proteinaceous aggregations, whereas other types of nerve cells, often directly in the vicinity of those involved, maintain their integrity both morphologically and functionally. This means that the neuronal damage and loss in the brain during IPD are not haphazard, but, on the contrary, produce a distinctive lesional distribution pattern *(4,6)*. Although the reasons for the pronounced susceptibility of some neuronal types as opposed to the decided resistance of others are still not fully understood, the degree to which involved axons have undergone myelination and/or differences in axon length probably predispose some nerve cells to greater stress than others.

4. ANATOMICAL EXCURSUS

Recognition of the nonrandom topographical pattern and multi-system aspects of the disease process is made possible by using diagrams that show the superordinate centers

of the visceromotor, somatomotor, and limbic systems. Normal function of these systems largely depends on the performance of the cerebral cortex, the preeminent controlling and executive entity of the human brain.

The cerebral cortex is composed of a small allocortex (Fig. 2A, black) and a far-reaching neocortex (Fig. 2A, deep gray, light gray, white) *(56–58)*. Summarily stated, the neocortex is principally responsible for relationships to the outside world. It constantly receives data from beyond the individual organism via somatosensory, auditory, and visual neuronal pathways, whereas, at the same time, regulating somatomotor impulses that impact on the outer environment (Fig. 2B). The allocortex is composed of the olfactory bulb and related areas, as well as supervening centers of the limbic system, including the entorhinal region and hippocampal formation (Figs. 2B,C) *(59,60)*. The nuclear complex of the amygdala is closely linked to the allocortex.

Transitional zones exist between the mature neocortex and the allocortex proper. These constitute the mesocortex, a unique architectonic entity that is remarkably well developed only among higher primates, above all humans (Figs. 2A, cross-hatching, c) *(57,61)*. The neocortex of the parietal, occipital, and temporal lobes consists of a highly refined primary field that receives particularly heavy input from specific thalamo-cortical projections (Fig. 2A, dark gray). These primary fields are flanked by somewhat less highly differentiated first-order sensory association areas (Fig. 2A, light gray), which, in turn, are accompanied by extensive and relatively simply organized high order processing areas (Fig. 2A, white) *(62)*. The frontal lobe is similarly subdivided into a primary motor field (Fig. 2A, dark gray) and adjoining premotor fields (Fig. 2A, light gray) followed by prefrontal areas (Fig. 2A, white).

Somatosensory, visual, and auditory information arrives at the respective primary sensory field and proceeds via the first-order association areas to related high-order processing areas. The exteroceptive data then is conveyed via long cortico-cortical projections to the prefrontal cortex (Fig. 2C). These connections are outward projections that terminate in layer 4 of the target fields *(63)*. Minor pathways leading away from the prefrontal cortex are provided by cortico-cortical backward projections that terminate in layer I of their target areas (broken arrows in Fig. 2C) and transmit the information via premotor areas to the primary motor field. The striatal and the cerebellar loops, however, provide the major routes for this return pathway and integrate the basal ganglia, lower brainstem nuclear grays, and the cerebellum into the regulation of cortical output (Fig. 2C) *(64–66)*.

The superordinate components of the limbic loop also participate in data transfer and are involved at the nodal point where information is transferred from sensory association areas to the prefrontal cortex *(67–69)*. One contingent of information leaves the mainstream and proceeds through multiple neocortical relay stations and the anteromedial temporal mesocortex to converge on the entorhinal region and lateral nucleus of the amygdala, thereby making the neocortex the chief source of input to the human limbic system (for the afferent trunk of the limbic loop, *see* Fig. 2C). In this context, it should be noted that the components of the limbic loop that process neocortical data are late developments both phylogenetically and ontogenetically. In the course of evolution from macrosmatic mammals to microsmatic higher primates, including humans, the neocortex not only undergoes a remarkable degree of expansion, but a thoroughgoing internal reorganization of the limbic loop also takes place. One hallmark of this evolutionary process is the massive increase of components that receive input from and generate output to the

neocortex. These internal changes occur at the expense of the initially predominant areas and subcortical nuclei involved in processing olfactory data.

The entorhinal region, hippocampal formation, and amygdala are strongly interconnected and generate important projections that terminate in the ventral striatum (accumbens nucleus and "limbic" subdivisions of the putamen). This input is supplemented by projections from the thalamic midline nuclei. From the ventral striatum, the data are transferred via the ventral pallidum and mediodorsal thalamus to medial and orbital portions of the prefrontal neocortex. All of these projections exert "limbic" influence on the prefrontal cortex (for the efferent trunk of the limbic loop, *see* Fig. 2C) *(65,67,69)*. As such, the predominance of input from and output to the neocortex is a central feature of the limbic loop in the human brain. Limbic loop components can well be viewed as a neuronal bridge that link the external and internal worlds (Fig. 2B).

5. SIX STAGES IN THE EVOLUTION OF LNs AND LBs

The pathology does not evolve simultaneously at all of the susceptible sites. Instead, it commences at predisposed locations and progresses from there in a predetermined and predictable manner. The topographic advance of the lesions is so typical and displays so little variation from one case to another that the brain changes that develop in the course of the presymptomatic and symptomatic phases of IPD can be assigned to one of six neuropathological stages (Fig. 1A–D) *(5,6)*.

The initial lesions usually develop at two predilection sites:

1. The olfactory bulb and/or anterior olfactory nucleus, and
2. The dorsal visceromotor nucleus of the vagal nerve and adjoining intermediate reticular zone (Fig. 3B) *(5)*.

The lesions in olfactory structures gradually become more severe and eventually extend into adjoining olfactory sites without advancing into non-olfactory areas of the neocortex or non-olfactory subcortical grays *(7)*. Accordingly, the disease process takes as its main starting point the dorsal visceromotor nucleus of the vagal nerve and pursues, from there, an essentially ascending path until it reaches the cerebral cortex (Fig. 1A–D) *(5,6,53)*.

5.1. Stages 1 and 2: Brainstem Pathology Is Confined to the Medulla Oblongata and Pontine Tegmentum

5.1.1. Stage 1

The first LNs and LBs in the brainstem consistently originate in the dorsal visceromotor nucleus of the vagal nerve (stage 1, Fig. 3B) *(6)*. Moreover, conspicuous spindle-shaped LNs appear in the thin, long, and unmyelinated axons of the visceromotor cells that represent preganglionic fibers of the vagal nerve (Fig. 3D) *(70,71)*. The myelinated viscerosensory fibers lead to the small-celled nuclei surrounding the solitary tract (Fig. 4A,B). Likewise, powerfully myelinated axons that belong to the special visceromotor projections of the vagal nerve originate from the ambiguus nucleus (Fig. 4A,B). These axons and their parent neurons remain free of inclusion bodies for the duration of the disease process.

Select types of nerve cells in the enteric nervous system also develop the same forms of inclusion bodies as the central nervous system. LBs and LNs appear in the presymptomatic and symptomatic phases of IPD in the vasoactive intestinal peptide (VIP) neurons of the Auerbach plexus (Fig. 4A,B) *(72,73)*.

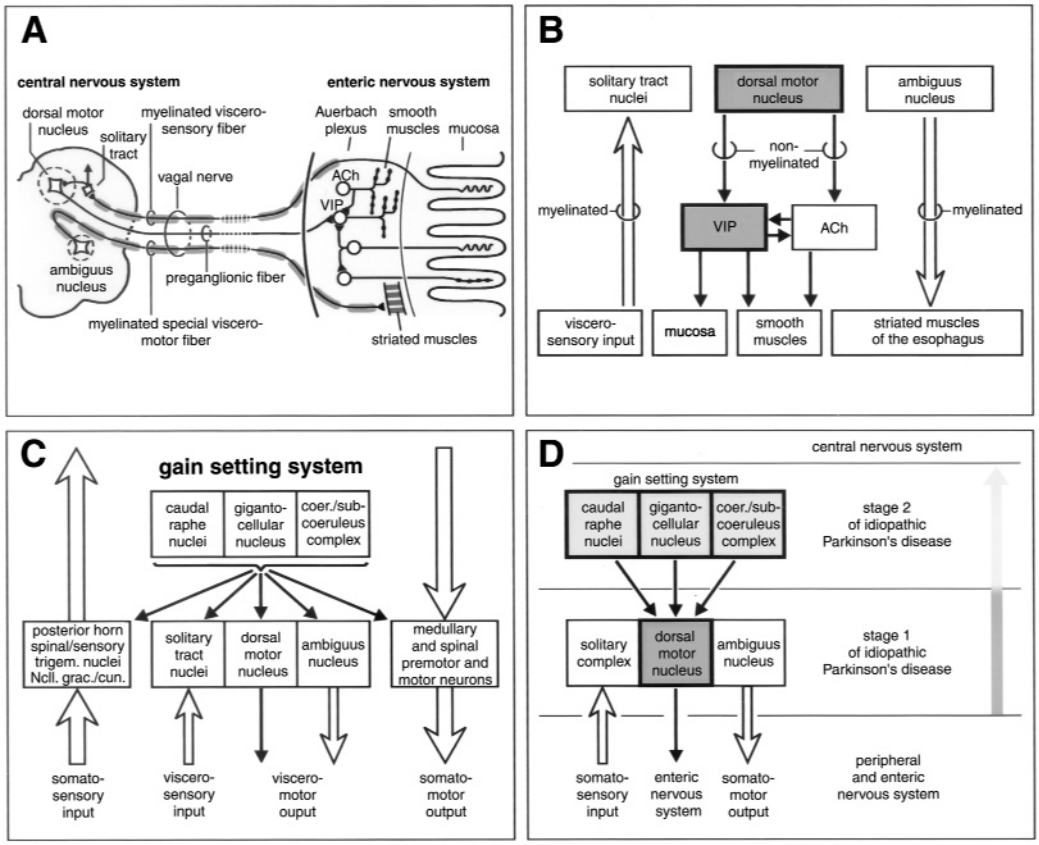

Fig. 4. **(A)** Fiber pathway connecting the enteric nervous system and central nervous system via the vagal nerve. Myelinated viscerosensory fibers terminate in the small-celled nuclei that surround the solitary tract. Myelinated visceromotor fibers from the ambiguus nucleus innervate striated muscles of the upper esophagus. Unmyelinated preganglionic fibers originate from the dorsal visceromotor nucleus of the vagal nerve and contact ganglion cells of Auerbach's plexus. **(B)** Pathological involvement in stage 1. Affected nuclear grays are indicated by boldface framing. In contrast to the unmyelinated preganglionic fibers of the vagal dorsal visceromotor nucleus, the well-myelinated viscerosensory input and visceromotor fibers that stem from the nucleus ambiguus do not develop α-synuclein immunoreactive aggregates. **(C)** Centers influenced by the nuclear grays of the gain-setting system. The coeruleus–subcoeruleus complex, gigantocellular reticular nucleus, and caudal raphe nuclei (magnus, obscurus, pallidus) modulate the excitability levels of the spinal and medullary centers for somatosensory and viscerosensory input together with those for visceromotor and somatomotor output. **(D)** In stage 2, only the gain-setting nuclei (boldface frames) become involved and supplement the affection of the dorsal visceromotor nucleus of the vagal nerve (boldface frame). **(B–D)** Thick white arrows represent strongly myelinated fibers, thin black arrows unmyelinated or poorly myelinated axons. ACh, acetylcholine-containing neurons; coer.–subcoeruleus complex, coeruleus–subcoeruleus complex; Ncll. grac./cun., gracile and cuneate nuclei; spinal/sensory trigem. nuclei, spinal and sensory trigeminal nuclei; VIP, vasoactive intestinal peptide-containing neurons. Figure 4C reproduced from ref. *79* with permission from Landes Bioscience Press.

5.1.2. Stage 2

In stage 2, the lesions within the dorsal vagal area increase in the severity (Fig. 3C), and additional pathology develops in the nucleus raphes magnus, gigantocellular nucleus of the reticular formation, and the coeruleus–subcoeruleus complex (Fig. 3E–G). It is important to note that the disease process does not begin in the substantia nigra *(6,7)*. On the contrary, during the first two stages it remains confined to the medulla oblongata and pontine tegmentum. Apart from the dorsal visceromotor nucleus of the vagal nerve and adjacent intermediate reticular zone, the only other nuclei that sustain additional damage at this point are those that function closely together as components of the so-called "gain setting" or "level setting" system *(74–78)*.

The potential functional consequences of the neuronal damage that develops in stages 1–2 are illustrated in Fig. 4C *(79)*. The lower row depicts the spinal and medullary centers of ascending sensory and descending motor pathways. All of these relay nuclei are regulated by the nuclei of the gain-setting system (upper row in Fig. 4C) *(79)*. The thinly myelinated descending tracts of the gain-setting nuclei form a pain control system that partially inhibits or even entirely blocks the relay nuclei for somatosensory and viscerosensory input. In addition, these nuclei serve as a motor control system for both somatomotor and visceromotor output. They regulate the sensitivity as well as excitability levels of medullary and spinal premotor and motor neurons. The gain-setting system is capable of limiting the conduction of incoming pain signals in a given situation and places the organism's motor neurons in a heightened state of preparedness for action *(74,76)*. Sensitive techniques to assess dysfunctions of the vagal dorsal visceromotor nucleus and/or gain-setting nuclei might one day be used for early clinical diagnosis of the pathological process already at stages 1–2.

Major components of the cerebellar loop are displayed in somewhat greater detail in Fig. 8A (red nucleus, inferior olive, pontine gray, cerebellum, ventral intermediate nucleus of the thalamus) *(79)*. Not only do they commence myelination relatively early and prenatally *(80–82)*, but all of these nuclear components of the loop and the cerebellar cortex resist the development of IPD-related lesions.

5.2. Stages 3 and 4: Pathology Advances Into the Mesencephalic Tegmentum, Basal Forebrain, Mesocortex, and Allocortex

5.2.1. Stage 3

In stage 3, the steadily ascending process crosses the upper limit of the pontine tegmentum and progresses into basal portions of the midbrain and forebrain. Severe neuronal damage develops in the substantia nigra, tegmental pedunculopontine nucleus, nonthalamic nuclear grays with diffuse projections, and amygdala, particularly in the central nucleus of this nuclear complex (Fig. 5A). At the same time, the severity of the pathology at previously involved sites increases, and inclusion bodies begin to appear in melanoneurons of the dorsal vagal area and intermediate reticular zone *(6)*.

The hallmark of stage 3 is the involvement of the substantia nigra (Figs. 5A, 6A,B) *(6,83–85)* and pedunculopontine nucleus (Figs. 5A, 6C–F) *(87–93)*. At first, a few isolated, and in part very long, LNs develop within both nuclear grays, thereafter increasing rapidly in number. Subsequently, LBs emerge within both the cholinergic projection cells of the pedunculopontine nucleus and the neuromelanin-containing dopaminergic neurons of the subnucleus posterolateralis of the substantia nigra (Fig. 6B,E,F). Next, the posterosuperior

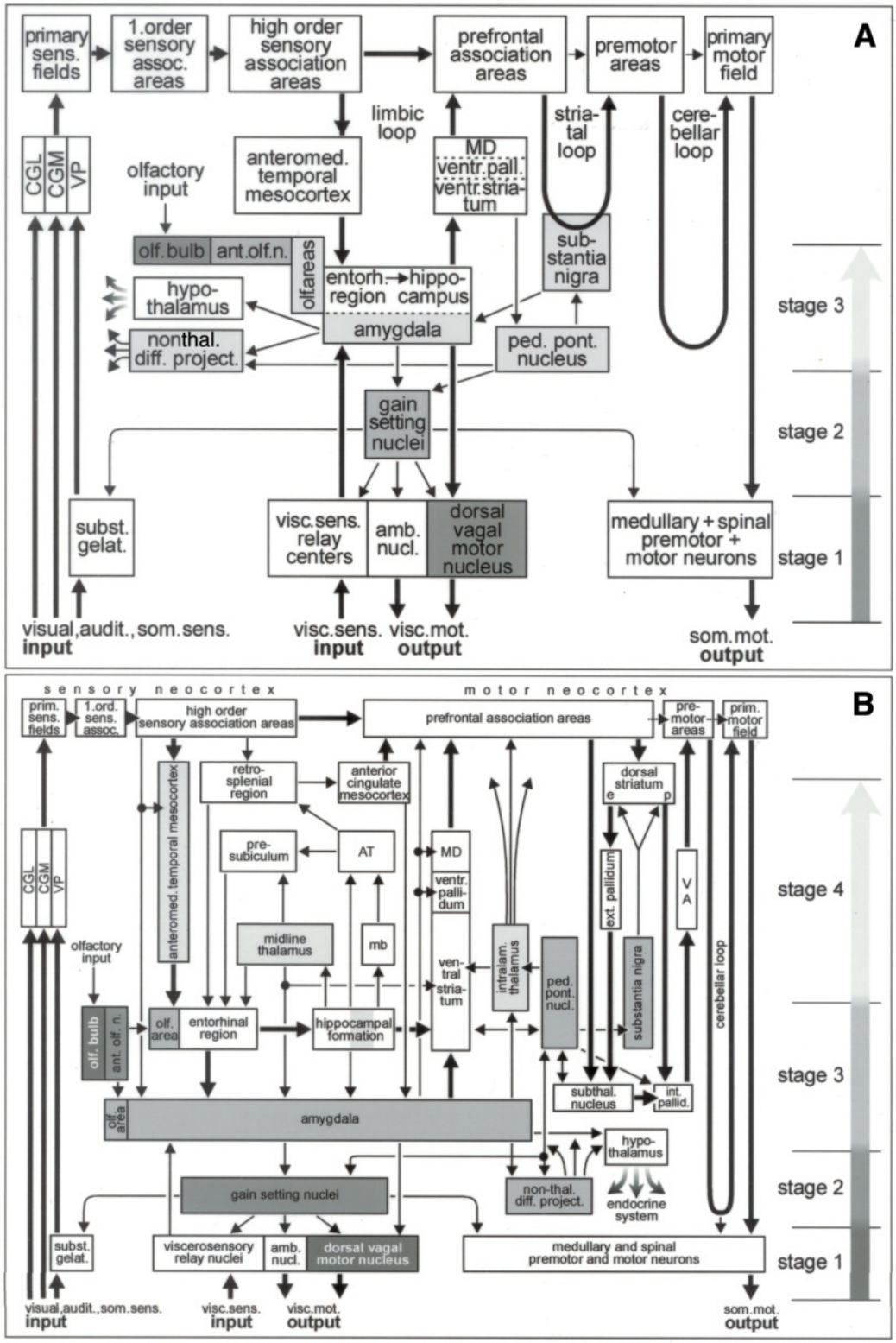
A
primary sens. fields
1.order sensory assoc. areas
high order sensory association areas
prefrontal association areas
premotor areas
primary motor field
limbic loop
striatal loop
cerebellar loop
CGL
CGM
VP
olfactory input
anteromed. temporal mesocortex
MD
ventr.pall.
ventr.striatum
olf.bulb
ant.olf.n.
olfareas
substantia nigra
hypothalamus
entorh. region
hippocampus
amygdala
nonthal. diff. project.
ped. pont. nucleus
gain setting nuclei
subst. gelat.
visc.sens. relay centers
amb. nucl.
dorsal vagal motor nucleus
medullary + spinal premotor + motor neurons
stage 3
stage 2
stage 1
visual,audit.,som.sens. input
visc.sens. input
visc.mot. output
som.mot. output
B
sensory neocortex
motor neocortex
prim. sens. fields
1.ord. sens. assoc.
high order sensory association areas
prefrontal association areas
pre-motor areas
prim. motor field
retro-splenial region
anterior cingulate mesocortex
dorsal striatum
e
p
anteromed. temporal mesocortex
pre-subiculum
AT
MD
ventr. palli-dum
ext. pallidum
VA
midline thalamus
mb
ventral striatum
intralam. thalamus
ped. pont. nucl.
substantia nigra
cerebellar loop
olfactory input
olf. bulb
ant. olf. n.
olf. area
entorhinal region
hippocampal formation
subthal. nucleus
int. pallid.
amygdala
hypothalamus
gain setting nuclei
non-thal. diff. project.
endocrine system
subst. gelat.
viscerosensory relay nuclei
amb. nucl.
dorsal vagal motor nucleus
medullary and spinal premotor and motor neurons
stage 4
stage 3
stage 2
stage 1
visual,audit.,som.sens. input
visc.sens. input
visc.mot. output
som.mot. output

Fig. 5.

Fig. 5. Further progress of the ascending Idiopathic Parkinson's disease related pathology in stage 3 **(A)** and stage 4 **(B)**. Growing density of the pathology in involved nuclear grays is indicated by increasing degrees of gray shading. Thick arrows indicate the chief interconnectivities and can facilitate recognition of the principal pathways sketched in Figs. 2B,C. **(A)** In stage 3, the central nucleus of the amygdala, the tegmental pedunculopontine nucleus, and substantia nigra, as well as most of the nonthalamic nuclear grays with diffuse projections become the focus of initially subtle and, then, more severe changes. The sparsely myelinated, descending projections that originate in the central nucleus of the amygdala influence both the gain setting nuclei and the dorsal visceromotor nucleus of the vagal nerve. In stage 3, for the first time, the pathological process reaches a superordinate relay nucleus of the limbic loop (amygdala) and directive nuclei of the striatal loop (substantia nigra, pedunculopontine nucleus). Note that the pedunculopontine nucleus serves as a link between the limbic and striatal loops. **(B)** The hallmark of stage 4 is the initial penetration of the cerebral cortex by the disease process at a very circumscribed region, namely, the anteromedial temporal mesocortex (the eye of the needle, as it were, within the afferent trunk of the limbic loop). The diagram shows more details than the previous ones and includes the chief components of both the limbic and striatal loops. The interconnectivities of the entorhinal region, hippocampal formation, and amygdala are shown as well as the three limbic circuits (1. subiculum—midline thalamic nuclei—entorhinal cortex; 2. subiculum—mamillary body—anterior thalamic nuclei—presubiculum—entorhinal cortex; 3. subiculum—anterior thalamic nuclei—retrosplenial region—anterior cingulate cortex—amygdala). Observe the insertion of the anteromedial temporal mesocortex into the afferent trunk of the limbic loop and that of the ventral striatum, ventral pallidum, and mediodorsal thalamic nuclei into the efferent trunk. amb. nucl., ambiguus nucleus; ant. olf. n., anterior olfactory nucleus; AT, anterior thalamic nuclei; CGL, lateral geniculate body; CGM, medial geniculate body; dorsal striatum e, encephalin-containing projection neurons of the dorsal striatum; dorsal striatum p, substance P-containing projection neurons of the dorsal striatum; entorh. region, entorhinal region; ext. pallidum, external pallidum; int. pallid., internal pallidum; intralam. thalamus, intralaminar nuclei of the thalamus; mb, mamillary body; MD, mediodorsal nuclei of the thalamus; nonthal. diff. project., nonthalamic diffusely projecting nuclei; olf.areas, olfactory areas; olf. bulb, olfactory bulb; ped. pont. nucleus, pedunculopontine nucleus; prim. motor field, primary motor field; prim. sens. fields, primary sensory fields; som. mot. output, somatomotor output; subst. gelat., substantia gelatinosa; subthal. nucleus, subthalamic nucleus; ventr.pall., ventral pallidum; ventr.striatum, ventral striatum; visc. sens. input, viscerosensory input; visc. mot. output, visceromotor output; visc. sens. relay centers, viscerosensory relay centers of the brain stem; visual, audit., som. sens. input, visual, auditory, somatosensory input; VA, ventral anterior nucleus of the thalamus; VP, ventral posterior nuclear complex of the thalamus; 1. order sens. assoc., first order sensory association areas.

and posteromedial nigral cell groups become involved. The magnocellular and anterior subnuclei (Fig. 6A) remain nearly intact or show only mild affection *(6,84)*. The vulnerable neurons of both nuclear grays generate thin and poorly myelinated axons *(54,77)*.

The most important centers of the striatal loop are depicted in Figs. 5B and 8A *(79)*. Cortico-striatal projections terminate on the predominating medium-sized projection neurons of the striatum. Substance P-containing cells of this type preferentially project upon the internal pallidum, whereas neurons that contain encephalin target the external pallidum. The projection neurons of the two pallidal portions dispatch their axons to different nuclear grays, thereby furnishing a short and a long pathway. Efferent projections of the internal pallidum reach the ventral anterior nucleus of the thalamus via the short pathway, whereas those of the external pallidum terminate in the subthalamic nucleus and, in so doing, arrive at the thalamic target via the long pathway. The performance of nimble precision movements necessitates the temporary activation of the centers of the

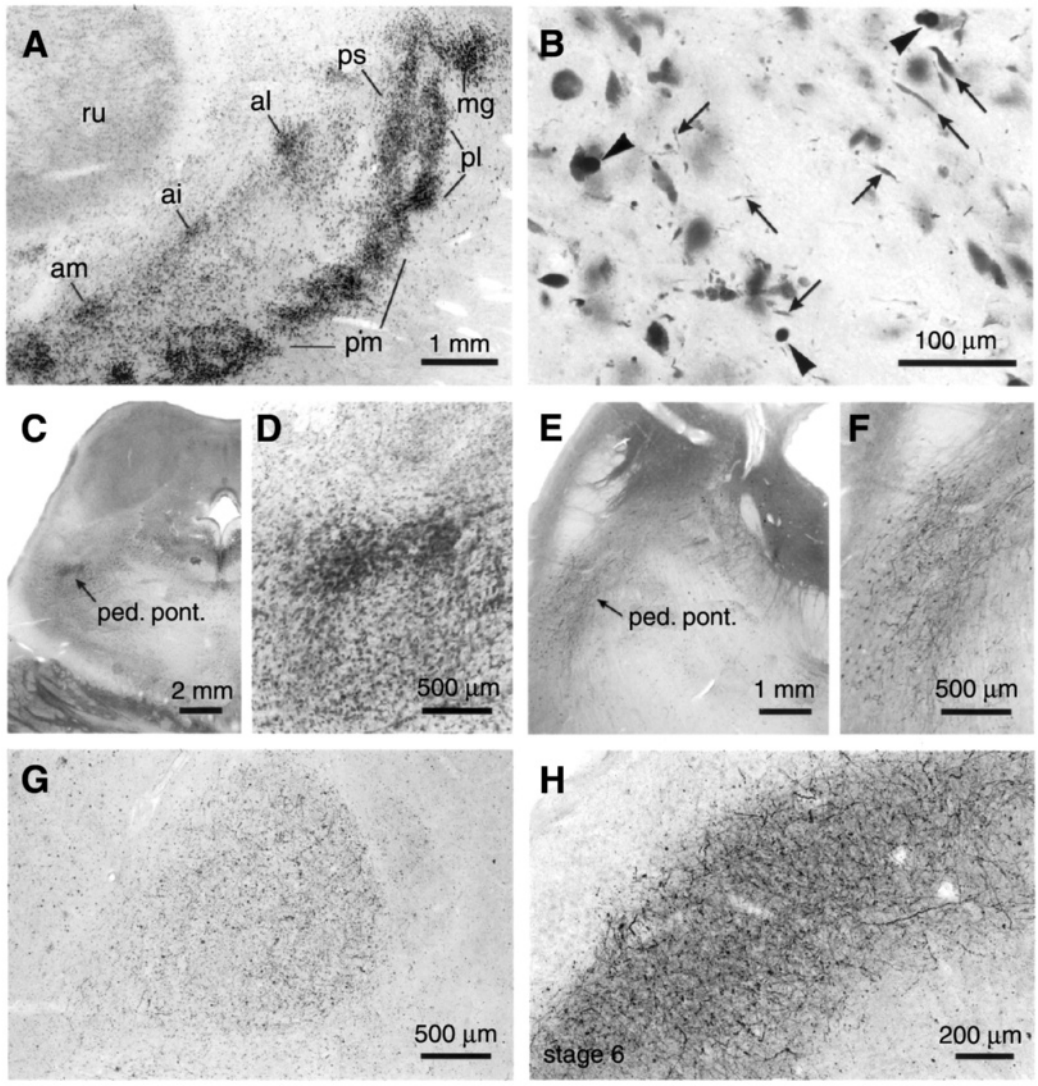

Fig. 6. Topographical anatomy and Idiopathic Parkinson's disease-related inclusion body pathology of vulnerable nuclear grays (100 µm sections stained for lipofuscin pigment and Nissl material, or immunoreactions for α-synuclein; ref. *23*). **(A)** Normal substantia nigra showing the seven subnuclei of the pars compacta. ai, anterointermediate subnucleus, al, anterolateral subnucleus, am, anteromedial subnucleus, mg, magnocellular subnucleus, pl, posterolateral subnucleus, pm, posteromedial subnucleus, ps, posterosuperior subnucleus, ru, red nucleus *(13)*. **(B)** In stage 3, the pathology reaches the substantia nigra (posterolateral subnucleus) for the first time. Notice the variously shaped Lewy neurites (LNs) (arrows), and Lewy bodies (LBs) in melanoneurons (arrowheads). **(C,D)** Topographical location of the tegmental pedunculopontinus nucleus (ped. pont.) lateral to the decussatio of the upper cerebellar peduncle and situated between the coeruleus/subcoeruleus complex posteriorly and substantia nigra anteriorly in a control case. **(E,F)** Dense network of LNs as seen in this nucleus at stage 5. **(G)** The very dense latticework of LNs and small LBs characterizes the central nucleus of the amygdala and permits its differentiation from surrounding nuclear grays (here shown with advanced pathology in stage 5). **(H)** In stages 4–6, a plexus of LNs develops in the second sector of the Ammon's horn. It is loosely woven in stage 4, thickens visibly in stage 5, and reaches its maximum, as shown here, in stage 6. Figure 6a reproduced from ref. *6* with permission from Steinkopff Verlag.

short pathway, which guarantees the uninterrupted flow of information that passes from the neocortex through the striatal loop. Following the brief dominance of the short pathway, the long pathway promptly takes over the lead (Fig. 5B). It is the dopaminergic neurons of the substantia nigra that essentially regulate this seesaw phenomenon or balancing act between the two routes *(64,65)*.

The pedunculopontine nucleus receives input from the ventral striatum and ventral pallidum. Via mostly bidirectional interconnectivities, it influences portions of the striatal loop, i.e., subthalamic nucleus, internal pallidum, and, above all, the pars compacta of the substantia nigra (Fig. 5B). Other projections reach the intralaminar nuclei of the thalamus, the nonthalamic nuclei with diffuse projections, and the gain-setting nuclei of the lower brainstem. As such, the nuclear gray occupies a strategic position between the limbic and striatal loops (Fig. 5B) and is not only involved in cognitive processing but also regulates locomotor activity *(91–95)*. Together with the thalamic intralaminar nuclei, the pedunculopontine nucleus is part of the "ascending reticular activating system" *(96)*. In tandem with the gain-setting nuclei, it furnishes a rhythmogenic complex which, among other functions, influences the sleep–wake cycle *(97)*. Except for the substantia nigra and pedunculopontine nucleus, which give off thin and sparingly myelinated axons, most centers of the striatal loop (dorsal striatum, external and internal pallidum, subthalamic nucleus, and ventral anterior nucleus of the thalamus) generate sturdily myelinated projections and resist the development of LNs/LBs (Figs. 5B, 8A) *(79)*.

The chief projection neurons of additional groups of vulnerable nuclear grays share in common the conspicuous feature of generating relatively long, thin, and sparsely myelinated axons that establish diffuse projections toward a large number of subcortical nuclei and nearly the entire cerebellar and telencephalic cortex. These nuclear grays include the coeruleus–subcoeruleus complex, the nuclei of the oral raphe system, the paranigral and parabrachial pigmented nuclei of the mesencephalic tegmentum, the hypothalamic tuberomamillary nucleus, and the magnocellular nuclei of the basal forebrain, which include the medial septal nucleus, interstitial nucleus of the diagonal band, and basal nucleus of Meynert (Fig. 5A) *(98–101)*. Each of these nuclear grays is highly susceptible to the lesions, with the first subtle changes generally beginning to appear in the coeruleus-subcoeruleus complex in stage 2 cases, and in the other nuclear grays in stage 3 cases *(4,6,18,102,103)*.

A further remarkable development at this stage is the early affection of the central nucleus of the amygdala *(104,105)*. A network of filiform LNs and small LBs gradually fills the nucleus and distinguishes it from the surrounding nuclei (Fig. 6G). The central nucleus receives an admixture of interoceptive input from viscerosensory relay nuclei and exteroceptive data from the amygdalar basolateral complex. It controls subordinate centers that regulate endocrine and autonomic functions together with all of the nonthalamic nuclei with diffuse projections. Furthermore, the central nucleus sends poorly myelinated projections to both the gain-setting nuclei and the dorsal visceromotor nucleus of the vagal nerve (Fig. 5A) *(104–110)*.

Following the lead of the central nucleus, the disease process reaches the basolateral nuclei of the amygdala. This nuclear complex maintains reciprocal connections with the hippocampal formation (Fig. 5B). The lateral nucleus receives multiple input from various sources, particularly from the sensory neocortex via the anteromedial temporal mesocortex and allocortical entorhinal region (Figs. 5B, 8A) *(79)*. The amygdalar basal

and accessory basal nuclei chiefly dispatch projections that terminate in the ventral striatum, ventral pallidum, mediodorsal thalamus, insular, and prefrontal cortex (Fig. 8A) *(79,106,108)*. The basal and accessory basal nuclei develop higher densities of LBs and a thinner network of LNs than the lateral nucleus *(104)*.

5.2.2. Stage 4

The lesions in all of the previously involved nuclear grays worsen. The loss of melanoneurons in the coeruleus–subcoeruleus complex becomes visible to the naked eye. Within the thalamus, initial lesions appear in the intralaminar and midline nuclei (Fig. 5B) *(111)*. The key feature at this stage, however, is the encroachment of the disease process for the first time on the cerebral cortex. Here, it is the anteromedial temporal mesocortex that consistently represents the most vulnerable site and exhibits the earliest cortical LNs and LBs (Fig. 5B). At the same time, but subject to somewhat greater variation, subtle changes also appear in the second sector of the Ammon's horn *(6)*.

The thalamic intralaminar nuclei produce sparsely myelinated and diffusely arranged fibers that extend in a nonspecific manner throughout more than one cortical area and terminate in layers 1 and 6. The thalamic midline nuclei likewise generate poorly myelinated axons that furnish thalamo–allocortical circuits and solid projections to the ventral striatum (Figs. 5B, 8A) *(79)*. The relay nuclei of the thalamus, by contrast, provide sturdily myelinated, specific projections to the neocortex that form small columnar arborizations in layers 2–5 of defined areas. Thalamic relay nuclei are exempt from the pathology, whereas the intralaminar and midline nuclei develop lesions commencing in stage 4 cases (Fig. 5B) *(111)*.

Following directly on the heels of the basolateral amygdala, the disease process penetrates a specific portion of the cerebral cortex: the anteromedial temporal mesocortex. This component of the human brain is exceptionally well developed and mediates between the entorhinal allocortex medially and the temporal neocortex laterally *(61)*. Data in transit from neocortical high-order sensory association areas are siphoned through this mesocortical region and transported via the entorhinal region and hippocampal formation, ventral striatum, ventral pallidum, and mediodorsal thalamus to the prefrontal cortex (Figs. 5A,B, 8A) *(79)*. Bilateral impairment of this stream of information opens the way for the appearance of memory dysfunction and intellectual decline. A network of LNs emerges in layers 2–3 and abundant LBs appear in layers 5–6 (Fig. 7A) *(79)*.

5.3. Stages 5 and 6: Pathology Reaches the Neocortex

5.3.1. Stage 5

The density of the pathological changes increases in all of the previously involved sites. The substantia nigra appears blanched on macroscopic inspection. The network of LNs in the second sector of the Ammon's horn is visible to the unaided eye in immunostained sections and tends to advance into adjoining portions of the first and third sectors. Figure 6H displays this plexus at stage 6 *(112)*.

The subcortical pathology becomes supplemented by involvement of the ventral claustrum and ventral striatum. Like the intralaminar thalamic nuclei, the claustrum establishes bidirectional and nonspecific connections to the cerebral cortex (Fig. 8A) *(79)*. The ventral claustrum chiefly is linked to the entorhinal region, hippocampal formation, and thalamic intralaminar nuclei (Fig. 8A) *(79,113)*.

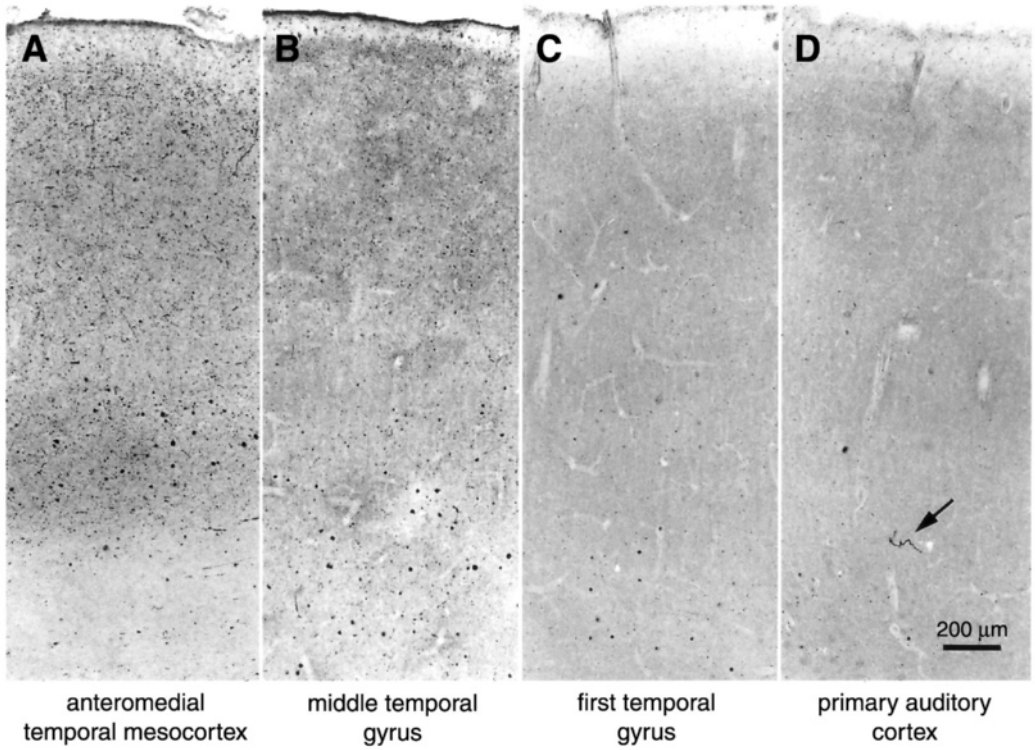

Fig. 7. Cortical pathology as seen in stages 4–6 of Idiopathic Parkinson's disease (α-synuclein immunoreactions, 100 µm ref. *22*). **(A–D)** Affection of the cerebral cortex. With the anteromedial temporal mesocortex as its point of departure in stage 4 **(A)**, the cortical pathology encroaches upon the adjoining neocortical high-order sensory association areas and prefrontal fields in stage 5 **(B)**. Notice the presence of many Lewy body-containing pyramidal cells within the deep layers. In stage 6, the pathology advances further into the first-order sensory association areas and premotor fields **(C)**, and eventually encroaches upon the primary areas of the neocortex, here exemplified by the primary auditory area **(D)**. (Reproduced from ref. *79* with permission from Landes Bioscience Press.)

The cortical lesions now include subtle involvement of the entorhinal allocortex with a few LBs in the deep layers. It is characteristic of this stage, however, that the disease process, once it has gained a foothold in the anteromedial temporal mesocortex, engulfs additional mesocortical areas in insular, subgenual, and anterior cingulate regions and then progresses into the adjoining prefrontal and high-order sensory association areas of the neocortex (Figs. 7B, 8A) *(79)*. The insular and subgenual mesocortex represent topically organized viscerosensory and visceromotor regions (Fig. 8A) *(79,114–117)*. With the transition from the mesocortex to the mature neocortex, the density of LNs in layers 2–3 gradually becomes less, and LNs no longer occur in layers 5–6. LBs are found mostly within the small pyramidal cells that reside in the deep layers 5–6 *(118)*.

5.3.2. Stage 6

In the final stage, all of the earlier affected nuclear grays and cortical areas are full of pathology. From the prefrontal and high-order sensory association areas, the disease process advances into premotor and first-order sensory association areas, and occasionally

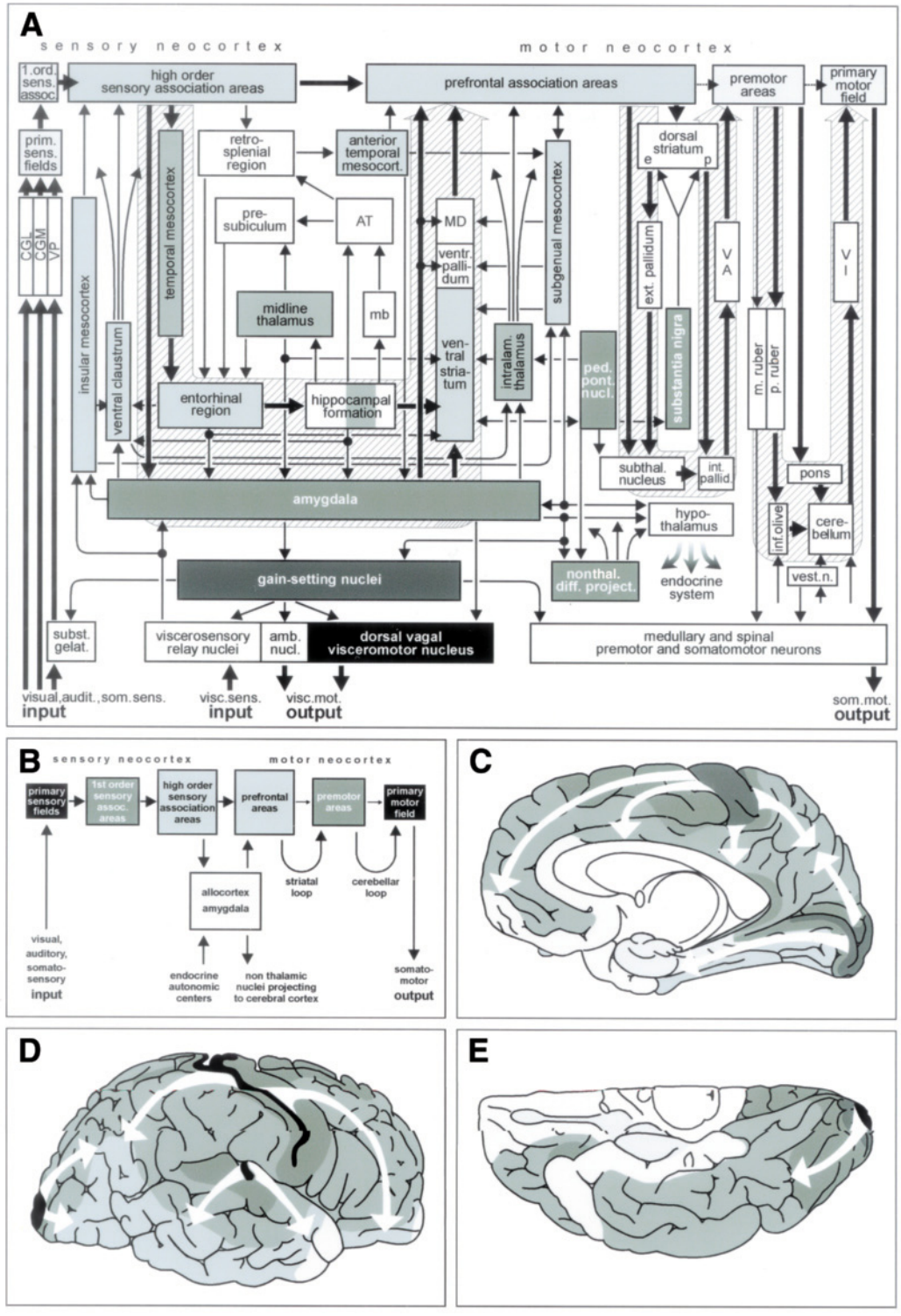
A
sensory neocortex
motor neocortex
1.ord. sens. assoc.
high order sensory association areas
prefrontal association areas
premotor areas
primary motor field
prim. sens. fields
retro-splenial region
anterior temporal mesocort.
dorsal striatum
e
p
CGL
CGM
VP
pre-subiculum
AT
MD
ventr. palli-dum
temporal mesocortex
subgenual mesocortex
ext. pallidum
V A
V I
insular mesocortex
ventral claustrum
midline thalamus
mb
ven-tral stria-tum
intralam. thalamus
ped. pont. nucl.
substantia nigra
m. ruber
p. ruber
entorhinal region
hippocampal formation
subthal. nucleus
int. pallid.
pons
amygdala
hypo-thalamus
inf.olive
cere-bellum
gain-setting nuclei
nonthal. diff. project.
endocrine system
vest.n.
subst. gelat.
viscerosensory relay nuclei
amb. nucl.
dorsal vagal visceromotor nucleus
medullary and spinal premotor and somatomotor neurons
visual,audit.,som.sens.
input
visc.sens.
input
visc.mot.
output
som.mot.
output
B
sensory neocortex
motor neocortex
primary sensory fields
1st order sensory assoc. areas
high order sensory association areas
prefrontal areas
premotor areas
primary motor field
allocortex amygdala
striatal loop
cerebellar loop
visual, auditory, somato-sensory
input
endocrine autonomic centers
non thalamic nuclei projecting to cerebral cortex
somato-motor
output
C
D
E

Fig. 8.

Fig. 8. (A) Schematic representation of components of the limbic, visceromotor, and somatomotor systems. The most important centers of the cerebellar loop have been added to the scheme to make the somatomotor system more complete. Furthermore, additional details are supplied in the form of select cortical areas that regulate viscerosensory and visceromotor functions (insular and subgenual mesocortex). The three broad arrows in the background (cross-hatching) are intended to facilitate recognition of the limbic, striatal, and cerebellar loops. Even in this more complicated diagram, it becomes clear that normal functions of the limbic loop depend on the structural integrity of the anteromedial temporal mesocortex. Earmarks of stages 5–6 are that the lesions advance into the mature neocortex, initially making inroads into the extended prefrontal and high-order sensory association areas (in stage 5), followed by incursions into premotor and first-order sensory association areas, and eventually affecting primary fields (in stage 6). Damaged structures are marked by five degrees of shading: black (involved from stage 1 on), charcoal (from stage 2 on), dark gray (from stage 3 on), medium gray (from stage 4 on), light gray (first affected in stage 5), and very light gray (first affected in stage 6). It is precisely the superordinate centers of the limbic and striatal loops that are susceptible to the worst neuronal damage. The severe involvement of the anteromedial temporal mesocortex leads to a marked reduction of the data transfer from the sensory neocortex via entorhinal region, hippocampal formation, and amygdala to the prefrontal cortex. The gradual affection of the neocortex paves the way for cognitive decline. The dysfunctions of the visceromotor and somatomotor systems become supplemented by deterioration of cortically-controlled intellectual capabilities. **(B–E)** Neocortical myelination begins in the primary sensory and primary motor fields and progresses via first-order sensory association areas and premotor areas to the related high-order association and prefrontal areas (indicated by arrows). This results in very dense myelination of the primary sensory and primary motor fields in the human adult. With increasing distance from the primary fields, the average myelin content falls off gradually and is minimal in anterior portions of the mesocortex (shown by differences in black and gray shading). The myelination process bears a likeness to the reverse image of the gradual destruction of the neocortex that evolves in the late stages of Idiopathic Parkinson's disease (compare Fig. 8C–E with Fig. 1B–D). The lesions commence in the anteromedial temporal mesocortex. From there, the pathology extends into adjoining high-order association areas, eventually reaching the neocortical primary sensory and motor fields (indicated by arrows). amb. nucl., ambiguus nucleus; anterior cingulate mesocort., anterior cingulate mesocortex; AT, anterior thalamic nuclei; CGL, lateral geniculate body; CGM, medial geniculate body; dorsal striatum e, encephalin-containing projection neurons of the dorsal striatum; dorsal striatum p, substance P-containing projection neurons of the dorsal striatum; ext. pallidum, external pallidum; inf. olive, inferior olive; int. pallid., internal pallidum; intralam. thalamus, intralaminar nuclei of the thalamus; mb, mamillary body; MD, mediodorsal nuclei of the thalamus; m. ruber, magnocellular portion of the red nucleus; nonthal. diff. project., non-thalamic diffusely projecting nuclei; p. ruber, parvocellular portion of the red nucleus; ped.pont.nucl., tegmental pedunculopontine nucleus; prim. sens. fields, primary sensory fields; som.mot. output, somatomotor output; subst. gelat., substantia gelatinosa; subthal. nucleus, subthalamic nucleus; VA, ventral anterior nucleus of the thalamus; ventr. pallidum, ventral pallidum; vest. n., vestibular nuclei; VI, ventral intermediate nucleus of the thalamus; visc.mot. output, visceromotor output; visc.sens. input, viscerosensory input; visual, audit., som.sens. input, visual, auditory, somatosensory input; VP, ventral posterior nuclear complex of the thalamus; 1.ord. sens. assoc., first order sensory association areas. (Figure 8A reproduced from ref. *79* with permission from Landes Bioscience Press.)

even into neocortical primary fields (Figs. 7C,D, 8A) *(6,79)*. A marked reduction in the density of LBs becomes discernible with the gradual transition from the second to the first temporal gyrus. Neocortical primary areas usually display only a few scattered LNs/LBs (Fig. 7D) *(79)*.

5.4. Progression of the Lesions From Stages 4–6 Recapitulates the Process of Neocortical Myelination in Reverse Order

The remarkable consistency in the topographic expansion of the pathology in IPD remains enigmatic. One key to understanding the phenomenon may be the observation that the sequential appearance of LNs/LBs in the neocortex and the process of neocortical myelination are mirror images: the progression is the same, but the order is reversed (compare Fig. 1B–D with Fig. 8C–E) *(5)*. Late myelinating mesocortical and neocortical areas develop LNs/LBs earlier in the disease process and at higher densities than those that commence myelination early (Fig. 8B). Regressive brain changes often tend to repeat the maturation process, but in reverse order *(119–122)*.

Myelination represents the final step in brain maturation. Functional maturity of projection neurons usually is achieved only after axonal myelination is completed. Myelination of the neocortex is a late-onset and particularly prolonged process, which follows a predetermined sequence *(80–82,123)*. It commences in the neocortical primary fields and continues via the first order sensory association areas and premotor areas into high-order sensory association areas and prefrontal areas, eventually reaching the mesocortex (Fig. 8B–E). Exceptionally dense myelination of the primary fields is the end result in the human adult and, on the average, myelin density declines with increasing distance from the primary areas. The anteromedial temporal mesocortex is very sparsely myelinated *(57)*. Just as the primary neocortical fields are heavily myelinated and more or less impervious to the disease process, cortical areas that myelinate last are the most susceptible to the lesions *(124)*. As such, it should come as no surprise that the poorly myelinated anteromedial temporal mesocortex is the induction site of the earliest cortical LNs and LBs.

6. FUNCTIONAL CONSEQUENCES OF THE LESIONS

The collective profile of the pathological alterations associated with IPD, as shown in Fig. 8A, reveals particularly severe neuronal damage in all superordinate centers that regulate limbic, visceromotor, and somatomotor functions *(79)*. The diagram has yet to be completed; nonetheless, taken together with the earlier diagrams (Figs. 2B,C, 4B,D, 5A,B), it is just clear enough to convey an overall impression of the topographic extent and severity of the damage that gradually develops in these centers during the course of the disease. Neuropathological studies that take into account features of myelination, characteristics of vulnerable neuronal types, and the existence of anatomical pathways that interconnect susceptible nuclear grays could provide greater insight into the pathological process that underlies IPD, thereby helping to explain how the lesions progress from one brain region to another and, perhaps more importantly, clarifying whether the disease process commences within or beyond the central nervous system *(53)*.

6.1. Involvement of Olfactory Bulb, Related Olfactory Areas, and Nuclei

With the exception of the medial nucleus of the amygdala, all of the components of the olfactory system become involved in IPD. In fact, the olfactory bulb and anterior olfactory nucleus show considerable pathology already in most stage 1 cases *(7,125)*. These reliably occurring lesions are congruent with clinical protocols that make mention of early olfactory dysfunctions in IPD patients *(126–129)*.

6.2. *Affection of Superordinate Relay Centers That Control Visceromotor Functions*

The ascending pathological process successively destroys relay centers that influence autonomic functions: The affection of the vagal dorsal visceromotor nucleus (stage 1) is followed by growing destruction of the amygdalar central nucleus and the interstitial nucleus of the stria terminalis (stage 3), and eventually is complemented by involvement of the insular and subgenual mesocortical regions (stage 5). Because the original monograph by James Parkinson in 1817 *(130)*, visceromotor dysfunctions have been noted as early and frequently occurring phenomena in IPD *(3,131–135)*. Because the leading causes of death, which are more prevalent in Parkinson's patients than in age-matched controls, include bronchopneumonia, myocardial infarction, and other cardiac dysfunctions, one could surmise that the damage sustained by most autonomic system centers predisposes such individuals to these lethal events *(136)*.

The highest superordinate center for control of visceromotor and viscerosensory functions is represented by the subgenual and insular mesocortical regions. Their structural and functional integrity is needed to maintain the sympathetically mediated increase in heart rate during stress, the resting level for the gain of the cardiac component of the baroreflex response, and the appropriate response of skin conductance to emotional stimuli *(137–139)*. Neuronal damage to these centers results in deficient autonomic responses to the effects of stress, cognitive challenge, or emotionally momentous stimuli. Severe impairments could lead to the complete absence of affect-related visceromotor responses, asymbolia for pain, partial or total interoceptive agnosia, and apraxia *(140)*.

6.3. *Involvement of the Ventral Claustrum, Intralaminar Nuclei of the Thalamus, and Nonthalamic Nuclei With Diffuse Projections*

The superordinate components of the limbic loop regulate the ventral claustrum, intralaminar nuclei of the thalamus, and influence, chiefly by way of the central nucleus of the amygdala, all of the other nonthalamic nuclei that send diffuse projections to the cerebral cortex (Fig. 8A). All of these nuclei develop severe pathology *(4,6,18,79,102,103)*. Normally, they influence cortical processing and most probably modulate the level of activity of cortical projection neurons in accordance with external and/or internal conditions and input. The damage inflicted on these nuclei, therefore, exacts a considerable toll on the general input to the cerebral cortex and results in curtailment of the versatility with which cortical functions adapt to the ever-changing demands placed upon the organism. Such damage can prepare the ground for a universal reduction of the higher cognitive functions in the human brain.

6.4. *Involvement of the Anteromedial Temporal Mesocortex: Disruption of Data Flow From the Sensory Neocortex Via Components of the Limbic Loop to the Prefrontal Neocortex*

All three high-order centers of the limbic loop (entorhinal region, hippocampal formation, amygdala), as well as many of the cortical fields and subcortical nuclei that are connected with them sustain considerable damage (Fig. 8A) *(79)*. Within this context, it should be emphasized again that the highest organizational level of the human limbic system is markedly neocortex-oriented and not only receives abundant sensory data via the anteromedial temporal mesocortex from the parietal, occipital, and temporal neocortex

but also sends powerful projections to the prefrontal neocortex (Figs. 2B,C, 5A,B, 8A) *(79)*. As pointed out previously, the anteromedial temporal mesocortex consistently exhibits the maximal cortical lesional density. The second point of entry for neocortical data into the limbic loop, the lateral nucleus of the amygdala, also shows pronounced neuronal disease-related alterations. On the efferent side, there are the seriously damaged basal and accessory basal nuclei of the amygdala that generate major projections to the ventral striatum, ventral pallidum, mediodorsal thalamus, and prefrontal cortex. In short, the widespread damage to the anteromedial temporal mesocortex and amygdala undermines the data transfer from the sensory neocortex via the pivotal entorhinal region and amygdala to the prefrontal cortex (Fig. 8A) *(79)*.

The centers of the limbic loop are ideally positioned to select information from the streams of exteroceptive and interoceptive data, to evaluate the significance of environmental and cognitive events, and to direct emotional responses and behavior (Fig. 2B). The limbic loop plays an integral role not only in regulating motivation and the initiation of affect-related movement but also in the maintenance of emotional equilibrium, social behavior, learning abilities, and memory functions *(141–144)*. At the same time, the limbic system influences the somatosensory and somatomotor systems since its activation leads to inhibition of nociception and to enhanced susceptibility of motor neurons for excitatory input. In fact, the limbic influence on the prefrontal cortex explains why the organism's motor activity reflects the individual's emotional state. The degree of damage that develops in the limbic loop is detrimental to all of these functions, eventually leading to a dissociation between the voluntary and emotional motor systems and causing personality changes in addition to diminished cognitive performance *(146)*.

ACKNOWLEDGMENTS

This study was supported by the Deutsche Forschungsgemeinschaft (DFG) and the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie. The authors wish to express their appreciation to Jürgen Bohl, MD (Department of Pathology, Johannes Gutenberg University, Mainz), Prof. Hansjürgen Bratzke, MD (Institute for Forensic Medicine, J.W. Goethe University, Frankfurt am Main), and Ms. Inge Szász-Jakobi for her expertise in preparing the illustrations. Figures 4C, 7, and 8A appear with the permission of Landes Bioscience (Georgetown, TX, 2003), and Figs. 1A-D, 3B, and 6A with the permission of Steinkopff Press (Darmstadt, Germany, 2002).

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21

Dopamine and Glutamate in Parkinson's Disease

Biochemistry, Clinical Aspects, and Treatment

Heinz Reichmann, Bernd Janetzky, and Peter Riederer

1. BIOCHEMISTRY

In 1817 the London physician James Parkinson described in his well-known essay on the shaking palsy, a disease that is characterized by tremor (mainly at rest), muscular rigidity, which leads to difficulties in walking, writing, speaking, and masking of facial expression, bradykinesia, a slowness in initiating and executing movements, and stooped posture and instability. Many of these clinical features are also manifested by other basal ganglia (BG) disorders and, thus, often referred to as parkinsonian syndromes. Today we know three main subgroups of parkinsonian syndromes: To the main group belongs idiopathic Parkinson's disease (IPD) including the known hereditary forms (e.g., α-synuclein- and PARK-mutations); "parkinsonism-plus" syndromes are characterized by progressive supranuclear palsy, multiple system atrophy, and the Parkinson–amyotrophic lateral sclerosis (ALS)–dementia complex that include parkinsonian movement abnormalities in addition to other neurological deficits. The secondary, symptomatic forms of parkinsonisms are formed by infectious diseases, tumors, metabolic disturbances, drugs, and toxins.

Like Huntington's disease and ballism Parkinson's disease (PD) is a movement disorder caused by lesions of the BG. For the better understanding of the functional role of the relevant neurotransmitters and neuromodulators, it is important to consider the neuronal circuits and connection of the BG. The BG consist of several large, anatomically distinct masses of gray matter. They consist of the substantia nigra pars compacta (SNc) and pars reticulata (SNr), the striatum comprised of caudate and putamen, and the pallidum, composed of the inner and outer parts of the globus pallidus. These areas form many complex afferent and efferent connections to each other and other parts of the brain, forming the so-called motor circuit and regulating sensomotor activities (Fig. 1). As can be seen from Fig. 1, BG are the central part of a cortical-thalamic-cortical feedback. The direct pathway contains two inhibitory γ-aminobutyric acid (GABA)ergic synapses between the striatum and the medial pallidum, respectively—the SNr and the medial pallidum or the SNr and the thalamic ventroanterior and ventrolateral nuclei. An activation of this pathway produces a disinhibition of the excitatoric glutamatergic thalamic input to the sensory, motor, and associated cortical areas.

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

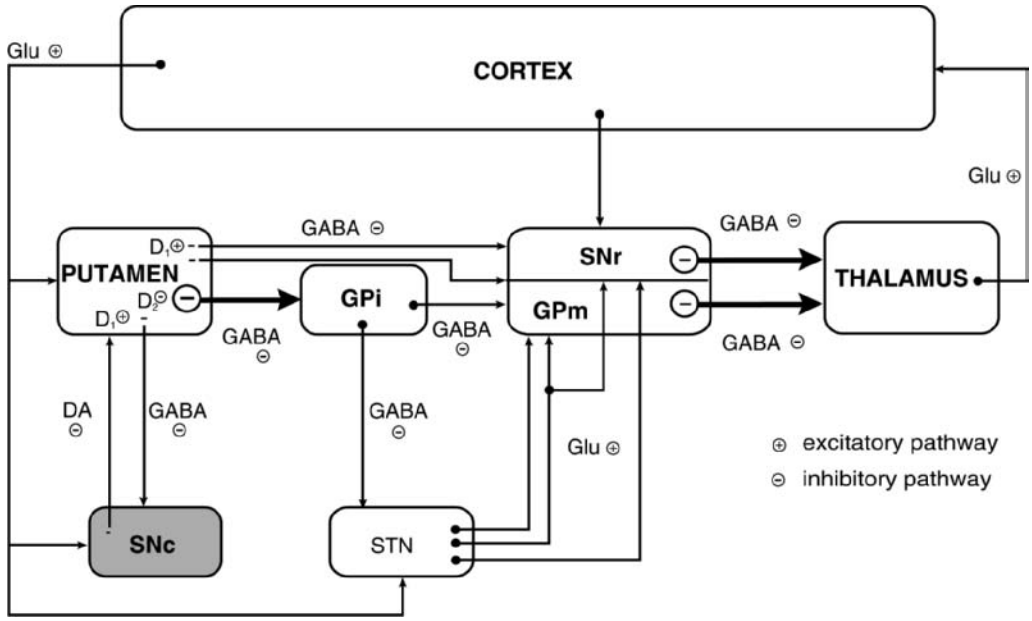

Fig. 1. Motor loop in the brain (adapted from ref. *12*). SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; GPI, globus pallidus external; GPm, globus pallidus internal; DA, dopamine; Glu, glutamate; GABA, γ-aminobutyric acid.

The indirect pathway between striatum and pallidum and between pallidum and the subthalamic nucleus (STN) respectively the thalamus includes one excitatory glutamatergic and three inhibitory GABAergic projections. In contrast to the direct pathway, the three inhibitory outflows result in net inhibition of the thalamic-cortical projections when the indirect circuit is activated. Beside other factors the activity in these two pathways depends on the balance of excitatory and inhibitory receptors activated on the striatal GABAergic neurons.

Dopamine (DA) has, however, a differential effect on these two pathways. Whereas DA excites striato-nigral neurons through D_1 receptors, it inhibits striato-pallidal neurons through D_2 receptors (for review, *see* ref. *1*). Dopaminergic fibers have therefore an inhibitory action on the striatal GABAergic/encephalinergic cells projecting to the external pallidal segment, but an excitatory action on the GABAergic/substance P-containing neurons projecting directly to the internal segment of the GP and the SNr.

Among the most important modulators of these circuits are the dopaminergic innervations from the SNc and the intrastriatal cholinergic neurons to the striatum. In the striatum, a well-balanced equilibrium between inhibitory DA effects (at D_2 receptors) and excitatory glutamine effects (at *N*-methyl-D-aspartate NMDA receptors) does exist. Therefore excitatory D_1 receptors are found mainly on the direct pathway and inhibitory D_2 receptors on the indirect pathway.

The catecholamine DA is the most predominant monoamine and the main neurotransmitter in the BG. It is synthesized from the amino acid tyrosine by tyrosine hydroxylase (TH) via 3,4-dihydroxy-L-phenylalanine (levodopa [L-DOPA]) by DOPA carboxylase where TH is the rate-limiting enzyme. The homotetrameric enzyme TH is found in all

cells that synthesize catecholamines and is a mixed-function oxidase that uses molecular oxygen and tyrosine as its substrates and biopterin as a cofactor. Ordinarily, low concentrations of catecholamines are free in the cytosol, where they may be metabolized by enzymes including monoamine oxidase (MAO).

Thus, conversion of tyrosine to L-DOPA and L-DOPA to DA occurs in the cytosol. In normal brain the highest concentrations of DA, about 80% of total brain DA, are found in the striatal areas *(2)*. The second-highest DA levels are found in the SN, followed by the GP and the STN. Interestingly the DA metabolite homovanillic acid (HVA) is not distributed in the same manner. Whereas in the striatum HVA/DA ratios are only slightly increased, ratios in SN are three times, in GP about 9 times, and even 11 times higher in STN than DA *(3)*. These differences most likely reflect differences in either the number of DA transporter, with the striatum having the highest and GP the lowest number, or the neuronal DA turnover in the corresponding regions. The basal ganglia are also rich in all DA-associated proteins such as the DA synthetic enzyme TH and DOPA decarboxylase, as well as specific DA and monoamine transporter and pre- and postsynaptic DA receptors (for review, *see* ref. *3*).

The amino acid glutamate is the principal excitatory neurotransmitter in the brain. It has been estimated that about 40% of all synapses in the brain are glutamatergic *(4,5)*. Glutamate is stored in synaptic vesicles within nerve terminals from which, on polarization, it is released into the synaptic cleft in a Ca^{2+}-dependent manner. Its action is terminated by removal from the synaptic cleft via an Na^{+}-dependent, high-affinity uptake system, which is located on neurons and glial cells *(6)*. Glutamate exerts its physiological actions via the activation of several classes of receptors, which are primarily located on postsynaptic neurons and which are present in virtually all areas of the central nervous system (CNS). Glutamate receptors were originally classified by pharmacological means into NMDA and non-NMDA receptors. Non-NMDA receptors were further divided into those preferring α-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA) or kainic acid (KA) as agonists *(7)*. The activation of these receptors leads to opening of associated ion channels ("ionotrophic receptors"). An additional class of glutamate receptors linked to G proteins. Their activation produces changes in cyclic nucleotides or phosphoinositol metabolism.

As mentioned earlier neuromelanine containing dopaminergic neurons in the SNc and their axonal projections in the striatum are the primary neurotransmitter population lost in PD resulting in imbalance of the DA depending neurotransmission systems. Decreased DA activity would lead to a reduced activity of the direct pathways and therefore to an increase of the inhibitory actions of the BG on thalamo-cortical and brainstem mechanisms. In the indirect pathway, the glutamatergic neurons projecting from the subthalamic nucleus would become overactive. Both effects result in higher activation of the internal pallidal segment and increased inhibition of the thalamus (Fig. 2). The tonic inhibition by BG output structures would therefore be exacerbated. This may explain the slowness of movements that is one of the cardinal symptoms of PD.

Vice versa, an increase of the DA activity in the striatum, for instance, by drug-induced high L-DOPA levels, would decrease the inhibitory effect that the internal pallidal segment has on the thalamo-cortical and brainstem systems. This disinhibition of the thalamus would have a facilitatory effect on movements generated by cortical or brainstem activity and lead to a hyperactive state of the patient.

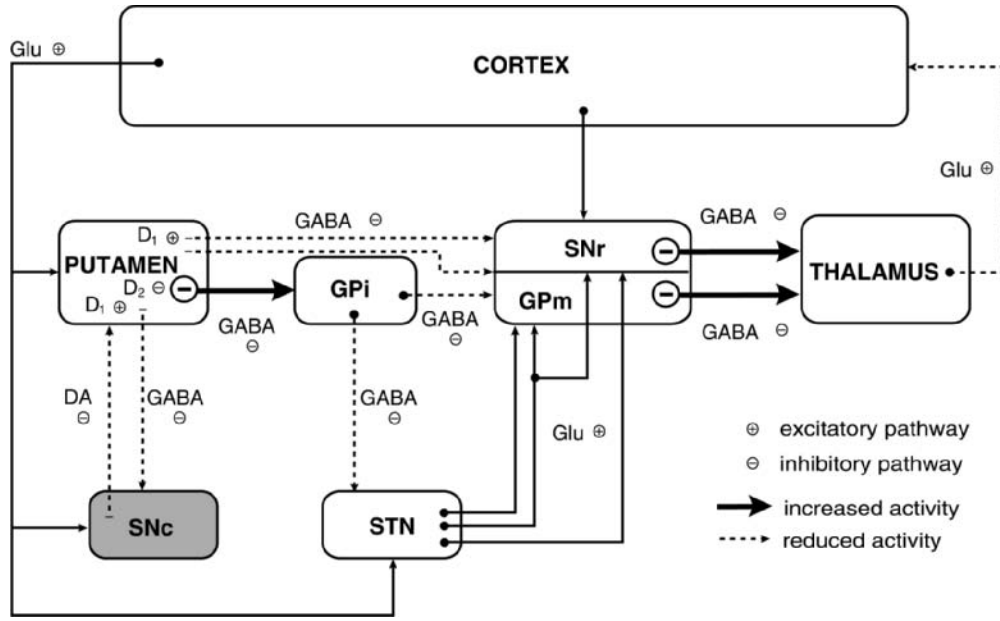

Fig. 2. Altered motor loop in Parkinson's diseased brain (adapted from ref. *13*). For abbreviations *see* Fig. 1.

Besides DA, all other biochemical markers for the presynaptic striatal DA terminals, such as the levels of the major DA metabolite HVA, the DA synthetic enzymes TH and L-DOPA-decarboxylase, and the DA transporter (DAT) sites are reduced (for review, *see* ref. *8*). In the advanced stages of PD, the striatal DA loss exceeds the 80% mark, with DA levels in the putamen being consistently more reduced than the caudate nucleus. This putamen–caudate difference is a result of the uneven pattern loss of the melanin containing DA perikarya in the SNc. Although the degree of the striatal DA loss correlates significantly with the degree of nigral cell loss, the latter is distinctly less than would be expected from the degree of the striatal DA loss *(9)*. The striatal DA changes remain clinically silent until the threshold value of 60–80% DA loss is reached. Within the symptomatic range of DA loss (>60% reduction), a correlation is seen between the degree of DA loss and the severity of PD symptoms. In every clinical case of PD, the DA loss in the putamen, but not in the caudate nucleus, exceeds the critical threshold value of 60–80% *(8)*.

The high threshold for the striatal DA loss to induce clinical manifestations of PD may be explained by compensation of the remaining DA neurons. The basis for this compensation in early stages of PD may be a metabolic overactivity of the remaining DA neurons, evidenced by the shifting of striatal HVA/DA ratio in favor of HVA, indicating an increase in DA turnover in the remaining neurons *(10)*. In the advanced stages of the disease (>90% DA loss) an increase of striatal D2 receptor sites was observed maximizing the therapeutic efficacy of the DA substitution treatment *(11)*. This adaptive capacity may also explain the observation that in order to become clinically relevant as a motor disorder the striatal DA loss has to reach this critical high value of 80%. A smaller DA loss remains clinically of little consequence. These observations

on functional compensation of DA neurons have been also observed in several animal models of PD *(12–14)*.

Although changes in other neurotransmitter systems can be found in the PD brain, the striatal DA loss is by far the most profound neurotransmitter alteration and therefore the rational basis for the DA substitution therapy that includes drugs, such as L-DOPA and the direct-acting DA agonists, as well as brain grafting.

Although significant progress has been made in the understanding of the PD pathophysiology, the main reasons and steps of the specific degeneration processes of nigral dopaminergic neurons are still unknown. An accidental contribution came in the early 1980s, when numerous young adults presented with a PD-like syndrome. Extensive investigation demonstrated that this phenomenon was caused by the unintentional self-administration of MPTP, a product of the illicit synthesis of meperidine analogs *(15)*. MPTP freely crosses the blood–brain barrier. Once in the brain, this "protoxin" is oxidized to 1-methyl-4-phenylpyridinium (MPP^+), the active toxin, by monoamine oxidase B. MPP^+ is then taken up selectively by nigral neurons via the DAT. Furthermore it is accumulated in mitochondria, where it can reach concentrations in the millimolar range. Finally toxicity results from inhibition of mitochondrial respiration, as a consequence of MPP^+ binding to the rotenone-sensitive site of complex I (NADH-ubiquinone oxidoreductase), the largest and most complicated protein complex of the mitochondrial electron transport chain *(16–18)*. In the brain, MPP^+ has an affinity for complex I in the low-millimolar range *(19)*. And complex I is highly vulnerable to oxidative damage *(20)*. In addition, partial inhibition of complex I leads to free-radical formation, which may cause irreversible damage to the enzyme complex *(21)*. MPP^+ has since been widely used to reproduce a clinical and neuropathological picture of PD in primates and nonprimates *(22)*.

The greater vulnerability of DA neurons to impairment of energy metabolism has been suggested by several findings. Dopaminergic neurons in mouse mesencephalic cultures were three times more sensitive than mesencephalic GABA neurons or striatal GABA or cholinergic neurons to a sequential exposure to rotenone, a plant-derived complex I inhibitor, and glutamate *(23)*, also suggesting an inherent vulnerability of dopaminergic neurons to energy impairment. Similar results have been obtained in rat mesencephalic cultures *(24)*. In addition the chronic exposure of small amounts of rotenone to rats results in a more or less specific degeneration of dopaminergic neurons *(25,26)*. It is thought that the partial inhibition of complex I results in an enhanced production of free radicals. In addition DA neurons of the SNc are thought to belong to a group of highly predisposed cells with high energy expendition because of their morphological specifications and metabolic attributes *(27)*. Keeping in mind that PD is characterized by a mild 15–30% reduction of complex I *(28–30)*, that only 20–30% partial inhibition of complex I is needed for a significant increase in free-radical production *(21)* and that especially dopaminergic neurons and their terminals may be more sensitive to these processes, it may be noteworthy to consider similar endogenous factors, too.

In recent years, increasing evidence has suggested that oxidative stress may play a central role in the pathophysiology of PD *(31,32)*. The substantia nigra is exposed to a high degree of oxidative stress as a consequence of formation of cytotoxic oxygen radicals. The activities of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine synthesis, and monoamine oxidase, which catabolyzes catecholamines, cause the formation of H_2O_2 as a normal byproduct. Auto-oxidation of DA, which leads to the production of

melanin, also yields H_2O_2 (for further details, *see* Chapter 18). H_2O_2, toxic *per se*, slowly decomposes to $^{\bullet}OH$, the most reactive free radical. This nonenzymatic reaction is accelerated in the presence of iron (particularly when it is in the free, ferrous form, Fe^{2+}), which is abundant physiologically in the SNPc. That iron can be toxic has been confirmed by the fact that, in rats, it causes selective damage to nigral neurons when injected locally. Also, the iron chelator desferrioxamine can prevent the toxic effect of 6-hydroxyDA on nigral dopaminergic neurons *(33,34)*. In fact, abnormally elevated levels of iron have been reported in the substantia nigra of PD patients *(35,36)*.

In addition in PD, the subthalamic neurons are disinhibited and their excessive firing could trigger the nigral dentrites to produce harmful amounts of DA, creating additional stresses on themselves and their neighbors *(37)*.

It has been suggested that excitotoxicity may play a role in the pathophysiology of numerous neurologic diseases. The term "excitotoxicity" was initially used to describe neuronal death provoked by administration of very high concentrations of exogenous glutamate, or compounds with agonistic actions onto glutamate receptors. This phenomenon was first described by Olney, who coined the term "excitotoxicity" after the correlated the neurotoxic and the excitatory properties of various glutamate analogs *(38)*.

Acute neurodegeneration was observed in those areas not well protected by the blood–brain barrier. However, thus far, a direct role for endogenous glutamate-mediated toxicity has been demonstrated only in hypoxic/ischemic brain damage, in which a large increase in extracellular glutamate and a concomitant depression of the uptake (inactivation) system seem to be responsible for neuronal death *(39,40)*. Periods of anoxic insult to neuronal tissue that last more than a few seconds, such as during cardiac arrest or thrombotic stroke, often result in neurotoxicity. Oxygen deprivation precipitates a depletion of energy stores within neuronal and glial cell compartments with a concomitant acidosis and release of free radicals. Depletion of energy stores affects cellular metabolism, energy-dependent ionic pumps and the ability of cells to maintain resting membrane potential. Consequently, depolarization of cells results in action potentials and the release of glutamate from presynaptic terminals, which activates postsynaptic AMPA and NMDA receptors. Entry of Ca^{2+} through glutamate receptors and voltage-sensitive Ca^{2+} channels increases the intracellular concentration of Ca^{2+}. An elevation of intracellular Ca^{2+} will trigger a cascade of second-messenger systems, many of which remain activated long after the initial stimulus is removed. The inability of a population of cells to maintain a resting potential thus precipitates a positive feedback loop, leading to neuronal cell injury or death.

Disorders of excitatory amino acid transmission have been implicated in ALS and the chronic neurodegenerative diseases olivopontocerebellar atrophy and Huntington's chorea, too. Neurolathyrism is a spastic disorder occurring in East Africa and India. It is associated with the dietary consumption of the legume *Lathyrus sativus*. The glutamate-like excitant α-*N*-oxalylamino-L-alanine has been identified as the toxin in this plant. Its action at AMPA receptors in the spinal cord may be responsible for the observed degeneration of lower and upper motor neurons.

The high incidence of ALS observed in residents of the Pacific island of Guam was determined to be because of the dietary ingestion of the cycad *Cycas circinalis*. This seed contains an amino acid, α-*N*-methylamino-L-alanine, which, in the presence of bicarbonate, becomes excitotoxic through a mechanism involving the activation of AMPA

and NMDA receptors. Its action can be blocked by the NMDA receptor antagonist D-AP5.

The brain is remarkably resistant to very high concentrations of glutamate. Thus, the normal brain possesses the instruments necessary to deal with a potential neurotoxin like glutamate. But that may be different if, for any reason, neuronal energy production is impaired. An important feature of the interaction between excitotoxicity and bioenergetic defects is that they are synergistic *(41)*. That is, in the setting of a mild metabolic disturbance as may be seen, for example, by genetic deficits in radical detoxification or exogenous factors, nontoxic concentrations of glutamate and other agonists produce widespread, severe damage. This indicates that when neuronal mitochondria are not functioning optimally, neurons are sensitized to the toxic effects of glutamate.

Thus, excitotoxic cell death can occur even in the absence of abnormally elevated glutamate levels, and NMDA receptors seem to be central to the synergistic interaction between glutamate and bioenergetic defects. Beneficial effects of glutamate receptor antagonists in models of neurological disorders are often used to support the notion that endogenous excitotoxicity (i.e., resulting from extracellular accumulation of endogenous glutamate) is a major contributor to neuronal death associated with these conditions. By means of local infusions of glutamate receptor antagonists into the striatum, it has been shown that glutamate through NMDA, but not through AMPA receptors drives these striato-pallidal neurons and thus NMDA receptor antagonists are able to reverse parkinsonian symptoms *(42,43)*.

Neurotoxicity mediated by the NMDA receptor is apparently caused by a massive influx of extracellular Ca^{2+} *(44)*. The increase in cytoplasmic Ca^{2+} activates a number of Ca^{2+} dependent enzymes, including protein kinase C, phospholipase A_2, phospholipase C, Ca^{2+}/calmodulin dependent protein kinase II, nitric oxide synthase, and various proteases and nucleases. Ca^{2+}-induced activation of enzymes involved in the catabolism of proteins, phospholipids, and nucleic acid may lead to cell death through different pathways. The relative contribution of these pathways is still unclear. For example, activation of phospholipase A_2 might result in extensive membrane breakdown *(45)*, whereas Ca^{2+}-mediated activation of proteases seems to determine changes in the microtubular organization of the cytoskeleton that lead to characteristic cytoskeletal alterations *(46)*. On the other hand, activation of phospholipase A_2 and subsequent production of arachidonic acid lead to the generation of cytotoxic oxygen radicals *(47)*. Also, in certain neurons, Ca^{2+}-mediated activation of nitric oxide synthase causes the release of nitric oxide, which is lethal to surrounding neurons *(47)*. Such an effect may be caused by the generation of peroxynitrite anion from the reaction of nitric oxide with superoxide anion ($\bullet O_2^-$) and the subsequent decomposition to hydroxyl radical ($^\bullet OH$) *(48)*.

This relationship between excitotoxicity and bioenergetics may play a role in the pathophysiology of a variety of neurodegenerative diseases and may be relevant to PD pathophysiology, too. The oxidative damage and death of DA neurons associated with the toxin, methamphetamine, can be blocked with an NMDA antagonist. A mitochondrial bioenergetic defect may also be central to the etiology of PD. There is increasing evidence indicating that excitatory amino acids are involved in the neurotoxic effects of MPTP (49). It could be demonstrated that NMDA antagonists also protect against the neurotoxic effects of mitochondrial poisons, including MPP^+ or intrastriatal administered malonate *(50–52)*. The fact that NMDA receptor antagonists can prevent neuronal death induced by mechanisms that are believed to be relevant to the pathogenesis of PD may

support that excitotoxicity in conjunction with a mitochondrial impairment could be a cofactor in the neurodegeneration characteristic of this disorder.

2. CLINICAL ASPECTS AND TREATMENT

2.1. L-DOPA

Because patients with IPD show a dopaminergic deficit in the striatum, it was meaningful to test the application of DA for treatment. Soon it became obvious that DA causes too many side effects and does not cross the blood–brain barrier. For this reason, L-DOPA, the precursor of DA, is used. In spite of the fact that it is now generally accepted that L-DOPA causes side effects, such as motor fluctuations and dyskinesia, it is still considered to be one of the greatest achievements ever reached in clinical treatment in neurology.

In 1961, the Viennese scientists Birkmayer, a neurologist, and Hornykiewicz, a biochemist, started to treat patients suffering from IPD intravenously with 20–50 mg L-DOPA. They observed considerable improvement of motor symptoms *(53)*. Interestingly, however, some double-blind studies performed at that time did not show any significant improvement under L-DOPA therapy. If "evidence-based medicine" had existed at that time L-DOPA might never have become the most frequently used drug for motor symptoms in IPD.

A major breakthrough was the addition of a decarboxylase inhibitor (benserazide) to L-DOPA preparations, which decreased the peripheral side effects. Cotzias succeeded in 1969 in treating patients by administering 4–16 g/d of L-DOPA orally. In most countries the ratio of L-DOPA to decarboxylase inhibitor is 4:1 and a dosage of at least 75 mg is needed to completely inhibit peripheral decarboxylase. Thus, in some patients who receive, for instance, only 3×50 mg of L-DOPA, there might be not enough inhibition of this enzyme.

Most neurologists still consider L-DOPA as the gold standard for IPD treatment. This would imply that L-DOPA is highly effective and causes not many side effects; but this is no longer true, since studies with DA agonists have shown that in initial phases of the disease DA agonists like ropinirole are as effective as L-DOPA. In spite of the good tolerability and efficacy L-DOPA does not help against freezing, falls, malfunction of the autonomic nervous system, or psychiatric symptoms such as depression, anxiety, or dementia.

Meanwhile, many open and double-blind studies have underlined the effectiveness of L-DOPA. Besides the normal formulation there are soluble and slow-release preparations available. The soluble tablet starts to work 15–30 min after intake compared with 45–90 min with the standard formulation and with 50–150 min with the slow-release formulation. Patients with early-morning akinesia particularly appreciate the speed, but patients with wearing-off and swallowing problems also benefit. We sometimes treat patients with only soluble L-DOPA since it has the same duration of action as the standard formulation *(54)* and causes no higher cmax (the minimal effective concentration is 1000 ng/mL in plasma). In a cross-over study with 13 patients, Ziegler and colleagues showed that a switch from standard to soluble L-DOPA shortened the off-periods in 10 patients when exchanged 1:1 *(55)*. The soluble formulation is best used in the morning as an adjunct to the standard formulation or during the day to the slow-release formulation. Stocchi et al. showed that the combination of soluble and slow-release formulations is a very efficient treatment for wearing-off *(56)*. Their patients presented with an improvement of motor function. Another advantage of the soluble formulation is its use for diagnosis of IPD. Patients who are older than 50 yr of age take 200 mg soluble L-DOPA and the Unified Parkinson's Disease Rating Scale

(UPDRS) is used before and 30–60 min after drug intake. If there is an improvement of at least 30%, the diagnosis of Parkinson's can be made.

The slow-release formulation acts for about 6 h. Rinne reported in 1990 on 40 *de novo* patients whom he treated with standard or slow-release formulation *(57)*. He selected a 15% higher dosage for the slow-release formulation, but this might still be low, since in our experience, the slow-release formulation shows only 60% effectiveness compared to the standard formulation. The study lasted 2 yr and Rinne showed that the slow-release formulation caused fewer fluctuations and less dyskinesia compared to the standard formulation. Kinnunen and colleagues *(58)* supported these findings when they showed a significantly lower occurance of fluctuations after a study period of 3 yr when using slow-release formulation. Dyskinesia was the same in both groups. Koller and coworkers *(59)* conducted a double-blind multicenter study, again comparing standard with slow-release L-DOPA for 5 yr. None of the patients had ever received any dopaminergic drug. Patients were in Hoehn and Yahr grade I to III and between 30 and 75 yr of age. A total of 618 patients were screeened and 306 were randomly recruited for the standard and 312 for the slow-release formulation. After 5 yr 60% of patients were still being studied. At the end of the study patients took 426 mg/d of standard L-DOPA formulation and 736 mg/d of slow-release formulation. Koller notes that the latter dosage is equivalent to a bioavailability of 510 mg/d. Motor fluctuations occurred after 5 yr in 20.6% of the patients with the standard formulation and in 21.8% with the slow-release formulation. According to the patients' diaries both formulations resulted in 16% motor complications. The slow-release L-DOPA group was slightly better with respect to activities of daily living and cognitive function, which may be explained by the higher dosage compared to standard formulation. It is of special interest that after 5 yr only 20% showed motor complications, which contradicts other studies in which after 5 yr up to 50% of patients had developed motor complications *(60,61)*. The reason for this might be that in this study, the dosage of L-DOPA was relatively low. After 5 yr 20% of all patients were still taking their initial L-DOPA dose, which underlines the fact that low dosages do not cause motor complications. Each group suffered limited side effects and only 9% of patients dropped out in each arm. Up to 30% of patients complained of nausea, whereas dizziness was reported by 10% and minor psychiatric complications. These data again support the good tolerability of L-DOPA.

Another application of slow-release formulations is their use overnight to avoid early-morning dystonia or akinesia. Both a German study group *(62,63)* and an English one *(64)* were able to show that patients with nightly motor fluctuations presented with better sleep quality after switching to a slow-release formulation. Patients could turn better in bed, had fewer cramps and less dystonia, and used fewer sleeping pills. However, to avoid early-morning dystonia sufficient dosage has to be applied. This is applicable only if patients do not develop hallucinations or peak-dose dyskinesia after high doses. A point of concern is the unpredictable resorption and reponse when slow-release preparations are used during the day. There is a lot of interference with food, which causes unpredictable differences in resorption.

Typical side effects of L-DOPA are nausea, dizziness, and psychiatric complications such as hallucinations. Contraindications are pregnancy, breast-feeding, and age of less than 25 yr. Although L-DOPA is considered to be very effective and especially helpful in older patients with various diseases it holds some problems. The early and high-dose use

of L-DOPA results in a high percentage of dyskinesia, motor fluctuations and psychiatric complications. Cedarbaum et al. *(61)* reported that 45% of patients suffered dyskinesia after 5 yr of L-DOPA use, 66% after 10 yr and 88% after 15 yr. Kostic and colleagues showed that young patients are especially prone to developing dyskinesia *(60)*.

There are anecdotal reports on so-called priming, which means that patients younger than 40 yr of age after having taken a single dose of L-DOPA develop dyskinesia years later following another application. This explains the slogan "low and slow" recommending the use of L-DOPA as late as possible, as low as possible, and as high as necessary *(65)*. In our view this recommendation does not result in a disadvantage for our patients, because nowadays we have more alternatives, such as DA agonists, which are effective and have very few motor side effects. At later stages all of our patients receive L-DOPA.

Although there is a controversy over the possibility of L-DOPA toxicity (e.g., ref. *66*) the physician has to wonder how L-DOPA functions when almost all dopaminergic neurones in the substantia nigra are gone. Hirsch and coworkers *(67)* have shown that glia can convert L-DOPA to DA and certainly detoxify at least some of the radicals produced by DA degradation.

In conclusion L-DOPA is a very beneficial drug for the treatment of IPD. It is, however, of the utmost importance to be careful with respect of early use and high dosages.

2.2. *Catechol-O-Methyl-Transferase Inhibitors*

A decarboxylase inhibitor (benserazide, carbidopa) is added to all L-DOPA preparations to avoid degradation of levodopa to dopamine in the gut and blood. Before Birkmayer invented the decarboxylase inhibitors, this degradation of L-DOPA used to cause serious side effects. There is, however, a second pathway that causes conversion of levodopa to dopamine in the gut via catechol-*O*-methyl-transferase (COMT), and until now this pathway was neglected. Thus, the invention of two COMT inhibitors, tolcapone and entacapone, was a major improvement in the avoidance of L-DOPA degradation in the gut and in this way allows an increase in the amount of L-DOPA passing through the blood–brain barrier. COMT inhibitors thus lead to a longer stimulation of the dopamine receptors (better area under the curve) without a major increase in c max. Entacapone, for instance, extends the plasma half-life of L-DOPA by 75% and improves the area under the curve (time and extent of action of L-DOPA at the dopamine receptor) by 48%. Initially two COMT inhibitors were available but tolcapone was withdrawn in the EU in 1998 because of lethal liver failure in some patients. In the same year entacapone, which functions exclusively peripherally, was licensed. It is a reversible COMT inhibitor. Its absorpiton time is about 45 min. Each tablet contains 200 mg of entacapone, a concentration that led to 60% inhibition of COMT in red blood cells. There are several big international studies that have demonstrated improvements with this medication, especially in on-periods and avoidance of wearing-off. The Scandinavian Nordic Multicenter Entacapone COMT study included 85 patients with entacapone and 86 with placebo in addition to L-DOPA *(78)*. Disease duration was about 10 yr and most patients had been treated with L-DOPA for about 8 yr and experienced motor fluctuations for about 4–5 yr before entering the study. Mean L-DOPA dosage was 700 + 300 mg L-DOPA per day. After 24 wk of addition of 200 mg entacapone to each tablet of L-DOPA there was a reduction in off-time from 5.5 to 4.2 h/d and a concomittant increase in daily on-time from 9.3 to 11 h. Similar results were obtained by the American study *(79)*. In a more recent study *(80)* the efficacy

and safety of entacapone, used as an adjunct to L-DOPA, was demonstrated in a double-blind trial that included 301 PD patients, the majority of whom had motor fluctuations. Thus, entacapone is safe and efficacious in patients who present with motor fluctuations, such as wearing-off. Typical side effects are coloring of the urine (red color), diarrhea, and dopaminergic side effects such as nausea. Liver problems with increased transaminase activity do not seem to occur with entacapone therapy. Because of its short half-life, it has to be administered with each L-DOPA tablet.

More recently, a tablet combining L-DOPA, entacapone, and the decarboxylase inhibitor has been developed and is marketed (Stalevo™).

2.3. Selegiline

Selegiline is an irreversible MAO-B inhibitor that slows down the degradation of DA, which can therefore act longer at the DA receptor. Selegiline is a very potent neuroprotective substance both in animals treated with MPTP and in cell culture. It leads to an induction of radical scavangers, is itself antioxidative, and inhibits apoptosis. Besides MAO-B inhibition, selegiline also inhibits DA reuptake, and inhibits presynaptic dopaminergic autoreceptors, thus causing increased production and release of DA. Whether or not selegiline is neuroprotective in PD patients is open to debate (81). In the initial Deprenyl and Tocopherol Antioxidative Therapy for Parkinson's Disease (DATATOP) study report *(82)* a delay in the onset of disability in early-onset PD patients, untreated except for receiving selegiline was demonstrated, and was cited by some as proof of neuroprotection. Meanwhile, an extension of the DATATOP study revealed no superiority of deprenyl treatment with respect to the end point of disability requiring L-DOPA, suggesting that the initial advantages of deprenyl were not sustained *(83)*. Nonetheless, there are indications from a study by *(84)* that examined the long-term effect of selegiline on the progression of PD. One hundred and sixty-three patients were treated with L-DOPA and benserzide, combinded with selegiline or placebo for 5 yr in a double-blind randomized protocol followed by a 1-mo wash-out of selegiline or placebo. Results indicated that patients who were treated with both L-DOPA and selegiline developed markedly less severe parkinsonism and required lower doses of L-DOPA during the 5-yr study period than patients with L-DOPA and placebo. There was no trend toward worsening during wash-out among patients previously treated with selegiline, which may not be explained by a pure symptomatic effect of selegiline.

Unequivocal results of published studies demonstrate that selegiline can delay the beginning of L-DOPA therapy by 9 mo *(85)* and that it reduces the amount of L-DOPA necessary for good symptomatic treatment *(86,87)*. The latter is especially true in *de novo* patients with Hoehn and Yahr L-DOPA-induced motor fluctuations occur less in selegiline treated patients and there might even be a reduction in the occurance of the freezing phenomenon. Based on a study by *(88)* most physicians administer 1 mg selegiline per 10 kg of body weight. Typical side effects are fatigue, dizziness, constipation, restlessness, dry mouth, depression, nausea, sweating, orthostatic problems, anxiety, and palpitation. Lees et al. *(89)* have reported that selegiline may cause serious cardiac problems, a finding that was not accepted by the rest of the PD specialists (e.g., ref. *90*).

A new formulation of selegiline, which allows sublingual resorption within seconds and avoids first-pass effect in the liver *(91)*, is an innovative development. This allows a dose reduction to 1.25 mg and produces 90% less metabolites. Up to now there are no comparative studies with selegiline.

Finally, rasagiline, which is a further MAO-B inhibitor, will be licensed within the near future in several countries. Compared to selegiline it lacks tyramine sympathomimetic potentiation and amphetamine-like metabolites, which could be a therapeutic advantage *(92)*. The Parkinson Study Group just recently *(93)* published a study in which they tested the safety and efficacy of rasagiline in a multicenter, 26-wk, parallel-group, randomized, double-blind, placebo-controlled trial. They included 404 early-PD patients not requiring dopaminergic therapy. Patients were randomized to rasagiline dosages of 1 or 2 mg per day or matching placebo. Compared to placebo, both dosages of rasagiline resulted in a –4 units improvement in the UPDRS. There was no difference with respect to side effects, underlining the safety of this new drug. Further studies have to be performed to test the effects of rasagiline in long-term treatment.

2.4. DA Agonists

DA agonists are drugs that directly stimulate the DA receptors, thus mimicking DA. Whereas L-DOPA has to be metabolized to DA in dopaminergic neurones in the substantia nigra, DA agonists don't have to be metabolized before action. They can be subdivided into ergoline and nonergoline derivatives. Apomorphine, ropinirole, and pramipexole are nonergoline derivatives whereas cabergoline, bromocriptine, lisuride, pergolide, and α-dihydroergocriptine are ergoline derivatives. There are at least five subtypes of DA receptors, which are named D_1, D_2, D_3, D_4, and D_5. D_1 receptors are located in the striatum (direct pathway), the nucleus amygdala, accumbens, and tuberculum olfactorium. D_2 receptors are found in the striatum (indirect pathway) and seem to be most important for locomotion. D_3 receptors are found in the limbic system and might be important for emotion, motivation, and cognition. D_4 receptors are also linked to neuropsychiatric relevant functions in the prefrontal cortex and hypothalamus. Finally, D_5 receptors are also located in the hippocampus, prefrontal cortex, and hypothalamus; their function is still unknown. The DA agonists that are available differ in their affinity to DA receptor subtypes. Though all of them stimulate D_2 receptors, cabergoline, apomorphine, and particularly pergolide stimulate D_1 receptors. Up to now it is not known whether this is an advantage over DA agonists, which don't stimulate D_1 receptors. It is also unknown whether the effectiveness of pergolide in patients with urinary incontinence stems from this D_1 stimulation *(94)*. We have recently shown that the D_3-stimulating pramipexole shows an antianhedonica and an antidepressant function in PD patients *(95)*. It is commonly agreed that ergoline derivatives may cause Raynaud syndrome, lung fibrosis, and erythromelalgia. In addition, we would not recommend using those drugs in patients with coronary heart problems.

For most physicians these considerations are not as important as the effectiveness and tolerability of these drugs. Common side effects include hypotension, nausea, orthostatic problems, and sometimes psychosis and hallucinations in the elderly. Frucht et al. *(96)* have for the first time described the occurrence of so-called sudden sleep attacks in patients who took pramipexole or ropinirole. New data indicate that all dopaminergic drugs (including L-DOPA) are associated with an increase in daytime somnolence but that there might be a slightly higher incidence of this with nonergoline derivatives (survey of the German Parkinson Lay Organisation). Effectiveness is also a matter of debate since there are no good comparative studies among the DA agonists. Thus, it is difficult to decide whether pramipexole shows the best action against tremor *(97,98)*, since other agonists such as ropinirole have also shown their effectiveness with respect to tremor

(99) in the meantime. The so-called equivalence doses for L-DOPA and DA agonists, which is a prerequisite to compare these drugs, are also a matter of debate.

In recent years, the concept of continous DA receptor stimulation has been used to explain why DA agonists cause almost no dyskinesia whereas L-DOPA with its plasma half-life of 2 h causes dyskinesia in 50% of patients after 5 yr *(60)*. For this reason cabergoline, which has a plasma half-life of 68 h, might be advantageous. Finally, physicians are very fond of DA agonists with an easy and fast titration scheme, which is especially true for pramipexole and cabergoline.

A matter of debate has been the potency of DA agonists in comparison to L-dopa treatment, which is still considered by many as a "gold-standard." In a double-blind, randomized study Rascol and colleagues *(100)* compared L-DOPA with ropinirole monotherapy and not only showed once again that L-DOPA caused dyskinesia in 50% percent of patients after 5 yr and that 30% percent of patients could stay on monotherapy with this agonist, but also that in the Hoehn and Yahr stages I–II.5, outcome (improvement of motor symptoms in UPDRS III) was equal between L-DOPA and ropinirole. Meanwhile, studies involving other agonists have also shown that long-term treatment with agonists prevents dyskinesia.

Neuroprotection is an important issue because all DA agonists have been shown to be neuroprotective in tissue culture and animal models (e.g., refs. *101,102*). It is somewhat problematic to establish studies in humans to evaluate neuroprotection. The best methods available seem to be imaging techniques such as single photon emission computerized tomography (SPECT) and position emission tomography (PET). Recently, two studies addressed the question whether L-DOPA or a DA agonist might better protect neurons from the disease process by the use of SPECT and PET. Marek and colleagues used the DAT SPECT method and demonstrated that pramipexole is superior to L-DOPA by about 35% after 2, 4 *(103)*, and 5 yr (unpublished data). Whone et al. *(104)* published data from a 2-yr double-blind study in which they compared L-DOPA with ropinirole by use of Fluoro-DOPA-PET and again it was shown that the agonist was superior to L-DOPA with respect to decrease in Fluoro-DOPA-signal by 35%. These two studies raised a very controversial debate on the question as to whether neuroprotection with DA agonists is on the horizon. Unfortunately, ethical reasons forbid the comparison of these two active arms with a placebo arm. It is still not certain whether drugs influence PET and SPECT and particularly to what extent these data reflect the supposition that neurons that appear to function well correlate with the number that are still alive.

Taken together, DA agonists have some distinct advantages over L-DOPA in PD treatment. They do not require carrier-mediated transport for absorption or entry into the brain; they act directly at the DA receptor without prior metabolizing or storage; they are not stored in the dying neurons of the substantia nigra and thus don't cause an increase in oxidative stress. They have better plasma half-lives, which allows continous dopaminergic stimulation and prevention from dyskinesia. Lastly, some of the DA agonists can be administered in a patch or parenterally.

2.5. NMDA Receptor Antagonists

2.5.1. Amantadine

Amantadines have experienced a real renaissance in the treatment of IPD. This is based on the fact that their mode of action has been clarified and that new data have indicated good symptom control and dyskinesia prevention. As explained, the mode of

action is the antiglutamatergic function *(68,69)*, which prevents influx of calcium ions in the small spiny neurons located in the striatum. A Harvard physician, Bob Schwab, listened in 1969 to a patient who told him that she had taken amantadine hydrochloride to protect her from influenza *(70)*. After winter she had stopped the intake of amantadine to recognize only then that this drug had considerably improved her parkinsonian symptoms. One year later Schwab had treated more than 150 patients suffering from IPD with this drug. His findings soon were repeated in Germany and Austria by Fünfgeld and Danielczyk, respectively. The latter also reported that amantadine sulfate is an excellent drug to act significantly against akinetic crises *(71)*. In different countries either amantadine hydrochloride or amantadine sulfate is available. In our hands, amantadine sulfate is a potent drug for correcting the mismatch between DA and glutamate. It has a half-life of 10–30 h and normally 100 mg twice daily is sufficient for early phases of the disease.

Danielczyk *(72)* and Uitti et al. *(73)* have reported that patients who were treated with amantadine have a better life expectancy than those who were not treated with the drug. Uitti's study was retrospective, which limits its value, but nonetheless it may be useful to check for neuroprotection by initiating a PET study. As indicated above early phases of IPS are sufficiently treated with 100 mg twice a day, but in later phases 200 mg three times a day or 150 mg four times a day may be necessary. Higher dosages are not advisable since they may cause seizures.

Amantadine sulfate is the most potent drug in cases of akinetic crisis. Normally 200 mg amantadine sulfate in 500 mL NaCl solution are administered intravenously over 3–4 h. A maximum of six infusions can be given per day. Most patients improve, however, if one infusion of 200 mg amantadine sulfate is administered daily for 3–5 d. The infusion should last at least 3 h to avoid concentrations that are too high and might then cause side effects such as hallucinations. In case of kidney disfunction, lower concentrations are advisable.

A relatively new field is the application of amantadine for treating L-DOPA-induced dyskinesia. Its effectiveness has been demonstrated in both animal and clincal studies. Rajput et al. *(74)* and Verhagen-Metmann *(75)* presented controlled studies in which 200 mg amantadine per day significantly improved dyskinesia. Rajput included 19 patients with hyperkinesia of whom 13 were found to have choreatic peak-dose dyskinesia. Fourteen out of 19 patients improved within 2 wk with respect to dyskinesia and seven also improved with respect to motor symptoms. Verhagen-Metmann et al. *(75)* achieved a decrease of 60% in peak-dose dyskinesia, which was still the case when patients were analyzed again 1 yr later. The rate of dyskinesia was decreased by 56% and the on-time was significantly prolonged.

Typical side effects of amantadine are sleep disturbance, nervousness, general restlessness, nausea, loss of appetite, livedo reticularis and ankle edema, optic hallucinations, and occasionally seizures and supraventricular tachycardia. To avoid delirium, amantadine has to be tapered off slowly. In most instances amantadine can be combined successfully with all other antiparkinsonian drugs, the only exception being budipine. Simultanoeus use of amantadine and anticholinergics may cause hallucinations.

2.5.2. Budipine

As indicated earlier, it would be too simple to consider IPD as being caused only by a dopaminergic deficit. The glutamatergic and cholinergic systems are overactive and there are also impairments of the serotonergic, noradrenergic, and other systems. In contrast to most other antiparkinsonian drugs, budipine is a so-called "dirty drug," which means that

it acts on many transmitter systems. Budipine has anticholinergic effects that are weaker than those achieved by classical anticholinergic drugs, such as bornaprine and others. In addition, it causes the secretion of DA from presynaptic vesicles, is a reversible inhibitor of MAO-B, and corrects adrenergic and noradrenergic dysfunction. The most important mode of action of budipine seems to be its antiglutamatergic effect *(76)*.

Budipine passes the blood–brain barrier, is metabolized by hydroxylation, and is excreted both in urine and feces within 24 h.

In Germany budipine has been licensed since 1997 for combination therapy in patients without motor fluctuations. Initially, most neurologists concentrated on the antitremor effects of budipine since it shows anticholinergic effects and diminished tremor in animal models of PD. So far, there are two double-blind studies with budipine. One study (FK 004) was created to study the effectiveness and safety of budipine *(77)*. Budipine was administered in doses between 40 and 60 mg/d in 99 patients with inital IPD who had previously taken either L-DOPA or bromocriptine. After 16 wk of treatment there were improvements not just in tremor (using the Columbia University Rating Scale) but also in rigidity and hypokinesia. The positive effect of budipine was not related to the age of the patients or the dosage of L-DOPA. These data imply that the addition of budipine to so far well-adjusted patients may still bring some benefit for the patients owing to its antiglutamatergic action. Unfortunately, there are no data on neuroprotection because we believe that the use of budipine should be in early stages of IPD.

In light of these results it was desirable to check for the use of budipine in monotherapy in the initial stages of IPD (study 290191). Again, 40–60 mg of budipine were administered. After 6 mo 55% of patients who had received placebo needed L-DOPA whereas in the budipine arm only 25% needed L-DOPA treatment. In an open study with 2234 patients, the safety and tolerability, as well as the effectiveness of budipine was demonstrated (unpublished data).

In general budipine is tolerated well. It is important to build up budipine dosage fairly slowly. It is best to increase the dose by 10 mg/wk, to use no more than 60 mg/d and to split the dose into three smaller doses per day. Some patients need up to 6 wk before they show improvement after sufficient budipine treatment. We had some patients who showed optimal benefit only after 16 wk of treatment. The slow increase is important because hallucinations occur if the drug is escalated too quickly. Reducing or stopping budipine treatment stops hallucinations.

In 1999 there were isolated cases of patients who experienced QT prolongation in electrocradiogram (ECG) and tachycardia in form of "torsades de pointes" after budipine application. QT prolongation is usually harmless, but if certain drugs are coadministered it could become dangerous. There are therefore strict rules on how to use budipine, meaning that personal allowance to prescribe the drug is necessary. ECGs are warranted before and during budipine therapy. If patients complain about palpitations, dizziness, or syncopes, budipine should be stopped.

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Dopamine and Glutamate in Motor and Cognitive Symptoms of Parkinson's Disease

Werner J. Schmidt

1. INTRODUCTION

Parkinson's disease (PD) is characterized by motor and cognitive deficits. The main motor symptoms of PD are on the one hand hypokinetic signs, such as bradykinesia, akinesia, rigidity, and loss of postural reflexes, and on the other hand hyperkinetic signs, such as tremor, (*see* Chapter 21; ref. *1*). Cognitive deficits in nondemented PD patients comprise deficits in sensitivity to reward *(2)*, deficits in procedural and habit learning *(3)*, and deficits in switching arbitrarily from one behavioral activity to another, possibly owing to a deficit in attentional set shifting *(4)*. It has even been argued that PD patients are deficient in so-called executive functions of human mind *(5,6)*. "Executive function is a neuropsychological construct that has been used to capture the highest order of cognitive abilities," e.g., flexibility of thought in the generation of solutions to novel problems *(7,8)*. The reason why the cognitive aspects are not well perceived may be that in humans, the cognitive functions in PD are mainly unconscious; they are thus not experienced and cannot be explicitely communicated. Another reason that renders analysis of PD symptoms so difficult is that the diseased human brain is able to use (cortical) loops to bypass or compensate for basal ganglia (BG) dysfunctions, however, with the disadvantage of a loss of parallel processing and reduced velocity.

PD is a neurodegenerative disease that affects, not exclusively, but mainly the BG (*see* Chapters 20 and 21). Therefore, this chapter will focus on the BG-mediated deficits in PD. Traditionally, BG have been considered to subserve the generation of simple motor behavior and supportive postural control. As it was the case with PD, this traditional view also has changed in recent years. A closer look at the role that BG play shows that BG have no direct output to spinal motor neurons and thus are not involved in some direct motor control. They are "several synapses away" from the motor output and this makes analysis of their functions so difficult. Furthermore, it became increasingly clear that BG are involved not only in generation of primitive motor activity, but also in the generation and selection of higher order motor programs. Further, BG represent the substrate for extraction of reward information from a large variety of stimuli and events *(9)*; they are also the site of representation of unconscious egocentric (body-centered) orientation

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

as well as the substrate for implicit forms of learning such as incentive and procedural learning *(10,11)*.

These "higher" or cognitive functions of the BG will be addressed in this chapter and an attempt will be made to relate them to the BG transmitters dopamine (DA) and glutamate (GLU).

2. THE BASAL GANGLIA

2.1. The Action Selection Model of BG Functions

The functional substrate that mediates information processing through the BG is represented by the two main pathways (loops): the direct pathway from striatum via substania nigra pars reticulata (SNr) to the thalamus and the indirect pathway from striatum via Globus pallidus external segment (GPe), subthalamic nucleus (STN), and globus pallidus internal or medial segment (GPi) to the thalamus (*see* Chapters 20 and 21). A cortical signal, when processed through the direct pathway disinhibits the thalamus; through the indirect pathway it inhibits the thalamus. Thus, the direct and the indirect pathway have opposing effects on thalamic activity. The action selection model postulates that, by way of this dual influence on the thalamo–cortical projection, the BG are able to inhibit "unwanted" behavior and to disinhibit (facilitate) "wanted" behavior. This means that the BG are able to evaluate a stimulus–reaction chain and to decide, on the basis of past and present, internal and external conditions, which behavior is wanted and which is unwanted. Indeed, the neurons of the main input structure of the BG, the spiny I cells of the striatum, are characterized by their pronounced context-dependency. This means that the neuronal network of the striatum is able to represent specific contextual memories for past and present, internal and external events *(12)*. This "action selection hypothesis" of BG functions is most attractive and postulates that the BG act to select a behavior and inhibit competing behaviors that would otherwise interfere with the "wanted" behavior *(1)*. This action selection hypothesis nicely conforms to what was proposed by Hassler many years ago *(13)*.

3. DOPAMINE

DA is a powerful modulator of the direct and the indirect pathway of the BG, primarily at the level of the striatum (caudate-putamen) *(14)*. In PD, dopaminergic neurons in the SNc degenerate and this results in a reduction of DA concentrations in the SNc and in the ventral tegmental area (VTA), but mainly in the projection area in the striatum and, to a lesser extent, in the nucleus accumbens and prefrontal cortex. A loss of DA in the striatum results in reduced activation of D1 receptors on the spiny neurons, giving rise to the direct pathway, and in a loss of inhibition by D2 receptors on spiny neurons, giving rise to the indirect pathway. Because these neurons also receive an excitatory glutamatergic input from the cortex, the glutamatergic tone predominates and they became overactive. GLU, *N*-methyl-D-aspartate (NMDA) receptor antagonists infused directly into the striatum are able to block this neuronal hyperactivity *(15)*.

In PD, the loss of DA results in an inhibition of thalamic nuclei and in turn to a reduced output of the BG to the brainstem and to the cortex. The discovery that in PD there is a reduced Bereitschaftspotential above the supplementary motor area *(16)* conforms to this view. The reduced outflow form the BG is considered as a main factor leading to akinesia in PD.

Table 1
Stability of Behavior Against Dopamine Loss

With decreasing dopamine-activity, behaviors are affected in the following order:
– spontaneous, internally generated behavior
– conditioned behavior
– externally guided behavior
– fixed action patterns elicited by key stimuli

3.1. DA and Akinesia

Bradykinesia and akinesia, the cardinal symptoms of PD, can be induced in any mammal and even in submammalian species, by reducing DA activity in the BG. Experimentally, systemic DA hypofunctioning can be achieved by various drugs that reduce DA concentrations (reserpine, α-methyl-P-tyrosine) or by drugs that block DA receptors (haloperidol and other neuroleptics). Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to monkeys destroys dopaminergic neurons and is widely used as an animal model for PD. Recently rotenone has been shown to destroy, with some selectivity, dopaminergic neurons and produce parkinsonian symptoms *(17)*. Selective DA loss in specific brain structures can be achieved by infusing the neurotoxin 6-hydroxydopamine (6-OHDA) directly into the respective structures.

All these treatments induce akinesia and bradykinesia; however, it has been known for a long time that there is no global unselective inhibition of motor behavior, but rather a selective pattern of inhibition of behavior. From animal experiments and from observations in PD, the following generalizations may be deduced (Table 1).

With decreasing DA activity, spontaneous, internally guided behavior, is the first to disappear whereas externally guided behavior can still be elicited. For example, an untreated lactating mouse, sitting on its litter in the nest, exhibits several spontaneous activities and reacts to the calls of pups that have fallen out of the nest, with pup retrieval behavior; i.e., the mouse searches for the pup and takes it back to the nest. If such a mouse was treated with the DA receptor-blocking drug haloperidol, the mouse became completely akinetic and exhibited no spontaneous activity. However, if the mouse heard the call of a pup, the akinetic mouse was able to immediately leave the nest to search for and retrieve the pup *(18)*. Several anecdotal reports from parkinsonian patients are in line with these findings: Strong external stimuli, for example, the outbreak of fire in a room, can elicit a coordinated flight in previously akinetic patients (kinesia paradoxa). With decreasing DA activity also a learned behavior is more liable to disappear than stimulus elicited behavior *(19)*.

From these considerations it may be concluded that parkinsonian patients do better in an environment that guides them completely by external signs or instructions. Indeed, this has been demonstrated: The daily life performance of PD patients improved remarkably when they were guided by declarative sequences of instructions for components of movements *(20)*.

3.2. DA and the Action Selection Model

There is much evidence that DA is crucially involved in action selection. Increasing the DA activity enhances the behaviors that an animal exhibits *(19,21)*. It enhances the

occurrence of behaviors that compete with ongoing behavior (i.e., collateral behaviors) and therefore increases the rate of switching from one behavior to another *(22)*. If, by increasing DA activity, a maximal switching rate has been achieved, a further increase in DA activity results in stereotypies that are performed in a high frequency *(21)*.

A gradual reduction of DA activity gradually strengthens the suppression of behaviors that compete with ongoing behavior *(23)* and as a consequence the rate of switching from one behavior to another gradually declines *(22)*. For example, the innate predatory behavior of ferrets, which is very stable against DA depletion, is executed in a much more focused manner when the ferrets were treated with neuroleptic drugs, since under reduced DA acitivity, behaviors that compete with predation are rather selectively suppressed *(19,23)*. With further (nearly complete) reduction of DA activity, however, predatory behavior also was suppressed and the animals became akinetic.

A DA deficit impairs the ability to switch from one behavioral activity to another arbitrarily, i.e., in the absence of an external stimulus. Indeed, a switching deficit could be predicted from the statements above. The so-called switching deficit has been extensively studied in animals *(22)* and has been shown in humans suffering from PD *(4)*. A definition of PD by the World Federation of Neurology takes account of this in defining PD as "a disorder characterized by poverty and slowness of initiation and execution of willed and associated movements and difficulty in changing one motor pattern to another in the absence of paralysis." The switching deficit in PD clearly appears to be DA-dependent since the magnitude of the switching deficit was related to the degree to which levodopa (L-DOPA) ameliorated the patients' motor response *(24)*. More recent studies clearly show that the switching deficit not only impairs the switching from one motor activity to another; the switching deficit has been shown on several levels of behavioral integration—a deficit in switching of cognitive strategies *(4)* and of attention has been shown *(25)*.

3.3. DA and Reward

The experience of reward seems to be changed in PD; rewarding events are less rewarding for these patients and this correlates with less activation in the striatum *(26)*. There are also reports that rewarding drugs are less rewarding to parkinsonian patients *(27,28)* and, therefore, these patients are supposed to be less liable to develop addiction *(29)*.

A sound concept of reward (*see* Chapter 14), primarily based on animal studies but increasingly extended to humans, has emerged. In the frame of this concept, primary reward can be thought of as the motivational value that an organism attributes to an event (for a discussion of reward and reinforcement *see* ref. *30*). The attribution of reward to behaviours such as feeding drinking, grooming, and so on, ensures the survival of the individuum. The attribution of reward to behaviors such as courtship, nest building, sex, maternal or parental behavior and social behavior ensures the survival of the species.

There is extensive pharmacological and electrophysiological evidence (for review, *see* ref. *12*), that DA plays a crucial role in the mediation of reward. Indeed, the dopaminergic neurons originating in the VTA and projecting to the nucleus accumbens are considered to constitute a scalar, global reward signal that is broadcast to the majority of postsynaptic neurons. However, reward signals not only reach the nucleus accumbens but also the dorsal striatum and the other dopaminergically innervated structures in the BG and in the prefrontal cortex. A reduced DA activity abolishes the rewarding properties of rewards and of stimuli that announce reward. Therefore, reduced DA activity results in

Table 2
Parallel Learning Systems Building Up Memories

Explicit memory	Implicit memory
Knowledges	Motor, cognitive, perceptual skills, sensitization
Adaptive	Rule-like, habits
Rapid extinction and forgetting	Very resistant against extinction and forgetting
In humans	
Conscious	Unconscious
Declarative	Nondeclarative
Structures involved	
Hippocampus cortex: temporal lobe	Basal ganglia

extinction- like decrease of rewarded behavior; for example, under reduced DA activity, lever pressing for food reward is not immediately abolished, but the frequency of lever pressings declines in an extinction-like fashion *(10,31)*.

The dopaminergic reward signal has a very unique characteristics: it depends on the unpredictability of the rewarding stimuli or of the stimuli associated with reward. Predicted reward, or predicted stimuli that announce a reward, do not activate the dopaminergic neurons *(12)*. Recent studies are well on the way to elucidating how neurons in the caudate nucleus transform expected reward into a spatially selective behavior *(32)*.

3.4. DA and Learning

From animal experimentation evidence has accumulated that there are parallel learning systems in the brain that are clearly separated (for review, *see* ref. *33*). This concept seems to also be verified in the human brain. Two main parallel learning systems in the brain are the explicit (declarative) learning and implicit (nondeclarative) learning (*see* Table 2). Whereas explicit learning is cortically (including hippocampus) mediated and is experienced consciously in humans, implicit learning is mediated by the BG and is unconscious. Implicit learning refers to learning how to do a task in the absence of knowledge of what has been learned. Forms of implicit learning are incentive learning, procedural and stimulus–response habit learning, and sensitization. Also, association of a context to drug effects that eventually cause placebo effects is a form of implicit learning. Procedural- and habit-learning deficits have been reported for PD patients *(3)*. In-depth research on these forms of learning clearly attributed them to BG (for review, *see* ref. *34*). Knowlton et al. *(3)*, who have tested nondemented PD patients and compared them to amnesic patients, pointed out that the habit-learning deficit of PD patients "is not restricted to motor aspects of behavior but also to higher aspects, such as acquiring nonmotor dispositions and tendencies that depend on new associations. These nonmotor habits presumably include a wide range of dispositions and tendencies, which are shaped by reward, specific to particular stimuli, and which guide behavior and cognition."

3.5. The Influence of Context/Expectation/Placebo on Parkinsonian Symptoms and Dopamine Release

It is known from the placebo effect that the context can play an important role. Context means here all circumstances surrounding drug administration and experience of

drug effects. The placebo effect is well documented in the field of pain. A context induces expectations these are able to activate the endogenous opioid system. Naloxone abolishes the placebo effect *(35)*. Now it seems proven that in PD patients a placebo effect also can occur: PD patients can exhibit a marked improvement in symptoms when receiving placebo treatment, i.e., if they are in a context that precisely mimics the situation under which they previously got L-DOPA *(36)*. A recent study addressed the biochemical substrate of this placebo responses. It was found that patients expecting their normal medication (L-DOPA) react with enhanced DA release when receiving a placebo *(37)*. Obviously, during repeated drug (L-DOPA) taking, an association between the context and relief of symptoms has taken place. When placebo is administered, the context induces expectations of relief of symptoms. This expectation or prediction is able to enhance DA activity that indeed counteracts PD symptoms.These findings are in accordance with electrophysiological studies in monkeys: It has been found that DA neurons are critically involved in reward predictions. A learned stimulus can activate DA neurons *(11)*.

Animal experimentation indicates that the opposite can also take place; that means that a context is able to elicit, or to worsen, PD symptoms. Upon repeated measuring of neuroleptic-induced parkinsonism in rats (catalepsy), the context—i.e., the experimental setup and the environment—is sufficient to elicit catalepsy, known as the repeated measures effect *(38)*. Also intensification of catalepsy in the rat is totally context-dependent; it develops only when the animals are tested for catalepsy daily in the same context. For example, an experimental reduction (to 46%) of DA activity was induced by 6-OHDA lesion of the SNc. This lesion induced only minimal catalepsy (akinesia and rigidity). However, daily testing of these rats (in the same context) resulted in a day-to-day increase of catalepsy and finally, after about 5–7 d, in complete akinesia and rigidity. A change of the context abolished the sensitized response *(39)*. We labeled this intensification of catalepsy "sensitization of catalepsy," because it follows in all aspects the rules that were found to govern sensitization caused by psychostimulants (*40*; for review, *see* ref. *41*). Thus PD symptoms seem to result from, to a considerable degree, sensitization of akinesia.

In PD patients the loss of DA occurs gradually with progression of the disease. Therefore sensitization of akinesia cannot directly be observed. However, from clinical medication with neuroleptics it is known that parkinsonian symptoms do not occur simultaneously with onset of DA receptor blockade, but develop gradually (sensitize) over several days of treatment. The gradual intensification of akinesia has also been postulated from a more theoretical point of view: Within the framework of the action selection model, DA is considered to be a teaching signal indicating whether a certain situation is "wanted" or "unwanted." Because of the lack of DA in PD, there is a persistent state of negative reinforcement, signaling that a certain situation/action is "unwanted" and this may gradually lead to intensification of akinesia *(1,40)*.

Overall it seems that akinesia is not a purely motor symptom of PD; the previous consideration shows that akinesia is, at least partly, built up by sensitization learning.

3.6. DA and Egocentric Orientation

The striatum seems to be a key structure for the representation of egocentrically body-centered based localization. For example, in maze tasks requiring the finding of goals relative to the rats starting point, striatally lesioned rats perform weakly *(42)*. In contrast,

if the rats had to find the goals according to cues in the room, striatally lesioned rats show no deficits. Hippocampally lesioned rats showed just the opposite pattern of results; they perform poor when they have to navigate according to spatial cues but they have no problems to orienting egocentrically *(43)*. Obviously, the caudate processes egocentric spatial working memory and this concept applies to humans too *(7,44)*. Parkinsonian patients often show orientation deficits when dependent on egocentric cues as diagnosed as disturbed perception of extrapersonal space *(45,46),* as well as amnesia for spatial locations *(47,48)*.

3.7. Genetically Altered Dopaminergic Transmission

3.7.1. Behavioral Consequences of DA-Receptor Knockout

To date, five different DA receptors have been identified and characterized. All DA receptors belong to the family of G protein-coupled or metabotropic receptors. The D1-like family comprises D1 and D5 receptors, which are positively coupled to adenylyl cyclase; the D2-like family comprises the D2, D3, and D5 receptors, which are negatively coupled (*see* Chapter 2).

A D2 knockout mouse has been created that lacks both isoforms of the receptor. This knockout mouse shows typical behavioral abnormalities such as reduced locomotion, slower movements, and abnormal gait with sprawled hind legs. A reduced intake of food and water resulted in a slight reduction of body weight. Biochemically, the knockout mouse shows an elevation of enkephalin levels in the striatum, and decrease of substance P expression, whereas the level of dynorphin expression was unaltered. Both the behavioral and the biochemical findings show striking similarities with striatally 6-OHDA-lesioned rats. All this shows the close connection between the D2 receptor and PD; this connection does not exist between the D1 receptor and PD, therefore the D2 knockout mice have already been used as animal model of PD. Further, absence of D2 receptors cannot be compensated for other members of the DA receptor's family.

Electrophysiological research on D2 receptor knockout mice supports the concept of an additional autoreceptor function of the D2 receptor: Recordings revealed that in contrast to wildtype, dopaminergic cells of D2 receptor knockout mice do not change their electrical activity when the DA agonist quinpirol or DA were administered (for review, *see* ref. *49*). In summary, research with DA receptor knockout mice strongly supports the view that PD symptoms are closely connected with reduced stimulation of DA D2 receptors.

3.7.2. Behavioral Consequences of Dopamine Transporter Knockout

In the normal physiological situation, dopamine transporter (DAT) inactivates synaptically released DA by reuptake and thus constitute a synaptic transmitter-inactivation mechanism. In DAT Knockout mice, DA persists about 300 times longer in the synaptic cleft than in normal mice. In turn, the mice exhibit hyperdopaminergic behavior such as increased locomotor behavior. Drugs that target the DAT did not work in DAT Knockout mice. In fact, the mice did not show hyperactivity to cocaine and amphetamine. By feedback mechanisms, the enhanced DA concentration reduces tyrosine hydroxylase activity and DA concentration in the dopaminergic neuron. Also the DA autoreceptor is affected: The impulse-, synthesis-, and release-regulating autoreceptor in knockout mice revealed nearly complete loss of function. All these findings may provide insight into the consequences of hyperdopaminergia (50,51).

In PD there is a degeneration of DAT and of the vesicular monoamine transporter *(52)*.

3.8. Reversal of Parkinsonian Symptoms by DA-Substitution Therapy

It is of utmost importance and interest whether or not the currently used DA-substitution therapy of PD is able to reverse the cognitive symptoms of PD. Charbonneau et al. *(53)* have tested treated PD patients for incentive learning and paired-associate learning. They showed that PD patients are deficient in incentive motivational learning but not in paired-associate learning. All patients that were tested were taking L-DOPA, deprenyl, and/or bromocriptine. This suggests that the DA substiution therapy is not able to restore incentive learning, possibly since the DA substitution is ineffective in restoring the phasic signals necessary for incentive learning to occur. Czerneky et al. *(2)* report on the existence of apathy in PD in the absence of depression and dementia. L-DOPA treatment counteracted apathy. However, the stimulus–reward learning deficit in PD was not reversed by L-DOPA. Also, deficits in reversals, i.e., deficits in flexibility in a gambling task, are not sensitive to L-DOPA.

In conclusion, the DA substitution therapy significantly counteracts the motor symptoms of PD and has significantly enhanced life quality of PD patients. However, some cognitive deficits in PD seem not to be reversed by DA substitution. The development of therapeutic concepts aimed at the restoration of motor and cognitive abilities in PD are a challenge for future research worthy of being addressed.

4. GLUTAMATE

4.1. GLU in the Basal Ganglia

GLU is the transmitter of most (perhaps all) corticofugal neurons. Thus the BG also receive glutamatergic inputs. The striatum (nucleus caudatus and putamen), which is the main input station of the BG, receives prominent inputs from prefrontal, limbic, and other cortical areas, as well as glutamateric inputs from the midline and intralaminar nuclei of the thalamus. The other BG nuclei also receive glutamatergic input from the cortex but to a minor extent. All GLU-receptor types are present in the nuclei of the BG (*see* Chapter 3) *(54)*.

4.2. GLU and Akinesia

A DA deficit, such as that found in PD, results in transmitter imbalances throughout the BG circuits. A loss of DA in the striatum results in a loss of dopaminergic inhibition of the neurons giving rise to the indirect pathway. Thus, there is a relative overactivity of GLU, rendering these neurons overactive. The overactivity of these neurons produces catalepsy in the rat, and infusions of the NMDA receptor antagonist AP-5 into the striatum counteract catalepsy *(15)*. Conversely, infusions of NMDA into the striatum of normal rats produced some akinesia *(55)*. From these findings it can be concluded that DA via D2 receptors and GLU via NMDA receptors exert opposite effects on striatal output neurons (for review, *see* refs. *56* and *57* and Chapters 3–5).

It is very well established that a DA loss results in overactivity of STN neurons projecting to the BG output nuclei GPi and SNr. This projection is glutamatergic. Reducing overactivity in the STN with GLU antagonists has been shown to counteract catalepsy in rats *(58)*. Lesioning the STN, or inhibiting its activity by high-frequency stimulation, has already been used to treat PD patients.

Because of the opposite effects that DA and GLU exert on akinesia in various BG nuclei, systemic administration of NMDA receptor antagonists should also be able to reverse catalepsy in rats. Indeed, it was found that the NMDA receptor blocker dizocilpine

(MK801) reversed haloperidol-induced catalepsy in rats *(59)*. Also, competitive NMDA receptor antagonists exert anticataleptic effects in rats. However, AMPA receptor antagonists, when given systemically, were not able to counteract catalepsy *(60)*.

In conclusion, PD, which primarily is a DA-deficiency disease, could be considered as a secondary GLU hyperactivity syndrome. Reducing GLU activity therefore can be considered as a potential therapeutical principle to treat motor symptoms of PD, alternative to the DA-substitution therapies. Only uncompetitive NMDA receptor antagonists and GLU release inhibitors have been tested so far in humans. Amantadine, memantine, and budipine have been reported to exert antiparkinsonian effects. Most of the studies with new drugs show no or only very poor antiparkinsonian effects. However, the design and sample size make it difficult to draw any final conclusion (for review, *see* ref. *60*).

4.3. GLU and Reward

The DA–GLU interaction plays a very critical role in reward mediated by the BG. GLU is a main transmitter of the so-called reward circuitry encompassing the VTA, the nucleus accumbens, and frontal cortex (for review, *see* ref. *41*). Rewarding properties for NMDA receptor antagonists have been proposed since some are self-administered by animals and since the NMDA antagonist phencyclidine (PCP) has been used as a drug of abuse. Measuring reward in the conditioned place preference paradigm reveals some rewarding effects but only in a very small dosage range (for review, *see* ref. *61*).

4.4. GLU and Learning

GLU is most important in explicit learning, which is mediated by the hippocampus–cortex system (*see* Table 2). However, this system does not seem to be primarily affected in PD *(3)*. Little is known about the role of GLU in procedural learning.

But there are some data on sensitization and on dyskinesias. It may appear somehow surprising that sensitization and dyskinesias are mentioned in the same chapter, but there are indications that both phenomena are owing to similar mechanisms of plasticity taking place in the BG *(62)*.

4.4.1. Sensitization

Catalepsy sensitizes in rats; i.e., there is experience-dependent intensification on repeated elicitation. Thus sensitization may also contribute to the severity of PD symptoms in humans. As discussed in Subheading 3.4., the sensitization of catalepsy follows precisely the rules that govern psychostimulant-induced sensitization of locomotion *(40)*. Since it has been reported that some forms of psychostimulant-induced sensitization are blocked by NMDA receptor antagonists *(41)*, it was tested whether NMDA receptor antagonists are able to block the sensitization of catalepsy too. Uncompetitive (MK801) *(63)* competitive, and antagonists with some preference for the NR2B receptor type (eliprodil and Ro25-6981) *(64)* have been tested; none of these drugs had any effect on the development of catalepsy sensitization. However, they make the expression of the sensitized component state-dependent. This means that the sensitized response was only expressed under the NMDA receptor antagonist. Thus, so far we cannot block development of catalepsy sensitization pharmacologically.

4.4.2. Dyskinesias

Progression of PD in combination with L-DOPA therapy over years eventually leads to shortenings of L-DOPA actions, which are labeled wearing-off fluctuations. Subsequently,

when the effect of an L-DOPA dose ceases abruptly and unpredictedly, the term on–off phenomenon is used. In addition to these fluctuations in motor functions, PD patients also develop involuntary movements called dyskinesias (primarily chorea and dystonia). Dyskinesias can become extremely distressing for the patients. Emanating from the fact that long-term potentiation and long-term depression occur in the striatum and that there is a GLU overactivity, Chase *(65)* demostrated that NMDA recpetor antagonists are able to normalize the shortening of L-DOPA response in rats. Amantadine too was able to attenuate response fluctuations resulting from repeated L-DOPA treatment *(66)*. In studies with monkeys, some, but not all, NMDA antagonists showed a significant antidyskinetic activity, as well as AMPA receptor antagonists *(67)*. In PD patients, the NMDA receptor antagonist dextrorphan consistently showed a very good effect against dyskinesias in several studies. Also amantadine had an impressive antidyskinetic action, which is maintained for at least 1 yr (for review, *see* ref. *68*).

Overall, it appears that increased synaptic GLU activity, leading to plastic changes in the striatum, contributes to the development and expression of dyskinesias. Reducing the striatal GLU tone with NMDA receptor antagonists may provide a promising strategy to prevent the development of dyskinesias.

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XI Huntington's Disease

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Dopamine and Glutamate in Huntington's Disease

Carlos Cepeda, Marjorie A. Ariano, and Michael S. Levine

1. INTRODUCTION

The striatum is the main input structure of the basal ganglia. It is a centrally located region where afferents from the cerebral cortex, thalamus, and substantia nigra converge and interact. Glutamate is released from cortical and, to a lesser extent, thalamic terminals *(1,2)*. Dopamine (DA) is released from nigrostriatal terminals *(3)*. Because glutamate and DA inputs terminate on the same spines of striatal medium-sized spiny neurons (MSSNs), these sites offer the potential for physiological interactions between the glutamate and DA transmitter systems *(4)*.

Glutamate and DA interactions in the striatum support major sensory, motor, cognitive, and motivational functions *(5–9)*. Alterations in DA and glutamate pathways are also important for various pathological conditions such as Parkinson's disease, schizophrenia, Tourette syndrome, and attention-deficit hyperactivity disorder, to name a few. In Huntington's disease (HD) there is a progressive loss of striatal and cortical neurons. In particular, MSSNs are preferentially affected *(10,11)*, although recent data have emphasized cortical pathology as well *(12–14)*. The mechanisms leading to selective cell loss in HD are unknown, but several hypotheses involving glutamate, DA, or their interaction have been proposed. In this chapter we are going to review recent advances in our knowledge of glutamate and DA interactions in HD. The first section provides an overview of the literature, the second highlights results from our laboratories using different mouse models of HD, the third discusses possible mechanisms of selective neuronal vulnerability in HD, and the last focuses on diverse treatments for this disease. Because of space limitations the review of the literature is not exhaustive and we apologize if relevant papers were not included.

1.1. Huntington's Disease

HD is an autosomal, dominantly inherited neurodegenerative disease pathologically characterized, as pointed out above, by neuronal loss in striatum and cortex (for review, *see* ref. *15*). The symptoms include abnormal dance-like movements (hence the name chorea), dementia, and disorders of mood, particularly depression. Its incidence varies considerably worldwide. It affects approx 1 in 20,000 people of western European descent, whereas the incidence is much lower in Asian (1 in 300,000) and African populations (1 in

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

million). In the United States about 1 in 10,000 people have the disease, with about 30,000 known cases. By comparison, in Barranquitas, a small town near Lake Maracaibo in Venezuela, the prevalence is as high as 50%. The high incidence in this population was instrumental in the discovery of the HD gene and the mutation (an expansion of CAG repeats) that causes HD *(16)*. The HD gene (*IT15*) is located on the short arm of chromosome 4. An increase in the normal number of CAG repeats (generally >40) leads to the development of the disease. HD is typically a late-onset disease although juvenile variants occur, usually when more CAG repeats are present. In young children with HD, the symptoms almost invariably include epileptic seizures *(17,18)*.

The protein coded by the HD gene, *huntingtin* (~330 kDa), is expressed ubiquitously throughout the body *(19)*. In the brain, it is predominantly found in neurons *(20)* but its function remains a mystery *(21)*. Huntingtin is a cytoplasmic protein closely associated with vesicle membranes and microtubules, suggesting it may have a role in vesicle trafficking *(22)*. Its distribution is very similar to that of synaptophysin *(23)* and it has been shown to associate with various proteins, in particular PSD95, a scaffolding protein found at the postsynaptic density that also is involved in anchoring *N*-methyl-D-aspartate (NMDA) receptors *(24)*.

The mechanism by which mutant *huntingtin* causes lethality in neurons is also unknown. In molecular terms, it has been proposed that proteins with more than 40 glutamine residues precipitate as insoluble fibers *(25)*, allowing the formation of protein aggregates. Aggregates of mutant *huntingtin* localize in the nucleus and dystrophic neurites and may be part of the pathogenic mechanisms in HD *(26)*. Neuropil aggregates appear to be more common than nuclear aggregates and are more prevalent in cortex than in striatum *(27)*. Electron microscopic studies reveal many neuropil aggregates in axon terminals, which are colocalized with synaptic vesicles suggesting they may affect synaptic transmission *(28)*. *Huntingtin* expression also overlaps with clathrin, a coat protein involved in endocytosis. DA treatment alters the subcellular localization of huntingtin and increases its expression in clathrin-enriched membrane fractions *(29)*.

Finally, an interesting yet puzzling feature of HD is the selective vulnerability of striatal projection MSSNs compared to striatal interneurons *(10)*. Furthermore, MSSNs that project to the globus pallidus (enkephalin-positive) appear to deteriorate first, followed by those that project to the substantia nigra (substance P-positive) *(30,31)*.

1.2. Glutamate and DA Receptors: Classification and Interactions

Glutamate receptors are divided into two main subtypes *(32,33)*: ionotropic receptors, which are ligand-gated channels, and metabotropic receptors (mGluRs), which primarily are coupled to various signal transduction processes generally involving G protein activation, although alternative signaling pathways have been described *(34)*. The ionotropic receptors are further subdivided into NMDA receptors and two types of non-NMDA receptors, kainate (KA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) *(35)*.

Eight mGluRs have been cloned and characterized. They are subdivided into three groups on the basis of sequence similarity, signal transduction mechanisms, and pharmacological profile group I mGluRs (mGluR1 and 5) are thought to mediate stimulation of phospholipase C and the generation of an intracellular Ca^{2+} signal, whereas group II (mGluR2 and 3) and group III (mGluR4, 6, 7, and 8) mGluRs mediate an

inhibition of adenylyl cyclase activity, and hence cyclic adenosine monophosphate (cAMP) levels *(36,37)*.

There is also diversity among DA receptors. Five different receptor subtypes have been cloned. These have been classified in two main families: the D1 family (D_1 and D_5 receptor subtypes) and the D2 family (D_2, D_3 and D_4 receptor subtypes) *(38,39)*. In this chapter, subscripted notation will indicate DA receptor subtypes and nonsubscripted notation will indicate DA receptor families.

A significant number of glutamate and DA receptors are strategically located on the spines and dendrites of MSSNs, so that glutamate inputs can be modulated postsynaptically by activation of different subtypes of DA receptors. D_2 receptors are also present on glutamate inputs to the striatum allowing presynaptic modulation of neurotransmitter release *(40)*. Finally, DA terminals can also be modulated by glutamate activity *(41)*. Glutamate and DA receptor interactions are complex and depend on a number of factors including receptor subtype, site of action, i.e., pre- or postsynaptic, timing of inputs, and concentration of neurotransmitter, to name a few (for review, *see* ref. *42*).

In the striatum, the region most affected in HD, DA modulates glutamate inputs producing either potentiation or attenuation of glutamate-evoked responses *(43,44)*. In the model we have proposed, the way MSSNs respond to cortical inputs is mainly dictated by the DA and glutamate receptor subtype preferentially activated. According to our first iteration of this model, DA via activation of D1 receptors potentiates responses induced by activation of NMDA receptors, whereas DA via activation of D2 receptors attenuates responses mediated by activation of ionotropic non-NMDA receptors *(42–44)*. The other interactions are less predictable, although there is a tendency for D1 receptors to enhance non-NMDA receptor-mediated responses and for D2 receptors to decrease NMDA responses. Subsequent studies from our laboratory and others have expanded the model to include pre- and postsynaptic actions, as well as a number of different intracellular mechanistic pathways. Studies in D_2 knockout mice revealed that glutamate release is facilitated in the corticostriatal pathway, providing electrophysiological support for the presence of functional D_2 presynaptic receptors potentially regulating release of glutamate under physiological conditions *(40)*.

As for the intracellular mechanisms involved in the potentiation of NMDA-evoked responses by DA, our studies support the view that a complex and redundant system allows the occurrence of this interaction. On the one hand, DA, via D1 receptors, enhances Ca^{2+} conductances mediated by "L"-type channels *(45,46)*. The increase in Ca^{2+} currents adds to the enhancement of NMDA currents by D1 receptors. We demonstrated this effect by showing that nifedipine, a selective "L"-type Ca^{2+} channel blocker, reduces the enhancement of NMDA currents by DA or D1 receptor agonists *(47)*. On the other hand, intracellular mechanisms involving the cAMP–protein kinase A (PKA) cascade play an important role because forskolin, an activator of the PKA cascade, also enhances NMDA-evoked responses *(48,49)*. Furthermore, this enhancement is significantly reduced in striatal neurons from DARPP-32 knockout mice *(50)*, indicating that this phosphoprotein is critically involved. Forskolin also increases NMDA-R1 subunit phosphorylation *(51,52)*. There are other potential mechanisms involved in NMDA response enhancement, as recent evidence indicates that D1 receptor activation alters trafficking of NMDA receptor subunits *(53)* and, conversely, NMDA receptor activation recruits D1 receptors into the membrane *(54)*. Finally, in striatal neurons D1 receptors are colocalized

with NMDA receptors in the postsynaptic density and they coimmunoprecipitate with NMDA receptor subunits, indicating clustering of D1 and NMDA receptors and the possibility of direct interactions, in particular with NMDA-R1 subunits *(55)*.

We tested the heuristic value of our model of DA and glutamate interactions using a cell-swelling assay *(56)* in visualized striatal neurons from young rats. Cell swelling, an early sign of excitotoxicity, is produced by prolonged bath application of NMDA or non-NMDA receptor agonists. We examined the effects of DA and selective D1 and D2 family receptor agonists and antagonists on cell swelling. Activation of DA receptors in the absence of NMDA did not produce cell swelling. However, DA and the D1 receptor agonist SKF 38393, increased the magnitude of NMDA-induced cell swelling. This effect was reduced in the presence of the D1 receptor antagonist SCH 23390. In contrast, activation of D2 family receptors with quinpirole resulted in decreased cell swelling. Quinpirole also attenuated cell swelling induced by activation of KA receptors *(57)*. These results provided evidence that DA receptors have the potential to modulate excitotoxicity in the striatum, a process that has been suggested to be responsible for cell dysfunction and, ultimately, cell death in HD. As reviewed in the next sections, there is an extensive body of literature showing that DA has a major role in striatal toxicity.

1.3. Glutamate, HD, and Energy Metabolism

The discovery that excitatory amino acids (EAAs) produced selective axon-sparing lesions prompted replication of the cell loss observed in HD. Striatal injections of KA reproduced some of the anatomical and neurochemical changes observed in HD *(58–61)*. Moreover, selective NMDA receptor agonists, e.g., quinolinic acid, better replicated the cell loss characteristic of HD *(62)*.

Based on these observations the excitotoxicity hypothesis of cell death in HD was developed. This hypothesis postulates that excessive glutamate release and/or receptor stimulation leads to striatal neuronal degeneration (*see* refs. *11* and *63*). A study using proton magnetic resonance spectroscopy in HD provided some evidence supporting the excitotoxicity hypothesis. Patients with early HD had increased glutamate in the striatum *(64)*. However, other studies found no change in glutamate levels *(65)*. There is also evidence that no overall change in glutamate receptor binding occurs in HD brains *(66)*.

The relative importance of NMDA vs non-NMDA receptors in HD has been controversial and evidence for a greater role of one or the other receptor subtype has been provided. Most evidence suggests a predominant role for NMDA receptors *(67)*. For example, glycine, a coagonist of NMDA receptors, is increased in platelets of HD patients *(68)*. However, there is also evidence to indicate that striatal cells containing non-NMDA receptors may be at least as or more susceptible to excitotoxic insults. One observation supporting this claim is that cortical and striatal cell loss is more prominent in areas enriched with non-NMDA receptors *(69)*. Finally, there also is an important role for mGluRs, particularly group I mGluRs, in excitotoxicity. Injections of group I antagonists can protect against striatal lesions produced by NMDA or quinolinate *(70)*.

Although the excitotoxicity hypothesis of cell death has provided important insights into mechanisms underlying neurodegeneration in HD, it has major limitations (cf. ref. *11*). A more integrative hypothesis should include other factors known to influence excitotoxicity. It has been long recognized that oxidative stress is a causal or, at least, ancillary factor in neurodegenerative disorders which, together with excessive activation of glutamate

receptors, provides a final common pathway for neuronal vulnerability *(71)*. Similarly, alterations in energy metabolism probably have a role in cell death *(72)*. For example, some animal models of HD use substances that interfere with energy metabolism. Chronic applications of the mitochondrial toxin 3-nitropropionic acid (3-NP), an irreversible inhibitor of complex II succinate dehydrogenase (SDH), causes striatal neuropathology similar to that seen in HD *(73,74)*. It has been postulated that metabolic impairment, as that produced by 3-NP treatment, reduces the threshold for glutamate receptor-mediated neurotoxicity *(75,76)*.

One of the strengths of the energy impairment/excitotoxicity hypothesis is that striatal damage can occur without the elevation of striatal glutamate levels or alterations in glutamate receptors usually associated with excitotoxicity. This is particularly important because glutamate levels do not appear to be increased in HD *(65)*. However, a weakness of this hypothesis is that SDH activity in all neurons of the brain is affected similarly by 3-NP, although extrastriatal regions appear to be less vulnerable to this toxin *(74,77–79)*. As a result, the current hypothesis does not fully account for the striatal selectivity of 3-NP neurotoxicity. It seems likely that other factors in the striatum must be important for the induction of 3-NP lesions.

It has been suggested that the energy impairment/excitotoxicity hypothesis for 3-NP toxicity should be expanded to include a role for DA because the vulnerability of striatal neurons to 3-NP depends on an intact DA input *(80)*. It is likely that the striatal selectivity of 3-NP lesions is attributable to the striatum being a major target for both DA and glutamate inputs, making it one of the more vulnerable regions in the 3-NP-treated brain *(80,81)*.

1.4. DA and HD

As described previously, DA has an important role in the modulation of excitotoxicity. DA itself can be lethal to cells. At high concentrations, DA can cause striatal cell death both in vitro and in vivo *(82–85)*. DA is oxidized to produce quinones, which can react covalently with cysteinyl residues on proteins, as well as other reactive oxygen species *(86)*. Generation of reactive oxygen species via DA metabolism has been implicated in DA-induced cell death *(87)*. In addition, levodopa (L-DOPA) is also a weak excitotoxin *(88)*.

DA plays a major role in exacerbating neuronal damage initiated by a number of insults. For example, substantia nigra lesions protect against striatal damage induced by ischemia or EAAs *(89,90)*. Removal of DA input also reduces 3-NP toxicity *(80,91)*. Depletion of striatal DA by 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway significantly attenuates malonate toxicity and completely blocks the malonate-induced generation of hydroxyl radicals *(92)*. Conversely, administration of amphetamine, which increases striatal DA levels, potentiates 3-NP toxicity *(93)*. Increased striatal DA release enhances the formation of 3-NP lesions, whereas decreased DA levels prevent lesion formation in the striatum *(80)*. Blocking D2 receptors with sulpiride does not prevent 3-NP/methamphetamine-induced lesions. In contrast, fewer lesions were induced in rats pretreated with the D1 antagonist SCH 23390, and when both D1 and D2 antagonists were administered simultaneously, the number of rats presenting with lesions was greatly reduced *(80)*. Pharmacological blockade of D2 receptors or 6-OHDA lesions of the substantia nigra also reduce quinolinic acid lesions *(94)*. Unfortunately, in this study haloperidol was used to block D2 receptors. Haloperidol has

also been shown to be an NMDA receptor antagonist *(95)*, which could help explain the reduced lesion size.

There is an increasing body of evidence implicating the DA system in HD. Studies suggest that the DA system may be overactive in HD *(96)*. Oxidative stress has been demonstrated to impair DA uptake *(97)*, which could account for increased levels of DA in the cerebrospinal fluid of patients with HD *(96)*. Elevated concentrations of DA or a toxic metabolite could tip the balance of a system already under oxidative stress and lead to preferential striatal cell death *(98)*. In contrast, there is also evidence for a progressive loss of DA content during the course of HD *(99,100)*.

Alterations in DA receptors have also been observed in HD. There is general agreement that DA receptors are decreased in HD patients *(101–107)*. Furthermore, the density of D1 receptors appears to be a sensitive positron emission tomography (PET) marker for early brain degeneration in HD *(101)*. D2 binding has also been observed to be decreased in HD patients *(108)*. Loss of striatal D1 and D2 receptors may be associated with rigidity *(109)* and bradykinesia in early HD *(110)*.

Similar alterations in DA function and receptor density have been reported in animal models. An early role for dysfunctional DA signaling in HD occurs in at least one transgenic mouse model of HD, the R6/2 *(111)*. DA release also is reduced in R6/2 mice at 6 wk and this deficiency appears to be important in generating hypoactivity *(112)*. Reductions in DA receptors have also been found. For example, there is a loss of D2 binding and decrease in 6-[18F]fluoro-L-DOPA uptake in a primate model of HD *(113)*. DA receptors are decreased in multiple transgenic HD mouse models *(114,115)*. The function of the decrease in DA receptors is not known, but one of the consequences of these changes may be an increase in DA release (via a decrease in presynaptic autoinhibition by D_2 receptors). Mutant striatal neurons exposed to a neurotoxic concentration of DA exhibit elevated cell death *(116)*. Interestingly, in contrast to the reductions in $D_{1–4}$ receptors, D_5 receptor expression is enhanced in mouse models *(115)*. Furthermore, in spite of reductions in D_1 receptors, cAMP expression is also elevated in R6/2 and other mouse models (ref. *115*, *see* Subheading 2.6.).

Stimulation of D1 or D2 receptors in the R6/2 model of HD produces differential effects on early gene induction, with an unexpected hyper-responsiveness of D1- containing neurons, probably reflecting huntingtin-induced toxicity or a compensatory mechanism for decreased DA input *(117)*. If this is the case, an increased responsiveness of D_1 receptors, combined with upregulation of D_5 receptors and cAMP, may enhance toxicity by activation of NMDA receptors *(42)*, particularly if there were an underlying energy deficit in HD *(80)*.

2. MOUSE MODELS OF HD

2.1. *Cellular Alterations in Mouse Models of HD*

The generation of mouse models of HD has helped to understand the neuronal dysfunction underlying behavioral phenotypes and neurodegeneration in HD. At present, a number of transgenic, knock-in, and conditional mouse models have been developed. Several recent reviews of the phenotypes in many of these models have been published *(118,119)*. Our laboratories have examined electrophysiological and morphological cellular alterations extensively, using different mouse models of HD. We have primarily utilized transgenic animal models, including the R6/1 and R6/2 *(120)*, YAC72 *(121)*, and the Tg100 *(122)*, as well as two knock-in models, CAG71 and CAG94 *(123)*.

One of the most studied models is the R6 line of transgenic mice generated by Gill Bates *(120)*. In particular, the R6/2 mice, with approx 150 CAG repeats, manifest a very aggressive form of HD, somewhat similar to the juvenile variant. Transgenic animals display overt behavioral symptoms as early as 5 wk of age and die of unknown causes at about 15 wk. Affected animals display a number of alterations including, the formation of neuronal intranuclear inclusions *(124)*, changes in transmitter and receptor expression *(114,115)*, and altered signaling mechanisms *(111,125,126)*. There are also metabolic deficits *(127)* and a generalized reduction in lactate dehydrogenase activity in transgenic animals *(128)*. These alterations produce characteristic motor *(129)* and learning deficits *(130,131)*.

Our studies have used standard electrophysiological techniques including intracellular and whole-cell patch clamp recordings to examine neuronal properties and correlated immunohistochemistry to determine protein expression. When electrophysiological recordings are done, biocytin, an intracellular marker, is routinely included in the pipette to examine the morphology of the cell after the experiment *(132)*.

2.2. *Morphological Changes*

Neuronal death is not prominent in most HD mouse models, although it does occur. It is a late event that seems dependent on which transgenic or knock-in model is examined. In the R6 line neuronal loss is modest and occurs very late in the life of the animal *(133)*. However, we have observed early and significant changes in striatal somato-dendritic morphology *(123,134)*. Somatic areas and dendritic fields are reduced. Recurving dendrites are apparent in striatal neurons, similar to those found in HD patients *(10)*. Loss of spines may be an early morphological change. Alterations in cortical pyramidal neurons also occur *(134)*. In contrast to the R6 line, the YAC72 model displays selective degeneration of MSSNs in the lateral striatum around 12 mo of age *(121)*.

2.3. *Alterations in Passive and Active Membrane Properties*

One of the earliest and most consistent alterations in the basic membrane properties of MSSNs in the R6/2 transgenic mouse model is an increase in input resistance. This increase probably reflects loss of conductive membrane channels owing to morphological changes such as reduced membrane area possibly as a consequence of loss of spines. Consistent with this observation, cell capacitance is significantly reduced in symptomatic animals. The increase in membrane input resistance appears to be followed by a reduction in K^+ conductances and decreases in specific K^+ channel subunit protein expression (*see* Subheading 2.6.). In R6/2 transgenics there is a significant decrease in inward rectification. As a consequence, many MSSNs have a depolarized resting membrane potential *(134)*. This alteration is particularly relevant because membrane depolarization can remove the Mg^{2+} block of the NMDA receptor. Other K^+ conductances may also be affected, because alterations in firing patterns occur in some cells *(134)*. Another cellular dysfunction is a reduction in voltage-gated Ca^{2+} conductances *(111,135)*. This effect appears to occur after 40–50 d of age in the R6/2 transgenics, and is also likely to affect the firing patterns of MSSNs. However, in recent preliminary observations we have also observed an increase in voltage-gated Ca^{2+} conductances in younger R6/2 mice. Therefore, the changes in Ca^{2+} conductances may be a complex biphasic effect.

2.4. Increased Responsiveness to NMDA

In all models of HD examined thus far we found that a subset of MSSNs are more sensitive to application of NMDA. We first examined NMDA-induced cell swelling in R6/2 and two knock-in mouse models of HD, CAG 71, and CAG94 *(123)*. There was an overall increase in cell swelling in transgenic and CAG94 mice compared to controls indicating cells from these HD models are more sensitive to NMDA. Interestingly, the increase in sensitivity was limited to NMDA receptors, as sensitivity to KA was not affected. Electrophysiological and Ca^{2+} imaging studies supported these observations. Cells from transgenic animals (R6/2, YAC72, and Tg100) displayed larger NMDA currents and Ca^{2+} influx than cells from littermate controls *(122,135)*. Furthermore, cells from transgenic animals also displayed reduced Mg^{2+} sensitivity. Similar increases in NMDA receptor sensitivity have been observed in other animal models *(136)* and treatment with SDH inhibitors (e.g., 3-NP) augments NMDA-mediated corticostriatal excitation in striatal MSSNs *(137)*.

How this increase in sensitivity occurs remains unknown. One explanation is that huntingtin with expanded polyQ tracts interferes with the binding of PSD95 to the NR2 NMDA and GluR6 KA receptor subunits, causing both receptors to become hypersensitive to glutamate *(24)*. Another possible explanation will be developed in the following sections.

2.5. Synaptic Responses

Cellular alterations in mouse models are not limited to changes in intrinsic membrane properties and receptor function. The connections between the cortex and the striatum are also affected. Cortical changes accompany degeneration in the striatum. There is clear evidence for a progressive thinning of the cortical ribbon and pyramidal neuron loss in HD patients *(13,14,138–142)*. Early degeneration of the corticostriatal pathway may occur in conjunction with the accumulation of mutant huntingtin in axonal swellings in striatal neuropil and in the cytoplasm of cortical neurons *(12,13)*. These changes in cortical projection neurons may lead to alterations in synaptic function and receptor responsiveness. Defective neurotransmision in HD is also supported by observations suggesting early impairment of proteins involved in the control of neurotransmitter release, such as complexin II, synaptobrevin, and synapsin I *(143–145)*.

One of the first indications of electrophysiological changes in the corticostriatal pathway is the observation that the stimulus intensity necessary to evoke an excitatory postsynaptic potential in MSSNs is significantly increased in R6/2 and Tg100 transgenic mice *(122,134)*. Subsequently we described transient and progressive changes in spontaneous synaptic activity in transgenic R6/2 mice *(146)*. Spontaneous excitatory postsynaptic currents show a progressive reduction in frequency that becomes more evident as the neurological phenotype advances. We interpreted these effects as a progressive disconnection between the striatum and its cortical inputs.

In R6/2 animals there was a transient expression of large synaptic currents (~5 wk of age) that coincided with the onset of behavioral symptoms *(146)*. We hypothesized that these large events reflect dysregulation of glutamate release and/or an increase in cortical synchronization. The fact that R6 mice often develop epileptic seizures could imply that the cortex in HD becomes hyperexcitable. Interestingly, synchronous cortical input, similar to that produced by local application of picrotoxin in the cortex, appears to target

enkephalin-positive neurons preferentially *(147)*. As pointed out previously, these neurons are more vulnerable in HD *(30,31,148)* and enkephalin expression seems to depend on intact cortical inputs *(149)*.

In addition to possible increased cortical activation, alterations in the number of presynaptic D2, mGluRs, and adenosine receptors regulating glutamate release could contribute to the occurrence of large synaptic events *(114,115)*. These types of receptors are involved in the presynaptic regulation of glutamate release *(40,150,151)*.

The progressive disconnection between cortex and striatum observed in R6/2 transgenics has important implications. First, it casts doubts on the belief that chronic excess glutamate release is implicated in striatal cell death. Indeed, release studies have been inconclusive. Studies report either no change or a reduction of glutamate in the striatum *(152–154)*. Second, the progressive disconnection between MSSNs and their cortical inputs may deprive these cells of important trophic factors such as brain-derived neurotrophic factor (BDNF) *(155)*.

Finally, this progressive disconnection could help explain the surprising and seemingly paradoxical observation that, in some mouse models of HD, lesions produced by injections of quinolinic acid or KA are dramatically reduced compared to control animals *(156,157)*. Reduced receptor sensitivity to these EAAs can be ruled out because immediate early gene responses do not appear impaired, suggesting that resistance may be conferred by other processes further along the toxic cascade *(158)*. We propose that the progressive loss of cortical inputs explains neuroprotection at least in R6/2 mice. It has long been recognized that in order to produce an excitotoxic lesion in the striatum the integrity of the excitatory cortical projection is required *(70,159,160)*. The integrity of this projection is severely compromised in R6/2 mice, which then contributes to the neuroprotection. This hypothesis is supported by the observation that young transgenic animals and other mouse models are not protected against excitotoxic lesions *(161)*, indicating that the HD mutation *per se* is not neuroprotective.

Because neuroprotection develops against other insults such as cerebral ischemia *(162)*, 3-NP *(163)*, DA-induced toxicity *(164)*, and methamphetamine *(158)*, it is likely that other factors may also be involved. Thus, reduced DA function in R6 transgenics may aid in neuroprotection *(165)*.

2.6. Protein Expression

Correlative immunohistochemical studies have demonstrated marked changes in protein expression, which may underlie some of the functional changes observed electrophysiologically. For example, reduced resting membrane potentials in R6/2 transgenics is correlated with a decrease in expression of Kir channels, involved in inward rectification. Examination of Kir2.1 and Kir2.3 channel proteins revealed significant decreases in the HD mice. Alterations were also found in the expression of the K^+ channels regulating membrane repolarization. The expression of Kv2.1, a channel protein associated with the delayed rectifiers in the striatum, was also reduced *(166)*. Increased responsiveness to NMDA correlated with an increase of NR1 subunit expression and reduced NR2A/B expression *(135)*. Similarly, reduced glutamatergic synaptic activity was correlated with a marked reduction in synaptophysin and PSD95 expression *(146)*.

Changes in expression of DA receptors also occur in HD transgenic mice. Immunohistochemical analysis has revealed that expression of D_1, D_2 and D_3 receptors is markedly

reduced in multiple types of transgenic mice whereas expression of D_5 receptors is increased *(115)*. Parenthetically, the human *DA* D_5 receptor gene is located in chromosome 4, centromeric to the location of the HD locus, suggesting the possibility that cis-position effects could be responsible for alterations in D1 family receptors *(167)*. The increase in D_5 receptor expression is accompanied by heightened cAMP levels *(115)*. This is a significant finding because based on the greater expression of the D_1 receptors in the striatum of wildtype mice one would predict a substantial decrease in cAMP levels in transgenic mice owing to the loss of D_1 receptors. In contrast, the elevation in cAMP staining indicates that alternative means to activate striatal adenylyl cyclase exist after significant D_1 receptor losses. Possibly the cAMP increase is linked to the increase in D_5 receptor expression. Alternatively, the receptor could become uncoupled from its transduction system. According to our model of DA and glutamate receptor interactions, the enhancement of NMDA responses in transgenic animals could be provided not solely by hypersensitive D_1 but also by D_5 receptors *(168)*.

The expression of tyrosine hydroxylase is also diminished substantially in mouse models of HD as the phenotype progresses *(115)*. Previous studies in rats *(169,170)* show that the loss of nigrostriatal inputs produces a substantial morphological alteration in striatal MSSNs, similar to some of our findings in R6/2 mice *(134)*. Thus, multiple changes in the DA system may exacerbate the dysfunctions that occur in MSSNs in HD. The changes in DA signaling produce many other effects in MSSNs. Among the most notable is the diminished capacity of these neurons to modulate other afferent activity following losses in DA *(170)*. This produces significant cellular stress, which may further contribute to the elevations in cAMP detected as an early event in the transgenic mice. Changes in cAMP-mediated signaling pathways will produce many downstream changes in phosphorylation events mediated by PKA. Two of these PKA substrates, which are richly expressed in the striatum and change in the mouse models, are DARPP-32 *(171)* and NMDA receptors *(135,136)*.

3. SELECTIVE NEURONAL VULNERABILITY IN HD

One of the greatest puzzles in HD is the selective vulnerability of MSSNs and the resistance of striatal interneurons. Clearly, multiple factors must contribute to this selective vulnerability including differing levels of expression of huntingtin, differences in the density of NMDA receptors, and the degree of cortical innervation, to indicate just a few (*see* refs. *148* and *172*).

One hypothesis is that huntingtin expression differs in various types of neurons and this may account for selective vulnerability. Thus, high levels of expression are confined to neurons and neuropil within the matrix compartment, with lower levels of expression in the patch compartment. Furthermore, large cholinergic interneurons do not appear to express huntingtin *(173)*. These findings are controversial, however *(174)*. What is consistent is that corticostriatal neurons are enriched in huntingtin, suggesting that the HD mutation may render corticostriatal neurons dysfunctional first and potentially destructive upon some MSSNs, rather than render striatal neurons vulnerable *(174)*.

There is little doubt that NMDA receptors play an important role in degeneration of MSSNs in HD. Since the cellular distribution, density, and subunit composition of NMDA receptors are not equal throughout the striatum *(175),* these factors could help explain

differential vulnerability. For example, striatal interneurons have reduced density of NMDA receptors and the subunit composition is different from that expressed by MSSNs *(176)*.

Taking advantage of cell identification with infrared videomicroscopy we examined cell swelling induced by NMDA in MSSNs compared with large, putative cholinergic, interneurons. We observed that, in contrast to MSSNs, cell swelling was not induced in large interneurons by bath application of NMDA *(177)*. This effect was not because of the inability of large interneurons to swell, because KA application could induce cell swelling. Electrophysiological experiments confirmed reduced NMDA current density in large interneurons. Although previous reports suggested that cholinergic interneurons were less responsive to all glutamate receptor agonists *(178)*, our results demonstrated that the reduced sensitivity was not indiscriminate, but specific to NMDA responses.

Another factor that could contribute to the selective vulnerability of MSSNs is the degree of cortical innervation *(174)*. Our observations suggest that a progressive disconnection between cortex and striatum occurs in HD. We could expect that striatal neurons that receive less cortical inputs would be more resistant to degeneration. At least one class of striatal interneuron, the cholinergic large aspiny cell, which has been shown to be less densely innervated than the MSSN *(177,179,180)*, is spared in HD. This conclusion lends support to the idea that a critical determinant of neuronal vulnerability is the extent to which cells receive input from cortical and other huntingtin-rich glutamate neurons *(174)*.

The question then becomes what is the mechanism of MSSN degeneration in human HD? One potential scenario is that early changes in cortical projection neurons alter their ability to release glutamate and possibly BDNF into target areas. This decrease induces changes in postsynaptic receptor density, distribution, or subunit composition leading to denervation supersensitivity. Although studies reporting this phenomenon in the striatum are relatively rare, one set of experiments on striatal glutamate receptor expression after cortical ablations found evidence of EAA receptor changes in gene expression, supporting the concept of denervation supersensitivity *(181)*. In addition, there is evidence that the composition of postsynaptic NMDA receptors is under tight presynaptic control *(182)*. Alterations in presynaptic activity thus may affect the types of postsynaptic NMDA receptors activated.

Recent studies are attributing an increasingly important role to extrasynaptic NMDA receptors *(183)*. In view of the fact that the number of synaptic contacts may be reduced in HD, the role of these extrasynaptic receptors may be increased. In normal conditions extrasynaptic NMDA receptors appear to signal glutamate spillover, i.e., extrasynaptic diffusion of neurotransmitter *(183,184)*. Receptor subunit composition is different between synaptic and extrasynaptic receptors. Thus, in hippocampal neurons, extrasynaptic NMDA receptors contain NR1 and NR2B subunits, whereas synaptic NMDA receptors also contain the NR2A subunit *(185)*. This has led to the suggestion that synaptic and extrasynaptic NMDA receptors may have differing roles in excitotoxicity *(186)*. In support, there is evidence that synaptic and extrasynaptic NMDA receptors have opposing effects on CREB (cAMP response element binding protein), gene regulation, and neuronal survival *(187)*. Thus, whereas Ca^{2+} entry through synaptic NMDA receptors induces CREB activity and *BDNF* gene expression, Ca^{2+} entry through extrasynaptic NMDA receptors activates a dominant CREB shutoff pathway that blocks induction of

BDNF expression *(187)*. These results imply that synaptic NMDA receptors have anti-apoptotic activity, whereas stimulation of extrasynaptic NMDA receptors causes loss of mitochondrial membrane potential and cell death *(187)*.

Considering that there is a progressive disconnection between the cortex and the striatum, associated with reductions in synaptophysin and PSD95, and knowing that the density of NMDA receptors is not reduced in HD, one reasonable assumption is an increased role of extrasynaptic NMDA receptors as the disease progresses. Enhanced activation of extrasynaptic NMDA receptors may facilitate cell dysfunction and eventual death. Indeed, recent studies have indicated that reduced expression of PSD95 in neurons may be responsible for neuronal vulnerability *(188)*.

Finally, another factor that affects neuronal vulnerability is the presence or absence of dendritic spines. We do not know yet what causes the progressive loss of spines in transgenic HD mice *(134)*. We can only speculate that early dysregulation of glutamate release, manifested by the presence of large synaptic events, in conjunction with an increase in cortical excitability, may induce changes at the postsynaptic level. Studies of hippocampal neurons show that exposure to glutamate or NMDA for short periods of time can produce a rapid loss of dendritic spines *(189)*. However, a decrease in synaptic activity observed in later stages of the disease could also cause elimination of spines *(190)*. Whatever the mechanism of spine elimination in R6/2 transgenics, one consequence of spine loss is to make these neurons more vulnerable to subsequent excitotoxic stimuli *(189)*. In that sense spines, as well as normal levels of synaptic activity, can be viewed as neuroprotective *(190)*. Supporting this suggestion, it has recently been shown that environmental stimulation can increase the life expectancy of R6/2 *(191,192)* and R6/1 *(193)* mice and prevents the occurrence of seizures. Environmental stimulation could thus increase spine density *(194)* and possibly reduce the rate of MSSNs spine loss in HD.

4. FUTURE DIRECTIONS AND THERAPY

It is clear that arresting expression of mutated huntingtin may someday provide a means to directly attack the roots of HD. For example, recent research highlights the possibility of rescuing polyglutamine-mediated cytotoxicity by RNA interference *(195)*. However, the vast majority of therapeutic approaches have been pharmacological, using compounds directed at treating the symptoms. Although a large number of drug trials has been performed, no cure for HD has as yet been discovered. Excellent reviews of this area have been already published *(196,197)*.

The recent generation of transgenic mouse models of HD has opened new venues for therapeutic development *(198)*. One of the therapeutic approaches in transgenic animals has been the attempt to prevent protein aggregation. Congo red and chrysamine G modulate aggregate formation and delay the onset of symptoms in R6/2 mice *(199,200)*. Creatine also inhibits aggregate formation *(199)*. Tetracycline derivatives, in particular minocycline, interfere with activation of caspases and exert a neuroprotective effect *(201–203)*. Transglutaminase activity is increased in HD brains and cystamine, a transglutaminase inhibitor, extends survival in R6/2 mice *(204)*. Cystamine inhibits caspases and increases the level of antioxidants, such as glutathione *(205)*. Benzothiazoles are also potential inhibitors of polyglutamine aggregation *(206)*.

More relevant in the context of the present chapter is a discussion of treatments related to glutamate and DA. If the excitotoxicity theory, or a variant of it, is correct, one would

expect that blockade of glutamate receptors, in particular the NMDA subtype, would be beneficial in preventing neurodegeneration. Several studies have demonstrated neuroprotective effects of amantadine and memantine, two antagonists of NMDA receptors *(207)*. Amantadine also reduced dyskinesias in HD patients *(208,209)*. Coenzyme Q10, an essential cofactor of electron transport, and remacemide, another NMDA receptor antagonist, ameliorate motor deficits in transgenic mice *(210,211)*. If it is true that extrasynaptic NMDA receptors play a role in HD, selective blockers of these receptors also should be beneficial *(187)*. Ifenprodil preferentially blocks NR2B containing NMDA receptors and is thus a potential target for therapy.

mGluR antagonists have also shown potential therapeutic effects, though the effects are different depending on which group of mGluRs is activated *(212,213)*. Adenosine receptors (A_1 agonists or A_{2A} antagonists) may also exert neuroprotective effects because of their modulatory role on glutamate release in the corticostriatal pathway *(214,215)*.

Other drugs that affect cellular excitability and glutamate release have also been tested. Riluzole prolonged survival time in R6/2 mice *(216)*. Lithium has also been evaluated with positive results in neuroprotection *(217)*. In addition, some histone deacetylase inhibitors such as suberoylanilide hydroxamic acid have been shown to be beneficial in the R6/2 model of HD *(218)* and arrest neurodegeneration in *Drosophila (219)*.

As reviewed in the previous sections, DA is important in the neurodegenerative processes underlying HD. Its role needs to be re-evaluated because modulation of the DA system might provide a target for therapy *(80)*. Because DA release may be compromised in HD, and DA receptors are decreased early in the disease, attempts to restore or enhance DA function have been assessed. Apomorphine, a D1/D2 receptor agonist, seemed to ameliorate HD symptoms *(220,221)*. In the R6/2 model, replacement therapy with L-DOPA caused short-term behavioral improvements but long-term treatment was deleterious on survival and rotarod performance *(112)*.

On the other side of the spectrum anti-DA therapy has also been considered *(222)*. D2 blockers do not appear to affect the long-term progression of HD. Bromocriptine, rather than improving chorea, induced an exacerbation *(223)*. However, dose-dependent effects were also observed. Low doses produced clinical improvement but higher doses potentiated the symptoms *(224)*. Finally, another D2 blocker, sulpiride, produced no functional improvement but reduced abnormal movements *(225)*, supporting the view that choreatic movements correlate with overactivity in DA systems, although others found no signs of hyperactivity of DA neurons *(226)*.

Dietary changes may also affect the progression of the disease. For example, essential fatty acids have therapeutic potential in HD *(227)* and dietary restrictions slow the progression of the disease *(228)*. Creatine supplementation has also proven beneficial *(229,230)* and tauroursodeoxycholic acid, an endogenously produced hydrophilic bile acid, is neuroprotective in the R6/2 model *(231)*.

However, most of these therapeutic approaches produce only transient effects *(232)* and some produce undesirable side effects. One recent promising area involves transplantation of fetal striatal tissue. Early trials in baboons have been attempted *(233)* and fetal transplants restore electrophysiological sensitivity to DA in the lesioned striatum of animals with experimental HD *(234)*.

Although there have been trials using fetal striatal transplantation in HD patients *(235,236)*, the risks involved in transplantation weighed against mild benefits must be

carefully considered (*see* refs. *237* and *238*). Many factors have to be evaluated before fetal neural grafts become more universally usable as a treatment for HD *(239)*. In addition, because HD is manifested in many cerebral regions, one has to consider the potential benefits of targeting other areas aside from the striatum. For example, transplantation of donor cortex from wildtype mice into the anterior cingulate cortex of neonatal R6/1 mice delayed onset of motor deficits *(240)*.

Another promising venue is to restore trophic factors lost because of the decrease in cortical inputs. For example, microspheres loaded with nerve growth factors (NGF) can be used *(241)*. Alternatively, adenovirus-mediated ciliary neurotrophic factor gene transfer may be a useful delivery system for neuroprotection *(242,243)*. The neurotrophin BDNF can prevent a subpopulation of striatal neurons from undergoing NMDA-induced cell death *(244)* and biologically delivered NGF can attenuate striatal damage caused by 3-NP *(245)*. BDNF inhibits apoptosis and DA-induced free-radical production in striatal neurons but does not prevent cell death *(246)*. DA depletion by 6-OHDA can also increase striatal levels of neurotrophins *(247,248)* and expression of their receptors *(249)*. Riluzole stimulates NGF and BDNF synthesis in cultured mouse astrocytes *(250)*. Finally, dietary restriction may normalize BDNF levels and slow disease progression *(228)*. Other factors may be coadjuvant to BDNF restoration. For example, environmental enrichment can slow the progression of the disease in R6/2 mice *(192)* presumably, among other factors, by increasing BDNF levels.

One of the most important issues is when to begin treatment. Many of the pharmacological approaches may have been started too late, after physiological changes became irreversible. If alterations in the cerebral cortex precede postsynaptic changes in the striatum, a logical target would be to try to prevent the development of alterations in the cortex and the corticostriatal pathway. Thus, in order to prevent the development of motor symptoms, very early intervention may be necessary.

5. CONCLUSIONS

We have reviewed evidence pertaining to some of the pathophysiological mechanisms that are involved in HD, especially those involving glutamate and DA. In particular, the recent development of mouse models of HD has provided invaluable tools to better understand this devastating disease. Although the excitotoxicity hypothesis has provided a useful framework to begin to understand the mechanisms that lead to cell degeneration in HD, especially if alterations in energy metabolism are taken into account, it is clear that other factors are involved. In particular, DA, and its modulation of glutamate transmission, seems to play a critical role. Early alterations in corticostriatal neurotransmission are also relevant and may induce events that trigger cascades of postsynaptic alterations resulting in striatal neuronal dysfunction. The progressive loss of cortical inputs, along with a reduction of important trophic factors, could be a potential target for early therapeutic intervention. Finally, the early occurrence of glutamate dysregulation, manifested by the presence of large synaptic events, may represent another possible target.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Hereditary Disease Foundation and USPHS grant NS 41574 for providing support for the studies performed in our laboratories.

Miriam A. Hickey and Oanh K. Nguyen provided valuable comments on the manuscript and helped to organize the references.

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XII Dementias

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Dopaminergic and Glutamatergic Systems in Alzheimer's Disease

Paul T. Francis

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative condition affecting approximately 450,000 people in the United Kingdom and some 2.5 million in the United States and it is the most common cause of dementia in the elderly. The prevalence increases from 0.2/100 below 60 yr of age, through 0.3/100 below 70 and 3.2/100 to 10.8/100 below 80 years of age *(1)*. The clinical syndrome is characterized by higher cognitive dysfunction, behavioral disturbance, and loss of activities of daily living *(2)*. Although cognitive impairment is a defining characteristic of the disease, behavioral changes such as aggressive behavior, depression, and psychosis, together with loss of abilities such as dressing and feeding, cause most distress to carers *(3)*. The sufferer goes from mild impairment to an almost vegetative state over a period of 8–10 yr with increasing demand on carers for the first few years followed by increased service use, both of which have huge economic, personal, and social burdens on society. Suffers usually die of an intercurrent illness, such as bronchopneumonia. The diagnosis of AD during life is often one of exclusion and is based on psychometric testing and standardized criteria and in many cases is supported by imaging *(4)*. The only definitive diagnosis is made from a microscopic examination of brain tissue that has rarely been obtained at neurosurgical biopsy *(5)* or more commonly at postmortem examination, again using standardized criteria *(6)*.

Brains of patients suffering from AD show very substantial, but regionally selective, atrophy reflected in sulcal and ventricular widening. The main areas affected are the medial temporal lobe structures including the hippocampus, together with the temporal, frontal, and parietal cortex with sparing of the primary motor and primary sensory cortical areas *(7)*. The atrophy is considered to be as a result of neuronal loss from these structures affecting primarily pyramidal neurons with sparing of cortical interneurones *(8)*. Subcortical nuclei that project to the cortex, such as the nucleus basalis of Meynert, locus coereleus, and raphe nuclei, also suffer cell loss *(9–11)*. Neuronal loss is accompanied by extensive synapse loss *(12)*.

In addition to cell loss there are characteristic histopathological changes occurring, which include the presence of intracellular neurofibrillary tangles (NFTs: consisting of a

From: *Dopamine and Glutamate in Psychiatric Disorders*
Edited by: W. J. Schmidt and M. E. A. Reith © Humana Press Inc., Totowa, NJ

hypophosphorylated form of the microtubule associated protein τ) and extracellular deposits of a 40–42 amino acid peptide, Aβ (derived from amyloid precursor protein), which can be found as amorphous deposits or as a central core of senile or neuritic plaques *(12)*. Cell and synapse loss, together with NFTs, correlate strongly with severity of dementia, whereas the relationship with plaques is less robust. From a neurochemical standpoint acetylcholine, glutamate, serotonin, and noradrenaline are the major transmitter systems affected with relative sparing of dopamine, γ-aminobutyric acid (GABA), and most peptides *(13–15)*.

2. DOPAMINE AND AD

Dopaminergic neurotransmission in the prefrontal cortex is involved in working memory and disorders of its function have been implicated in mental disorders such as schizophrenia and depression *(16–18)*. Thus, specific lesion of dopaminergic inputs into this region has been shown to impair cognitive tasks *(19)*. Furthermore, the predominant receptor subtype present in human frontal cortex appears to be D_1 *(20,21)* and the actions of dopamine at this receptor are believed to be essential to working memory function in man *(22)*. Studies of messenger RNA indicate that D_1 receptors are located on both pyramidal neurons and interneurons and may have a complex effect on activity depending on the level of stimulation *(23)*. In addition there is evidence that dopamine facilitates the release of both glutamate and acetylcholine release in the hippocampus and frontal cortex of animals *(24–26)*, both transmitters have established roles in cognitive function *(14,15)*. Studies of D_1 knockout mice indicate that these receptors are involved in aspects of spatial learning in the absence of visual and motor impairment *(27)*. In addition to a relationship to cognitive symptoms there is also recent evidence that genetic variation in the D_1 (and D3) receptor gene may predispose AD patients to develop psychotic or aggressive symptoms *(28)*.

Reports of the status of the dopaminergic system in AD lack some consistency, probably because of problems with differential diagnosis, but generally indicate a relative preservation in AD. It is likely that changes to the nigrostriatal system may only occur in those patients with motor disorders or where there is overlapping Parkinson's disease or dementia with Lewy bodies (DLB) *(29)*. Consistent with this interpretation, reductions in the dopamine transporter in the striatum have been reported for both Parkinson's disease and DLB but not for AD *(30)*. There are many older studies reporting loss of dopamine and homovanillic acid (HVA) from the striatum and cerebrospinal fluid but questions remain about the differential diagnosis as DLB was not recognized at that stage *(31)*. There is significant cell loss in the ventral tegmental region, which provides dopaminergic innervation of the cerebral cortex *(32)*. Although no loss of dopamine was reported, changes in HVA in frontal and temporal lobes have been observed *(31,33)*, which may imply that functional changes to this system occur. Our own recent studies are reported in Table 1 and show no change in either dopamine or the metabolites HVA or 3,4-dihydroxyphenyl-acetic acid (DOPAC) in three cortical regions.

In terms of dopamine receptors some post mortem studies show changes in striatal dopamine receptors in AD patients with DLB or those with prominent psychosis (e. g. refs. *34* and *35*), but this does not appear to be a general feature of AD. A recent study using positron emission tomography did show a reduction in D_1 but not D_2 receptors in the striatum and putamen of AD patients *(36)*. Few studies have been conducted outside the striatum, partly because of the relatively low concentration of receptors; however, loss

Table 1
Concentrations of Dopamine, Homovanillic Acid, and 3,4 Dihydroxyphenylacetic Acid in Cortex From Patients With Alzheimer's Disease and Controls[a]

	Temporal cortex (BA 21)			Frontal cortex (BA 11)		
	Dopamine	HVA	DOPAC	Dopamine	HVA	DOPAC
Control	0.34 ∀ 0.06 (15)	15.6 ∀ 4.4 (17)	0.49 ∀ 0.08 (16)	0.42 ∀ 0.12 (10)	0.70 ∀ 0.12 (15)	17.5 ∀ 4.6 (17)
Alzheimer's disease	0.41 ∀ 0.04 (16)	17.1 ∀ 2.2 (17)	0.62 ∀ 0.06 (18)	0.53 ∀ 0.09 (12)	0.71 ∀ 0.06 (20)	22.5 ∀ 2.6 (20)

[a]Values are mean pmol/mg protein ∀ Standard Error of Mean with *n* in parentheses. There are no significant differences between AD and control *(36a)*.

HVA, homovanillic acid; DOPAC, 3,4 dihydroxyphenylacetic acid.

of D_2 receptors was reported in both amygdala and hippocampus in a small study *(37)*. In our studies of D_1 receptors in three cortical regions no change was noted in AD (Table 2).

Overall, notwithstanding the possible links between disturbance of dopaminergic signalling and impaired cognition, there is little evidence for changes to this system in AD. However, the lack of change in this system in the face of substantial cholinergic changes has been suggested as a basis for psychotic symptoms which are sometimes found in AD patients, but more especially DLB patients *(38–40)*.

3. GLUTAMATE IN AD

The amino acid glutamate (and probably aspartate) is the principal excitatory neurotransmitter of the brain, being used at approximately two-thirds of synapses *(41)*. The majority of neurons and indeed glia are likely to be influenced by glutamate since they have receptors for glutamate. Glutamate is considered to be the main neurotransmitter of neocortical and hippocampal pyramidal neurons and is thus involved in higher mental functions, such as cognition and memory *(14)*. One of the main mechanisms by which glutamate may contribute to learning and memory functions is via long-term potentiation (LTP), a form of synaptic strengthening following brief, high-frequency stimulation *(42)*. Disturbance of excitatory glutamatergic neurotransmission is believed to be associated with many neurological disorders including AD *(14)*, ischemic brain damage *(43)*, motor neuron disease *(44)*, and epilepsy *(45)*.

Unlike other neurotransmitters (e.g., GABA, acetylcholine), glutamate is an integral part of protein, energy, and ammonia metabolism of all cells. Thus, the intracellular concentration is high (close to 10 mmoles/kg wet wt), which has made the study of presynaptic glutamatergic neurotransmission difficult *(14)*.

Glutamate is synthesized in nerve terminals by one of several possible enzymes. First, glutamine can be converted to glutamate by the action of the mitochondrial enzyme glutaminase *(46)*; second glutamate can be produced by transamination from aspartate in the cytosol. Glutamate is transported into vesicles by the action of recently identified vesicular glutamate transporters *(47)* at high concentration and released on depolarization as for other neurotransmitters. Once released glutamate is removed from the synapse by very rapid and efficient uptake systems. These glutamate transporters are located on the pre- and postsynaptic elements but the majority of glutamate is taken up into astrocytes where it is

metabolized to glutamine by the enzyme glutamine synthase. Glutamine is then released by these cells and may be taken up by neurons for possible recycling into transmitter glutamate (for an extensive review of all aspects of glutamate uptake, *see* ref. *48*).

The majority, approx 95%, of glutamate uptake in the cortex and hippocampus follow release is accounted for by the glutamate transporter (GLT), with other transporters (e.g., glutamate aspartate transporter [GLAST], excitatory amino acids carrier [EAAC]) apparently playing a minor role *(48)*. The location of each of these transporters has proved controversial but is best summarized as follows: GLT and GLAST protein are located in astrocytes in the normal adult central nervous system, whereas EAAC is present in neurons. Messenger RNA for GLT has been detected in neurons but protein has not, perhaps because of lack of sensitivity to very low levels.

Study of postsynaptic glutamatergic mechanisms has proved less difficult as a range of glutamate receptors has been identified, falling into two main classes, ionotropic (*N*-methyl-D-aspartate [NMDA] and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA]/kainate subtypes) and metabotropic *(49)*. The ionotropic subtypes have varying permeability to Na^+ and Ca^{2+} ions whereas the metabotropic subtypes couple to adenylyl cyclase, phospholipase C, or ion channels *(50)*.

3.1. Role of Glutamate in Learning and Memory

A role for glutamate and glutamate receptors in learning and memory is widely recognised. For example, NMDA antagonists impair learning and memory *(51–53)*, whereas NMDA agonists and facilitators improve memory *(54,55)*. Likewise AMPAKines (positive modulators of receptor function) facilitate learning and memory (e.g., ref. *56*). One intriguing finding, as yet not fully explained, is the observation that the uncompetitive NMDA antagonist memantine *improves* memory *(57,58)*. Circumstantial evidence also points to the involvement of glutamatergic pathways in learning and memory and derives from the well-known role of structures, such as the hippocampus *(59)*. More specifically, lesions of certain glutamatergic pathways impair learning and memory *(60)*.

In addition to the above, glutamate and glutamate receptors are involved in mechanisms of synaptic plasticity (LTP and long-term depression [LTD]), which are considered to underlie learning and memory *(42,61,62)*.

Genetically modified mice have been perhaps less useful in the glutamate field than others, not surprising given the ubiquity and importance of glutamate as a neurotransmitter. Generally speaking they confirm that glutamate receptors are involved in aspects of learning and memory *(63)*.

3.2. Excitotoxicity

A major feature of the glutamatergic system is the apparent delicate balance between too much and too little transmitter, both of which can impair neuronal survival *(64)* and cognition *(60)*. The concept of excitotoxic cell death caused by exogenously applied agents, such as NMDA and kainate, emerged in the 1970s through the work of Olney and Schwartz (e.g., ref. *65*). The mechanism involves excessive activation of receptors leading to raised intracellular Ca^{2+} and consequent activation of a cascade of enzymes, which ultimately result in cell death by necrosis or apoptosis *(66,67)*. During the 1980s it was also suggested that endogenous glutamate could build up and become excitotoxic, perhaps as a result of impaired glutamate clearance (as a consequence of disrupted transporter function

Table 2
Dopamine D1 Receptor Binding in Temporal (BA 21) and Frontal Cortex (BA9 & 46) of Control, AD Patients, and Mixed/Other Dementia Patients[a]

	BA 21		BA 46		BA 9	
	Bmax (fmol/mg prot)	Kd (n*M*)	Bmax (fmol/mg prot)	Kd (n*M*)	Bmax (fmol/mg rot)	Kd (n*M*)
Control (n = 25–30)	901 ∀ 73	6.22 ∀ 0.58	901 ∀ 76	6.37 ∀ 0.76	1186 ∀ 129	9.11 ∀ 0.96
Alzheimer's disease (n = 32–36)	805 ∀ 74	7.09 ∀ 0.88	865 ∀ 75	7.61 ∀ 1.00	967 ∀ 90	8.71 ∀ 1.01
Mixed/other dementia (n = 10)	817 ∀ 102	5.33 ∀ 1.09	723 ∀ 97	5.31 ∀ 0.77	1153 ∀ 185	9.71 ∀ 1.62

[a]Values are mean ∀ SEM; there are no significant differences *(36a)*.
BA, Brodmann area; K^d, receptor affinity.

or indirectly in conditions of reduced energy availability) *(68,69)*. Others have cautioned that there is no simple relationship between raised extracellular glutamate concentrations and cell death in vivo *(70)*.

3.3. Glutamatergic Changes in AD

The most persuasive evidence indicating a significant presynaptic glutamatergic deficit in AD is histopathological (*see* Table 3). Thus, brains from patients with AD invariably show considerable shrinkage of the temporal (particularly structures in the medial aspect), parietal, and frontal lobes of the cortex accompanied by sulcal widening and ventricular enlargement by gross neuropathological examination and using in vivo imaging techniques *(71)*. This atrophy is a consequence of loss of pyramidal neurons and their synapses together with surrounding neuropil. Pyramidal neurons of the neocortex forming corticortical and corticofugal together with those of the entorhinal and hippocampal CA1 region are lost in AD and, additionally, remaining neurons are subject to NFT formation *(8;72–74)* (*see* Table 3). The clinical significance of these changes is highlighted by the observation that these individual markers correlate with the degree of dementia *(12,75)*. Biochemical evidence implicates glutamate as the neurotransmitter of these pathways (as reviewed in ref. *14*) and therefore one may infer that glutamatergic neurons degenerate in AD.

Direct biochemical evidence for a presynaptic deficit in glutamatergic neurons has been more difficult to come by owing to the ubiquitous distribution of high concentrations of the amino acid and the lack of robust and selective markers (*see* Subheading 3) in comparison to, for example, the cholinergic system. However, despite these problems, several studies have shown reductions in the concentration of glutamate in AD tissue *(76–78)* and lumbar cerebrospinal fluid *(79)*, but *see* ref. *80*. Furthermore, glutamate-immunopositive neurons have been shown to be reduced in number and subject to NFT formation in sections from AD brain *(81)*. Direct measurement of phosphate-activated glutaminase activity (an enzyme involved in glutamate synthesis was unaf-

Table 3
Neuropathological Indices in Alzheimer's Disease[a]

	Temporal cortex	Frontal cortex	Hippocampus/entorhinal cortex
Atrophy[b] (range)	30% (17–45)	15% (0–31)	26%
Cell loss	58%[c]; 25–60%[d]	25–55%[d]	50–90%[e]
Synapse loss	52%[d]	35%[f]	—
Tangles (per field)	56[d]	35[d]	—

[a]Values represent mean (and range).
[b]Data taken from ref. *115*.
[c]Data taken from ref. *75*.
[d]Data taken from ref. *116*.
[e]Data taken from ref. *8*.
[f]Data taken from ref. *117*.

fected in AD; (ref. *46*). By contrast glutaminase-positive neurons were reduced in number and subject to NFT formation *(81)*.

Another possible marker of presynaptic system is GLT proteins; however, because these proteins are located pre- and postsynaptically in addition to a major localization to astrocytes, interpretation of results can be difficult *(48)*. Studies of D-aspartate binding to transporter sites in frozen postmortem tisssue revealed reductions in many cortical areas *(82,83)*; however, the relevance of this measure to glutamate uptake is doubtful because of possible sequestration of the ligand *(84,85)*. Functional measurement of Na^+-dependent D-aspartate uptake in fresh postmortem tissue also revealed significant reductions *(86)*. Studies using antibodies directed against the individual glutamate transporters reveal conflicting data. Reduced levels of GLT protein (but not mRNA) with normal levels of both GLAST and EAAC were reported by Li et al. *(87)* but not confirmed by Beckstrom et al. *(88)*. The latter authors suggest that postmortem proteolysis may be a problem in such studies. Our own studies indicate no significant reduction of GLT1 protein in parietal cortex of AD patients by Western blotting (Kirvell and Francis, unpublished observations). Even assuming there was no reduction of transporter protein, there is considerable evidence for oxidative damage of proteins including the glutamate transporters *(89,90)*. Thus there may be a functional deficit in glutamate uptake, which would be consistent with the reduced D-aspartate uptake and binding reported *(82,86)*.

We have recently begun to investigate the status of the newly discovered vesicular glutamate transporters (VGLUTs), VGLUT1 and VGLUT2 *(47,91)*. Preliminary studies indicate that there is a reduction in VGLUT1 (but not VGLUT2) by Western blotting in parietal cortex *(92)* but not in temporal cortex of AD patients (Kirvell & Francis, unpublished observations). This is in contrast to measurements of vesicular glutamate uptake rate, which were lower in the temporal cortex from AD patients *(93)*. This lack of change in protein with a reduction in a functional marker is reminiscent of the observation with glutamate transporter measurements and suggests a functional downregulation of the protein in both cases. The exact consequences of such changes remain to be determined.

Many receptors for neurotransmitters are relatively preserved in AD, however studies have demonstrated reductions in the NMDA receptor complex in the hippocampus *(94)* and neocortex *(95–97)*. Although kainate receptors are relatively spared *(98)*, AMPA receptors are reduced in several regions of the AD brain *(99,100)*.

Clearly there is considerable evidence for alterations in the pre- and postsynaptic glutamatergic system in AD that will compromise its ability to function. This contention is supported by the observation that the reduction of many markers of this system correlates with the degree of dementia *(14)*.

A contribution of excess endogenous glutamate to cell death in AD has been suggested *(101)* as a result of failure to remove glutamate from the synapse. This could occur if the energy-dependent transporters lack sufficient adenosine triphosphate (ATP) (reduced energy availability) or if oxidative processes damage the protein. In both cases the synaptic concentration of glutamate would rise and lead to excessive activation of postsynaptic glutamate receptors or a failure of the NMDA receptor to act as a coincidence detector. There is some evidence that energy levels may be reduced in AD owing to purturbed mitochondrial function *(14,102)* and considerable evidence for oxidative damage of proteins including the glutamate transporter *(89,90)*. It remains possible that changes in numbers of glutamate receptors or changes in ion selectivity may, over time, lead to cell death. For example, loss of basal forebrain cholinergic neurons in AD may be linked to the numbers of calcium-permeable AMPA receptors present on such cells *(103)*.

Glutamatergic hypoactivity may also contribute to the spread of pathology (and hence cell loss) along anatomically defined pathways. Lack of activation of receptors, as a consequence of cell loss, can lead to apoptosis of target neurons. In addition, pyramidal neurons are the site of the hyperphosphorylation of the microtubule-associated protein τ, which leads to tangle formation and are the main cell responsible for the metabolism of amyloid precursor protein to Aβ *(86)*. Activation of receptors linked to phospholipase C (such as some metabotropic glutamate receptors) has been shown to increase the secretion of neuroprotective forms of amyloid precursor protein and decrease Aβ *(104)* while at the same time reducing the phosphorylation state of τ *(105,106)*. If glutamate neurotransmission is reduced as a consequence of tangle formation, one may hypothesize that Aβ production may increase and tau become more hyperphosphorylated in neurons innervated by the affected neuron *(15)*. These changes could then contribute to the pathological cascade observed in AD.

4. TREATMENT STRATEGIES

Treatment strategies that increase the activity of remaining glutamatergic neurons, without causing excitotoxicity, continue to represent an important target for the symptomatic treatment of AD and may have a disease-modifying effect. Several approaches have been tried including positive modulation of both AMPA and NMDA receptors. AMPAKines, which are considered to work by increasing the sensitivity of these receptors, are currently in clinical trial for mild cognitive impairment *(107)*. Modulation of the NMDA receptor has been attempted via the glycine coagonist site with clear indication in preclinical studies that the partial agonist D-cycloserine improved learning and memory *(108,109)*. Clinical studies have suggested some benefit but full-scale trials have not been initiated *(56,110,111)*. There is currently no evidence that these drugs enhance excitotoxicity.

Perhaps the most surprising development is the success of the non-competitive NMDA antagonist memantine in clinical trials in moderate and severe AD *(58)*. One would normally consider that such an approach— the blockade of a receptor that would

normally be activated in learning and memory— would be counterintuitive. However, there is evidence that this molecule acts like magnesium ions rather than MK-801 and is therefore able to prevent background activation of the NMDA receptor (noise) but allow activation of this receptor for LTP formation *(57,112)*. There are also reports of a clinical trial by Forest Laboratories Inc. *(113)* that show a benefit of the combination of memantine with the most widely used acetylcholinesterase inhibitor (donepezil) in the treatment of AD. Because cholinesterase inhibitors are likely to act in part by increasing glutamate release *(15,114)* the benefit may be hypothesized to come from the combination of a reduction in noise (memantine) and an increase in signal (donepezil).

ACKNOWLEDGMENTS

Current work is supported by grants from The Wellcome Trust, The Medical Research Council and GlaxoSmithKline, and The Dunhill Medical Trust.

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