Advances in Neurobiology 11

Vladimir Parpura
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Alexei Verkhratsky *Editors*

Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain

Springer

Advances in Neurobiology

Volume 11

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ISSN 2190-5215 ISSN 2190-5223 (electronic)
ISBN 978-3-319-08893-8 ISBN 978-3-319-08894-5 (eBook)
DOI 10.1007/978-3-319-08894-5
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014948468

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

To our families

Acknowledgements

We would like to thank all the authors for their contributions.

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Vladimir Parpura, M.D., Ph.D. holds both a medical degree, awarded from the University of Zagreb in Croatia in 1989, and a doctorate, received in Neuroscience and Zoology from Iowa State University in 1993. He has held faculty appointments at the Department of Zoology and Genetics, Iowa State University and the Department of Cell Biology and Neuroscience, University of California, Riverside. He is presently a tenured Associate Professor in the Department of Neurobiology, University of Alabama Birmingham, as well as a tenured Full Professor in the Department of Biotechnology, University of Rijeka, Croatia. He has been elected as a Member of Academia Europaea (MAE) in 2012. His current research includes: (1) studying the modulation of calcium-dependent glutamate release from astrocytes in health and disease; (2) assessing the role of the enteric glia in gut functions; (3) visualization of vesicular/receptor trafficking; (4) examination of the nature and energetics of interactions between exocytotic proteins using single molecule detection approaches; (5) development of scaffolds and dispersible materials, most notably modified carbon nanotubes, which can be used in repair after brain injury; (6) development of biosensors (e.g., botulinum toxin and nanofabricated carbon-based detectors), and (7) biomimetic micro-robotics. He has been interfacing neuroscience with nanoscience/nanotechnology, synthetic biology and biomedical engineering.

Arne Schousboe has a Ph.D. in biochemistry from the University of Copenhagen in 1968 and after a post-doc period with Dr. Eugene Roberts at the Department of Neuroscience, City of Hope National Medical Center in Los Angeles in 1972–1973 he came back to University of Copenhagen to resume a tenured position as an Associate Professor at the Medical Faculty and in 1978 he got his doctorate in science (D.Sc.) from the University of Copenhagen. In 1990 he moved to the Royal Danish School of Pharmacy as a Full Professor of biochemistry and has remained in this position since then when this Institution changed to become an independent University and subsequently a Faculty of Pharmaceutical Sciences at the University of Copenhagen and most recently a part of the Faculty of Health and Medical Sciences at the same University. He served as the Department Chair from 2005 to 2010 and is now part-time Professor in the Department of Drug Design and Pharmacology at this Faculty. He has been working on astrocyte function focusing on amino acid neurotransmission during the past more than 40 years and is currently engaged in studies of glutamate and GABA homeostasis and metabolism. He has published over 500 papers on these topics. He has served on the editorial board on numerous neuroscience journals over the years and is currently the Editor-in-Chief of Neurochemical Research and Editor of the book series Advances in Neurobiology.

Alexei Verkhratsky Member of Academia Europaea, Member of the German National Academy of Sciences Leopoldina, Member of Real Academia Nacional de Farmacia (Spain), was born in 1961 in Stanislaw, Galicia, Western Ukraine. He graduated from Kiev Medical Institute in 1983, and received Ph.D. (1986) and D.Sc. (1993) in Physiology from Bogomoletz Institute of Physiology, Kiev, Ukraine. He joined the Division of Neuroscience, School of Biological Sciences in Manchester in September 1999, became a Professor of Neurophysiology in 2002 and served as Head of the said Division from 2002 to 2004. From 2007 to 2010 he was appointed as a visitor professor/Head of Department of Cellular and Molecular Neurophysiology at the Institute of Experimental Medicine, Academy of Sciences of Check Republic. In 2010 A. Verkhratsky was appointed as a Research Professor of Ikerbasque (the Basque Research Council), in 2011 as an Honorary Visiting Professor at Kyushu University, Fukuoka, Japan and from 2012 he acts as Adjunct Scientific Director of the Achucarro Basque Centre for Neuroscience (Bilbao, Spain). Prof. A. Verkhratsky is a co-editor-in-chief of *Cell Calcium* (2000) and *Membrane Transport & Signalling—Wiley Interdisciplinary Reviews* (2009), Receiving Editor of *Cell Death & Disease* (2009) and a member of editorial boards of numerous scientific journals.

Prof. Alexei Verkhratsky is an internationally recognised scholar in the field of cellular neurophysiology. His research is concentrated on the mechanisms of inter- and intracellular signalling in the CNS, being especially focused on two main types of neural cells, neurones and neuroglia. He made important contributions to understanding the chemical and electrical transmission in reciprocal neuronal-glial communications and on the role of intracellular Ca^{2+} signals in the integrative processes in the nervous system. Many of A. Verkhratsky's studies are dedicated to investigations of cellular mechanisms of neurodegeneration. In recent years he studies the glial pathology in Alzheimer disease. He authored a pioneering hypothesis of astroglial atrophy as a mechanism of neurodegeneration.

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Chapter 1
Glutamate and ATP: The Crossroads of Signaling and Metabolism in the Brain

Alexei Verkhratsky, Arne Schousboe, and Vladimir Parpura

Abstract ATP and glutamate have emerged as highly versatile molecules for cellular metabolism and intercellular communication in the brain. Their metabolic and signaling pathways are interlaced. We concisely outline the synthesis and metabolism of these precious molecules, as well as their use as neurotransmitters in autocrine, paracrine, and heterocellular signaling. Functional diversity of this glutamatergic and purinergic signaling is defined by an expression of a multitude of receptors on neurons and glial cells alike. Both ATP and glutamate play a role in neuropathology. This chapter is meant to introduce the chapters that follow in this book dedicated to the in-depth overview of the role of glutamate and ATP in the brain metabolism and signaling in health and disease.

Keywords Astrocyte • Microglia • Neurotransmitter release • Neurotransmitter receptors • Glutamate synthesis • Metabolism of ATP • Neuropathology

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_1

1.1 Introduction

Purines, pyrimidines, and ATP are those fateful molecules, which shaped the life on the Earth, as they occurred in the prebiotic period and became essential for biological evolution. Indeed, without purines and pyrimidines, construction of RNA and DNA would be impossible and hence the genetic code that sustains life familiar to us would never appear. ATP was selected very early as the main source of biological energy, and thus became an indispensable feature of life. This was a critical evolutionary choice because it shaped enzymatic systems to utilize ATP in energy-dependent reactions and preordained an appearance of the universal intracellular signaling system based on calcium ions as keeping cytosolic Ca^{2+} extremely low became vitally important, since otherwise insoluble Ca^{2+}-phosphates would preclude the cell energetics. Thus, all living cells on the Earth beginning from the most primitive ones had high cytosolic concentrations of ATP (or, occasionally, GTP) and there is little surprise that ATP was soon utilized by nature for another fundamental function of sending information from one living cell to another. Taken together, ATP acts as the main energy source and is pivotal for numerous signaling cascades both inside the cells (by fuelling various transport systems and donating phosphate groups) and between the cells (by chemical transmission). Similarly, glutamate acts as an important molecule for both intercellular signaling through glutamatergic transmission and cell energetics by contributing to ATP production.

1.2 Glutamate and ATP as Primary Neuronal and Glial Neurotransmitters in the CNS

Glutamate, ATP, and adenosine are among the most widespread neurotransmitters in the central nervous system (CNS). Indeed glutamate and ATP/adenosine can be released from neural cells (i.e., neurons and neuroglia) through several mechanisms that include exocytosis, i.e., release from specific vesicles that accumulate and store both types of molecules, and by transmembrane diffusion via several types of ion channels (Pankratov et al. 2006; Parpura et al. 2004; Lalo et al. 2011). When being released glutamate and purines interact with multiple receptors of both ionotropic and metabotropic varieties abundantly expressed in neurons and glia; activation of these receptors mediates numerous effects that include fast synaptic transmission, synaptic plasticity, multiple trophic, and proliferative responses, etc. In addition both glutamate and purines are fundamental players in neuropathology, the former being a primary mediator of excitotoxicity whereas the latter signal molecules are involved in activation of numerous defensive reactions, such as activation of microglia or initiation of astrogliosis (Franke et al. 2012; Kettenmann et al. 2011).

Despite high prevalence and multiple physiological and pathophysiological responses mediated through glutamate and purines, their role in neurotransmission was recognized relatively late, most likely because of the high presence of both

molecules in cells in general and in neural cells in particular (Burnstock 2012). It has been argued, for a long time, that glutamate is indispensable for cellular metabolism and biosynthesis, whereas ATP is a primary source of energy and hence cells should not easily part with neither of these molecules.

Nonetheless the neurotropic action of both glutamate and purines has been observed from early 1930s and for the very first time the neurotransmitter role for glutamate was suggested in 1954 by Takashi Hayashi who found that injection of glutamate in the brain provokes convulsions (Hayashi 1954). Several years later the first direct demonstration of neuronal excitation following iontophoresis of glutamate to neurons in spinal cord was made by D.R. Curtis, J.W. Phillis, and J.C. Watkins; they, however, found that similar excitation can be induced by other amino acids, and hence arrived to a conclusion that glutamate acts as a non-specific activator and does not appear as a chemical messenger for synaptic transmission (Curtis et al. 1959). However, the discovery of specific agonists and antagonists of glutamate receptors that mimicked various electrophysiological effects of glutamate led to an appreciation of glutamate as a legitimate neurotransmitter in the CNS (Evans et al. 1979; Watkins and Evans 1981). In depth characterization of specific mechanisms for biosynthesis, vesicular packaging and termination through uptake have cemented the concept of the neurotransmitter role of glutamate in vast majority of central neurons (for references, see Storm-Mathisen et al. 1995).

Development of a concept of purinergic neurotransmission was even more painful and long-lasting. Despite the fact that signaling action of purines was suggested in 1929 by Alan Drury and Albert Szent-Györgyi (Drury and Szent-Györgyi 1929) who found that intravenous injections of adenosine-5′-monophosphate (or adenylic acid) and adenosine evoked prominent negative chronotropy (up to a complete cardiac arrest), dilatation of coronary blood vessels that resulted in profound hypotensive actions, and also inhibited spontaneous activity of intestinal smooth muscle. Much further evidence for effects of adenine nucleotides on cardiovascular system and smooth muscle has been accumulated in 1940–1950s, and yet disbelief and outright animosity met the idea of purinergic neurotransmission and purinergic nerves promulgated by Geoffrey Burnstock (Burnstock 1972). Only at the beginning of 1990s, after purinergic receptors were cloned and quantal release of ATP was electrophysiologically recorded, the ATP was recognized as a *bona fide* neurotransmitter and the concept of purinergic neurotransmission was generally acknowledged (see Burnstock and Verkhratsky 2012 for details and extensive reference list).

Neurotransmission, that for a long time was believed to be a sole function of neurons, was, in last two decades, discovered in brain non-excitable cells, in astrocytes. First, multiple receptors for virtually all classical neurotransmitters (including all types of glutamate ionotropic and metabotropic receptors as well as all types of purinoceptors) were found in neuroglial cells (Verkhratsky 2010). Second, the discovery of a vesicular release mechanism for glutamate in astroglia (for references, see Parpura et al. 2010) has extended the classical concept of neurotransmission from being a synaptic/neuronal event to be a process involving an obligatory partnership between the pre- and postsynaptic nerve endings and the surrounding astrocytic membranes referred to as the tripartite synapse (Araque et al. 1999). Third, it

was demonstrated that astrocytes are releasing ATP in response to physiological stimulation and this release may occur by several mechanisms including quantal exocytosis (Lalo et al. 2011).

Chemical transmission in the CNS is regulated by several complex mechanisms responsible for synthesis of neurotransmitters, their accumulation into vesicles, their release and inactivation. In many cases these mechanisms are segregated between different cells types, which is particularly prominent for both glutamatergic and purinergic transmission. Therefore, we continue with brief description of the turnover of glutamate and ATP in the CNS.

1.3 Synthesis of Glutamate in the CNS

Glutamate metabolism is extremely complex (as shall be discussed in detail in the dedicated chapter by Schousboe et al. 2014). Here, we limit ourselves to a rather concise description of the differences between neuronal and astrocytic glutamate biosynthesis.

1.3.1 Neurons Cannot Produce Glutamate from Glucose

Classical studies of brain metabolism utilizing radioactively labeled glucose ([^{14}C] glucose) had provided compelling evidence that glutamate would extremely rapidly become labeled with carbon-14 (for references, see Schousboe 2012) demonstrating an intense glutamate synthesis from glucose, which according to a study by Okamoto and Quastel (1972) likely reflects neuronal synthesis of glutamate. For a number of years it was thought that glucose could act as a substrate for net synthesis of glutamate, a process that requires the presence of an anaplerotic mechanism allowing synthesis of a tricarboxylic acid (TCA) cycle intermediate (see, Schousboe et al. 2014). In the brain this process is catalyzed by the enzyme pyruvate carboxylase (PC) (Patel 1974) and the discovery that this enzyme is only expressed in astrocytes (Yu et al. 1983; Shank et al. 1985) led to a revision of this notion. Since neurons do not express pyruvate carboxylase they are incapable of performing a net synthesis of glutamate which is derived from the TCA cycle intermediate α-ketoglutarate (see, Schousboe et al. 2014). This is not a contradiction to the intense labeling from radioactively labeled glucose as carbon-14 labeling does not represent a net synthesis. The labeling is a result of the fact that the two carbon atoms entering the TCA cycle as acetyl-CoA and which originate from glucose remain in the cycle during the first turn and therefore appear in α-ketoglutarate and hence in glutamate (see, Schousboe et al. 2014).

The direct precursor for glutamate is glutamine which by the action of phosphate activated glutaminase (PAG) being expressed in neurons is deamidated to form glutamine (Kvamme et al. 2001). Interestingly, neurons are also incapable of

performing a net synthesis of glutamine as they do not express the enzyme required to convert glutamate to glutamine, glutamine synthetase (GS) which is an astrocytic enzyme (Norenberg and Martinez-Hernandez 1979). Altogether this means that the glutamatergic neurons are completely dependent on an astrocytic partnership in order to maintain the glutamate neurotransmitter pool (see, Schousboe et al. 2014).

1.3.2 Synthesis of Glutamate in Astrocytes

Neurons lack the two key enzymes which are obligatory for a cell to perform a net synthesis of glutamine from glucose, i.e., PC and GS. The latter enzyme is not important for glutamate synthesis, but rather this astrocyte-specific enzyme is of importance for maintaining the glutamate content in neurons. Namely, as discussed above, neurons import glutamine from astrocytes in order to produce glutamate by hydrolytic deamidation of glutamine. This is a consequence of the design of the glutamate neurotransmission which to a large extent operates by recycling glutamate and glutamine between the glutamatergic neurons and the surrounding astrocytes in the glutamate–glutamine cycle (for details, see Schousboe et al. 2014). The key enzymatic pathways allowing a net synthesis of glutamate from glucose involve conversion of two molecules of pyruvate originating from the glycolytic pathway to acetyl-CoA and oxaloacetate (OAA) catalyzed by the enzymes pyruvate dehydrogenase and PC, respectively. As stated above PC is only expressed in astrocytes, which makes this cell unique in the sense that in the brain it is the only cell type that can perform a net synthesis of glutamate. OAA and acetyl-CoA will condense to form citrate and in subsequent steps of the TCA cycle α-ketoglutarate is formed and this can in an aminotransferase reaction be converted to glutamate (Westergaard et al. 1996). In the case when glutamate is utilized in the astrocyte as a gliotransmitter it is packaged into vesicles in analogy to the glutamatergic neurons (reviewed in Montana et al. 2006). If it is to be used in the glutamatergic neuron as neurotransmitter, it is converted to glutamine by GS and subsequently transferred to the neurons. The transfer is based on the presence of sets of specific transporters for glutamine in the astrocytic and neuronal plasma membranes facilitating release from the astrocytes and uptake into the neurons (for details, see Schousboe et al. 2013).

1.3.3 Astroglia Control Glutamatergic Transmission by Glutamate–Glutamine Shuttle

Astrocytes are the main regulator of extracellular glutamate in the brain (see e.g., Eulenburg and Gomeza 2010 for review); perisynaptic astrocytes take up ~80 % of all glutamate released during synaptic transmission, with only ~20 % being accumulated into neurons (mainly postsynaptic). To ascertain continuity of neurotransmission astrocytes possess a special system that recovers glutamate to the presynaptic

terminal. As has been described above, glutamate, that enters astroglial cells is metabolized (by glutamine synthetase) into glutamine, which, subsequently, is transferred into presynaptic compartments; this system being generally referred to as glutamate–glutamine shuttle. Transport of glutamine from astrocytes and into neurons is mediated by specific amino acid transporters (Edwards 2007). The Na^+/H^+ dependent sodium coupled neutral amino acid transporters SN1/SNAT3/SLC38A3 and SN2/SNAT5/SLC38A5, also known as an N transport system that mediates glutamine efflux is expressed exclusively in astrocytes. In contrast, neurons express the system A represented by the sodium coupled neutral amino acid transporters ATA1/SNAT1/SLC38A1 and ATA2/SNAT2/SLC38A2 which act as influx transporters mediating glutamine accumulation into the presynaptic terminals. After entering these terminals glutamine is hydrolyzed to glutamate, which afterwards is accumulated into synaptic vesicles.

1.4 Metabolism of ATP: The Primary Role of Astroglial Adenosine Kinase

After being released into the extracellular space ATP is very rapidly degraded to its derivatives ADP, AMP and finally to adenosine by multiple ectonucleotidases that keep ambient ATP level in brain interstitial fluids at a range of <10 nM. Extracellular adenosine, in turn, is regulated by the astroglial adenosine cycle. Astrocytes accumulate adenosine by two equilibrium transporters and one Na^+-dependent concentration transporter; subsequently adenosine is catabolized intracellularly by adenosine kinase, which is predominantly expressed in astrocytes (Boison et al. 2010). Astroglia-localized adenosine kinase acts as a dynamic controller of adenosine levels in the CNS, and transgenic deletion of this astroglial enzyme is lethal.

1.5 Mechanisms of Glutamate and ATP Release

Glutamate and ATP can be released from neural cells (i.e., from neurons, both at synaptic locations and extrasynaptically, and from astroglia) through multiple mechanisms (see Parpura and Zorec 2010; also Vardjan et al. 2014). Arguably the most abundant and possibly the most physiological release mechanism is represented by Ca^{2+}-regulated exocytosis.

1.5.1 The Ca^{2+}-Regulated Exocytosis

The Ca^{2+}-regulated exocytosis is controlled by a complex molecular machinery that includes the core ternary Soluble N-ethyl maleimide-Sensitive Fusion protein (NSF) Attachment Protein (SNAP) REceptor or SNARE complex composed of the

vesicle-associated membrane protein synaptobrevin 2, and proteins located at the plasma membrane: syntaxin and synaptosome-associated protein of 25 kDa (SNAP25) in neurons; astroglial homologue of the latter is known as SNAP23 (Montana et al. 2006; Parpura et al. 2010). This complex is further assisted by several associated proteins represented, for example, by the Ca^{2+}-sensor synaptotagmin 1 in neurons and its homologue, albeit in mammals Ca^{2+}-insensitive, synaptotagmin 4 in astrocytes.

The uptake of neurotransmitters into vesicles is carried out at the level of vesicle membrane by dedicated transporters, which generally utilize an electrochemical proton gradient provided by the V-type H^+-ATPase localized in the vesicular membrane. Glutamate accumulation is mediated by the vesicular glutamate transporters (VGLUTs), that exist in three isoforms VGLUT 1, 2, and 3 expressed in both synaptic vesicles and in vesicles in astroglial cells. ATP is transported by vesicular nucleotide transporter VNUT, which along with VGLUTs belongs to the family of SLC17 family of solute carrier transporters; the VGLUT isoforms designated as SLC17A6-8 and VNUT as SLC17A9 (Sawada et al. 2008). The release of ATP accumulated and stored in lysosomes was observed in cultured astrocytes (Zhang et al. 2007), although operation of this mechanism in physiological conditions in cells in healthy tissue remains to be confirmed.

1.5.2 Diffusional Release of Glutamate and ATP: The Role for Membrane Channels

Alternatively, both glutamate and ATP could be released by diffusion through plasmalemmal ion channels. This type of release is supported by steep transmembrane concentrational gradient established for both glutamate and ATP. The intracellular concentrations for glutamate and ATP are in mM range, whereas extracellular concentrations of these molecules are much lower (~25 nM for glutamate and <10 nM for ATP).

First, ATP and glutamate, which both are present in anionic form, could be secreted via various types of anion channels. These channels may include (1) the so-called "maxi" or large-conductance ATP-permeable anion channels activated by cell swelling, (2) Volume-Regulated Anion Channels or VRAC (which are also known as volume-sensitive outwardly rectifying, VSOR Cl^- channels) activated upon osmotic shock; (3) Tweety anion channels which have relatively large conductance and are ATP permeable; (4) Cystic fibrosis transmembrane conductance regulator (or CFTR) anion channels and possibly other types of anion channels such as γ-aminobutyric acid and glycine receptors, Ca^{2+}-activated Cl^- channels of anoctamins family, acid-activated Cl^- channels etc., although their role in neurotransmitter release has not been thoroughly explored. Second, glutamate and ATP can be released through $P2X_7$ purinoceptors that upon strong activation form large-conductance membrane pore (which can pass molecules with molecular weight up to 900 Da). Third, glutamate and ATP can be released through large permeability

pores produced by opening of unpaired connexins (or hemichannels) or pannexins (for detailed descriptions and references see Pelegrin and Surprenant 2009; Scemes et al. 2009; Fields 2011).

1.6 Multitude of Receptors Underlie Functional Diversity of Glutamatergic and Purinergic Signaling

Cellular functional responses initiated by glutamatergic and purinergic transmission are extremely diverse that is determined by the multitude of the receptors expressed on the target cells. Glutamate and purines interact with both ionotropic and metabotropic receptors abundantly expressed in neurons (both at postsynaptic densities and extrasynaptically) in all types of glia (astrocytes, oligodendrocytes, NG2 cells, and microglia) (see Verkhratsky and Burnstock 2014). The glutamate ionotropic receptors (Wisden and Seeburg 1993) mediate fast excitatory transmission, which is the main function of α-amino-3-hydroxy-5-methyl-isoxazole propionate (AMPA) and kainate receptor subtypes, as well as synaptic plasticity, the latter being regulated by *N*-methyl-D-aspartate (NMDA) glutamate receptors that require conditioning depolarization for activation and when activated mediate substantial local Ca^{2+} signals that affect synaptic efficacy (see e.g., Collingridge and Bliss 1995). The role of P2X receptors which are expressed in many central synapses is less well established, although they contribute to excitatory transmission, and provide for Ca^{2+} influx at hyperpolarized potentials (i.e., they do not require conditioning depolarization) which in turn is important for regulation of synaptic plasticity (Pankratov et al. 2009). Glial cells also express full complement of ionotropic glutamate receptors and several subtypes of P2X receptors that control glial ionic signaling (Illes et al. 2012; Verkhratsky et al. 2009). In astrocytes in particular both AMPA and NMDA receptors (the latter demonstrating a weak Mg^{2+} block at physiological membrane potentials) are reported to be activated by glutamate released during ongoing synaptic transmission (Verkhratsky and Kirchhoff 2007); they mediate Na^+ and Ca^{2+} fluxes, which control local ionic signaling in astroglial perisynaptic processes. Dynamic changes in the cytosolic concentration of these ions in turn regulate numerous astrocytic transporters that contribute to glial homeostatic response (Kirischuk et al. 2012; Parpura and Verkhratsky 2012). Ionotropic purinoceptors of $P2X_4$ and $P2X_7$ subtypes are also constitutively expressed in microglial cells and their activation control various aspects of microglial function (Kettenmann et al. 2011) for controlling motility and activation of microglia which are fundamental for CNS innate immunity and defensive response of the nervous tissue.

Both neurons and neuroglia express multiple metabotropic, glutamatergic, and purinergic receptors (see Verkhratsky and Burnstock 2014). Metabotropic glutamate receptors (mGluRs) are subdivided into three groups: group I includes mGluRs 1 and 5 that are functionally linked to phospholipase C and synthesis of 1,4,5-inositol-trisphosphate and diacylglycerol; group II (mGluRs 2, 3); and group III (mGluRs 4, 6, 7, 8); the latter two groups are coupled to adenylate cyclase.

In neuroglia the mGluR3 and mGluR5 are seemingly the most abundant. Similarly, neuroglial cells express numerous types of metabotropic purinoceptors including all four types (A_1, A_{2A}, A_{2B}, and A_3) of adenosine receptors and several types of P2Y receptors with predominant expression of $P2Y_{1,4,6}$ receptors. Activation of these receptors regulates multiple and diverse neuronal and glial functions from regulation of synaptic plasticity to numerous metabolic, trophic and cell differentiation, growth and survival responses (see Hertz et al. 2014 and Burnstock and Verkhratsky 2012).

1.7 Glutamatergic and Purinergic Transmission in Neuropathology

Glutamate and ATP are also fundamental mediators of brain pathology. Conceptually, the appearance of high concentrations of both molecules in the extracellular space is indicative of neural tissue damage, because all cells contain high intracellular concentrations of glutamate and ATP. Glutamate, excessively released during brain ischemia (see chapters by Sayre et al. 2014; Baltan 2014; and Ding 2014) acts as a neurotoxin and stimulates cellular death (both necrotic and apoptotic; see Ouyang et al. 2014). The nature of excitotoxicity lies in an excessive activation of NMDA receptors and cellular overload with Ca^{2+}, which in turn compromises mitochondria and activates numerous proteolytic enzymes. ATP, which is also released during various types of CNS insults, exerts more complex actions (see Franke and Illes 2014). Excessive concentrations of ATP activate astrogliotic response and control activation of microglia, both types of reactions being fundamental for brain defense and development of brain pathology.

1.8 Envoi

The intent of this chapter was to make introductory remarks to the chapters within this book dedicated to the in-depth overview of the role of glutamate and ATP in the brain metabolism and signaling in health and disease. In the collection of papers that follow, written by the leading experts in the field of cell metabolism and energetic, intracellular, and intercellular signaling, we cover various aspects of the interfacing these two fundamental molecules. We adopt integrative approach and discuss the ATP-glutamate interactions at various levels of living mater organization from single molecules to the organismal physiology. We trust that this book will be valuable for the researchers and workers in the fields of neurobiology, neurology, and psychiatry.

Acknowledgments Authors' research was supported by Alzheimer's Research Trust (UK) Programme Grant (ART/PG2004A/1) to A.V. and by the National Institutes of Health (The Eunice Kennedy Shriver National Institute of Child Health and Human Development award HD078678) to V.P.

Conflict of Interest The authors declare no conflict of interest.

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Chapter 2
Glutamate Metabolism in the Brain Focusing on Astrocytes

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Abstract Metabolism of glutamate, the main excitatory neurotransmitter and precursor of GABA, is exceedingly complex and highly compartmentalized in brain. Maintenance of these neurotransmitter pools is strictly dependent on the de novo synthesis of glutamine in astrocytes which requires both the anaplerotic enzyme pyruvate carboxylase and glutamine synthetase. Glutamate is formed directly from glutamine by deamidation via phosphate activated glutaminase a reaction that also yields ammonia. Glutamate plays key roles linking carbohydrate and amino acid metabolism via the tricarboxylic acid (TCA) cycle, as well as in nitrogen trafficking and ammonia homeostasis in brain. The anatomical specialization of astrocytic endfeet enables these cells to rapidly and efficiently remove neurotransmitters from the synaptic cleft to maintain homeostasis, and to provide glutamine to replenish neurotransmitter pools in both glutamatergic and GABAergic neurons. Since the glutamate–glutamine cycle is an open cycle that actively interfaces with other pathways, the de novo synthesis of glutamine in astrocytes helps to maintain the operation of this cycle. The fine-tuned biochemical specialization of astrocytes allows these cells to respond to subtle changes in neurotransmission by dynamically adjusting their anaplerotic and glycolytic activities, and adjusting the amount of glutamate oxidized for energy relative to direct formation of glutamine, to meet the demands for maintaining neurotransmission. This chapter summarizes the evidence that astrocytes are essential and dynamic partners in both glutamatergic and GABAergic neurotransmission in brain.

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_2

Keywords Glutamate • Glutamine • GABA • Metabolic compartmentation • Astrocytes • Glutamate–glutamine cycle • Pyruvate carboxylase • Glutamine synthetase • Phosphate activated glutaminase • Glutamate dehydrogenase

2.1 Introduction

Glutamate plays a key role in intermediary metabolism in all organs and tissues linking carbohydrate and amino acid metabolism via the tricarboxylic acid (TCA) cycle. The reason is that it serves as a co-substrate in all reactions catalyzed by aminotransferases being converted to α-ketoglutarate, a key intermediate in the TCA cycle (McKenna et al. 2012). In the brain, glutamate metabolism extends beyond this general view as it serves as the immediate precursor for γ-aminobutyric acid (GABA) which is formed by decarboxylation of glutamate catalyzed by glutamate decarboxylase (GAD) as first shown by Roberts and Frankel (1950). The fact that GABA is metabolized by GABA-transaminase (GABA-T) and further to the TCA cycle intermediate succinate, catalyzed by succinic semialdehyde dehydrogenase, provides a deviation of the TCA cycle reactions called the GABA-shunt which circumvents succinyl CoA (see Schousboe et al. 2013). As both glutamate and GABA serve dual roles in the brain as metabolites and important neurotransmitters mediating excitatory and inhibitory signals, respectively (for references see Schousboe et al. 2012, 2013), their metabolic pathways are of significant interest. The immediate precursor for neuronal synthesis of glutamate is glutamine. This reaction is catalyzed by phosphate activated glutaminase (PAG) which hydrolytically deamidates glutamine to form glutamate and ammonia (for further details, see below). Interestingly, this enzymatic reaction was extensively investigated by Krebs (1935) in several tissues including the brain. Later, detailed studies of glutamate and glutamine metabolic pathways in the brain performed in the laboratories of H. Waelsch and J.H. Quastel (e.g., Quastel 1975; Berl and Clarke 1969) provided evidence that glutamate metabolism in the brain is extremely complex. It was noted that using radioactively labeled glucose as a precursor higher specific radioactivity was seen in glutamate; whereas, with the radioactive precursors leucine, acetate, and bicarbonate, glutamine exhibited a higher specific radioactivity than its precursor glutamate (for references, see Berl and Clarke 1969; Quastel 1975). A higher specific radioactivity in a compound (e.g., glutamine) than that seen in its precursor (e.g., glutamate) indicates that the precursor exists in separate metabolic pools having different turnover rates or in other words this is referred to as "metabolic compartmentation" (for further details, see McKenna et al. 2012). This finding led to the concept of metabolic compartmentation of glutamate in the brain, with at least two compartments (van den Berg and Garfinkel 1971; Garfinkel 1966) which were subsequently defined as representing neurons and astrocytes. This concept is based on the finding that glutamine synthetase (GS), the enzyme that converts glutamate to glutamine, is exclusively localized in astrocytes (Norenberg and Martinez-Hernandez 1979), together with the finding that higher specific radioactivity in glutamine is observed with the precursors acetate, bicarbonate, and leucine (see above).

It is of interest that the de novo synthesis of glutamate, the precursor of glutamine, is also restricted to astrocytes as the anaplerotic enzyme pyruvate carboxylase (PC) is exclusively localized in astrocytes (Shank et al. 1985; Yu et al. 1983). This will be discussed in further detail below.

2.2 Enzymatic Reactions Involving Glutamate as Substrate or Product

2.2.1 Aspartate Aminotransferase

This enzyme catalyzing the reversible interconversion of aspartate, α-ketoglutarate, oxaloacetate, and glutamate (Fig. 2.1) is present in all tissues, and has the highest specific activity among aminotransferases in the brain (for references, see Cooper 1988). The equilibrium constant for the enzyme is close to unity (Krebs 1953), hence, the reaction proceeds easily in both directions and is generally considered an exchange reaction. This is important considering the fact that the two keto acid substrates (oxaloacetate and α-ketoglutarate) are constituents of the TCA cycle. Thus, the corresponding amino acids aspartate and glutamate are in equilibrium with these TCA cycle intermediates and reflect the metabolic status of the keto acids at any given time due to the high activity of this enzyme (for references, see McKenna et al. 2012). This forms the basis for using the appearance of labeled carbon in glutamate and aspartate, from labeled precursors that provide substrates for the TCA cycle as a surrogate for determining the activity of the TCA cycle (see, McKenna et al. 2012). It should be noted, that aspartate aminotransferase (AAT) can reversibly bind to the inner mitochondrial membrane, which influences the functional activity of this enzyme (for further details, see McKenna et al. 2000, 2006).

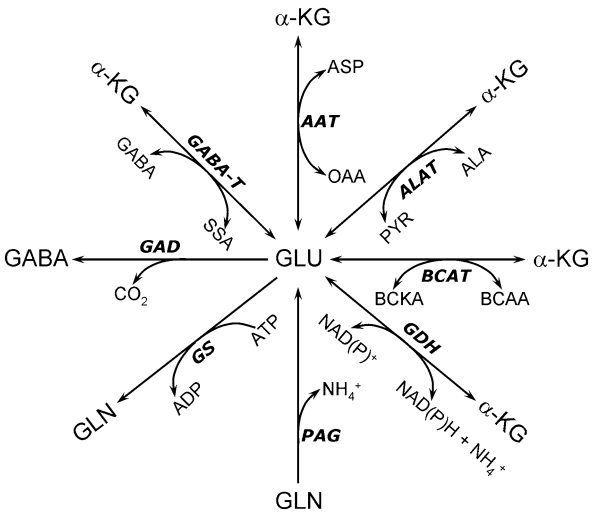

Fig. 2.1 Reactions involving glutamate (Glu) in astrocytes. *Ala* Alanine, *α-KG* α-ketoglutarate, *Asp* aspartate, *BCAA* branched chain amino acid, *Gln* glutamine. See text for other abbreviations

2.2.2 Alanine Aminotransferase

This aminotransferase catalyzes the reversible interconversion of alanine, α-ketoglutarate, pyruvate, and glutamate (Fig. 2.1) and its activity in the brain as well as in cultured brain cells is two orders of magnitude lower than that of AAT (Benuck et al. 1972; Larsson et al. 1985; Westergaard et al. 1993). The equilibrium constant for alanine aminotransferase (ALAT) is close to unity (Krebs 1953). Since pyruvate, one of the substrates for this enzyme is a product of glycolysis, ALAT couples the glycolytic pathway to amino acid metabolism and carbon label from glucose can be monitored by its appearance in alanine, which has a slower turnover rate than pyruvate. This provides a method to monitor glycolytic activity although it should be kept in mind that this relationship is not a direct correlation since the pyruvate pool has been shown to be compartmentalized (for further details, see Bakken et al. 1998; Obel et al. 2012; Waagepetersen et al. 2000).

ALAT is present in both astrocytes and neurons and may have a role in transfer of ammonia nitrogen between these cells as suggested by Waagepetersen et al. (2000). The ALAT reaction in concert with glutamate dehydrogenase (GDH) may be a particularly important mechanism for fixation of ammonia during hyperammonemic conditions when the normal activity of glutamine synthetase (see below) is inhibited (Dadsetan et al. 2011, 2013). This aspect will be discussed in further detail below.

2.2.3 Branched Chain Amino Acid Aminotransferase

The three branched chain amino acids (valine, leucine, and isoleucine) are metabolized by transamination with α-ketoglutarate, catalyzed by a common aminotransferase forming glutamate (Fig. 2.1) and the three keto acids α-ketoisovalerate, α-ketoisocaproate, and α-keto-β-methylvalerate, respectively (Cooper 1988). The branched chain amino acid aminotransferase isozymes (BCATs) are compartmentalized in brain cells; with the mitochondrial form being selectively localized in astrocytes and the cytosolic form localized in neurons (see, Lieth et al. 2001). This selective localization plays an important functional role as the branched chain amino acids also mediate shuttling of ammonia nitrogen between astrocytes and neurons (Bak et al. 2013; Lieth et al. 2001). This reaction may also contribute the amino group for de novo glutamate synthesis in astrocytes, which requires CO_2 fixation (Lieth et al. 2001). The branched chain amino acids have also been proposed to play a role in removal of ammonia during hyperammonemic conditions as seen in hepatic encephalopathy (Ott et al. 2005). Additionally, since these amino acids ultimately are metabolized to propionyl CoA, acetyl CoA, succinyl CoA, and acetoacetate, the latter three compounds can fuel the TCA cycle and facilitate glutamine production during hyperammonemia thereby ameliorating some of the excess ammonia (Ott et al. 2005). This functional role has been questioned since studies

using isolated neurons and astrocytes suggest that the branched chain amino acids are only modestly metabolized in brain (see, Bak et al. 2013). However, it should be noted that supplementation with branched chain amino acids was neuroprotective after traumatic brain injury (Cole et al. 2010).

2.2.4 Glutamate Dehydrogenase

This mitochondrially localized (Salganicoff and Derobertis 1965) enzyme catalyzes the redox-based interconversion of glutamate and α-ketoglutarate using $NAD(P)^+$ as coenzyme (Fig. 2.1). The thermodynamic equilibrium constant (6×10^{-15} M) favors the reductive amination of glutamate (Engel and Dalziel 1967). However, in the brain the normal direction of the reaction favors oxidative deamination of glutamate since the NAD^+/NADH ratio is high and the K_m, for ammonia (14–26 mM) is orders of magnitude higher than the prevailing ammonia concentration (Zaganas et al. 2001, 2009). The enzyme is highly regulated by ADP and leucine both of which function as allosteric activators and by GTP, which acts as an allosteric inhibitor (Spanaki et al. 2012). Emerging evidence suggests that GDH can be allosterically inhibited by mitochondrial SIRT4 (silent information regulator 4) that is highly expressed during brain development (Komlos et al. 2013; Lavu et al. 2008). Humans express two isoforms of the enzyme (GDH1 and GDH2) that differ dramatically with regard to the allosteric regulation; other mammals express only the housekeeping isoform, GDH1 (Spanaki et al. 2012). The enzyme is present in both neurons and astrocytes albeit the expression may be higher in astrocytes (Lovatt et al. 2007), particularly those from brain regions with high glutamatergic activity (Aoki et al. 1987).

As pointed out above, GDH in combination with aminotransferases is important for transferring ammonia to form amino acids from keto acids. Since reductive amination can only occur when the ammonia concentration approaches the K_m value for ammonia, it is likely that enzymatic complexes between GDH and the respective aminotransferases must exist to facilitate this reaction in vivo and there is some experimental evidence to suggest that this is indeed the case (Fahien et al. 1977; Islam et al. 2010; McKenna 2011).

2.2.5 Phosphate Activated Glutaminase

This enzyme which hydrolyzes glutamine to glutamate (Fig. 2.1) was first described by Krebs (1935), and its phosphate dependence was reported shortly thereafter by Errera and Greenstein (1949). The name, phosphate activated glutaminase or PAG, originates from this latter publication. The brain enzyme has been purified and extensively characterized by Kvamme and co-workers and is highly enriched in neurons (for review, see Kvamme et al. 2001). Using a preparation of cultured astrocytes, which may reflect the properties of these cells in situ (Lange et al. 2012), it

was shown by Schousboe et al. (1979) that this enzyme is also found in astrocytes. This finding has been controversial but a transcriptomic analysis of acutely isolated astrocytes has recently confirmed that PAG is indeed expressed by astrocytes (Lovatt et al. 2007). PAG is essential for synthesis of transmitter glutamate in glutamatergic neurons and it also plays a significant role in the synthesis of neurotransmitter GABA, since NMR studies revealed that glutamine synthesized in astrocytes and deamidated by neuronal PAG is preferentially used for GABA synthesis (see, Schousboe et al. 2013; Sonnewald et al. 1993c).

2.2.6 Glutamate Decarboxylase

The presence of GABA in the brain and its synthesis by α-decarboxylation of glutamate (see Fig. 2.1) was first described by Roberts and Frankel (1950). An enzyme, glutamic acid decarboxylase, catalyzing the conversion of glutamate to GABA was first identified in plants and bacteria. Therefore, it was concluded that formation of GABA from glutamate by α-decarboxylation in the brain was likely to be catalyzed by this enzyme. Glutamic acid decarboxylase (GAD) was finally purified to homogeneity from mouse brain more than 20 years later by Wu et al. (1973). Cloning studies subsequently showed that GAD exists in two isoforms with molecular weights of 65 and 67 kD; these isozymes are referred to as GAD65 and GAD67, respectively (for references, see Soghomonian and Martin 1998). In the brain these GAD isozymes are restricted to neurons and primarily GABAergic cells (Saito et al. 1974). The enzymes are also expressed in other tissues such as the pancreas (Okada et al. 1976). Both isoforms of the enzyme are located in the cytosol, but their subcellular localization differs, with the GAD67 isoform having a widespread expression compatible with a function related to general metabolism of the GABA pool; whereas the GAD65 isoform is mainly present in nerve endings and thought to be involved in the synthesis of neurotransmitter GABA (Walls et al. 2011; Martin and Rimvall 1993).

2.2.7 Glutamine Synthetase

As already pointed out, glutamate metabolism in the brain is highly complex and compartmentalized with different enzymes located in different cell types. The enzyme glutamine synthetase (GS) is responsible for conversion of glutamate to glutamine (Fig. 2.1) and is expressed exclusively in astrocytes (Norenberg and Martinez-Hernandez 1979). The functional importance of GS can most conveniently be assessed by use of the irreversible inhibitor methionine sulfoximine (MSO; Ronzio et al. 1969). The GS reaction adds a second nitrogen atom in the form of ammonia to glutamate to form glutamine and requires ATP for energy (Fig. 2.1). The K_m values for the three substrates glutamate, ammonia, and ATP are 2.5, 0.2, and 2.3 mM, respectively (Pamiljans et al. 1962), which means that the enzyme in situ is unlikely to be saturated with any of the substrates (see, Schousboe et al. 2012).

One of the most essential roles of astrocytes is removal of neurotransmitter glutamate from the synaptic cleft after depolarization (to maintain the low resting glutamate concentration of 1–10 μM) and conversion to glutamine (Bergles et al. 1999; Matsui et al. 2005). It should be noted, that the de novo synthesis of glutamine takes place only in astrocytes. De novo synthesis of glutamine from glucose requires the concerted action of glycolytic enzymes, pyruvate carboxylase, the TCA cycle, and conversion of α-ketoglutarate to glutamate, which via the action of GS can be converted to glutamine (Fig. 2.2). Pyruvate carboxylase like GS is dependent on

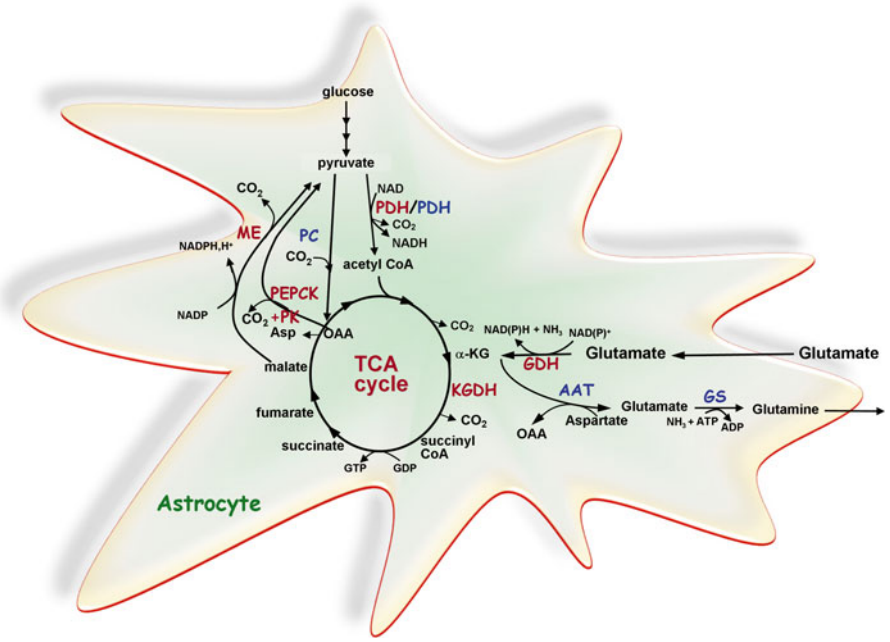

Fig. 2.2 Schematic representation of complete oxidation of glutamate via the "pyruvate recycling pathway" (essential enzymes in *red*) and de novo synthesis of glutamate and glutamine from glucose via pyruvate carboxylase (PC) (essential enzymes in *blue*) in astrocytes. For de novo synthesis of glutamate and glutamine, glucose is metabolized to acetyl CoA and oxaloacetate via pyruvate dehydrogenase (PDH) and pyruvate carboxylase, respectively. Acetyl CoA and oxaloacetate condenses to citrate and subsequently, α-ketoglutarate (α-KG) is formed. α-Ketoglutarate is converted to glutamate catalyzed by aspartate aminotransferase (AAT) and via an amidation, catalyzed by the energy requiring enzyme glutamine synthetase (GS), glutamine may be formed. The complete oxidation of glutamate is initiated by oxidative deamination catalyzed by glutamate dehydrogenase (GDH) and oxidative decarboxylation of α-ketoglutarate to succinyl CoA catalyzed by α-ketoglutarate dehydrogenase (KGDH). The carbon skeleton is subsequently converted to malate via multiple steps in the tricarboxylic acid (TCA) cycle and pyruvate via malic enzyme (ME). Alternatively, the concerted action of phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate kinase (PK) converts oxaloacetate (OAA) into pyruvate. The operation of ME or PEPCK plus PK in the direction of pyruvate is necessary for complete oxidation of glutamate. Pyruvate re-enters the TCA cycle via PDH and the carbon skeleton originating from glutamate may through this pathway be completely oxidized to CO_2 in the TCA cycle. *Asp* aspartate

ATP and is only expressed in astrocytes (Yu et al. 1983). Pyruvate carboxylation adds a carbon to pyruvate to form oxaloacetate and serves to replenish intermediates that would otherwise be drained from the TCA cycle by the conversion of α-ketoglutarate to glutamate and subsequently glutamine. ^{13}C-NMR studies show compartmentation in this process as the carbon added via pyruvate carboxylase is preferentially found in newly synthesized glutamine (Sonnewald et al. 1993a). Due to the selective cellular localization of glutamine synthetase and pyruvate carboxylase only astrocytes are able to perform a net synthesis of glutamine, which is the major precursor for the two most important neurotransmitters, glutamate and GABA (see, McKenna 2007; McKenna et al. 2012). A further discussion of this important aspect of brain function based on cellular specialization is provided below.

2.3 Overview of the Glutamate–Glutamine Cycle and Its Role in Glutamatergic Neurotransmission

Based on a series of elegant studies of radioactive labeling of glutamate and glutamine using different ^{14}C-labeled precursors such as glucose, acetate, and bicarbonate it was concluded that the brain contains *at least* two separate cellular compartments of these amino acids each having a distinct turnover of the amino acid pools (for references, see McKenna et al. 2012; Schousboe 2012). The seminal demonstration of the astrocyte-specific localization of glutamine synthetase (Norenberg and Martinez-Hernandez 1979) together with the multiple compartments of glutamate revealed by the labeling studies, led to the firm conclusion that the proposed glutamate–glutamine cycle (see Fig. 2.3) must be the mechanism allowing intercellular exchange of these amino acids (see, Öz et al. 2012, and Schousboe et al. 2012 for references). The selective cellular localization of specific glutamate and glutamine transporters on neuronal and glial plasma membranes is consistent with the functional importance of this cycle (see, Schousboe et al. 2012).

When the concept of the glutamate–glutamine cycle was first introduced it was thought that essentially *all* glutamate being released as neurotransmitter would be transferred to the astrocytes where it would quantitatively be converted to glutamine and subsequently transferred back to the glutamatergic neurons (Cotman et al. 1981). The fact that exogenous glutamate taken up by astrocytes can be oxidatively metabolized (see below for further details) demonstrates that the glutamate–glutamine cycle does not operate in a stoichiometric manner as first proposed (see, McKenna 2007; McKenna et al. 2012). If the glutamate–glutamine cycle exclusively operated in a stoichiometric fashion, then none of the glutamate taken up would be used to offset the high energy cost of glutamate transport into astrocytes, i.e., one ATP are required for transport of each glutamate molecule and activity of Na^+-K^+-ATPase to restore the sodium gradient (Attwell and Laughlin 2001; McKenna 2013).

Since the glutamate–glutamine cycle is an open cycle that loses intermediates to other pathways, the de novo synthesis of glutamine in astrocytes helps to maintain the operation of this cycle. Numerous studies have been aimed at obtaining a quantitative measure of the anaplerotic de novo synthesis of glutamate and glutamine; this information

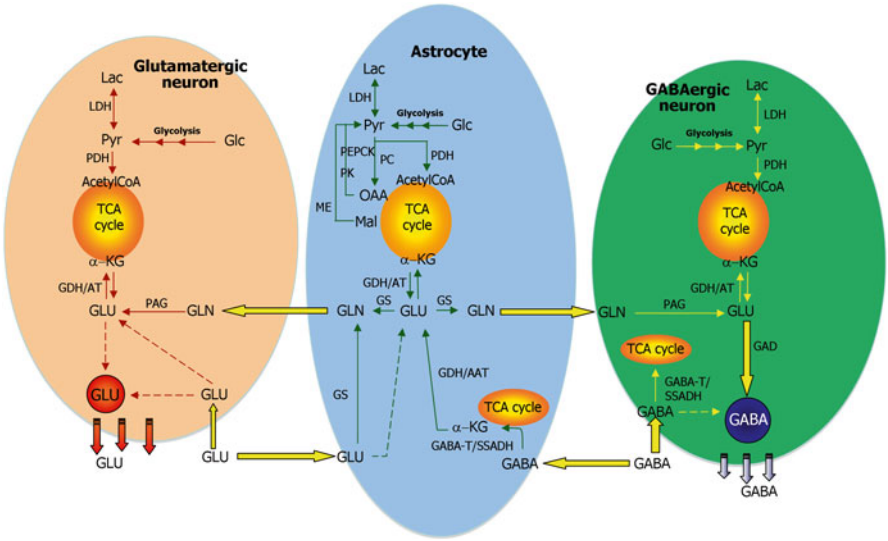

Fig. 2.3 Neurotransmitter glutamate (GLU) is mainly taken up by surrounding astrocytes subsequent to interaction with receptors in the synapse. In the astrocyte, glutamate is either converted to glutamine (GLN) catalyzed by glutamine synthetase (GS) as part of the glutamate–glutamine cycle or metabolized in the TCA cycle. Glutamine is transferred to the glutamatergic neuron to be used for synthesis of glutamate catalyzed by phosphate activated glutaminase (PAG). Glutamate enters the TCA cycle by the activity of GDH or an aminotransferase (AT), and the carbon skeleton may either be completely oxidatively metabolized via pyruvate recycling including malic enzyme (ME) activity or phosphoenolpyruvate carboxykinase and pyruvate kinase. Alternatively, the carbon skeleton supports the pool of TCA cycle intermediates and in that way potentially increases the oxidation of acetyl CoA in the TCA cycle. De novo synthesis of glutamate and glutamine from glucose occurs via the concerted action of PDH and pyruvate carboxylase (PC) making a net synthesis of TCA cycle intermediates. *CIT* citrate, *OAA* oxaloacetate, *PYR* pyruvate

has been summarized by Öz et al. (2012). The rate of anaplerosis mediated by pyruvate carboxylase ranges from 6 to 35 % of the rate of the TCA cycle and fulfills the need for de novo synthesis of glutamine (Öz et al. 2004, 2012). Since glutamine also serves as the main precursor for GABA (Sonnewald et al. 1993c) and some of the GABA released by neurons is taken up and metabolized by astrocytes, maintenance of GABAergic neurotransmission also relies on anaplerosis (Schousboe et al. 2013).

2.4 Influence of the Concentration of Glutamate on the Metabolic Pathways for Glutamate Metabolism

Early studies of glutamate metabolism in astrocytes using tracer concentrations of ^{14}C-labeled precursors reported a high proportion of the glutamate was converted to glutamine (Farinelli and Nicklas 1992; Zielke et al. 1990). However, a study by Yu et al. (1982) showed that a substantial fraction of exogenous glutamate was

metabolized via GDH and subsequently oxidized to CO_2 in astrocytes. Sonnewald et al. (1993b) using ^{13}C-NMR spectroscopy reported that a significant proportion of the label from glutamate metabolism in astrocytes was found in lactate. This labeling can only occur when glutamate is converted to α-ketoglutarate and further metabolized via the TCA cycle to malate, which can be converted to pyruvate and subsequently to lactate by a partial pyruvate recycling pathway (Fig. 2.4).

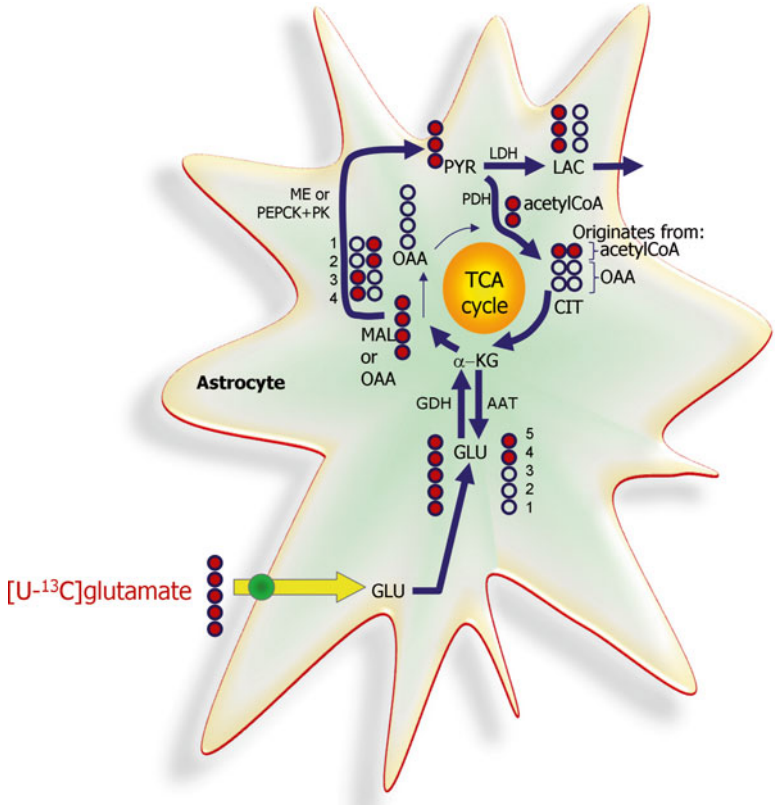

Fig. 2.4 [U-^{13}C]Glutamate (GLU) may be completely metabolized to CO_2 via the "pyruvate recycling pathway." Initially [U-^{13}C]glutamate is converted to α-ketoglutarate (α-KG) catalyzed mainly by GDH. Via the TCA cycle [U-^{13}C]malate (MAL)/oxaloacetate (OAA) is formed. [U-^{13}C]Malate may be oxidatively decarboxylated to [U-^{13}C]pyruvate (PYR) catalyzed by malic enzyme (ME). Alternatively, [U-^{13}C]oxaloacetate may be metabolized to pyruvate via [U-^{13}C]phosphoenolpyruvate catalyzed by the concerted action of phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate kinase (PK). The carbons originating from glutamate can subsequently re-enter the TCA cycle by oxidative decarboxylation of [U-^{13}C]pyruvate to [1,2-^{13}C]acetyl CoA catalyzed by PDH. Unlabeled oxaloacetate may condense with [1,2-^{13}C]acetyl CoA forming [1,2-^{13}C]citrate (CIT). From metabolism in the TCA cycle [4,5-^{13}C] α-ketoglutarate is formed and consequently [4,5-^{13}C]glutamate due to the activity of AAT. The symmetric succinate molecule causes scrambling of labeling and therefore both [1,2-^{13}C] and [3,4-^{13}C] oxaloacetates are formed. Oxaloacetate is in equilibrium with [1,2-^{13}C] and [3,4-^{13}C]aspartate due to the activity of AAT. Alternative to oxidative decarboxylation of pyruvate, [U-^{13}C]pyruvate may be converted to [U-^{13}C]lactate (LAC) catalyzed by lactate dehydrogenase (LDH)

These studies confirmed that a significant amount of glutamate was oxidatively metabolized for energy in astrocytes (Sonnewald et al. 1993b; McKenna 2013). McKenna et al. (1996) demonstrated that when the exogenous glutamate concentration was increased from 0.1 to 0.5 mM the proportion of glutamate oxidized by the TCA cycle in astrocytes greatly increased (from ~15 to 43 %) and the percent converted to glutamine decreased correspondingly (from ~85 to 57 %). This demonstrated that the metabolic fate of glutamate was shifted away from conversion to glutamine and towards oxidative metabolism as the concentration of glutamate in the extracellular milieu increased. Since the amount of glutamate in the synaptic cleft increases several orders of magnitude during depolarization (from ~1–10 μM to 1 mM) this provides astrocytes with the metabolic flexibility to readily form ATP from glutamate oxidation in the TCA cycle which offsets the high cost of glutamate transport into astrocytes (for further details, see McKenna 2013).

Recent studies show that the astrocyte glutamate transporter GLT-1 (in humans EAAT2) forms a complex with other proteins including hexokinase, several specific mitochondrial proteins including GDH and mitochondria that together serves to effectively channel glutamate towards energy producing oxidative metabolism rather than the energy utilizing reaction of conversion to glutamine in the cytosol (Genda et al. 2011).

2.5 Glutamate and Ammonia Homeostasis Under Normal and Hyperammonemic Conditions

2.5.1 Physiological Conditions

In glutamatergic and to some extent in GABAergic neurons ammonia is generated by the action of PAG, which removes the amido nitrogen from glutamine to form glutamate and subsequently GABA in GABAergic neurons (Fig. 2.5). Since GS, the primary enzyme capable of ammonia fixation in the brain, is located in astrocytes there needs to be a way by which ammonia nitrogen can be transferred between the neurons and the surrounding astrocytes. The specific mechanism(s) for returning ammonia removed by PAG in neurons to astrocytes has/have not been defined. For years it was assumed that the ammonium ion (being the prevailing form of ammonia) can diffuse freely through plasma membranes or perhaps through channels (Cooper and Plum 1987). Recently, two shuttle mechanisms based on exchange of an amino acid have been proposed (Lieth et al. 2001; Waagepetersen et al. 2000; Yudkoff et al. 1996; Zwingmann et al. 2000). Both models operate on the basis of ammonia being fixed into the amino group of glutamate by the action of GDH in neurons, and subsequently being transferred to either alanine or a branched chain amino acid, which serves to return the amino group to astrocytes (Fig. 2.5). In astrocytes the amino groups are transferred to glutamate by the respective aminotransferases (see above and Fig. 2.1) forming branched chain keto acids and pyruvate from the branch chain amino acids and alanine, respectively. The amino group transferred

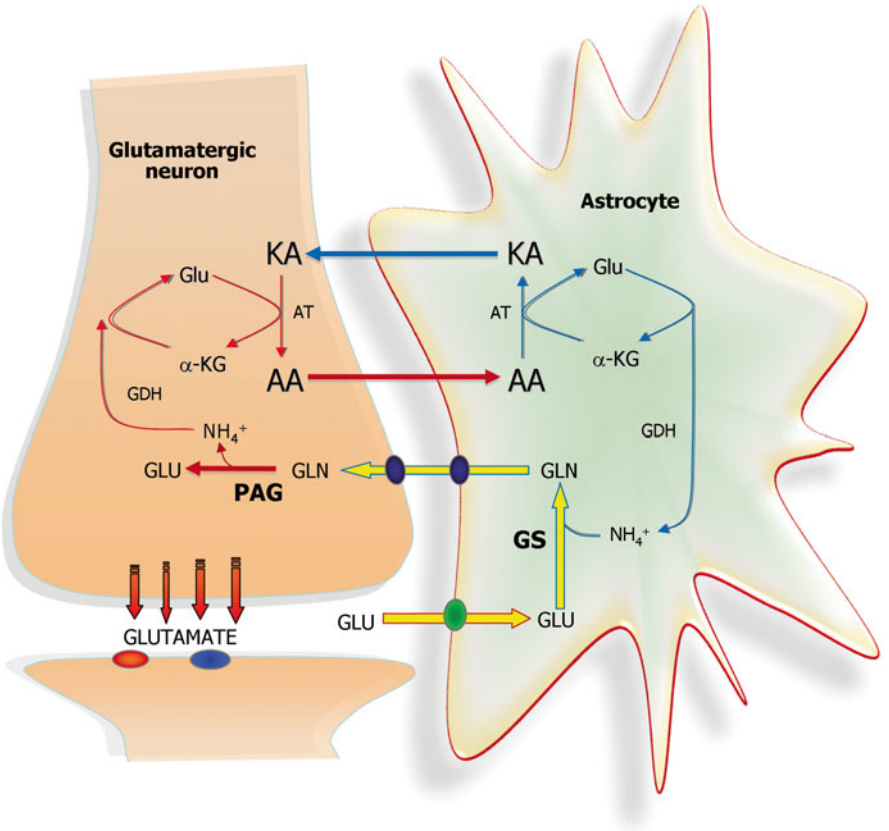

Fig. 2.5 Neurotransmitter glutamate is subsequent to receptor interaction primarily taken up mainly by astrocytes. Glutamate (GLU) is amidated to glutamine (GLN) via glutamine synthetase (GS) and glutamine is subsequently released from the astrocyte via specific transporters followed by uptake into the neuron. In the neuron, glutamine is deamidated by PAG to glutamate, which completes the glutamate–glutamine cycle. A net transfer of nitrogen from the astrocyte to the neuron occurs as part of the glutamate–glutamine cycle. This nitrogen transfer may be counteracted by transport of a neuro-inactive amino acid (AA) likely either alanine or one of the branched chain amino acids. The glutamate–glutamine cycle is coupled to an amino acid—keto acid (KA) cycle via the action of GDH and the relevant aminotransferase (AT)

to glutamate is subsequently removed by oxidative deamination via GDH making it available to glutamine synthetase for glutamine formation (see Figs. 2.1 and 2.5). A careful analysis of these mechanisms by modeling the rates of the reactions involved has, however, failed to provide evidence that the activity of these amino acid shuttles would be sufficient to match the actual need for transfer of ammonia back to astrocytes (Rothman et al. 2012). Hence, at the present time the quantitative importance of these proposed mechanisms for ammonia transfer in vivo has not been resolved.

2.5.2 *Hyperammonemia*

The most efficient pathway for ammonia fixation in the brain is the GS catalyzed conversion of glutamate to glutamine and a classical experiment by Cooper et al. (1979) using infusion of trace amounts of [^{13}N]ammonia into the brain showed that at least 98 % of the labeled ammonia could be found in the amide group of glutamine within seconds. When GS was inhibited by MSO, ammonia radioactivity was recovered in glutamate, albeit the amounts were small. This does, however, show that under appropriate conditions reductive amination mediated by GDH can occur (see above). In this context, it may be noted that during hyperammonemic conditions in neuronal-astrocytic co-cultures, the formation of both alanine and glutamine is increased (Leke et al. 2011). As pointed out above, de novo synthesis of glutamine requires CO_2 fixation by pyruvate carboxylase to form oxaloacetate; interestingly, hyperammonemic conditions have been shown to stimulate this pathway (Leke et al. 2011). On the other hand, when GS was inhibited and ammonia was fixed in alanine, the glycolytic pathway was accelerated while the anaplerotic pathway did not increase (Dadsetan et al. 2011, 2013). This shows that astrocytes are able to switch back and forth between anaplerosis and glycolysis depending on whether pyruvate or a TCA cycle intermediate is needed. This concept has been confirmed in cultures and in the brain in vivo since inhibition of GS by MSO during hyperammonemic conditions leads to a decrease in glutamine and an increase in alanine production (Dadsetan et al. 2011, 2013; Fries et al. 2014). This may be due in part to the fact that alanine does not function as an intracellular osmolyte in astrocytes, whereas glutamine does perform this function. It has been proposed that inhibition of GS may be a potentially useful therapeutic strategy to prevent the glutamine-associated glial swelling and the brain edema observed in hepatic encephalopathy (Brusilow et al. 2010; Dadsetan et al. 2011, 2013).

2.6 Concluding Remarks

It should be noted that glutamate metabolism is exceedingly complex and highly compartmentalized. Maintenance of the glutamate and GABA neurotransmitter pools is strictly dependent on the de novo synthesis of glutamine in astrocytes. The exquisite anatomical specialization of astrocytic endfeet enables these cells to rapidly and efficiently remove neurotransmitters from the synaptic cleft and provide glutamine to replenish neurotransmitter pools in glutamatergic and GABAergic neurons. This is possible due to the fine-tuned biochemical machinery including the distribution of pertinent transporters, as well as the presence of mitochondria and specific cytosolic enzymes in the astrocytic processes which ensheathe the synapses. This allows astrocytes to respond to subtle changes in neurotransmission by dynamically adjusting their anaplerotic and glycolytic activities to meet the demands for maintaining neurotransmission. Additionally, astrocytes can readily shift the

metabolic fate of glutamate removed from the synaptic cleft towards increased oxidative energy metabolism relative to direct formation of glutamine.

In conclusion, this chapter has summarized the evidence that astrocytes are dynamic partners in both glutamatergic and GABAergic neurotransmission in brain. Protoplasmic astrocytes cover and actively interact with hundreds of thousands to million synapses in human brain (Oberheim et al. 2006, 2009). Hence, astrocytes are essential partners in neurotransmission since they continuously monitor the extracellular milieu and modulate their metabolic activity in response to fluctuations in neuronal activity.

Conflict of Interest The authors declare no conflict of interest.

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Chapter 3
Glycogenolysis and Purinergic Signaling

Leif Hertz, Junnan Xu, and Liang Peng

Abstract Both ATP and glutamate are on one hand essential metabolites in brain and on the other serve a signaling function as transmitters. However, there is the major difference that the flux in the pathway producing transmitter glutamate is comparable to the rate of glucose metabolism in brain, whereas that producing transmitter ATP is orders of magnitude smaller than the metabolic turnover between ATP and ADP. Moreover, de novo glutamate production occurs exclusively in astrocytes, whereas transmitter ATP is produced both in neurons and astrocytes. This chapter deals only with ATP and exclusively with its formation and release in astrocytes, and it focuses on potential associations with glycogenolysis, which is known to be indispensable for the synthesis of glutamate. Glycogenolysis is dependent upon an increase in free intracellular Ca^{2+} concentration ($Ca^{2+}]_i$). It can be further stimulated by cAMP, but in contrast to widespread beliefs, cAMP can on its own not induce glycogenolysis. Astrocytes generate ATP from accumulated adenosine, and this process does not seem to require glycogenolysis. A minor amount of the generated ATP is utilized as a transmitter, and its synthesis requires the presence of the mainly intracellular nucleoside transporter ENT3. Many transmitters as well as extracellular K^+ concentrations high enough to open the voltage-sensitive L-channels for Ca^{2+} cause a release of transmitter ATP from astrocytes. Adenosine and ATP induce release of ATP by action at several different purinergic receptors. The release evoked by transmitters or elevated K^+ concentrations is abolished by DAB, an inhibitor of glycogenolysis.

Keywords Adenosine • Astrocyte • ATP • Calcium • Glycogenolysis • P2-ergic transmission

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_3

3.1 Introduction

Central nervous system function is profoundly affected by signaling exerted by adenosine, cyclic adenosine 3′,5′ monophosphate (cAMP), formed from adenosine triphosphate (ATP) (Rall and Sutheland 1962), and ATP itself, formed from adenosine via adenosine monophosphate (AMP) mainly in astrocytes (Matz and Hertz 1989; Studer et al. 2006). This chapter focuses on some of these functions as they relate to glycogenolysis, i.e., (1) purinergically-mediated cAMP generation and increase in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$); (2) glycogenolytic effects of adenosine and ATP; and (3) effects of glycogenolysis inhibition on stimulated release of ATP. In order to do so, regulation of glycogenolysis by the calcium ion (Ca^{2+}) and cAMP are briefly discussed. Purinergic effects on glycogenolysis represent a *direct* relationship between signaling by adenosine or ATP and brain metabolism. However, the metabolic rate of ATP–ADP turnover during energy metabolism is much higher than either ATP synthesis from adenosine or release of transmitter ATP. The rate of metabolic turnover can be calculated from a rate of oxidative metabolism of *glucose* that is essentially similar in astrocytes and neurons in the brain in vivo (reviewed by Hertz 2011) and in the rat/mouse amounts to ~0.6 μmol/min/g wet brain weight or 6 nmol/min/mg protein. This corresponds to an ATP/ADP turnover of ~180 nmol/min/mg protein or roughly 1,000 times the rate of ATP synthesis from adenosine via AMP in astrocytes (0.1–0.2 nmol/min/mg protein—see below). The rate of stimulated ATP release from astrocytes (~1 pmol/min/mg protein—see below) is again orders of magnitude lower. This represents a distinct difference from glutamate, which is produced and released at rates comparable to the rate of glucose metabolism in brain (Sibson et al. 1998; Rothman et al. 2011; Hertz 2013). It is also well known that glutamatergic signaling depends on glycogenolysis, which is required for de novo synthesis of glutamate from glucose, an entirely astrocytic process (Hertz et al. 2003; Gibbs et al. 2007). Glutamate synthesis is an essential process for signaling mediated by glutamate or γ-aminobutyric acid (GABA), where astrocytes and neurons are connected in a bidirectional glutamine–glutamate/GABA cycle (reviewed by Hertz and Zielke 2004; Hertz et al. 2007; Hertz 2013; Schousboe et al. 2014). No corresponding information is available about the possible involvement of glycogenolysis during adenosine or ATP signaling. This chapter shows that glycogenolysis is needed for ATP release from astrocytes during stimulation with transmitters or with elevated K^+ concentrations, and it discusses whether glycogenolysis may also be needed for its astrocytic synthesis from adenosine.

3.2 Glycogen

3.2.1 Glycogen and Glycogenolysis in Brain

Swanson et al. (1992) changed the historical concept of glycogen's role in the brain from that of an energy reservoir used in emergency situations to that of a compound consumed rapidly in response to enhanced neuronal activity by reporting that

glycogen is consumed during whisker stimulation. Since glycogen is located almost exclusively in astrocytes (Ibrahim 1975; Borke and Nau 1984), this must be an astrocytic response. Glycogenolysis is important for many signaling processes (e.g., Xu et al. 2013). It is ideal for such a role because it is rapidly degraded without initial phosphorylation (e.g., Hertz et al. 2007), and its Ca^{2+}-dependent degradation (Ozawa 1972) is initiated by many transmitters and by even small (and large) increases in extracellular K^+ concentration (Magistretti 1988; Hof et al. 1988; Obel et al. 2012).

Even when blood and brain glucose levels are normal, glycogenolysis occurs rapidly during and after brain activation (Dienel et al. 2002). Immediately after a one-trial aversive learning task the level of glycogen in forebrain of day-old chickens falls by 0.8 μmol glucose equivalent/min/g wet weight, and subsequently glycogen levels are restored to pre-training values at a rate of at least 0.2–0.3 μmol/min/g (Hertz et al. 2003). Inhibition of glycogenolysis by 1,4-dideoxy-1,4-imino-d-arabinitol (DAB) prevents glutamate synthesis and learning in this model (Gibbs et al. 2006, 2007). A facilitatory role on learning in the young chick by the ATP receptor agonist ADPβS and its blockade by the antagonist MRS2179 indicates an important role of ATP on memory consolidation via a P2Y1 (see below) receptor, which is abolished by DAB (Gibbs et al. 2011). Many experiments in that paper studied the ability of purinergic receptor activation to facilitate impaired learning, but it also showed inhibition of normal learning following administration of a purinergic antagonist. The importance of glycogen for learning has been confirmed by Suzuki et al. (2011) and Duran et al. (2013) in mammals and it has also been briefly reviewed (Hertz et al. 2013b).

During spreading depression, characterized by a very high extracellular K^+ concentration, the glycogen content in brain decreases by one quarter to one-third (Krivanek 1958; Lauritzen et al. 1990), with recovery within 10 min. This is probably related to the requirement for glycogenolysis during Na^+,K^+-ATPase-mediated astrocytic uptake of extracellular K^+ (Xu et al. 2013; Hertz et al. 2013c), an effect that also appears to be important during learning (Hertz et al. 2013b).

3.2.2 Glycogenolysis Is Stimulated by cAMP, But Only When Free Cytosolic Ca^{2+} Concentration ($[Ca^{2+}]_i$) Is Also Increased

Glycogenolysis is induced by activation of the enzyme glycogen phosphorylase with slightly different, although in principle identical, characteristics in brain, muscle, and liver. Briefly, the inactive phosphorylase *a* is converted to the active phosphorylase *b* by stimulation of another enzyme, phosphorylase kinase. Within the Sutherland group Rall et al. (1957) showed that increased formation of active phosphorylase in liver in the presence of noradrenaline and glucagon was mediated by a heat-stable factor, which accumulated when the hormones were incubated with ATP and particulate fractions of liver homogenates. Although the still widespread concept that the ATP product cAMP suffices to induce glycogenolysis is often

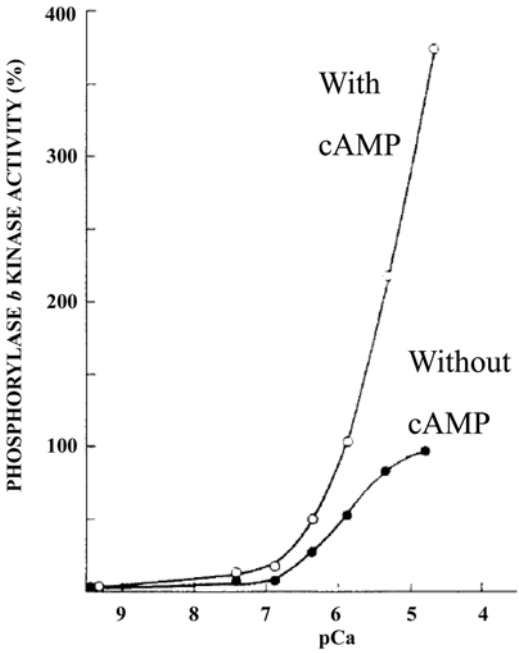

Fig. 3.1 Relationship between the activity of phosphorylase *b* kinase from cardiac muscle and the concentration of free calcium ion (Ca^{2+}) in the presence and in the absence of cyclic AMP (cAMP). Reaction mixtures contained (final concentrations): 4.0 mM ATP, 3×10^{-6} M cyclic AMP, 8×10^{3} units/mL phosphorylase *b*, appropriately diluted phosphorylase *b* kinase from cardiac muscle and specified concentrations of $CaCl_2$. The activity of the kinase is expressed as μg/mL of inorganic phosphate liberated in the assay medium of phosphorylase *a* after its conversion to phosphorylase *b*. *Closed circles* show the activities without cyclic AMP and *open circles* those with 3×10^{-6} cyclic AMP. From Ozawa (1972)

attributed to this group, this was not expressed in that paper. Ca^{2+} dependency of phosphorylase activation was demonstrated very clearly by Ozawa (1972) for muscle phosphorylase kinase (Fig. 3.1), which showed no activity at all in the absence of Ca^{2+}, even when cAMP was present. However, cAMP enhanced the stimulatory effect of Ca^{2+}. Based on these findings Ozawa (1972, 2011) concluded that adrenaline stimulates glycogenolysis by two mechanisms, an increase in $[Ca^{2+}]_i$, which is indispensable, and formation of cAMP which is facilitatory.

The phosphorylase kinase is a very complex protein kinase, with the holoenzyme consisting of four individual units, each having one catalytic (gamma) subunit and three different regulatory (alpha, beta, and delta) subunits (Brushia and Walsh 1999). The three regulatory subunits inhibit the phosphotransferase activity of the gamma subunit. Increase in $[Ca^{2+}]_i$ relieves inhibition via the delta subunit, which is identical to calmodulin. Phosphorylation of the alpha and beta subunits by cAMP also relieves inhibition of the gamma subunit and thereby activates the enzyme,

although only in the presence of an increase in $[Ca^{2+}]_i$ (Fig. 3.1), and phosphorylase kinase can also be activated by autophosphorylation (Brushia and Walsh 1999).

Results consistent with the conclusion by Ozawa (1972, 2011) that an increase in $[Ca^{2+}]$ suffices to stimulate glycogenolysis have been obtained in mouse brain slices by Ververken et al. (1982). They investigated the relative roles of cAMP and of Ca^{2+} for the activation of glycogen phosphorylase induced by noradrenaline (100 μM) and by depolarization (with 25 mM K^+) in mouse brain cortical slices. Short-term treatment with EGTA, a Ca^{2+} calcium chelator, or $LaCl_3$, competing with Ca^{2+}, abolished the noradrenaline-mediated activation of the phosphorylase, pointing to a critical role of extracellular Ca^{2+}, and the K^+-induced depolarization produced a rapid, fourfold activation of phosphorylase without increasing cAMP levels. These findings are in sharp contrast to a report by Choi et al. (2012) discussing cAMP formation as the important factor in K^+-mediated glycogenolysis. Also, during clearance of the increased extracellular K^+ by cultured astrocytes described above, the glycogenolytic stimulus was the elevation of the extracellular K^+ concentration itself, leading to an increase in $[Ca^{2+}]_i$, and the effect was prevented by inhibitors blocking the increase in $[Ca^{2+}]_i$ (Xu et al. 2013). K^+-stimulated glycogenolysis in cultured astrocytes is also abolished by interference of pathways interfering with increase in $[Ca^{2+}]_i$ (Xu et al. 2014b).

cAMP-independent activation of glycogenolysis has also been shown by Herlin et al. (1978) using human polymorphonuclear leukocytes and concluding that "activation of phosphorylase during phagocytosis is thus presumably due to an increase in cytosol Ca^{2+} and subsequent activation of phosphorylase kinase, and is independent of the simultaneous increase in concentration of cyclic AMP." This does obviously not negate transmitter- and cAMP-induced additional stimulation of glycogenolysis. Thus, during the extracellular K^+ undershoot following intense excitatory activity it appears that transmitter stimulation, primarily by β-adrenergic receptors, may lead to a glycogenolysis-dependent activation of the astrocytic Na^+, K^+-ATPase (Hertz et al. 2013c). However, like K^+-stimulated glycogenolysis (see above) isoproterenol-stimulated glycogenolysis is also dependent on increased $[Ca^{2+}]_i$, since it is prevented by inhibition of pathways shown in Fig. 3.2 (Du et al. 2010) leading to this increase via a G_s/G_i shift (Xu et al. 2014b). Analogously Sorg and Magistretti (1991) found only modest glycogenolysis in response to either dibutyryl cyclic AMP (dBcAMP), increasing intracellular cAMP, or phorbol dibutyrate (PdBU) a direct precursor of protein kinase C (PKC), stimulating the phosphoinositide system and thus Ca^{2+} release from the endoplasmic reticulum store. However, there was a pronounced glycogenolytic response to the two when given in combination (Fig. 3.3).

Fluoxetine, a $G_{q/11}$-linked specific 5-HT_{2B} receptor agonist (Li et al. 2008; Diaz et al. 2012), and elevated K^+ concentrations (Cai et al. 2011) activate pathways in cultured astrocytes very similar to that activated by β-adrenergic stimulation following its G_s/G_i shift (Du et al. 2010). Fluoxetine also activates glycogenolysis and after chronic treatment with this serotonin-specific reuptake inhibitor (SSRI) antidepressant, the transmitter-induced glycogenolytic response is increased

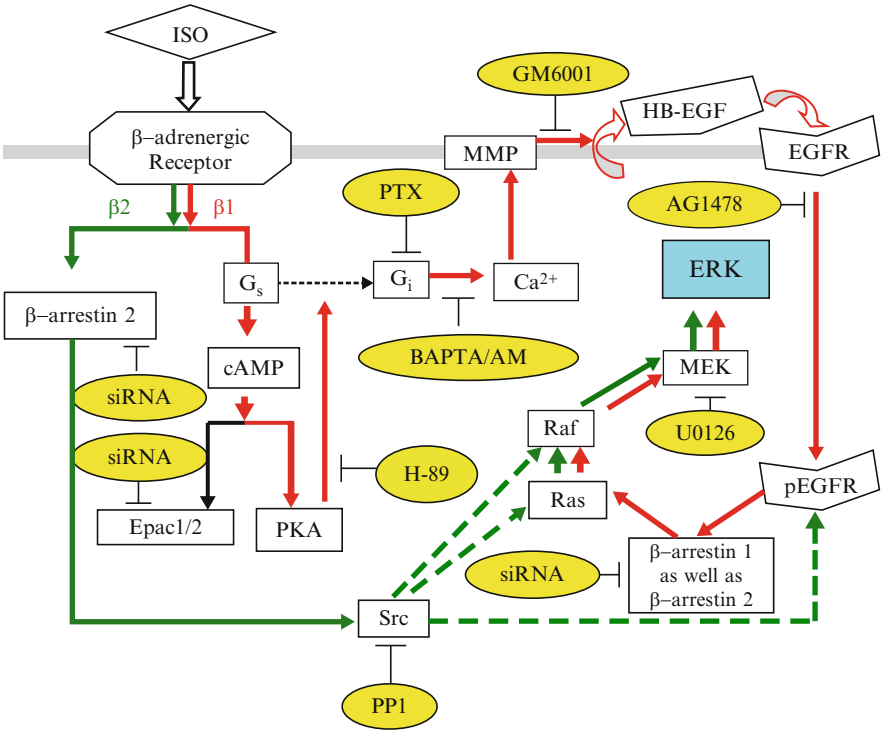

Fig. 3.2 β-Adrenergic pathways towards extracellular-signal-regulated kinase (ERK) phosphorylation and indication of specific inhibitors preventing the phosphorylation and used to delineate this pathway. At higher isoproterenol concentrations (≥1 mM), a β_1-induced, cyclic AMP- (cAMP) and protein kinase A-mediated G_s/G_i switch leads to an increase of $[Ca^{2+}]_i$, which stimulates a metalloproteinase and induces shedding of growth factor(s), leading to transactivation of the epidermal growth factor (EGF) receptor and subsequent $ERK_{1/2}$ phosphorylation. This is indicated by *red arrows* and the increase in $[Ca^{2+}]_i$ is essential for glycogenolysis, which is further enhanced by the formation of cAMP. Whether further downstream effects also are important for stimulated ATP release is not known and not dealt with in this paper. The same applies to lower concentrations (≤100 nM) of isoproterenol, which stimulate phosphorylation of $ERK_{1/2}$ by β_2-receptor and β-arrestin-mediated Src activation, without involvement of the EGF tyrosine kinase (*green arrows*). From Du et al. (2010)

(Kong et al. 2002). All of these pathways evoke transactivation of the epidermal growth factor (EGF) receptor leading to release of an agonist of this receptor (perhaps heparin-binding EGF). EGF alone has glycogenolytic effect in cultured astrocytes (Xu et al. 2014b) and it can cause phosphorylation of extracellular regulated kinase (ERK), stimulation of Ca^{2+}-dependent phospholipase A2 ($cPLA_2$) and increase in release of arachidonic acid (AA) and synthesis of its metabolites, as will be discussed later. EGF increases astrocytic $[Ca^{2+}]_i$, perhaps by promoting refilling of intracellular stores (Morita and Kudo 2010). Additional important questions regarding mechanisms of glycogenolysis in brain are discussed by DiNuzzo et al. (2013).

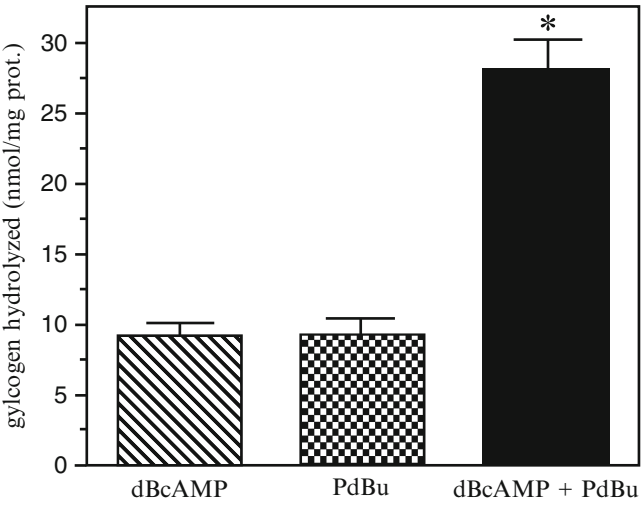

Fig. 3.3 Separate administration of either dibutyryl cyclic AMP (dBcAMP), which leads to an increase in intracellular cAMP, or phorbol dibutyrate (PdBU) a direct precursor of proteinkinase C (PKC), stimulating the phosphoinositide system and thus Ca^{2+} increase, have only little glycogenolytic effect on cultured astrocytes. However, a pronounced glycogenolytic response is seen when the two are given in combination. From Sorg and Magistretti (1991)

3.3 Purinergic Receptor Activity Connected with cAMP Increase and/or PKC-Mediated Increase in $[Ca^{2+}]_i$

3.3.1 Purinergic Receptors

The purinergic receptors are the nucleoside (P1) and nucleotide (P2) receptors. They have been thoroughly reviewed by Verkhratsky et al. (2009) and by Verkhratsky and Burnstock (2014). This chapter only reviews a few aspects, which are especially relevant for purinergic stimulation of glycogenolysis.

3.3.2 P1 Receptors

Almost 40 years ago the Hamprecht laboratory reported stimulation of cAMP formation in astrocyte-enriched cultures at relatively high adenosine concentrations (van Calker et al. 1979). In contrast, submicromolar concentrations of adenosine inhibited the increase in the intracellular level of cAMP caused by β-adrenoceptor agonists. These two effects were concluded to be mediated by two different adenosine receptors, which were named A1 and A2 receptors. This nomenclature is still valid, although the A2 receptor is now known to comprise an $A2_A$ and an $A2_B$

receptor (e.g., Jacobson and Gao 2006). A_{2A} adenosine receptors mediate their effects predominantly through coupling to adenylyl cyclase and cAMP formation, but in at least some cells their activation might also induce formation of inositol phosphates, rise in $[Ca^{2+}]$, and activation of PKC (Offermanns and Simon 1995). No such increase was shown by González-Benítez et al. (2002) in hepatocytes, although glycogenolysis was stimulated (discussed below), but Ca^{2+} entry from extracellular fluid had been prevented in their experiments. $A2_B$ receptors are positively coupled to both adenylyl cyclase and phospholipase C (PLC) (e.g., Jacobson and Gao 2006). The low adenosine concentrations stimulate mainly the A1 receptor-mediated increase in PLC activity and thus increase in $[Ca^{2+}]_i$ at least partly by release from intracellular stores (e.g., Jacobson and Gao 2006). A relatively small increase in hepatocytes has also been shown by González-Benítez et al. (2002), as shown in Fig. 3.4.

An additional adenosine receptor, showing neither A1 nor A2 specificity, and reducing the efficacy of intracellular Ca^{2+} signaling received its first thorough, albeit theoretical, description and naming as the A3 receptor by Ribeiro and Sebastião (1985). Its presence was later demonstrated in several tissues, including brain, by Zhou et al. (1992), Linden et al. (1993) and Sajjadi and Firestein (1993). Its activation reduces forskolin-stimulated cAMP accumulation in Chinese hamster ovary cells transfected with recombinant sheep A3 adenosine receptors (Linden et al. 1993). It couples to classical second messenger pathways such as inhibition of adenylyl cyclase, stimulation of PLC, and Ca^{2+} mobilization (Shen et al. 1995), and it also activates mitogen-activated protein kinases (e.g., Jacobson and Gao 2006).

Adenosine stimulates glycogenolysis in cultured astrocytes and brain slices, and at least in the cultured cells the stimulation was found to be dependent upon cAMP signaling (Magistretti et al. 1986; Sorg and Magistretti 1991). Stimulation of $A2_A$ receptors has been reported also to cause a delayed enhancement of glycogen synthesis in cultured astrocytes (Allaman et al. 2003), but it is unknown whether this is a specific effect of adenosine, or a response to adenosine as one of many transmitters causing morphological (Abe and Saito 1998) and functional (Meier et al. 1991) differentiation of cultured astrocytes. A similar claim for β-adrenergic receptors (Sorg and Magistretti 1992) could not be replicated in cultures already treated with the differentiating agent dBcAMP, where glycogen synthesis is stimulated by α_2-adrenergic signaling (Hertz et al. 2007).

It is unknown if adenosine activation of A2 receptors may be able to activate glycogenolysis in brain via a pathway related to that used after stimulation of β-adrenergic receptors (Fig. 3.2), i.e., after a possible G_s/G_i switch. Since stimulation of A1 receptor stimulates both PLC and ERK phosphorylation and thus probably also glycogenolysis, demonstration of this would ideally require use of a subtype-specific agonist. Moreover, ERK phosphorylation activated by an effect on A3 receptors (Shen et al. 2005) might also stimulate glycogenolysis. Effects of subtype receptor agonists have been carried out in isolated hepatocytes, reportedly comparable to normal hepatic parenchymal cells in situ in appearance and function (Berry and Friend 1969). Stimulation of glycogenolysis, measured after 45 min of

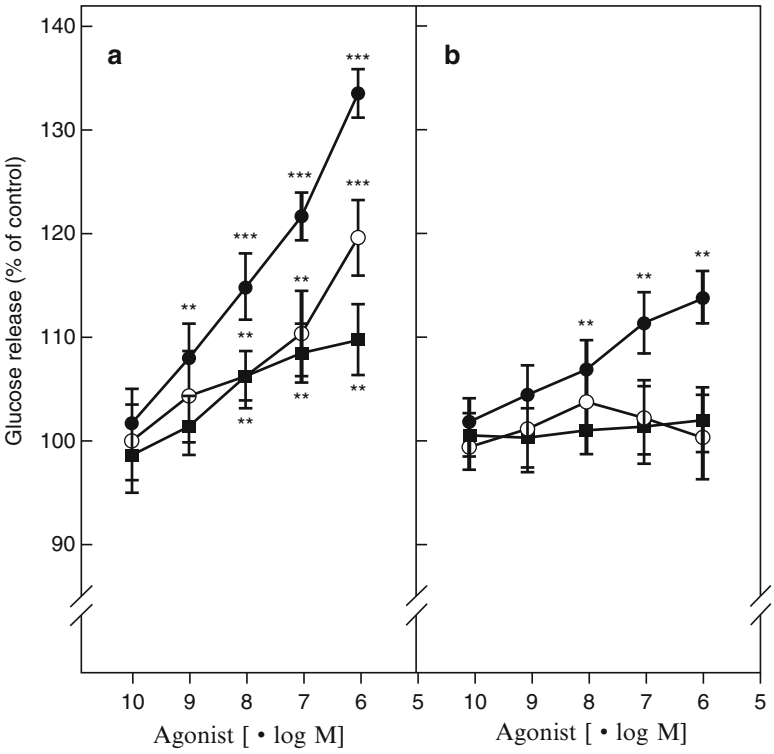

Fig. 3.4 Effect of adenosine receptor-selective agonists on glycogenolysis in hepatocytes. Hepatocytes from rats fed ad libitum were incubated in Krebs-Ringer in the presence (**a**) or absence (**b**) of 1.2 mM extracellular Ca^{2+} with a different agonist: CCPA (*filled circle*), an A1 agonist; CGS-21680 (*open circle*), an A2A agonist; or IB-MECA (*filled square*), an A3 agonist. Each value represents means ± S.E.M. Statistical significance vs. control is indicated: $*p<0.05$, $**p<0.01$, $***p<0.001$. From González-Benítez et al. (2002)

agonist exposure, was found in response to agonists of each subtype-specific receptor, but the largest effect was after A1 stimulation and the smallest in response to A3 stimulation, which had only little effect (González-Benítez et al. 2002). A2A stimulation stimulated glycogenolysis (Fig. 3.4), although it did not cause release of Ca^{2+} from intracellular stores (see above), but adenosine receptors are known to be permeable to extracellular Ca^{2+} (Neary et al. 1988).

Since van Calker et al. (1979) found A1 receptors to have much higher affinity for adenosine than A2 receptors we tested the glycogenolytic effect of 100 nM and 100 μM adenosine after 1, 5, and 15 min of exposure to adenosine. As seen from Fig. 3.5 the higher concentration (stimulating A2 receptors [and A1 receptors]) caused within 1 min an almost 50 % reduction in glycogen content, whereas the lower concentration (stimulating A1 receptors only) had virtually no effect at that time. However during the following 15 min it caused an additional gradual 10 %

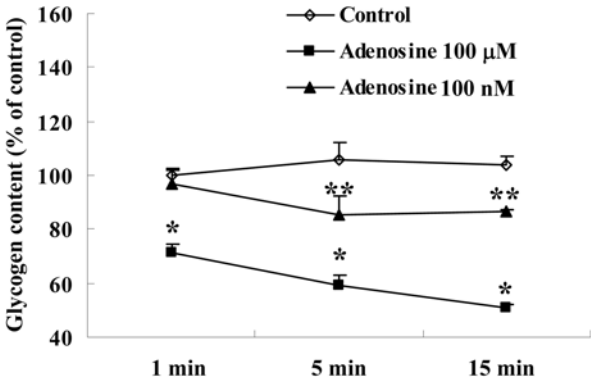

Fig. 3.5 Effect of adenosine on glycogenolysis, indicated as reduction of glycogen content, in astrocytes. Cultured astrocytes were incubated in Dulbecco's Minimum Essential Medium (DMEM) for 1, 5 or 15 min under control conditions (no drug added) (*open circle*), in the presence of 100 μM adenosine (*filled square*), activating both A2 and A1 receptors, or in the presence of 100 nM adenosine (*filled triangle*), activating A1 receptors. Note a large, immediate response to A2 stimulation and a much slower response to A1 stimulation. Each value represents means ± S.E.M of from three to five individual cultures. **Statistically significant ($p<0.05$) difference from control. *Statistically significant ($p<0.05$) difference from all other groups. Previously unpublished experiments by J. Xu, D. Song, Q. Bai and L. Peng

reduction, which obviously also was added to that caused by 100 μM adenosine, which must stimulate both receptors. Its very gradual effect is against the alternative possibility that A2-mediated glycogenolysis had not been completed after 1 min. Thus, in cultured astrocytes most glycogenolysis, and virtually the entire immediate response is due to A2 stimulation. The purpose of a slow, but continuing probably A1-mediated glycogenolysis is unknown.

3.3.3 P2 Receptors

In both neurons and astrocytes, ATP, as well as additional adenosine nucleotides and some non-adenosine nucleotides activate (1) ionotropic P2X receptors leading to Na^+ or Ca^{2+} entry and causing a multitude of Ca^{2+}-associated effects, and (2) metabotropic P2Y receptors (Illes et al. 2012). There are two subgroups of P2Y receptors: (1) $P2Y_{1,2,4,6,11}$, which all principally use G_q/G_{11} to activate the PLCβ/1,4,5-inositol trisphosphate pathway and release intracellular Ca^{2+}, and (2) $P2Y_{12,13,14}$, which almost exclusively couple to members of the $G_{i/o}$ family of G proteins (Abbracchio and Ceruti 2006). In spinal cord astrocytes, ATP stimulation of the P2Y1 receptor leads to increase in $[Ca^{2+}]_i$, transactivation of the EGF receptor, phosphorylation of $ERK_{1/2}$ (Xia and Zhu 2011), i.e., similar effects as those shown after activation of β_2-adrenergic cerebral receptors in Fig. 3.2. However, further downstream signaling

also activated $cPLA_2$, causing release of AA, beginning after 20 min, and synthesis of the prostaglandin PGE2 (Xia and Zhu 2011). Inhibitor studies showed interdependence of these effects, although Neary et al. (1988) had previously reported separate pathways for increase in $[Ca^{2+}]_i$ and ERK phosphorylation in cultured brain astrocytes. PKC may also be involved in some of the signaling pathways utilized by ATP (Priller et al. 1998; Tu et al. 2000). In intact tissue (rat optic nerve) P2Y and P2X receptor activation has been found to cause increase in $[Ca^{2+}]_i$ with the effect by P2Y agonists being most potent (James and Butt 2001; Hamilton et al. 2008). Stimulation of P2Y receptors causes glycogenolysis in cultured cerebral astrocytes (Sorg et al. 1995), and a similar stimulation by AA (Sorg et al. 1995) may suggest utilization of the same PGE2-dependent pathway as those described by either Xia and Zhu (2011) or Neary et al. (1988). This would cause a slow response. Alternatively, the glycogenolytic stimulation might be a direct effect of a probably faster increase in $[Ca^{2+}]_i$ by intracellular release and/or entry of extracellular Ca^{2+} (Neary et al. 1988). In an attempt to distinguish between these possibilities the time course of ATP-induced glycogenolysis was determined in primary cultures of mouse astrocytes. As can be seen from Fig. 3.6 more than one half of the glycogenolysis was completed after 1 min, but a slower glycogenolytic response continued for 15 min, suggesting both an immediate Ca^{2+}-mediated effect and a slower effect which might have occurred in response to AA or one or more of its metabolite(s). This interpretation needs pharmacological confirmation.

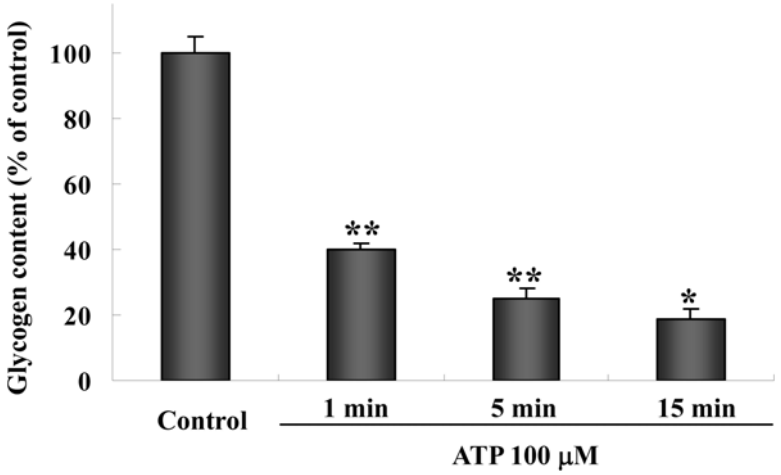

Fig. 3.6 Effect of ATP on glycogenolysis, indicated as reduction of glycogen content, in astrocytes. Cultured astrocytes were incubated in DMEM for 0 (control without any ATP), 1, 5, or 15 min in the presence of 100 μM ATP. Note a large, immediate response to stimulation with ATP followed by a much slower response. Each value represents means ± S.E.M of from three to five individual cultures. *Statistically significant ($p < 0.05$) difference from control. **Statistically significant ($p < 0.05$) difference from control and 15 min groups. Previously unpublished experiments by J. Xu, D. Song, Q. Bai and L. Peng

3.3.4 Other Nucleosides or Nucleotides

In addition to being activated by adenosine and adenosine nucleotides at least some purinergic receptors can also interact with other nucleosides or nucleotides, primarily guanosine and its nucleotides. Thus, in contrast to P2X receptors, P2Y receptors can also respond to UTP and UDP (Öhman and Erlinge 2012). In perfused liver, cyclic guanosine monophosphate has glycogenolytic effect (Glinsmann et al. 1969; Exton et al. 1971). However, effects on hepatocytes in intact liver could possibly be mediated by accessory hepatic cell types. Na^+-dependent, concentrative nucleoside uptake has been demonstrated in cultured astrocytes for only guanosine and adenosine, making uptake independent of intracellular metabolism, a mechanism described below (Peng et al. 2005). This is consistent with transmitter activity and need for a concentrative, and therefore Na^+-dependent, uptake in order to terminate this activity. Guanosine causes also an increase in $[Ca^{2+}]_i$, perhaps acting on the A3 receptor (Chen et al. 2001).

3.4 Cellular Uptake and Release of Purinergic Transmitters

3.4.1 Adenosine Uptake and Release Are Unlikely to Be Glycogenolysis-Dependent

The importance of glycogenolysis for other essential astrocytic functions (glutamate production; K^+ accumulation; learning) lead to the question whether it perhaps also could be essential for accumulation or release of adenosine and/or ATP as transmitters. In contrast to glutamate, adenosine does not need to be synthesized in a glycogenolysis-dependent process, because it is present in extracellular brain fluid (1–2 μM) (Zetterstrom et al. 1950), and it is also able to cross the blood–brain barrier (Pardridge et al. 1994). In cultured cells the measured cellular uptake rate (0.1–0.2 nmol/min/mg protein at 10 μM adenosine) is slightly higher in astrocytes than in neurons (Bender and Hertz 1986). Originally adenosine and other nucleotides and nucleobases were supposed by other authors to be transported into cells by facilitated diffusion only, mediated by equilibrative nucleoside transporters (ENTs), without any energy requirement, and measured during ultra-short incubation times (Geiger et al. 1988). Like simple diffusion, facilitated diffusion is only able to establish similar extra- and intracellular concentrations of the transported compound. Nevertheless, during uptake of, e.g., labeled adenosine the intracellular content of label might continue to increase, even if adenosine was accumulated exclusively by an ENT. This is because conversion of labeled adenosine to metabolites remaining in the tissue contributes to the total amount of label. Because these metabolites are no longer adenosine itself, additional labeled adenosine continues to diffuse into the cells as long as it is subsequently metabolized to different compounds. However, this process is not suited—at least not alone—for uptake of transmitters, which

must be accumulated even when they are not needed for cell metabolism. This can be accomplished by concentrative adenosine uptake, first shown by Hertz and Matz (1989) and Matz and Hertz (1990) after longer-time incubation in cultured astrocytes and neurons where subsequent metabolism had been partly inhibited. This uptake is mediated by concentrative nucleoside transporters (CNTs), which are able to accumulate adenosine against a concentration gradient, driven by co-transport with Na^+ along its electrochemical gradient. Since the ion gradients have been developed by glycogenolysis-dependent ion transport, this uptake is ultimately glycogenolysis-dependent. However, this does probably not become apparent during the concentrative uptake, because it is utilizing already established ion gradients. Classically, expression of both ENTs and CNTs' has mainly been detected in intact tissues, predominantly by in situ hybridization (determining mRNA), but occasionally also by immunohistochemistry (reviewed by Parkinson et al. 2011). This procedure seems to have gravely underestimated astrocytic expression, especially of the intracellular ENT3 (Li et al. 2013; Peng et al. 2013), which is indispensable for stimulated release of ATP (see below).

Release of a variety of gliotransmitters can occur using three general mechanisms/conduits: (1) plasma membrane channels; (2) plasma membrane transporters; and (3) Ca^{2+}-dependent exocytosis (Zorec et al. 2012). Lovatt et al. (2012) showed that active spiking neurons release adenosine through ENTs, leading to suppression of excitatory transmission. Matz and Hertz (1989) have similarly shown that adenosine release from both cultured neurons and astrocyte occurs by rapid diffusion.

3.4.1.1 ATP Release and Uptake

Nucleotides, including ATP, are not transported by either ENTs or CNTs. Channel-mediated efflux has been shown in cultured astrocytes (Cotrina et al. 1998; Kang et al. 1998). However, Guček et al. (2012) have convincingly demonstrated the advantage of vesicle-based vs. non-vesicle-based release, and morphological and biochemical evidence suggests that ATP as a gliotransmitter can be released by a Ca^{2+}-dependent exocytosis. Here we have only investigated whether this process, might be dependent upon glycogenolysis. We have, however, recently shown that release of ATP from cultured astrocytes in response to glutamate, adenosine, or an elevated K^+ concentration, while easily determined in normal cells, is completely abolished in astrocytes with greatly downregulated ENT3 expression following treatment with siRNA against ENT. Since ENT3 is mainly intracellular, this observation may suggest intracellular, perhaps intravesicular synthesis of gliotransmitter ATP from accumulated adenosine (Song et al. 2014).

For determination of the importance of glycogenolysis for stimulated release of gliotransmitter ATP, astrocyte cultures were incubated in colorless but otherwise normal Dulbecco's medium (with a glucose concentration of 7.5 mM). Release of ATP was triggered in some of the cultures by glutamate (100 μM), adenosine (100 μM), or a depolarizing K^+ concentration in the presence of an ecto-ATPase inhibitor (ARL67156). ATP in the medium was measured by a luciferin/luciferase

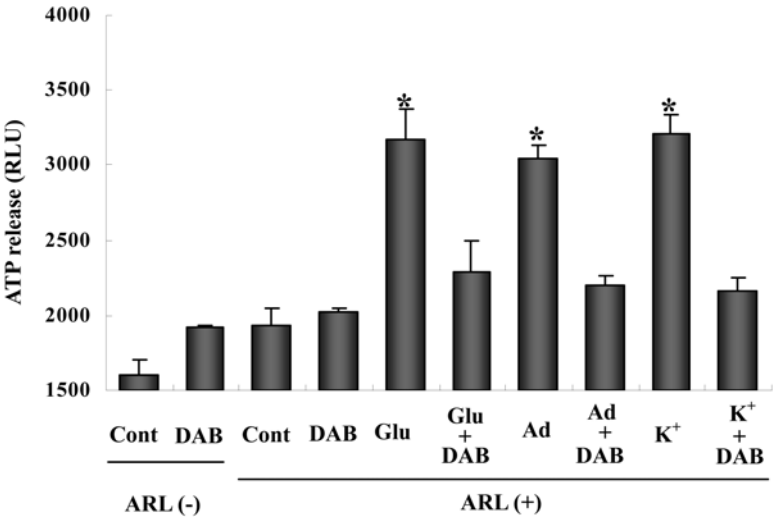

Fig. 3.7 Glycogenolysis is required for increase of ATP release above control (Cont) values from well-differentiated astrocyte cultures induced by addition of glutamate (Glu) to a final concentration of 100 μM, adenosine (Ad) to a final concentration of 100 μM or elevation of the extracellular K^+ concentration from 5 to 45 mM. The presence of any of these stimuli during incubation for 60 min in glucose-containing (7.5 mM) DMEM supplemented with the ecto-ATPase inhibitor ARL67156 (ARL) increased the content of ATP, measured as the relative light unit (RLU) values by a luciferin/luciferase technique. This increase was almost abolished in the additional presence of the glycogenolysis inhibitor (DAB). DAB may also inhibit ectonucleotidases as seen from the similar effects of ARL67156 and DAB and lack of additive effect when the two inhibitors are added together. Results are the averages of RLU values from three to five individual cultures. S.E.M. values are indicated by *vertical bars*. *Statistically significant ($p < 0.05$) difference from all other groups, but not from each other. From Xu et al. (2014a)

method in arbitrary units, and to obtain an estimate of released amounts in some cases converted to amounts of ATP by a calibration curve. The glycogenolysis inhibitor DAB was added 120 min before glutamate, adenosine, or K^+. As can be seen from Fig. 3.7, the effect of the ecto-ATPase inhibitor was relatively slight, although it did show an ATP release under control conditions. Each of the stimulatory agents caused a large stimulation of ATP release, which was greatly reduced by DAB. The stimulated release in Fig. 3.7 was estimated to amount to 1 pmol/min/mg protein. In the absence of any of the three stimulatory agents DAB had no effect. Figure 3.7 suggests that the ecto-ATPase also may be inhibited by DAB, since it had an effect similar to that of ARL67156 and the effects of the two drugs were not additive.

Since our cultures of astrocytes have been treated with the differentiating compound dBcAMP they react to extracellular K^+ concentrations exceeding 15 mM with depolarization-mediated L-type voltage-gated Ca^{2+} channel (VGCC) opening and $[Ca^{2+}]_i$ increase (Hertz and Code 1993; Zhao et al. 1996; Yan et al. 2013). Although the amount of Ca^{2+} that actually enters the cells is modest, the increase in $[Ca^{2+}]_i$ is enhanced by ryanodine receptor-mediated Ca^{2+} release, and a similar

process probably occurs in astrocytes in normal brain, which normally express the L-type VGCC genes $Ca_v1.3$ (Yan et al. 2013) and $Ca_v1.2$ (Du et al. 2014). However, since the control concentration of extracellular K^+ traditionally is slightly higher in the cultured cells (5.4 mM) than in the brain in vivo (~3 mM), the threshold for L-type VGCC opening may be slightly lower in vivo. The signaling pathway activated by L-type VGCC opening, which includes an increase in $[Ca^{2+}]_i$, it is inhibited by DAB (Xu et al. 2013). Activation of this pathway by addition of 10 mM K^+ or more is the likely reason for the stimulation of ATP release by K^+ concentrations ≥15 mM. The effect of addition of 10 mM KCl was found to be statistically significant, and it seemed to be slightly increased by 100 μM GABA (which is depolarizing in astrocytes [Ma et al. 2012] and had a small, nonsignificant effect on its own). This joint effect was significantly inhibited (abolished) by the dihydropyridine nifedipine, an inhibitor of L-type VGCC, which accordingly prevents the increase in $[Ca^{2+}]_i$ necessary for glycogenolysis (Fig. 3.8).

Inhibition of pathways activated by glutamate or adenosine in the absence of glycogenolysis is also the likely reason for the inhibitory effect of DAB on ATP release caused by glutamate and adenosine. This includes an effect on store-operated

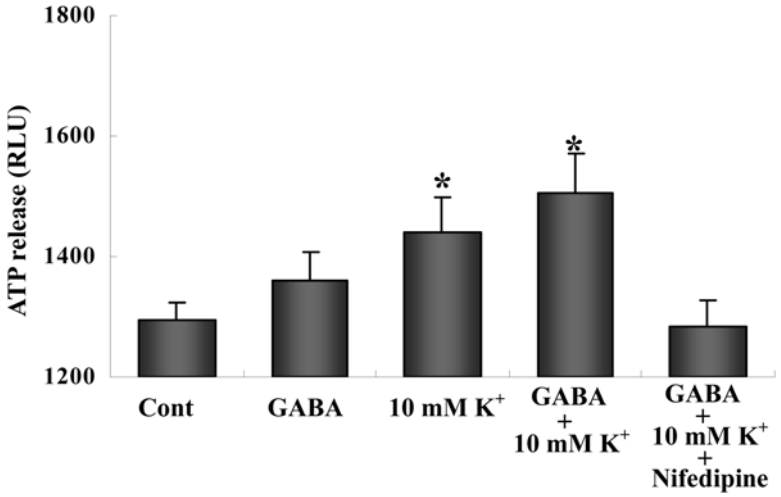

Fig. 3.8 Glycogenolysis-dependent and dihydropyridine-inhibited L-channel function is required for increase of ATP release from well-differentiated astrocyte cultures induced by addition of 10 mM K^+ to a final extracellular concentration of 15 mM. This effect may be enhanced by the simultaneous presence of γ-aminobutyric acid (GABA), which has a depolarizing effect on astrocytes. The cells were treated as in Fig. 3.7, and the inhibitor of ecto-ATPases, ARL67156 was present in all experiments. The increased ATP release was smaller than that evoked by the higher, more depolarizing K^+ concentration used in Fig. 3.7. The response required L-type Ca^{2+} channel activation, as indicated by its inhibition by 100 μM nifedipine, a dihydropyridine inhibitor of depolarization-opened L-type Ca^{2+} channels, leading to increase in Ca^{2+}, which is required for glycogenolysis to occur. Results are averages of RLU values from three to five individual cultures. S.E.M. values are indicated by *vertical bars*. *Statistically significant ($p<0.05$) difference from control, addition of GABA alone and of GABA + 10 mM K^+ + nifedipine. From Xu et al. (2013)

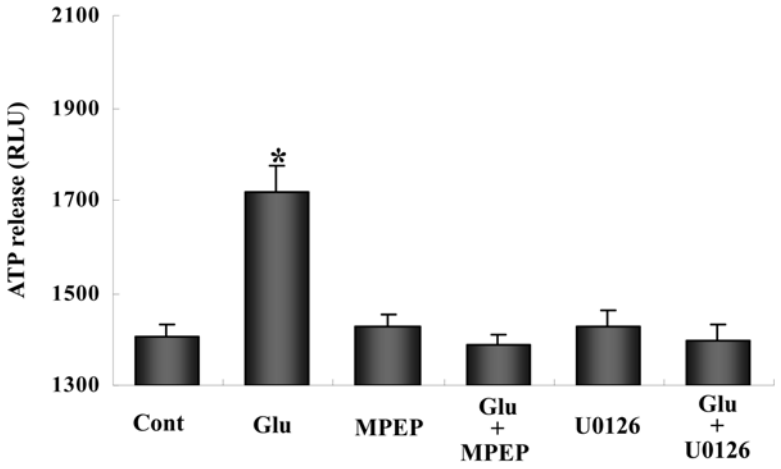

Fig. 3.9 Glutamate-stimulated ATP release from astrocytes above control (Cont) values requires GluK2 stimulation and $ERK_{1/2}$ phosphorylation. The cells were treated as in Fig. 3.7 with addition of glutamate to a final concentration of 100 μM glutamate (Glu), and the inhibitor of ecto-ATPases, ARL67156 was present in all experiments. Some experiments were performed in the additional presence of either 25 μM MPEP, an antagonist of mGluR5 and GluK2 receptors, or 10 μM U0126, a specific inhibitor of ERK phosphorylation (by inhibition of MEK). For undetermined reasons the ATP release in response to glutamate was smaller than in Fig. 3.1, but the main result is that it was completely prevented by either inhibitor. Literature data indicate that mGluR5 is not functioning in differentiated cells (see Fig. 3.11). The results should not lead to any conclusion with respect to possible involvement of $ERK_{1/2}$ in the inhibitory effect of DAB, about which there is no information. Results are averages of RLU values from three individual cultures. S.E.M. values are indicated by *vertical bars*. *Statistically significant ($p<0.05$) difference from all other groups. From Xu et al. (2013)

Ca^{2+} entry (Müller et al. 2014). The effect of glutamate on ATP release was in addition inhibited by 2-methyl-6-(phenylethynyl)-pyridine (MPEP), an inhibitor of mGlurR5 and GluK2 and by U0126, an inhibitor of $ERK_{1/2}$ phosphorylation (Fig. 3.9). An inhibitor of both GluK1 and GluK2, NS102 has previously been shown to inhibit glutamate-mediated increase in $[Ca^{2+}]_i$ (Li et al. 2011). Therefore the glutamate receptor involved is likely to be GluK2 rather than mGluR5. This conclusion is further supported by the observation by Sun et al. (2013) that GluR5 activation does not increase $[Ca^{2+}]_i$ in astrocytes in mature mice in vivo, as shown in Fig. 3.10, whereas the effect by ATP is maintained.

3.4.2 ATP Formation in Astrocytes Is Probably Not Glycogenolysis-Dependent

Profound differences exist between adenosine metabolism in neurons and astrocytes with nucleotide formation dominating in astrocytes (Fig. 3.11) and deamination in neurons (Matz and Hertz 1989). This difference has later been confirmed with the demonstration of astrocyte-specific expression of adenosine kinase,

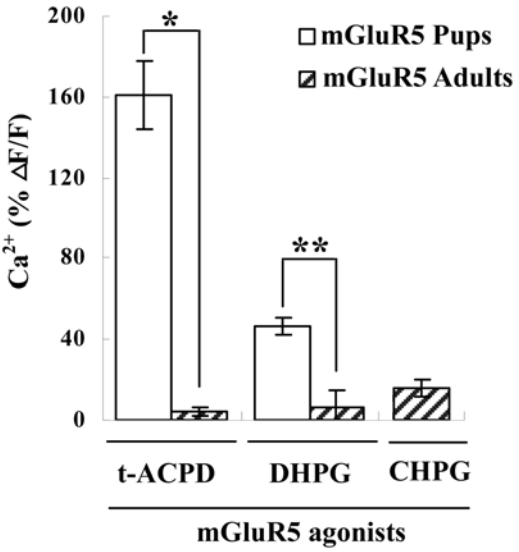

Fig. 3.10 Effect of the glutamate agonists t-ACPD, DHPG, and CHPG on glutamate induced increase in [Ca^{2+}] in astrocytes measured by two-photon microscopy of astrocytic Ca^{2+} signaling in vivo. The two former of these agonists increase [Ca^{2+}] in young (up to 3-week-old) animals but not in adult animals. The small effect of CPGH in adult mice might be due to additional agonist effect on other receptors. Modified from Sun et al. (2013)

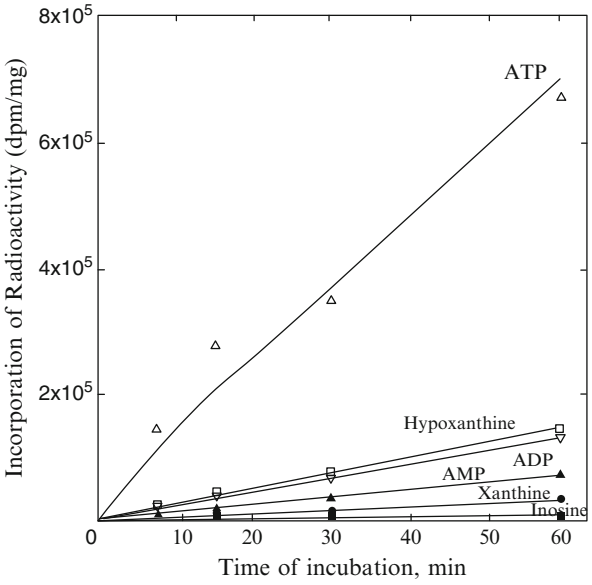

Fig. 3.11 Radioactivity incorporated from adenosine (0.5 μCi/mL) into the total pools (specific activity × pool size) of ATP, ADP, AMP, inosine, hypoxanthine, and xanthine in astrocytes in primary cultures incubated with 10 mM adenosine. Based on the incorporation of labeled adenosine into ATP during the 60-min period Matz and Hertz (1989) calculated a rate of ATP formation from adenosine of ~0.1 nmol/min/mg protein

catalyzing AMP production from adenosine in mature brain (Studer et al. 2006). That metabolism-mediated equilibrative uptake contributes greatly to adenosine uptake in vivo is indicated by the correlation between enhanced adenosine kinase activity and epileptic seizures (Boison 2013). It is also shown by the observation in cultured astrocytes that only ~25 % of total adenosine uptake (at 25 μM adenosine) is Na^+-dependent (Peng et al. 2005).

ENT function is highly unlikely to require glycogenolysis, since it is not energy-dependent and, as previously mentioned, CNTs depend on ion gradients, not directly on Na^+,K^+-ATPase activity. Boison (2013) has concluded that adenosine kinase activity is probably not dependent upon transmitter effects and signaling, although its gene expression may be (Pawelczyk et al. 2003). An absolute answer could be obtained by a study like that shown in Fig. 3.9 in the presence and absence of DAB. However, this is labor-intensive, and instead we have chosen to measure potential DAB effects on ATP content after previous loss of ATP. As shown in Fig. 3.12 the DAB effect was very small and nonsignificant, providing support to the concept that adenosine kinase activity does not depend upon glycogenolysis.

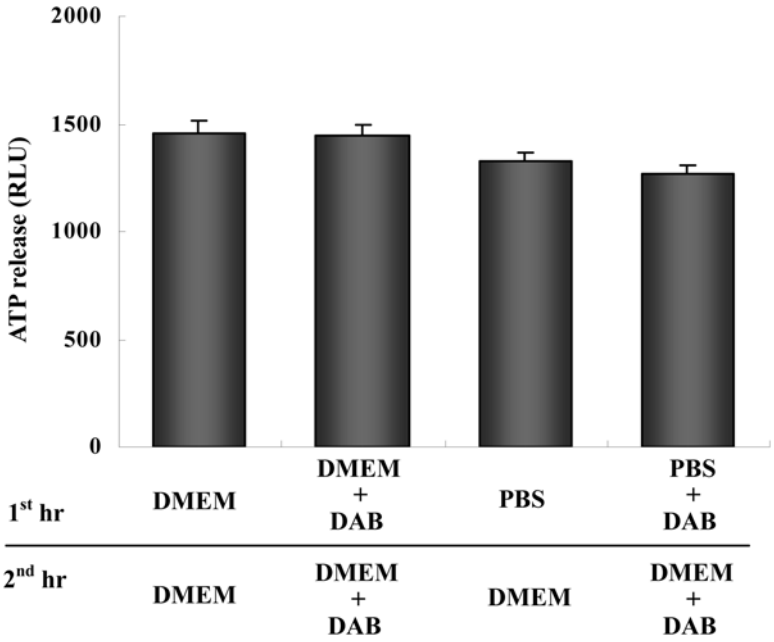

Fig. 3.12 Measurement of relative ATP contents in cultures of astrocytes after partial ATP-depletion. The two *left columns* show cultures incubated in normal tissue culture medium (DMEM) for 2 h with or without DAB. The two *right columns* show cultures loaded with 200 μL ice-cold PBS without glucose for 1 h (to trigger decline in ATP), one with DAB. Subsequently PBS was replaced with 200 μL DMEM in the continued presence or absence of a glycogenolysis inhibitor (DAB) and the incubation continued for another hour. Results are the averages of RLU from three individual groups. S.E.M. values are indicated by *vertical bars*. DAB had no significant effect. Previously unpublished experiments by J. Xu, D. Song, Q. Bai and L. Peng

3.5 Concluding Remarks

Both adenosine and ATP were found to simulate glycogenolysis by two kinetically distinguishable mechanisms. Adenosine probably did so by stimulation of two different P1 receptors. ATP stimulation of a P2 receptor may have stimulated glycogenolysis initially by an increase in $[Ca^{2+}]_i$ almost immediately after ATP administration and later by an effect of its downstream signaling products, including AA. The inclusion of AA and its metabolites in the ATP-stimulated signaling pathway shown by Xia and Zhu (2011) is very interesting because of the many harmful (Xia and Zhu 2011) and beneficial (Hertz et al. 2013c) effects of these compounds. Moreover both antidepressant drugs (fluoxetine) and anti-bipolar drugs (carbamazepine) upregulate gene expression of $cPLA_2$, the enzyme catalyzing release of AA, in freshly isolated astrocytes from drug-treated animals in vivo (Li et al. 2012; Song et al. 2012). Carbamazepine, but not fluoxetine has the opposite effect in freshly isolated neurons. This may be of interest in connection with a low ATP content observed by Cao et al. (2013) in the brains of mice that were susceptible to chronic social defeat. Administration of ATP induced a rapid antidepressant-like effect in these mice, whereas blockade of vesicular gliotransmission induced deficiencies in astrocytic ATP release and caused depressive-like behaviors that could be rescued via the administration of ATP. Based on different experiments the authors concluded that P2X2 receptors in the medial prefrontal cortex mediated the antidepressant-like effects of ATP, but this does not exclude a role of the P2Y-mediated signaling observed by Xia and Zhu (2011).

The inhibition of ATP release by DAB in response to elevated extracellular K^+ concentrations was expected, because of the glycogenolysis dependence of the signaling evoked by these K^+ concentrations in order to increase $[Ca^{2+}]_i$, a prerequisite for release of gliotransmitter ATP. The apparent enhancement by GABA of the potency of the K^+ effect was also expected, but has not previously been demonstrated. The abolishment of glutamate and adenosine-mediated stimulation of ATP release by inhibition of glycogenolysis adds yet another facet to the importance of glycogen in supporting essential astrocytic functions. A recent demonstration by Müller et al. (2014) that Ca^{2+} homeostasis in astrocytes requires glycogenolysis may possibly explain the abolishment of the stimulated ATP release known to be Ca^{2+}-dependent.

Acknowledgment The careful studies by Henry Matz of adenosine metabolism in cultured astrocytes and neurons 25 years ago are gratefully acknowledged.

Conflict of Interest The authors declare no conflict of interest.

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Chapter 4
Purinergic and Glutamatergic Receptors on Astroglia

Alexei Verkhratsky and Geoffrey Burnstock

Abstract Astroglial cells express many neurotransmitter receptors; the receptors to glutamate and ATP being the most abundant. Here, we provide a concise overview on the expression and main properties of astroglial glutamate receptors (ionotropic receptors represented by AMPA and NMDA subtypes) and metabotropic (mainly mGluR5 and mGluR3 subtypes) and purinoceptors (adenosine receptors of A_1, A_{2A}, A_{2B}, and A_3 types, ionotropic $P2X_{1/5}$ and $P2X_7$ subtypes, and metabotropic P2Y purinoceptors). We also discuss the role of these receptors in glial physiology and pathophysiology.

Keywords Astrocytes • Glutamate • ATP • Adenosine • NMDA receptors • AMPA receptors • Adenosine receptors • P2Y receptors • P2X receptors

4.1 Neurotransmitter Receptors in Neuroglia

Chemical transmission in the nervous system occurs as homocellular (neurons to neurons, or astrocytes to astrocytes) and/or heterocellular (neurons to astrocytes, astrocytes to neurons, or astrocytes to microglia, etc.) configurations. Signaling

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_4

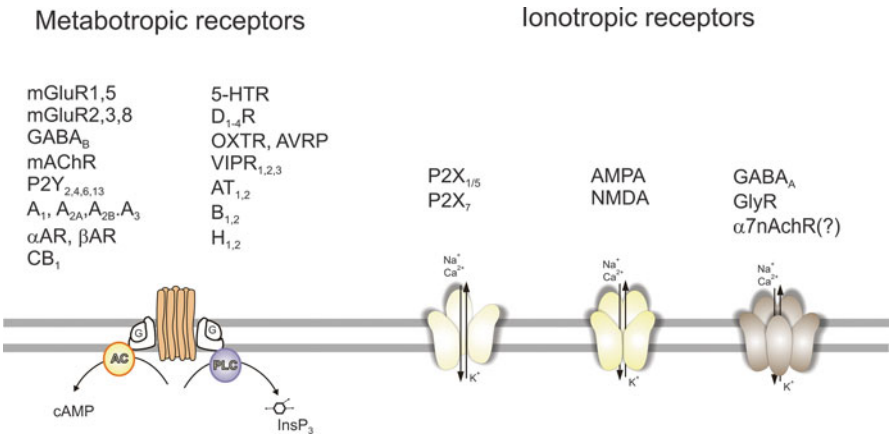

Fig. 4.1 Diversity of receptors to neurotransmitters and neuromodulators expressed by astroglia

molecules, mediating this chemical transmission, are classified into neurotransmitters, neuromodulators, and neurohormones, all being secreted (albeit in different quantities and at different rates) by both neuronal and neuroglial cells. At the receptive end of neurotransmission signaling (by all three types of molecules, the distinction between which is somewhat blurred) are specific receptors, many types of which are expressed in various portions of the nervous system.

Neuroglial cells of both macroglial and microglial varieties possess virtually all types of receptors to neurotransmitters, neuromodulators, and neurohormones present in the nervous system (Fig. 4.1) (Dave et al. 1991; McCarthy and Salm 1991; Shao and McCarthy 1994; Verkhratsky and Shmigol 1996; Gallo and Ghiani 2000; Verkhratsky and Steinhauser 2000; Verkhratsky et al. 2011, 2012b; Parpura et al. 2012). Expression of these receptors, however, is tightly regulated by developmental programs and brain region-specific environments. Astrocytes in the different regions of the central nervous system (CNS) have remarkably different sets of receptors, which often are congruent to main neurotransmitters released in the immediate vicinity and match (in their modalities) receptors expressed in neighboring neurons (Kirischuk et al. 1995, 1996a, b; Verkhratsky et al. 1998). For instance, astrocytes express glycine receptors exclusively in the spinal cord, where glycine acts as a major inhibitory neurotransmitter; similarly in the susbtantia nigra with predominant dopaminergic transmission, astroglial cells express dopamine receptors. This specialization can be manifested also at a subcellular level; when, for example, γ-aminobutyric acid (GABA) receptors are exclusively expressed in perisynaptic processes of Bergmann glial cells facing GABA-ergic synapses (for examples of regional specialization and appropriate references see Kirchhoff et al. 1996; Miyazaki et al. 2004; Verkhratsky 2010). A wide variety of neurotransmitter receptors are also expressed in microglial cells, which are, arguably, the most "receptive" cells in the CNS because in addition to numerous receptors to neurotransmitters and neurohormones, microglia are also in possession of multiple receptors to inflammatory and damage-related factors (Kettenmann et al. 2011).

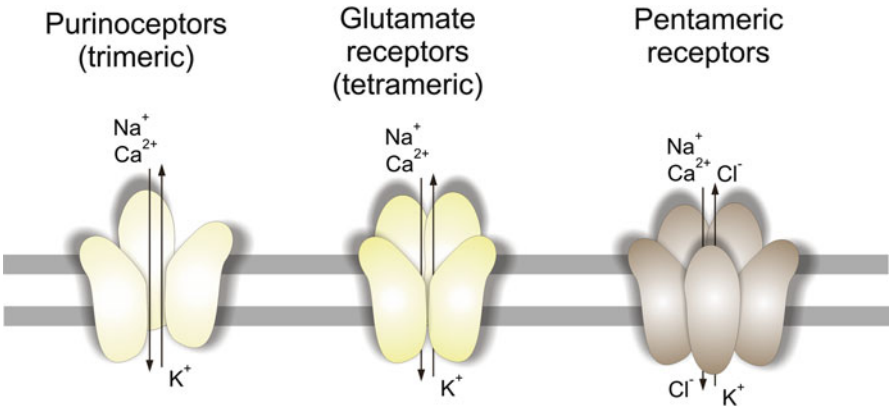

Fig. 4.2 Main classes of ionotropic receptors. Purinoceptors (trimeric P2X receptors; every subunit is assembled from two transmembrane domains), glutamate receptors (tetrameric AMPA, kainate, and NMDA receptors; each subunit is assembled of three transmembrane domains), and pentameric receptor channels for acetylcholine (ACh), GABA, glycine and serotonin (each subunit is composed of four transmembrane domains). Vertebrate P2X and ionotropic glutamate receptors are non-selective cation channels, whereas pentameric receptors are represented by non-selective cation channels (nicotinic ACh and serotonin receptors) or chloride channels ($GABA_A$, $glycine_A$ receptors). Invertebrate tissues express a range of pentameric channels of various modalities including GABA-gated cationic channels, ACh-gated Cl^- channels, glutamate-gated Cl^- channels, histamine-gated Cl^- channels, serotonin-gated Cl^- channels, and pH-gated Cl^- channels

Glutamate and ATP are the most abundant excitatory neurotransmitters in the CNS, which act through both metabotropic and ionotropic receptors. The latter belong to the extended superfamily of ligand-operated channels that include three major classes of tri- (purinoceptors), tetra- (glutamate receptors), and pentameric (acetylcholine, GABA, glycine, etc.) receptors (Fig. 4.2), whereas the former are represented by seven-transmembrane domain G protein-coupled receptors linked to intracellular second messengers. In this chapter we overview astroglial receptors for glutamate and ATP.

4.2 Astroglial Receptors for Glutamate

Astrocytes have several plasmalemmal glutamate sensors represented by ionotropic and metabotropic receptors and by glutamate transporters (Danbolt 2001; Parpura and Verkhratsky 2013). The latter not only removes glutamate from the extracellular space and acts as a part of the glutamate–glutamine shuttle but also acts as a source of the relatively large Na^+ influx that significantly contributes to astroglial Na^+ signaling (Kirischuk et al. 2012; Parpura and Verkhratsky 2012). Importantly, astroglial glutamate sensors have different sensitivities to glutamate; the *N*-methyl-D-aspartate (NMDA) receptors being the most sensitive (K_D ~2 μM); the plasmalemmal glutamate transporters have K_D's between 3–5 and 30–70 μM,

and the K_D for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and metabotropic receptors are 50 and ~130 μM, respectively (Verkhratsky and Kirchhoff 2007a, b).

4.2.1 Ionotropic Glutamate Receptors

Ionotropic glutamate receptors are classified according to their agonist specificity for AMPA, kainate, and NMDA receptors (for reviews see Wisden and Seeburg 1993; Mayer 2005). All these receptors are cationic channels with some differences in ionic selectivity, with glutamate being the only naturally occurring agonist; the sole exception is the NMDA receptor that also needs a co-agonist, L-glycine or D-serine. Members of all three types of glutamate receptors are expressed in astroglia, although this expression differs between brain regions.

The AMPA receptors are the most widely present in all neuroglial cells being detected in astrocytes (Condorelli et al. 1999; Verkhratsky and Steinhauser 2000; Seifert and Steinhauser 2001), oligodendrocytes (Bergles et al. 2000; Alberdi et al. 2005), NG2 glia (Lin and Bergles 2002) and in microglia (Noda et al. 2000). All four AMPA receptor subunits, GluA1-4 (GluRA-D or GluR1-4 according to previous classifications; see Bowie 2012) were identified in astrocytes from cerebellum, hippocampus, neocortex, retina, and brainstem (Gallo and Ghiani 2000; Seifert and Steinhauser 2001; Matthias et al. 2003; McDougal et al. 2011); expression of subunits, however, varies between brain regions. Subunit composition of AMPA homo- or heteromeric channels in turn defines their functional diversity (Bowie 2012), and hence astrocytes from different brain regions possess functionally distinct AMPA receptors. In some of these brain regions (such as cerebellum and brainstem) astroglial AMPA receptors do not contain the GluA2 subunit which underlies their (modest) Ca^{2+} permeability and sensitivity to polyamines; activation of these receptors therefore may trigger astroglial Ca^{2+} signals which have been detected both in vitro and in situ (Enkvist et al. 1989; Burnashev et al. 1992; Muller et al. 1992; Jabs et al. 1994; Porter and McCarthy 1995b; McDougal et al. 2011).

Kainate receptors (assembled as homo- and heteromers from five subunits, GluK1-5 (Perrais et al. 2010)) were identified in astrocytes at transcript and protein levels (Garcia-Barcina and Matute 1996; Brand-Schieber et al. 2004), although no functional evidence has been reported. Upregulation of expression of all five GluK subunits was observed in reactive astrocytes in the chemoconvulsive status epilepticus model of temporal lobe epilepsy (Vargas et al. 2013).

The NMDA receptors are heteromerically assembled from seven subunits (GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A, and GluN3B) of which the GluN1 subunit is obligatory for forming a functional ligand-gated channel. Subunit composition defines various properties of the NMDA receptor, including sensitivity to Mg^{2+} block and Ca^{2+} permeability. Initial evidence for astroglial expression of NMDA receptors was derived from immunocytochemistry and analysis of specific expression of mRNA and receptor protein (Conti et al. 1996, 1997; Schipke et al. 2001). Astroglial NMDA receptors have been subsequently functionally

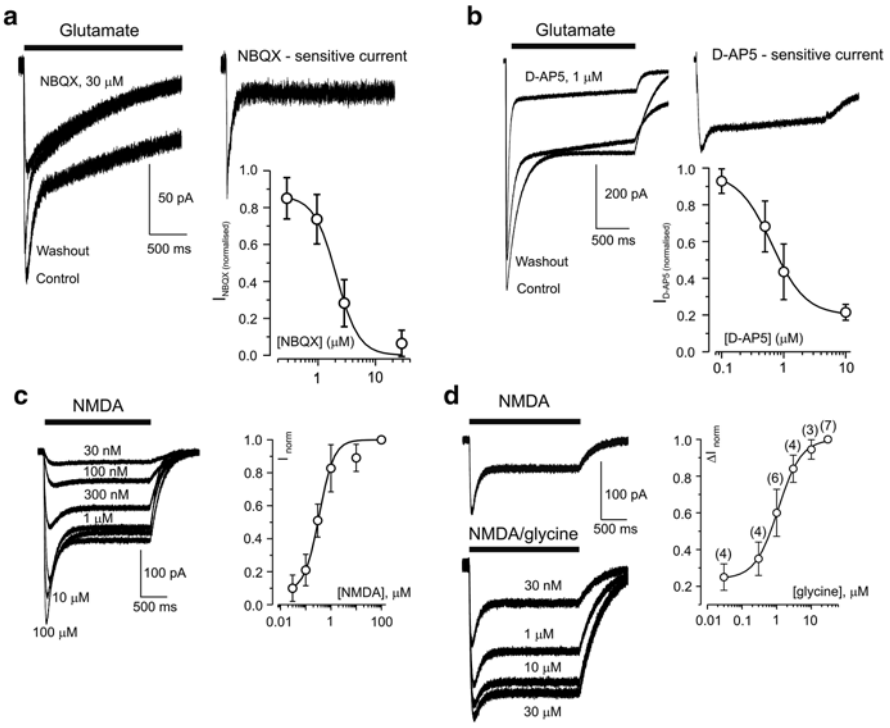

Fig. 4.3 AMPA and NMDA receptor-mediated currents in cortical astrocytes. (**a**) NBQX inhibits the fast component of glutamate-induced current. Representative traces illustrate the current before, during, and after application of 30 μM NBQX (*left panel*), and the NBQX-sensitive current obtained by subtraction (*right panel*). The concentration dependence of the block of the fast component for four cells ($IC_{50} = 2.2 \pm 0.4$ μM, Hill coefficient = 1.9) is shown in the inset. (**b**) D-AP5 inhibits the slow component of glutamate-induced current. Representative traces demonstrating the effect of 1 μM D-AP5 (*left panel*), and the D-AP5-sensitive component obtained by subtraction (*right panel*). The concentration dependence of the block for five cells ($IC_{50} = 0.64 \pm 0.1$ μM, Hill coefficient = 1.6) in the inset. (**c**) NMDA-induced (2 s application) currents in a single astrocyte and concentration-response curve constructed from six such experiments (EC_{50} 0.34 ± 0.06 μM, Hill coefficient = 1.5). (**d**) Glycine-dependent potentiation of astrocyte NMDA response. NMDA-induced currents in glycine-free normal extracellular solution are shown on the *top*; NMDA-induced currents in the presence of different glycine concentrations (30 nM, 1, 10 and 30 μM) are displayed below. The concentration-response curve (ΔI_{norm} represents the amplitudes of current increase normalized to the maximal increase at 30 μM glycine) constructed from seven experiments is shown on the right (EC_{50} 1.1 ± 0.07 μM, Hill coefficient = 1.2). Reproduced with permission from Lalo et al. (2006)

and pharmacologically characterized (Fig. 4.3) (Ziak et al. 1998; Schipke et al. 2001; Lalo et al. 2006; Verkhratsky and Kirchhoff 2007a, b). Ion currents through NMDA receptors were detected in astrocytes from cortex and spinal cord but not in astroglial cells from hippocampus. Functional astroglial NMDA receptors were further found in different species, including humans (Lee et al. 2010; Zhou et al. 2010; Gerard and Hansson 2012; Parfenova et al. 2012).

Astroglial NMDA receptors are functionally and most likely structurally different from receptors expressed in neurons. First, astroglial NMDA receptors show almost no Mg^{2+} block at characteristic resting potential levels (~−80 mV); the Mg^{2+} block of the astroglial NMDA receptor develops at much more negative potentials compared to neuronal receptors (about −120 vs. −60 mV) (Palygin et al. 2011). This feature seems to apply not only to astrocytes but also to oligodendroglia, in which NMDA-mediated currents and cytoplasmic Ca^{2+} signals were readily recorded at physiological extracellular Mg^{2+} concentrations of ~ 1 mM (Karadottir et al. 2005; Salter and Fern 2005; Micu et al. 2006). This could indicate that neuroglial cells have specific NMDA receptors which can be activated at resting membrane potentials without the need for membrane depolarization. Second, astroglial NMDA receptors demonstrate a substantially lower (about 2–3 times) Ca^{2+} permeability compared to neuronal receptors: the $P_{Ca}/P_{monovalent}$ for astrocytic NMDA receptors is ~3, neuronal NMDA receptors have a permeability ratio of Ca^{2+} to monovalent cations around 10 (Palygin et al. 2010). Although Ca^{2+} permeability of astroglial NMDA receptors is smaller than in neurons, opening of glial receptors results in reasonable Ca^{2+} influx, which may generate physiologically relevant Ca^{2+} signals in both freshly isolated astrocytes and in astrocytes residing in cortical slices (Fig. 4.4). Third, astroglial NMDA receptors are distinct in their pharmacology, being sensitive to the non-selective antagonist memantine and the GluNR2C/D subunit-selective antagonist UBP141 (Palygin et al. 2011). All these idiosyncrasies of astroglial NMDA receptors probably arise from specific subunit composition. The low Mg^{2+} sensitivity may be defined, for instance, by heteromerization with the GluN3 subunit, because it is known that incorporation of this subunit into dimeric GluN1/GluN3 or tri-heteromeric GluN1/2/3 receptors confers low sensitivity to Mg^{2+}-block. At the same time, astroglial NMDA receptor-mediated currents are effectively inhibited by D-AP5 and MK-801, indicating the presence of GluN2 subunits, which is also consistent with their sensitivity to UBP141. Therefore, the most probable composition of the astroglial NMDA receptor is heteromeric assembly of two GluN1, one GluN2, and one GluN3 subunits (see Lalo et al. 2011b for further details).

4.2.2 Metabotropic Glutamate Receptors

Metabotropic glutamate receptors are widely expressed in neuroglial cells, and are abundant in astroglia. Astrocytes express all the major types of metabotropic glutamate receptors (mGluRs), including mGluR 1 and 5 belonging to group I and linked to phospholipase C and inositol trisphosphate ($InsP_3$) production, as well as mGluRs 2,3 (group II) and mGluRs 4, 6–8 (Group III) that are coupled to adenylate cyclase. In astrocytes, mGluR3 and mGluR5 are the most abundant, being often concentrated in perisynaptic processes (Petralia et al. 1996; Aronica et al. 2000; Tamaru et al. 2001). Metabotropic receptors of the mGluR5 subtype are instrumental for generation of astroglial Ca^{2+} signals through stimulation of the production of $InsP_3$ and generation of subsequent $InsP_3$-induced Ca^{2+} release from the endoplasmic reticulum (ER), which underlie both local Ca^{2+} signals and propagating Ca^{2+} waves

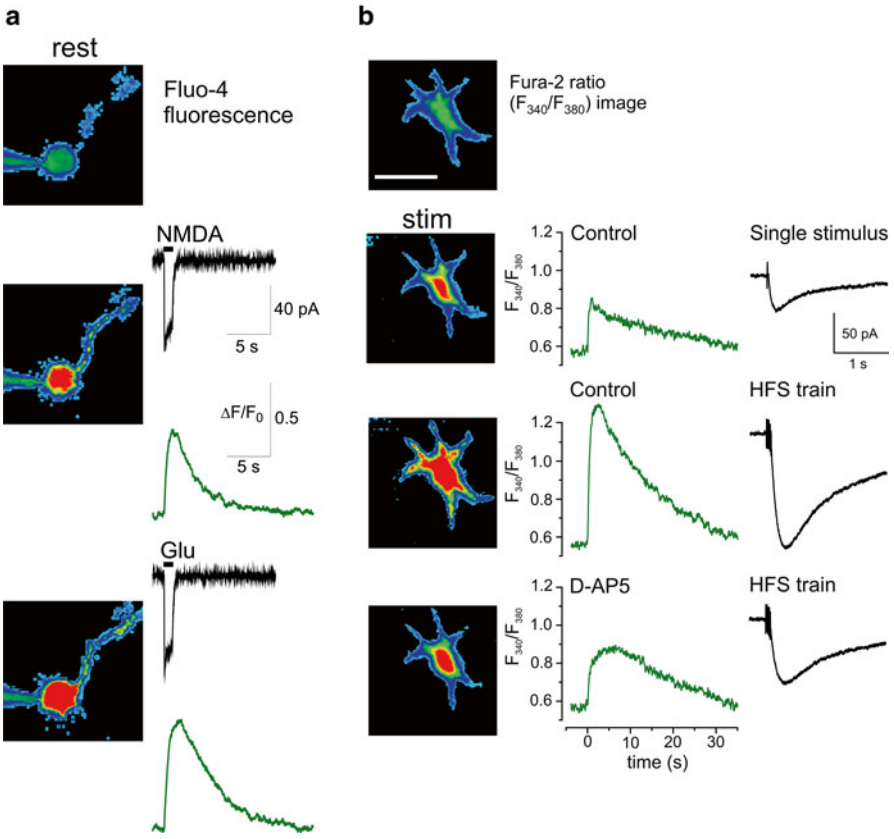

Fig. 4.4 Glutamate receptor-mediated Ca^{2+}-signaling in cortical astrocytes. (**a**). Acutely isolated astrocytes were loaded with Fluo-4 via a patch pipette. Fluorescent images were recorded simultaneously with transmembrane currents evoked by application of 100 μM NMDA or 100 μM glutamate $[Ca^{2+}]_i$ transients are represented as the $\Delta F/F_0$ ratio averaged over the cell soma (*green traces*). Holding potential was −80 mV. (**b**) Synaptically-induced ionotropic Ca^{2+} signals in protoplasmic cortical astrocytes in situ. Cortical layer II astrocytes were loaded with Fura-2 in situ via patch pipette. Fluorescent images were recorded simultaneously with glial currents evoked by neuronal afferent stimulation in presence of CNQX (control) and after application of 10 μM of AP-5, a selective antagonist of NMDA receptors. $[Ca^{2+}]_i$ transients (*green traces*) are expressed as F_{340}/F_{380} ratio. Modified from Palygin et al. (2010)

(Verkhratsky et al. 1998, 2012a; Parpura et al. 2011). Activation of mGluR5 by synaptically or extrasynaptically released glutamate induces astroglial Ca^{2+} signals independent from extracellular Ca^{2+} and sensitive to inhibitors of ER Ca^{2+} accumulation (thapsigargin or cyclopiazonic acid) and to the specific inhibitor of $InsP_3$ receptors, heparin (Kirischuk et al. 1999). There is evidence for age-dependent regulation of mGluR5 expression: experiments on mice of different ages demonstrated marked downregulation of these receptors in animals older than 3 weeks (Sun et al. 2013).

4.3 Astroglial Purinoceptors

Purinergic signaling, that utilizes ATP, adenosine and related purines as transmitter molecules, is arguably the most widespread and ubiquitous signaling system in living organisms and has ancient evolutionary roots (Burnstock 1996, 2007b; Burnstock and Verkhratsky 2009, 2012; Burnstock et al. 2010, 2011). Purinergic transmission is particularly important for neuroglia, because all types of glia are sensitive to ATP and its analogues (Abbracchio et al. 2009; Verkhratsky et al. 2009). ATP is released from neuronal terminals and from axons, from astrocytes and oligodendrocytes and also from microglia in both physiological and pathological contexts. There are multiple release mechanisms that include exocytosis of ATP-containing vesicles as well as diffusion through various types of ATP-permeable membrane channels (such as connexin hemichannels, volume-sensitive anion channels or $P2X_7$ purinoceptors (Pankratov et al. 2006; Lalo et al. 2011a)). Neuroglial cells express multiple purinergic receptors, that are classified into P1 receptors for adenosine and P2 receptors for ATP and related nucleotides (Burnstock 2007a).

4.3.1 Adenosine Receptors

Adenosine receptors are seven-transmembrane-spanning metabotropic receptors coupled to G_i, G_s, and G_o proteins (Burnstock and Verkhratsky 2012). All four types of adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) have been detected in astroglial cells (Table 4.1). Adenosine A_1 receptors were identified in astrocytes in vitro using pharmacological assays and analysis of mRNA expression (Woods et al. 1989; Peakman and Hill 1994, 1996). Functionally, adenosine was shown to trigger Ca^{2+} signals in primary cultured astrocytes (Peakman and Hill 1995; Pilitsis and Kimelberg 1998) in acute hippocampal (Porter and McCarthy 1995a) and olfactory bulb (Doengi et al. 2008) slices; these Ca^{2+} responses were mediated through activation of A_1 or A_{2A} receptors. In contrast, A_3 receptor-mediated Ca^{2+} signals were found in cultured mouse astrocytes (Chen et al. 2001). Adenosine, acting through either A_1 or A_{2B} receptors was also found to modulate parameters of glutamate-evoked Ca^{2+} signals in primary cultured astrocytes (Ogata et al. 1994; Toms and Roberts 1999; Ferroni et al. 2002; Alloisio et al. 2004). Activation of A_{2A} receptors was also shown to inhibit plasmalemmal glutamate (GLT-1) transporter in hippocampal astrocytes in a $[Ca^{2+}]_i$ and protein kinase A-dependent manner (Nishizaki et al. 2002).

In pathological conditions adenosine was reported to initiate reactive astrogliosis following direct injection into the rat cortex; this astrogliotic response was prevented by the A_2 receptor antagonist 1,3-dipropyl-7-methylxanthine (DPMX) (Hindley et al. 1994). Similarly, in primary cultured astrocytes, astrogliosis triggered by basic fibroblast growth factor was inhibited by A_{2A} receptor antagonists (Brambilla et al. 2003). Activation of adenosine receptors of all types has been reported to mediate neuroprotection, increase stress-induced survival of astrocytes

Table 4.1 P1 adenosine receptors in astroglia (modified from Verkhratsky et al. 2009)

Receptor type	Experimental preparation/species/technique	Properties/function	References
P1 receptors[a]	Cell culture/rat/electrophysiology	Cell hyperpolarization	Hosli et al. (1987)
	Hippocampal slices/Ca^{2+} imaging	Initiation of $[Ca^{2+}]_i$ transients due to Ca^{2+} release from the ER	Porter and McCarthy (1995a)
A_1 receptor	Cell culture/rat	Inhibition of cyclic AMP (cAMP) production	Peakman and Hill (1994)
	Cell culture/rat	Activation of PLC	Biber et al. (1997, 1999, 2001)
	Cell culture/rat forebrain/Ca^{2+} imaging	Initiation of $[Ca^{2+}]_i$ transients due to Ca^{2+} release from the ER, activation of Ca^{2+} entry and potentiation of histamine-induced Ca^{2+} release	Peakman and Hill (1995)
	Cell culture/rat cortex/Ca^{2+} imaging	Potentiation of acetylcholine-induced Ca^{2+} signaling	Ferroni et al. (2002)
	Cell culture/rat hippocampus/Ca^{2+} imaging	Potentiation of glutamate (mGluRs)-induced Ca^{2+} signaling	Ogata et al. (1994)
	Cell culture/rat cortex/Ca^{2+} imaging	Inhibition of $P2X_7$-mediated Ca^{2+} influx	Nobile et al. (2003)
A_{2A} receptor	Acute slices/mouse olfactory bulb/Ca^{2+} imaging	Initiation of $[Ca^{2+}]_i$ transients due to Ca^{2+} release from the ER	Doengi et al. (2008)
	Cell culture/slices/rat hippocampus/ electrophysiology	Inhibition of astroglial glutamate transporter GLT-1 and activation of astroglial glutamate release	Nishizaki et al. (2002)
	Cell culture/rat striatum	Inhibition of astrogliosis	Brambilla et al. (2003)
	Cell culture/rat	Inhibition of inducible nitric oxide synthase (iNOS) and NO production	Brodie et al. (1998)
A_{2B} receptor	Cell culture/rat	Stimulation of cAMP production	Peakman and Hill (1994)
	Acutely isolated cells/rat cortex/Ca^{2+} imaging	Initiation of $[Ca^{2+}]_i$ transients due to Ca^{2+} release from the ER	Pilitsis and Kimelberg (1998)
	Cell culture/rat cerebellum/Ca^{2+} imaging	Potentiation of glutamate (P2Y)-induced Ca^{2+} signaling	Jimenez et al. (1999)
A_3 receptor	Cell culture/mouse	Initiation of $[Ca^{2+}]_i$ transients due to Ca^{2+} release from the ER	Chen et al. (2001)
	Cell culture/rat	Activation of apoptosis at intense stimulation; protective effects at low/moderate stimulation	Abbracchio et al. (1997, 1998), Appel et al. (2001)
	Cell culture/mouse	Upregulation of CCL2 cytokine synthesis	Wittendorp et al. (2004)

[a]Refers to experiments where P1 receptors subtypes were not identified. PLC; phospholipase C

and also enhance glial resistivity to hypoxia (Choi et al. 2005; D'Alimonte et al. 2007a; Bjorklund et al. 2008). Overstimulation of A_3 receptors, however, triggered astroglial death (Abbracchio et al. 1997, 1998).

4.3.2 Ionotropic P2X Purinoceptors

Mammalian ionotropic (P2X) ATP receptors (Burnstock and Kennedy 1985) are homo- or heterotrimers assembled from individual subunits of which seven subtypes ($P2X_1$–$P2X_7$) have been cloned; the resulting channel is permeable to the major cations ($Na^+/K^+/Ca^{2+}$), with relative Ca^{2+} permeability determined by subunit composition (Burnstock 2007a; Burnstock et al. 2011; Burnstock and Verkhratsky 2012). Similarly, subunit composition determines receptor sensitivity to ATP; most homo- and heteromeric receptors are activated by low micromolar ATP concentrations; $P2X_{1/5}$ receptors are the most sensitive, with an EC_{50} for ATP ~50 nM, whereas $P2X_7$ receptors require millimolar concentrations of ATP for activation. All P2X receptor subunits are expressed in the nervous system, although their expression varies between different regions of the nervous system (Burnstock and Verkhratsky 2012).

Various P2X receptor subunits were identified in astroglial cells at mRNA and protein levels (Table 4.2). Functionally only $P2X_{1/5}$ and $P2X_7$ receptors have been characterized in astrocytes. In cortical astrocytes (both freshly isolated and studies in acute slices) ATP triggers ion currents, which have all the hallmarks of $P2X_{1/5}$ heteromeric channels (biphasic activation kinetics), "rebound" current in response to agonist washout, high sensitivity to ATP, little, if any, desensitization and inhibition by pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and 2′(3′)-*O*-(2,4,6-trinitrophenyl) ATP (TNP-ATP) (Fig. 4.5) (Lalo et al. 2008, 2011a). These astroglial $P2X_{1/5}$ receptors are Ca^{2+} permeable ($P_{Ca}/P_{monovalent}$ ~2) and their stimulation by agonist or through activation of neuronal inputs in slice preparations induce cytoplasmic Ca^{2+} signals (Fig. 4.6) (Palygin et al. 2010). The P2X receptors sensitive to the antagonist NF023 were also found to mediate $[Ca^{2+}]_i$ transients in astrocytes from the optic nerve (James and Butt 2001; Hamilton et al. 2008).

Astrocytes were also reported to express functional $P2X_7$ receptors, which were identified in freshly isolated human retinal Müller cells (Pannicke et al. 2000), in cultured astrocytes (Duan et al. 2003; Norenberg et al. 2010) and in cortical and hippocampal astrocytes in situ in acute brain slices (Fellin et al. 2006; Oliveira et al. 2011). The role of $P2X_7$ receptors in astroglial physiology remains speculative, although they may be important for pathological reactions of astroglia (Franke et al. 2012; Illes et al. 2012).

The pathological reactions related to activation of astroglial $P2X_7$ receptors may include, for example, release of neurotransmitters such as glutamate and ATP; these molecules may diffuse through the dilated pore of the $P2X_7$ receptor (Ballerini et al. 1996; Duan and Neary 2006; Suadicani et al. 2006). Long-lasting stimulation of $P2X_7$ receptors, for example, was shown to mediate sustained glutamate release in

Table 4.2 P2 receptors in astroglia (modified from Verkhratsky et al. 2009)

Receptor type	Experimental preparation/species/technique	Properties/function	References
P2X receptors			
$P2X_{1–5}$, $P2X_7$	Cell culture/rat cortex/RT-PCR	Specific mRNAs detected	Fumagalli et al. (2003), Dixon et al. (2004)
$P2X_{1–7}$	Tissue extracts/rat nucleus accumbens/RT-PCR	Specific mRNAs detected	Franke et al. (2001a)
$P2X_{2–4}$	Rat nucleus accumbens/immunostaining	Immunoreactivity detected	Franke et al. (2001a)
$P2X_{3–5}$	Acutely isolated Müller cells/rat retina/RT-PCR	Specific mRNAs detected	Jabs et al. (2000)
$P2X_1$, $P2X_5$	Acutely isolated cells/mouse cortex/RT-PCR	Specific mRNAs detected	Lalo et al. (2008)
$P2X_1$, $P2X_2$	Rat, guinea pig cerebellum/immunostaining	Immunoreactivity detected	Kanjhan et al. (1996), Loesch and Burnstock (1998)
$P2X_4$	Rat brainstem/immunostaining	Immunoreactivity detected	Ashour and Deuchars (2004)
$P2X_{1–4}$, $P2X_6$, $P2X_7$	Rat hippocampus/immunostaining	Immunoreactivity detected	Kukley et al. (2001)
$P2X_{1/5}$	Acute slices/mouse/electrophysiology	Specific currents through $P2X_{1/5}$ heteromeric receptors	Lalo et al. (2008)
P2X?, $P2X_7$?	Acutely isolated optic nerve/mouse/Ca^{2+} imaging	$[Ca^{2+}]_i$ transients associated with receptor-mediated Ca^{2+} entry	James and Butt (2002), Hamilton et al. (2008)
$P2X_7$	Acutely isolated Müller cells/human retina/electrophysiology	Specific mRNAs and immunoreactivity as well as currents through $P2X_7$ receptors were detected	Pannicke et al. (2000)
$P2X_7$	Cell culture/rat/Ca^{2+} imaging	$[Ca^{2+}]_i$ transients associated with receptor-mediated Ca^{2+} entry	Ballerini et al. (1996), Fumagalli et al. (2003), Nobile et al. (2003)
$P2X_7$	Cell culture/mouse cortex/electrophysiology	Specific currents through $P2X_7$ receptors; activation of $P2X_7$ receptors resulted in release of excitatory amino acids	Duan et al. (2003)
$P2X_7$	Cell culture/mouse	Activate synthesis of endocannabinoid 2-arachidonoylglycerol	Walter et al. (2004)
$P2X_7$	Cell culture/human	Stimulation of NO production	Narcisse et al. (2005)
$P2X_7$	Cell culture/human	Regulation of nuclear factor-κB signaling	John et al. (2001)

Table 4.2 (continued)

Receptor type	Experimental preparation/species/technique	Properties/function	References
$P2X_7$	Cell culture/rat	Upregulation of P2Y receptors expression	D'Alimonte et al. (2007b)
$P2X_7$	Cell culture/rat	Downregulation of aquaporin-4 expression	Lee et al. (2008)
P2Y receptors			
$P2Y_{1,2,4,6,12,13}$ and UDP-glucose $P2Y_{14}$ receptor	Cell culture/rat/RT-PCR	Specific mRNAs detected	Fumagalli et al. (2003), Dixon et al. (2004)
$P2Y_{1,4,6}$	Cell culture/rat cortex/RT-PCR	Specific mRNAs detected	Bennett et al. (2003)
$P2Y_{1,2}$	Cell culture/rat spinal cord/RT-PCR	Specific mRNAs detected	Fam et al. (2000)
$P2Y_{1,2,4}$	Cell culture/rat hippocampus/RT-PCR, Western blot	Specific mRNAs and proteins detected; ~50 % of cells express $P2Y_1$, some cells also express $P2Y_{2,4}$	Zhu and Kimelberg (2004)
$P2Y_{1,2,4,6}$	Acutely isolated Müller cells/rat retina/RT-PCR, immunostaining, electrophysiology	Specific mRNAs and immunoreactivity detected; stimulation of P2Y receptors triggered Ca^{2+}-dependent K^+ currents	Fries et al. (2004, 2005)
$P2Y_{1,2,4,6,11,13}$	Acutely isolated Müller cells/tiger salamander/Ca^{2+} imaging	$[Ca^{2+}]_i$ transients associated with Ca^{2+} release of the ER; receptors subtypes were identified using specific pharmacology	Reifel Saltzberg et al. (2003)
P2Y	Cell culture/rat/biochemical assays, Ca^{2+} imaging	Increase in $InsP_3$ production; $[Ca^{2+}]_i$ transients associated with Ca^{2+} release of the ER	Pearce et al. (1989), Kastritsis et al. (1992), Bruner and Murphy (1993b), Chen and Chen (1996), Ishimoto et al. (1997), Jimenez et al. (2000)
P2Y	Cell culture/rat, striatum/Ca^{2+} imaging	$[Ca^{2+}]_i$ transients associated with Ca^{2+} release of the ER	Centemeri et al. (1997)
P2Y	Cell culture/rat, neurohypophysis/Ca^{2+} imaging	$[Ca^{2+}]_i$ transients associated with Ca^{2+} release of the ER	Troadec et al. (1999), Uchiyama et al. (2001)

Receptor type	Experimental preparation/species/technique	Properties/function	References
P2Y	Cell culture/rat, spinal cord	$[Ca^{2+}]_i$ transients associated with Ca^{2+} release of the ER	Salter and Hicks (1994, 1995)
P2Y	Acute slices/mouse, cerebellum, Bergmann glial cells/Ca^{2+} imaging, electrophysiology	$[Ca^{2+}]_i$ transients associated with Ca^{2+} release of the ER	Kirischuk et al. (1995), Piet and Jahr (2007)
P2Y	Cell culture/rat, hippocampus/Ca^{2+} imaging	$[Ca^{2+}]_i$ transients associated with Ca^{2+} release of the ER	Bowser and Khakh (2004)
$P2Y_1$	Acute slices/mouse, olfactory bulb/Ca^{2+} imaging	$[Ca^{2+}]_i$ transients associated with Ca^{2+} release of the ER	Doengi et al. (2008)
$P2Y_1$, $P2Y_4$	Acutely isolated optic nerve/mouse/Ca^{2+} imaging	$[Ca^{2+}]_i$ transients associated with Ca^{2+} release of the ER	James and Butt (2001)
$P2Y_1$	Cell culture/mouse, cortex	Stimulation of ATP release through volume-sensitive anion channels	Anderson et al. (2004)
P2Y	Cell culture/rat	Stimulation of ATP release via exocytosis	Abdipranoto et al. (2003), Coco et al. (2003), Bowser and Khakh (2007), Striedinger et al. (2007)
P2Y	Cell culture/cell lines	Stimulation of ATP release through hemichannels	Cotrina et al. (1998, 2000)
P2Y	Cell culture/rat	Stimulation of glutamate release via exocytosis	Jeremic et al. (2001)
$P2Y_1$	Cell culture/rat hippocampus; acute slices/mouse hippocampus	Stimulation of glutamate release via exocytosis	Domercq et al. (2006)
P2Y	Cell culture/rat	Promotion of astroglial differentiation	Abbracchio et al. (1995), Bolego et al. (1997)
P2Y	Cell culture/rat, human	Initiation of astrogliosis	Ciccarelli et al. (1994), Brambilla et al. (1999, 2000, 2002)
P2Y	Retina preparations/rat, rabbit	Initiation of astrogliosis	Franke et al. (2001a, 2003), Uckermann et al. (2003)
$P2Y_1$, $P2Y_{12}$	Rat, nucleus accumbens, cortex/in vivo	Initiation of astrogliosis	Franke et al. (2001a, b)

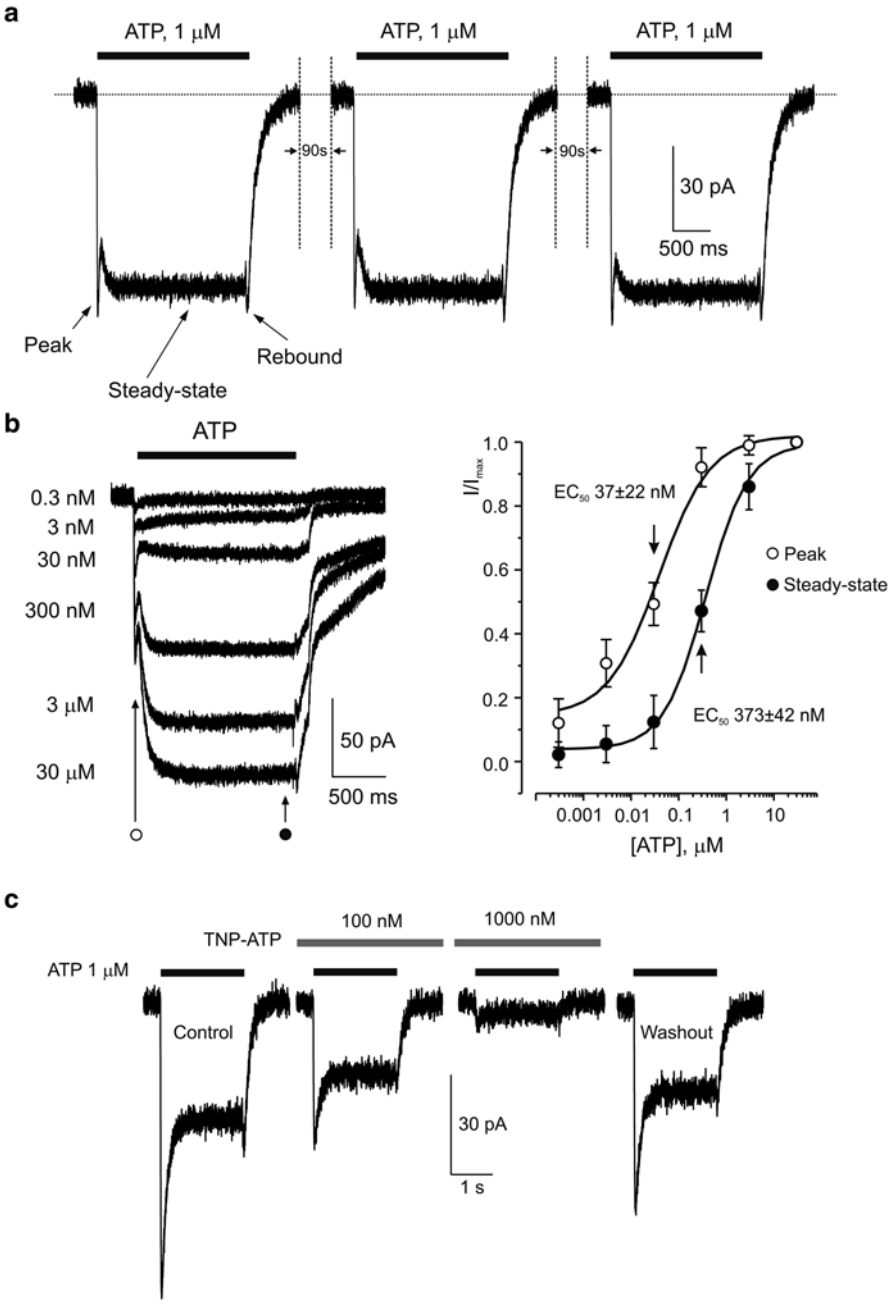

Fig. 4.5 $P2X_{1/5}$ receptor-mediated currents in acutely isolated cortical astrocytes. (**a**) The family of ATP currents evoked by repetitive applications of the agonist. The currents show no apparent desensitization. Current traces have a complex kinetics comprising the peak, the steady-state component, and the "rebound" inward current recorded upon ATP washout as indicated on the graph.

hippocampus (Fellin et al. 2006). ATP, when applied in millimolar concentrations (and thus most likely acting through $P2X_7$ receptors) also triggered release of endocannabinoid 2-arachidonoylglycerol from cultured astrocytes (Walter et al. 2004). Activation of $P2X_7$ receptors also induced release of pro-inflammatory molecules from astrocytes, including tumor necrosis factor (TNF)-α (Kucher and Neary 2005) or cysteinyl leukotrienes (Ballerini et al. 2005).

There are numerous indications that expression of $P2X_7$ receptors is significantly upregulated in neuropathology. For example, mechanical damage led to an appearance of $P2X_7$ immunopositive cells in nucleus accumbens (Franke et al. 2001a); similarly, expression of $P2X_7$ receptors was upregulated following focal cerebral ischemia in the rat cortex (Franke et al. 2004). Significant increase in the density of $P2X_7$-mediated currents was observed in human Müller cells isolated from patients with vitreoretinopathy (Bringmann et al. 2001); likewise, immunoreactivity for $P2X_7$ receptors was detected in reactive astrocytes from brain autopsies obtained from multiple sclerosis patients (Narcisse et al. 2005).

4.3.3 Metabotropic P2Y Purinoceptors

Metabotropic P2Y purinoceptors are subclassified into the $P2Y_{1,2,4,6,11}$ and $P2Y_{12,13,14}$ groups; this division reflecting G protein preference (Burnstock and Verkhratsky 2012). The $P2Y_{1,2,4,6,11}$ receptors are preferentially coupled to G_q/G_{11} proteins and thus regulate phospholipase C, production of $InsP_3$ and $InsP_3$-mediated Ca^{2+} release. The second group, $P2Y_{12,13,14}$ receptors, are linked to $G_{i/o}$ proteins, which in turn regulate ion channels and inhibit adenylate cyclase.

Data on the expression of P2Y receptors in astrocytes are summarized in Table 4.2. Generally astroglial cells are reported to express predominantly $P2Y_{1,4,6}$ receptors, although expression of other types has also been reported. Stimulation of P2Y receptors induces astroglial Ca^{2+} signaling, which results from Ca^{2+} release from the ER Ca^{2+} store and activation of secondary store-operated Ca^{2+} influx (Verkhratsky et al. 2012a). Such P2Y receptor-mediated Ca^{2+} signaling has been observed in astroglial cells in vitro (Kastritsis et al. 1992; Pearce and Langley 1994; Salter and Hicks 1994, 1995; Centemeri et al. 1997; Jimenez et al. 2000) and in situ in astrocytes from acute slices (Kirischuk et al. 1995; Bowser and Khakh 2004).

Fig. 4.5 (continued) (**b**) Concentration dependence of ATP-induced currents in cortical astrocytes. Membrane currents recorded from a single cell in response to different ATP concentrations are shown on the *left*. The *right panel* shows the concentration-response curves constructed from nine similar experiments; current amplitudes were measured at the initial peak and at the end of the current, as indicated on the graph. (**c**) Inhibition of ATP-induced currents by 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP). The graph shows ATP-induced currents measured from the cortical astrocyte in control conditions, in the presence of different concentrations of TNP-ATP and after the washout. Application of TNP-ATP started 2 min before application of ATP. All recordings were made at holding potential of −80 mV. Reproduced with permission from Lalo et al. (2008)

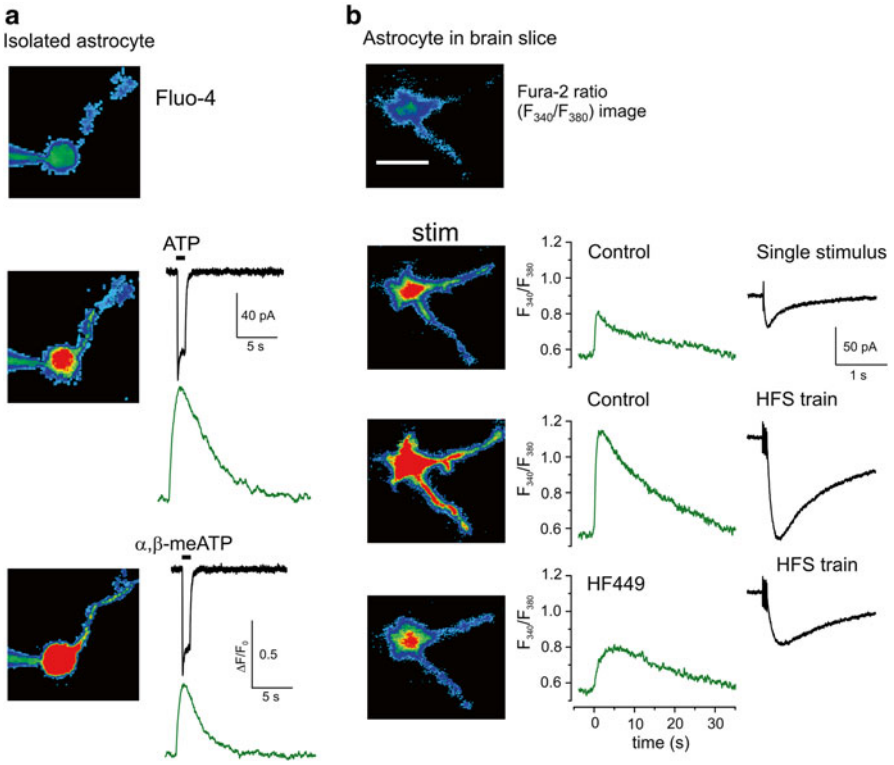

Fig. 4.6 $P2X_{1/5}$ receptor-mediated Ca^{2+}-signaling in cortical astrocytes. (**a**) Acutely isolated astrocytes were loaded with Fluo-4 via a patch pipette. Fluorescent images were recorded simultaneously with transmembrane currents evoked by application of 100 μM ATP or 10 μM α,β-methylene ATP. $[Ca^{2+}]_i$ transients are represented as the $\Delta F/F_0$ ratio averaged over the cell soma (*green traces*). Holding potential was −80 mV. (**b**) Synaptically-induced ionotropic Ca^{2+} signals in protoplasmic cortical astrocytes in situ. Cortical layer II astrocytes were loaded with Fura-2 in situ via patch pipette. Fluorescent images were recorded simultaneously with glial currents evoked by neuronal afferent stimulation in the presence of CNQX (control) and after the application of 10 nM of NF-449, a selective antagonist of P2X receptors. $[Ca^{2+}]_i$ transients (green traces) are expressed as F_{340}/F_{380} ratio. Modified from Palygin et al. (2010)

Activation of $P2Y_1$ receptors is known to be important for generating and maintaining propagating Ca^{2+} waves in cultured hippocampal astrocytes (Bowser and Khakh 2007). At the same time in primary cultured spinal cord astrocytes, both $P2Y_1$ and $P2Y_2$ receptors were required for Ca^{2+} waves to occur (Gallagher and Salter 2003; Bennett et al. 2006). Functional P2Y receptors mediating $InsP_3$-induced Ca^{2+} release were also identified in freshly isolated human Müller cells; Ca^{2+} release in these cells activated Ca^{2+}-dependent K^+ channels and Ca^{2+}-gated cationic channels (Bringmann et al. 2002).

P2Y receptor-stimulated Ca^{2+} signaling is known to stimulate astroglial release of neurotransmitters, most notably glutamate and ATP. For example, stimulation of

cultured rat cortical astrocytes with 10 μM UTP induced ATP release that was inhibited by the non-selective P2 receptor antagonist, suramin, by the inhibitor of ER Ca^{2+} accumulation, thapsigargin, by fragmentation of the Golgi complex with brefeldin A, by cytoskeleton disruption with cytochalasin D and by the exocytosis inhibitor, botulinum toxin A (Abdipranoto et al. 2003); all this evidence collectively indicates Ca^{2+}-dependent vesicular release. Release of ATP from astrocytes in the corpus callosum mediated through activation of P2Y receptors was identified as a primary mechanism of propagating Ca^{2+} waves between astroglial cells in the corpus callosum (Haas et al. 2006). Similarly, stimulation of P2Y metabotropic purinoceptors was demonstrated to induce release of glutamate and aspartate from primary cultured cortical astrocytes (Jeremic et al. 2001); this release was directly dependent on an increase in cytosolic Ca^{2+} originating from the ER.

Intracellular Ca^{2+} signals triggered following activation of $P2Y_1$ receptors also induced glutamate release from astrocytes in situ in acute hippocampal slices (Domercq et al. 2006). Activation of $P2Y_1$ receptors is also known to evoke release of TNFα and prostaglandins (Domercq et al. 2006). In astrocytes from mouse prefrontal cortex stimulation of $P2Y_4$ receptors induced vesicular release of glutamate (Wirkner et al. 2007). Activation of P2Y receptors increases synthesis of prostaglandins (Gebicke-Haerter et al. 1988) and eicosanoids, as well as mobilizes arachidonic acid (Bruner and Murphy 1990, 1993a). When $P2X_4$ receptors were activated in astroglial cells in culture, a significant increase in expression and release of synaptogenesis-associated factor thrombospondin-1 was described (Tran and Neary 2006). Finally P2Y receptors are instrumental in the induction of the astrogliotic response in various pathological conditions (Franke et al. 2012).

4.4 Conclusions

Astroglial cells express several types of ionotropic and metabotropic receptors to glutamate and to purines and pyrimidines. These receptors are activated in the course of intercellular chemical transmission in the CNS and regulate various aspects of astroglial responses in physiology and pathophysiology.

Conflict of Interest The authors declare no conflict of interest.

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Chapter 5
Regulated Exocytosis in Astrocytes is as Slow as the Metabolic Availability of Gliotransmitters: Focus on Glutamate and ATP

Nina Vardjan, Marko Kreft, and Robert Zorec

Abstract It is becoming clear that astrocytes, the most abundant type of glial cells in the mammalian brain, share many properties with neurons. One such property involves vesicles, which play a key role in cell-to-cell signaling. On the one hand, vesicles determine the signaling potential by delivering various receptors and transporters to the plasma membrane by vesicular exocytosis. On the other hand, vesicles are used in astrocytes for the release of vesicle-laden chemical messengers. This chapter compares the properties of Ca^{2+}-dependent fusion of the vesicle membrane with the plasma membrane in astrocytes and in neurons, monitored by membrane capacitance techniques. Moreover, we focus on membrane-bound vesicles that store gliotransmitters, glutamate, and adenosine 5′-triphosphate (ATP), to learn why regulated exocytosis in astrocytes is orders of magnitude slower than in neurons and the fact that these signaling molecules are also metabolites. The relatively slow kinetics of regulated exocytosis in astrocytes likely involves vesicle dynamics regulation and mechanisms governing the merger of the vesicle membrane with the plasma membrane, but may also depend on the availability of ATP and glutamate in metabolic pathways for packaging into vesicles via specific vesicle transporters.

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_5

Keywords Astrocyte • Glia • Neuroglia • Vesicle • Trafficking • Regulated exocytosis • Gliotransmitter • Antigen presentation • Neuroinflammation • Autism • Amyotrophic lateral sclerosis • Multiple sclerosis

5.1 Introduction

The paradigm that astrocytes, the most abundant glial cells in the central nervous system (CNS), play only subservient roles to neurons has shifted to a new paradigm in which astrocytes are active partners in cell-to-cell signaling. Many new functions have been described since the early 1990s, including regulation of synaptogenesis, synaptic transmission, brain microcirculation, roles in the formation and maintenance of the blood–brain barrier (BBB), roles in metabolic support for neurons, participation in pathologic immune responses, and contributions to the formation and resolution of brain edema (Dong and Benveniste 2001; Haydon 2001; Ke et al. 2001; Anderson and Nedergaard 2003; De Keyser et al. 2003; Nedergaard et al. 2003; Zonta et al. 2003; Abbott et al. 2006; Gordon et al. 2007; Nase et al. 2008; Stevens 2008; Risher et al. 2009). These new functions depend partly on the mechanisms by which astrocytes communicate with the surrounding cells. These include plasma membrane channels, receptors, transporters, and mechanisms that include exo- and endocytotic vesicles. The latter processes participate in maintaining the quality of the plasma membrane signaling capacity and in the release of signaling molecules—gliotransmitters—which may affect the function of neighboring cells in health and disease processes (Kreft et al. 2004b; Osborne et al. 2009; Parpura and Zorec 2010; Guček et al. 2012; Parpura et al. 2012; Zorec et al. 2012; Potokar et al. 2013b).

5.2 Types of Gliotransmitter Release: Advantages of Vesicle-Based Mechanisms

As in neurons, where criteria have been established for classifying a chemical being released from terminals as a neurotransmitter (Axelrod 1974), a similar approach has been adopted for neuroglia (Parpura and Zorec 2010; Parpura and Verkhratsky 2012). To identify a chemical released from glia/astrocytes as a gliotransmitter, the following criteria are usually considered: (i) synthesis by and/or storage in glia; (ii) regulated release triggered by physiological and/or pathologic stimuli; (iii) activation of paracrine or autocrine responses; and (iv) a role in (patho)physiological processes.

Several mechanisms of gliotransmitter release seem to coexist in a single astrocyte (Domingues et al. 2010; Hamilton and Attwell 2010). In addition to the vesicle-based mechanisms, non–vesicle-based release has been described to be mediated in glial cell through channels such as volume-regulated anion channels opening, through hemichannels (connexons/pannexons), and through ionotropic purinergic

receptors; through reverse uptake by membrane transporter (see references within Parpura and Zorec 2010). These mechanisms require a concentration gradient along which chemical messengers are transported to their targets, but the vesicle-based mechanisms, currently under active debate (Smith 2010), have certain advantages over the non–vesicle-based modes of release (Guček et al. 2012).

Vesicle-based transmitter and hormone release is based on exocytosis, a process that involves many stages including the merger between the vesicle and the plasma membranes (Coorssen and Zorec 2012). This universal process, an evolutionary trait of eukaryotic cells (Vardjan et al. 2010), emerged from a prokaryotic-like precursor cell by endosymbiosis (Cooper 2000). Thus, a hallmark of eukaryotic cells, including astrocytes, is a membrane-bound subcellular structure, including mitochondria, Golgi bodies, and secretory vesicles. When eukaryotic cells evolved 1,000–2,000 million years ago (Cooper 2000; Yoon et al. 2004), this was associated with an increase in cell volume three to four orders of magnitude. The increased cell size dictated a new organizational setup. A key reason for this is that signaling and communication within the relatively large volume of a eukaryotic cell could no longer be supported mainly by diffusion-based processes. Diffusion provides effective and rapid transport of molecules in the submicrometer range. Hence, the development of subcellular organelles presented a solution for this signaling problem of the large volume of eukaryotic cells. At least two important considerations support this notion.

First, relatively small-sized subcellular structures (i.e. secretory vesicles), in which chemical messengers can be stored at high concentration, present an advantage from the point of view of cell economy. Transport of molecules across the membrane against the concentration gradient, such as L-glutamate entering a vesicle, consumes energy in the form of ATP hydrolysis. It was estimated that the budget for recycling 4,000 glutamate molecules into a vesicle consists of 11,000 ATP molecules (Attwell and Laughlin 2001). In contrast, the budget for concentrating glutamate into the cytosol of a whole cell must be much higher, because the volume of a vesicle (a sphere of 50 nm in diameter) is at about nine orders of magnitude smaller than the volume of a typical cell (a sphere with a diameter of 15 μm). Therefore, a reduced more economical energy budget is required to attain a relatively high concentration of signaling molecules in the vesicle lumen versus that of the whole cytoplasm.

Second, secretory vesicles can be considered to represent a functional module, a high concentration-loaded compartment that can be placed in different locales within the cytoplasm, where a high concentration gradient of chemical messengers is required for diffusional delivery to the targets. Vesicles can thus be considered as signaling modules that can be strategically positioned within the cell, for example, into the nerve terminal adjacent to the presynaptic membrane in the proximity of the synaptic cleft. Such signal module positioning cannot be achieved with the machinery for the non–vesicle-based mechanisms of chemical messenger release, unless the non–vesicle-based structures are integrated into the specific plasma membrane locales by exocytosis.

Vesicle-based release mechanisms have played a key role in the development of multicellular organisms, especially in cases where rapid communication between cells is required. For example, neurotransmitters stored in secretory vesicles can be

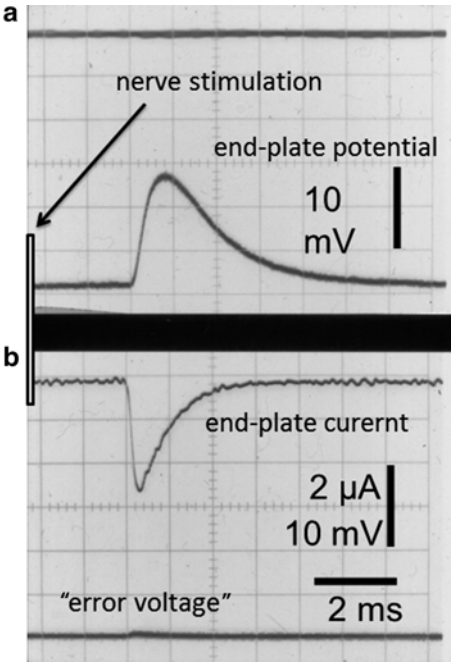

Fig. 5.1 (**a**) Postsynaptic end-plate potential and (**b**) end-plate current recorded by a two-microelectrode voltage-clamp method in the frog neuromuscular junction (Kordas and Zorec 1984). The vertical bar denotes the time of motor neuron stimulation and the error voltage after the voltage clamping was turned on, indicating the quality of voltage control of the postsynaptic membrane

discharged swiftly through the exocytotic fusion pore that is formed when vesicle membrane merges with the plasma membrane. The probability that a fusion pore is formed swiftly is higher if vesicles are placed adjacent to the plasma membrane, for example, in an active zone. The latency between the signal onset and the response at the postsynaptic membrane in a synapse can be as short as 100 μs (Sabatini and Regehr 1999). An example of the postsynaptic current (end-plate current) recorded in the frog neuromuscular junction is shown in Fig. 5.1. Note that there is a delay of about 2 ms, which is due to stimulation of the end of the nerve stump about 2 cm from the end plate. The delay is thus due to propagation of the action potential to the presynaptic terminal, entry of calcium into the presynaptic terminal, which triggers vesicle merger and fusion pore opening, diffusion of acetylcholine through the synaptic cleft to the postsynaptic membrane where it binds to the nicotinic receptors, opening the receptors and giving rise to the end-plate current (Fig. 5.1b).

The vesicle-based mechanisms of transmitter and hormone release can also exhibit much longer latencies. For example, the latency can be lengthened by delaying the vesicle delivery to the plasma membrane fusion sites (Potokar et al. 2013b). Moreover, the latency between the stimulus–secretion coupling can be increased by reducing vesicle discharge from the vesicle lumen by regulating vesicle fusion pore kinetics and/or fusion pore diameter (Vardjan et al. 2007). Very long delays between a stimulus and the vesicle merger with the plasma membrane may be achieved if the stimulus triggers synthesis of the signaling molecules that are released or are

incorporated into the plasma membrane. Astrocytes express many plasma membrane receptors, including major histocompatibility complex class II (MHC II), that play a role in antigen presentation, but only when exposed to interferon-γ (IFN-γ). When astrocytes are challenged with IFN-γ, it may take several hours for MHC II molecules to appear in secretory lysosomes (Vardjan et al. 2012). Therefore, a delay of a day or more can be achieved by controlling the expression of certain signaling molecules and their vesicle-based delivery to the plasma membrane or even their release from the vesicle lumen. Therefore, the vesicle-based mechanism is suitable not only for rapid communication, as in neuronal chemical synapses (Sabatini and Regehr 1999) but also in much longer spanning processes. The question can be asked whether regulated exocytosis in neurons and in astrocytes exhibits different properties.

5.3 Comparison of Regulated Exocytosis in Astrocytes and in the Neurons

The merger of the vesicle membrane with the plasma membrane, which leads to discharge of the vesicle content, is reflected in changes in the plasma membrane area: when a vesicle membrane fully integrates into the plasma membrane, the area of the plasma membrane increases by the area of the vesicle membrane. Therefore, fluctuations in membrane area are related to the secretory activity of a cell. An elegant way to monitor fluctuations in the plasma membrane area consists of measuring membrane capacitance (C_m), a parameter linearly related to the membrane area (Neher and Marty 1982; Rituper et al. 2013). This technique was used in a number of neuronal and neuroendocrine preparations (Neher 2012) and in cultured astrocytes (Kreft et al. 2004b). The advantage of this approach is that it allows us to test the hypothesis that an increase in cytosolic calcium ($[Ca^{2+}]_i$), increased by photolysis of caged calcium compounds (Neher and Zucker 1993), elicits an increase in the whole-cell C_m. This approach is presented in Fig. 5.2.

The Ca^{2+}-induced increase in C_m was blocked in astrocytes by tetanus neurotoxin, indicating that changes in membrane area are associated with a **s**oluble **N**-ethyl maleimide-sensitive fusion protein attachment protein receptor (SNARE)-dependent vesicular mechanism (Kreft et al. 2004b). A half-maximal response in C_m increase was attained at around 27 μM $[Ca^{2+}]_i$, which is similar to the Ca^{2+}-dependency of regulated exocytosis in neurons, recorded by a similar technique (Heidelberger et al. 1994; Bollmann et al. 2000; Kreft et al. 2003a). However, the kinetics of this response in astrocytes was at least two orders of magnitude slower in comparison with the rate of regulated exocytosis recorded by a similar technique in neurons (Kreft et al. 2004b). A comparison of UV flash-induced increases in C_m recorded in a neuron and in an astrocyte is shown in Fig. 5.3. By using the same recording system, the fastest component of the UV flash-induced increase in C_m was recorded in rod photoreceptors (400 s^{-1}), whereas it was two orders of magnitude smaller in astrocytes (2 s^{-1}) (Kreft et al. 2003a, 2004b).

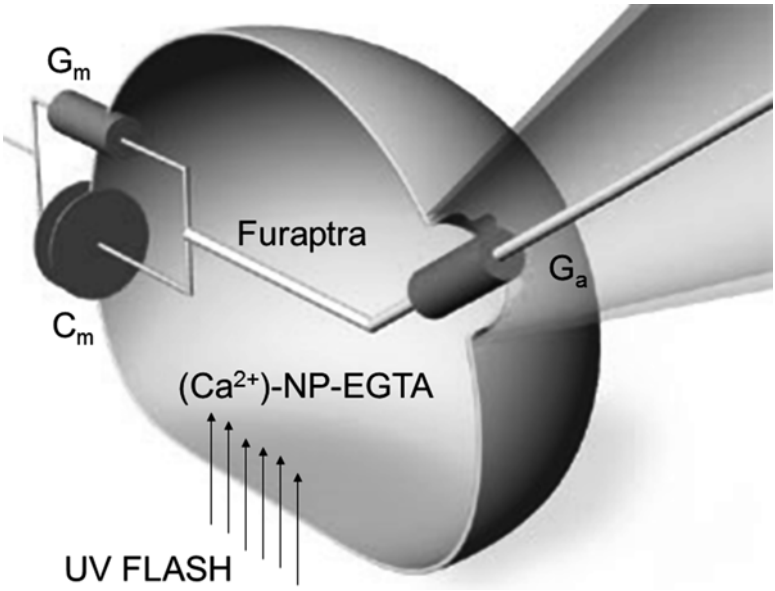

Fig. 5.2 A diagram of the whole-cell recording with an equivalent electrical scheme (parallel combination of membrane conductance (G_m) and membrane capacitance (C_m) coupled in series to access conductance (G_a), determined by electrical access of the patch pipette lumen into the cytoplasm). The advantage of this approach is that the composition of the cytosol can be controlled by dialysis of the pipette solution into the cytoplasm on the rupture of the plasma membrane at the rim of the patch pipette tip, establishing a diffusional continuum between the pipette and the cytoplasm. In this way, a calcium indicator (Furaptra) and caged calcium compounds (i.e. Ca^{2+}-loaded NP-EGTA) can be introduced into the cytoplasm of a cell. By delivery of a UV flash, Ca^{2+}-NP-EGTA is photolyzed and $[Ca^{2+}]_i$ is swiftly increased in a spatially homogeneous way (Neher and Zucker 1993)

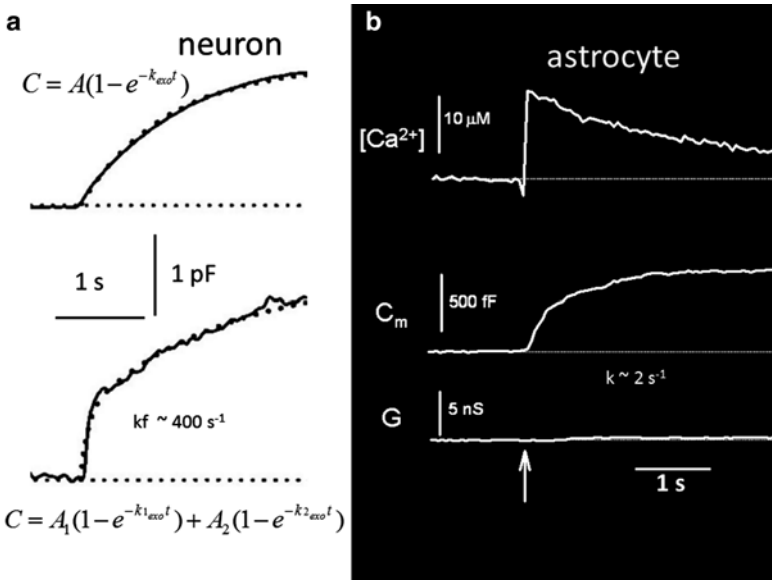

Fig. 5.3 Comparison of time-dependent changes in C_m recorded in (**a**) a neuronal cell (rod photoreceptor) and (**b**) an astrocyte. (**a**) Two kinetic types of Ca^{2+}-induced increases in C_m were recorded

Table 5.1 Ca^{2+}-dependence of the maximal rate of membrane capacitance (C_m) increase due to exocytosis (Max rate; s^{-1}) in various secretory cells

Cell type	Maximum rate (s^{-1})	K10% (μM)	Delay (ms)	References
Pituitary melanotrophs	25	20*	ND	Thomas et al. (1993); Rupnik et al. (2000)
	44	3		
Pancreatic β cells	70	1*	ND	Barg et al. (2001); Wan et al. (2004)
Chromaffin cells	1,500	40*	3	Voets (2000)
Rod photoreceptors	300	10*	ND	Kreft et al. (2003a); Thoreson et al. (2004)
	400	20		
Bipolar cells	3,000	80*	1	Heidelberger et al. (1994)
Inner hair cells	1,700	20*	1.5	Beutner et al. (2001)
Calyx of Held	6,000	30*	0.3–1	Bollmann et al. (2000); Schneggenburger and Neher (2000)
Inhibitory basket cells	5,000	20*	1	Sakaba (2008)
Astrocytes	0.1–2	20	30–300	Kreft et al. (2004b); Neher (2012)

*K10% denotes cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) at which the rate constant is 10 % of the maximal rate (Neher and Sakaba 2008). Values without an asterisk denote $[Ca^{2+}]_i$ at which the half-maximal rate of exocytosis was measured. Delay (ms) denotes the time between the stimulus and the increase in C_m. ND, not determined

Table 5.1 lists the maximal rate of regulated exocytosis, measured as a UV flash-induced increase in C_m, recorded in various secretory cell types. The fastest regulated exocytosis seems to be present in the calyx of Held with a rate of 6,000 s^{-1}. Even in endocrine cells, such as pancreatic β cells and pituitary melanotrophs, maximal rates of regulated exocytosis are higher than those measured in astrocytes. Therefore, one can conclude that astroglial cells exhibit Ca^{2+}-dependent regulated exocytosis with a time course orders of magnitude slower than that in neurons. Thus, astrocytes can be considered as signal integrators in the CNS; they listen to various signals and respond with a relatively long delay due to the slow responsiveness of the machinery for regulated exocytosis. However, the reason why regulated exocytosis is relatively slow in astrocytes is unknown and is considered in the next sections.

Fig. 5.3 (continued) in a photoreceptor. The *top trace* was best fitted by a single exponential function (*dotted line*); the *bottom trace* was best fitted by a sum of two exponential functions as shown by the equation below the *horizontal dotted line*. The fastest rate constant (k_f) was around 400 s^{-1} (modified from Kreft et al. 2003a). (**b**) The *top trace* shows time-dependent changes in $[Ca^{2+}]_i$ elicited by UV light flash photolysis of caged Ca^{2+} compound dialyzed into the cytosol of the astrocyte (Kreft et al. 2004b). UV flash was applied at the time indicated by the *arrow*. Note that the rapid increase in $[Ca^{2+}]_i$ after the UV flash application induced an exponential increase in C_m with a rate constant (k) of ~2 s^{-1} (Kreft et al. 2004b). *G* denotes the real part of the admittance signal

5.4 Why is Regulated Exocytosis Relatively Slow in Astrocytes?

The relatively slow kinetics of glial-regulated exocytosis (Fig. 5.3b) may be due to many factors, including the slow delivery of vesicles to the plasma membrane and/or distinct (slow) vesicle–plasma membrane fusion mechanisms, perhaps due to a distinct set of molecules. One such molecular distinction between neurons and astrocytes is the absence of synaptotagmin I in astrocytes, which is the key Ca^{2+} sensor in rapid synaptic transmission in neurons (Geppert et al. 1994). Consistent with this, it was shown that synaptotagmin I increases the probability of rapid regulated exocytosis at relatively low $[Ca^{2+}]_i$ in pituitary endocrine cells (Kreft et al. 2003b). What are the Ca^{2+} sensors for regulated exocytosis in astrocytes is still not clear and this is an important topic for future investigation.

The Ca^{2+}-induced increase in C_m shown in Fig. 5.3b is likely due to many vesicle types fusing with the plasma membrane. Membrane-bound vesicles in astrocytes carry several classes of molecules, such as amino acids, nucleotides, peptides, transporters, water channels, and receptors (Coco et al. 2003; Krzan et al. 2003; Bezzi et al. 2004; Pangrsic et al. 2007; Jean et al. 2008; Martineau et al. 2008, 2013; Parpura et al. 2010; Parpura and Zorec 2010; Vardjan et al. 2012; Potokar et al. 2013a). Their efficient delivery to the target destination in the cell is governed by vesicle mobility. It has been established that cytoplasmic vesicle mobility over distances of several micrometers is a tightly regulated process involving cell signalling pathways and alterations in cytoskeleton dynamics. Under pathologic conditions (ischemia, trauma, edema, neuroinflammation), different triggers alter vesicle mobility, as shown by several studies (Potokar et al. 2007, 2008, 2010, 2012, 2013a, b; Stenovec et al. 2007, 2011; Trkov et al. 2012; Vardjan et al. 2012) and reviewed by (Vardjan et al. 2013).

In this review, we focus on single-vesicle trafficking and release studies in astrocytes involving the gliotransmitters glutamate and ATP, which are also important metabolites. Although electron microscopy studies have revealed that astrocytes contain clear-core and, to a lesser extent, dense-core vesicles (Parpura and Zorec 2010; Guček et al. 2012), it is currently unknown whether the trafficking and vesicle content discharge of these are distinct, therefore here we highlight studies in which vesicle mobility and vesicle cargo discharge were monitored in real time in astrocytes, and we question whether the loading of these two gliotransmitters into vesicles may be limited by the availability of these two metabolites.

5.5 Mobility and Exocytosis Release of Glutamate and ATP Vesicles

The first spontaneous mobility of membrane-bound vesicles in astrocytes was described by (Potokar et al. 2005; Crippa et al. 2006), and subsequently confirmed by (Crippa et al. 2006). To determine vesicle mobility, several parameters such as

the total track length, the path a vesicle travels in a given period of time, the average velocity, the displacement, and the directionality index (ratio between the maximal displacement/total track length) can be measured. Maximal displacement represents a measure of the maximal net translocation of vesicles (Wacker et al. 1997). Consistent with other cell types (Burke et al. 1997; Tvaruskó et al. 1999; Duncan et al. 2003; Potokar et al. 2005), two distinct modes of vesicle mobility have been described in astrocytes: directional (vesicle tracks displaying a straight line) and nondirectional (vesicle tracks displaying a contorted line), and these modes of mobility were able to switch while a vesicle was being observed (Potokar et al. 2005). Moreover, directional mobility appeared to require an intact cytoskeleton (Potokar et al. 2007). These two forms of mobility likely determine the efficiency of vesicle delivery to the fusion site, leading to exocytotic release of vesicle content.

5.5.1 Glutamate-Loaded Vesicles

Glutamate-containing vesicles belong to the class of amino acid vesicles that likely contain glutamate and D-serine (Parpura et al. 1994; Bezzi et al. 2004; Kreft et al. 2004b; Montana et al. 2004, 2006; Martineau et al. 2008, 2013). Glutamate is packaged into vesicles by the vesicular glutamate transporters (VGLUTs) VGLUT1, VGLUT2, and VGLUT3 (Danbolt 2001; Parpura and Zorec 2010). Although the existence of VGLUT1 in mouse astrocytes has been questioned (Li et al. 2013), VGLUT1-containing vesicles in rat astrocytes are small and electron lucent, with an estimated diameter of ~30 nm in situ (Bezzi et al. 2004) and ~50 nm when they recycle (Stenovec et al. 2007), but larger sizes have also been reported (Chen et al. 2005; Malarkey and Parpura 2011). Why different vesicle diameters have been reported for glutamatergic vesicles is not known, but this may also be associated with different microscopy techniques used by different investigators. One way to resolve this question in the future would be to use new methods, such as stimulation emission depletion microscopy, a super-resolution fluorescence microscopy technique (Hell and Wichmann 1994; Jorgacevski et al. 2011) that presents new possibilities to resolve the question of the different diameters reported for gliotransmitter vesicles in astrocytes.

Glutamatergic vesicles are spontaneously mobile, but their mobility is increased under stimulated conditions. Glutamatergic vesicles were labelled in vivo using a novel approach (Stenovec et al. 2007) based on the fact that after Ca^{2+}-dependent exocytosis (Parpura et al. 1995; Jeftinija et al. 1996; Zhang et al. 2004a, b), exocytotic vesicles are endocytosed (Stenovec et al. 2007). Before entering the endocytotic pathway, exocytotic vesicles may enter several rounds of recycling, whereby the transient exocytotic fusion pore reopens several times. Vesicles that transiently expose their lumen to the extracellular space may take up fluorescently labelled antibodies against VGLUT1. These antibodies were raised against the amino acid residues that were thought to be present only in the cytoplasmic part of VGLUT1 transporter protein, but these are likely also present in the vesicle lumen

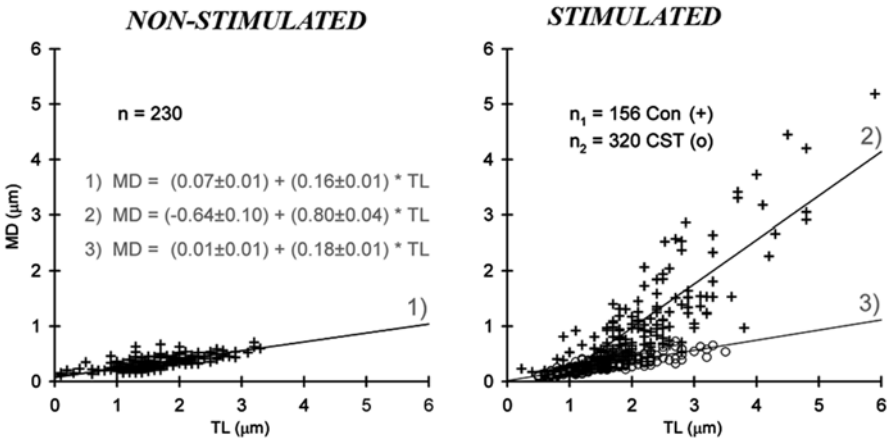

Fig. 5.4 Mobility properties of glutamatergic vesicles in astrocytes. Post-fusion mobility of retrieved VGLUT1-positive vesicles in non-stimulated and stimulated astrocytes. Glutamatergic vesicles were fluorescently labelled by exposing the astrocytes to the extracellular antibody recognizing the vesicle luminal domain of the VGLUT1 glutamate transporter. Vesicle mobility was analyzed by plotting vesicle maximal displacement (MD) as a function of the track length (TL). Numbered lines represent fits to the data (equations in *left panel* in *gray*). Stimulated cells (*right panel*) were treated with *Clostridium spiroforme* toxin (CST) to disintegrate actin filaments or left untreated (Con). Note that after the stimulation of cells by the addition of ionomycin to increase $[Ca^{2+}]_i$, increased vesicular mobility, seen as a steeper slope of the line (*line 2*). If cells were pretreated with CST, the slope of the line (*line 3*) remained unchanged in comparison with controls (*left panel*, *line 1*) (Modified from Stenovec et al. 2007)

in native vesicles, because anti-VGLUT1 antibodies label the luminal part of vesicles (Almqvist et al. 2007; Stenovec et al. 2007). At increased $[Ca^{2+}]_i$, immunolabelling was more pronounced and the directional mobility of VGLUT1 vesicles was increased (Fig. 5.4). Together with directionality, the fraction of fast-moving vesicles (>0.05 μm s^{-1}) increased at higher $[Ca^{2+}]_i$. These effects were absent in the cells preloaded with high-affinity Ca^{2+} buffer (BAPTA-AM). Microtubules, actin, and vimentin filaments likely play a role in the mobility process of VGLUT1 vesicles, because the disruption of actin attenuated their mobility (Stenovec et al. 2007). Regulation of vesicle mobility after vesicle retrieval may play several roles in synaptic plasticity (Aravanis et al. 2003), silent synapses (Gasparini et al. 2000), astrocyte-to-neuron communication (Haydon 2001; Volterra and Meldolesi 2005), and possibly more widely in cell biology in the genesis and removal of vesicles from the plasma membrane (Pelkmans and Zerial 2005). The stimulation-enhanced mobility of glutamatergic vesicles is in contrast to the stimulation-induced attenuation of mobility of peptidergic vesicles, which contain ATP in astrocytes (Pangrsic 2007) and endosomal structures, which likely plays an important role under pathologic conditions (Potokar et al. 2008, 2010, 2011; Vardjan et al. 2012).

The labelling of the vesicle lumen of glutamatergic vesicles by VGLUT1 antibodies indicates that the fusion pore dynamically opens and closes. These openings must be sufficiently wide to accommodate antibodies, which are much larger than

glutamate (Stenovec 2007). An insight into the stimulus–secretion coupling of glutamate release at the single-vesicle level comes from optical experiments where vesicle lumen was labelled by Acridine Orange (AO), which accumulates in acidic organelles. This dye co-localizes with VGLUT1-EGFP (Bezzi 2004). On stimulation of astrocytes by a metabotropic glutamate receptor agonist, AO-loaded vesicles exhibited light flashes that lasted ~500 ms, providing the time course of alkalinization of vesicles due to fusion pore openings. Subsequent to these light flashes, AO was observed to be released from single vesicles in ~100 ms (Bezzi 2004). In further experiments in which fusion pore opening at the single-vesicle level was monitored by a similar pH-sensitive detection approach, a VGLUT1-pHluorin construct was used, which revealed a time course of 200–300 ms for exocytosis (Marchaland 2008). An even slower time course of stimulation-induced fusion pore openings was recorded when the synapto-pHluorin construct, labelling synaptobrevin-laden vesicles, was used (Malarkey and Parpura 2011). The time course of these single fusion events is difficult to relate to the actual release of glutamate. The time course of fusion pore events is consistent with the whole-cell C_m measurements (Kreft et al. 2004a). The width to which the fusion pore opens in these single-vesicle measurements is not known. It is likely that the pH-dependent detection of fusion pore openings reports fusion pore diameters smaller than the glutamate molecule, because pH changes occur through a pore, which is much narrower than the molecular dimensions of glutamate. Therefore, better methods to monitor fusion pore diameter should be used in the future.

5.5.2 ATP-Loaded Vesicles

Extracellular ATP is a chemical messenger (Burnstock 1995). In astrocytes, it acts as a gliotransmitter (Parpura and Zorec 2010) and a major extracellular messenger for interastrocyte Ca^{2+}-mediated communication (Guthrie et al. 1999; Wang et al. 2000). In addition to non-vesicular modes of ATP release, such as the release of ATP from astrocytes mediated by the connexin hemichannel (Stout et al. 2002; Stehberg et al. 2012) and volume-sensitive organic osmolyte and anion channels (VSOAC) (Blum et al. 2010), Ca^{2+}-dependent exocytotic ATP release from astrocytes has also been confirmed (Parpura and Zorec 2010). ATP is loaded into vesicles by the vesicular nucleotide transporter (VNUT) (Oya et al. 2013), and was reported to be present in astrocytes (Sawada et al. 2008; Larsson et al. 2011; Oya et al. 2013). ATP-loaded astrocytic vesicles seem to be heterogeneous. In microglia, ATP is stored in non-lysosomal compartments (Imura et al. 2013). So far, the vesicular distribution of ATP in astrocytes has been shown to overlap with the marker of dense-core granules in the hippocampus, secretogranin II (Calegari et al. 1999; Coco et al. 2003) and with markers of lysosomes (Jaiswal et al. 2007; Zhang et al. 2007; Li et al. 2008; Verderio et al. 2012; Oya et al. 2013). Moreover, it seems to be co-stored together with peptides (Calegari et al. 1999; Belai and Burnstock 2000; Bodin and Burnstock 2001; Coco et al. 2003; Pangrsic et al. 2007).

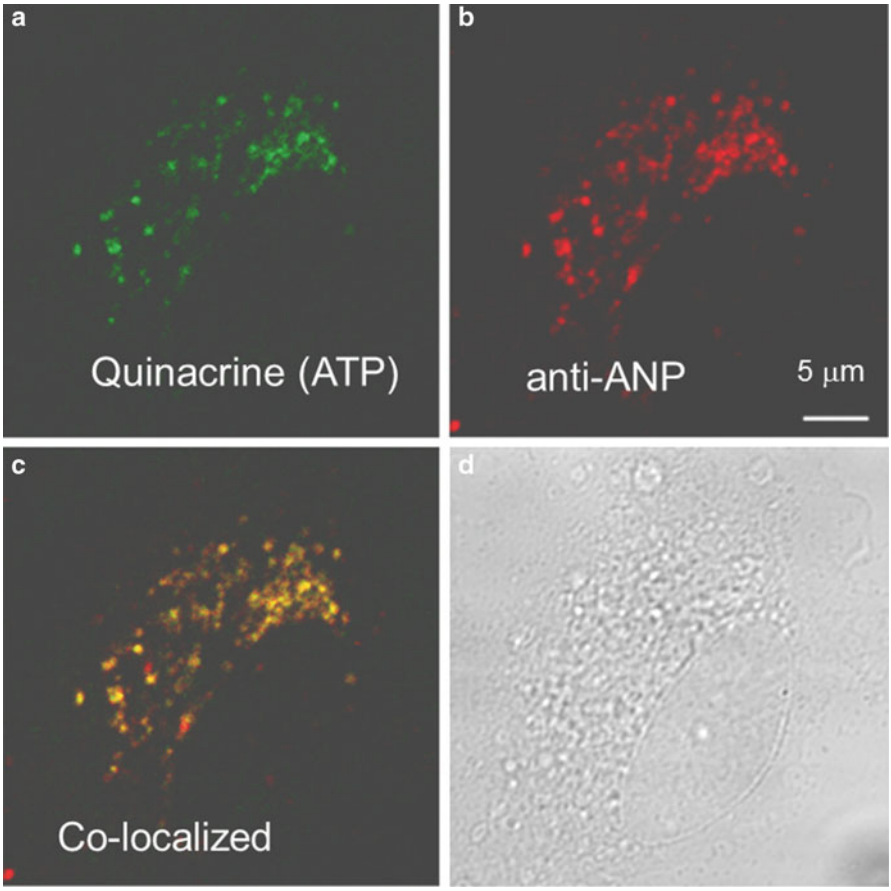

Fig. 5.5 ATP is co-stored in peptidergic vesicles of astrocytes. Rat astrocytes were labelled with quinacrine dihydrochloride (1 μM, 15 min, room temperature) and immunolabelled with antibodies against atrial natriuretic peptide (anti-ANP, 1: 1,000). Fluorescently labelled vesicles containing ATP (quinacrine dihydrochloride labelling, *green*) (**a**) and fluorescently immunolabelled vesicles containing ANP (*red*) in the same astrocyte (**b**). (**c**) Merged image shows a high degree of co-localization between both fluorescence signals, seen as *yellow*. The degree of fluorescence co-localization in this cell is 42 %. (**d**) Differential interference contrast image of the same cell. Bar, 5 μm (From Pangrsic 2007)

In neonatal cortical rat astrocytes, ATP-containing vesicles seem to substantially co-store atrial natriuretic peptide (ANP; 40 %; Fig. 5.5) (Pangrsic et al. 2007). Under spontaneous conditions, most of the ATP vesicles were located within 150 nm of the plasma membrane and this coincided with the observation that quinacrine-loaded vesicles displayed mainly nondirectional spontaneous mobility and only 4 % of vesicles were highly mobile (directional mobility). High $[Ca^{2+}]_i$ affected both types of vesicle mobility and completely abolished directional mobility. After a triggered increase in $[Ca^{2+}]_i$, less ATP vesicles were observed in the

cells, likely due to Ca^{2+}-activated discharge of the fluorescent cargo by regulated exocytosis. This effect was obstructed by the presence of the dominant-negative cytosolic tail of synaptobrevin 2, containing SNARE domain, (also used for astrocytes-specific expression in a so called dnSNARE transgenic mouse model; see below) which interferes with the formation of the SNARE complex (Zhang et al. 2004b; Pangrsic et al. 2007).

ATP is considered to be a major gliotransmitter in the propagation of calcium waves among astrocytes (Haydon 2001), and in the modulation of neuronal activity (Zhang et al. 2003; Pascual et al. 2005; Haydon and Carmignoto 2006), the exocytotic release of ATP may play a role in the delivery of this gliotransmitter to the extracellular milieu as a signaling messenger for intercellular communication in health and disease. In the dnSNARE mouse model, it was shown that ATP release is reduced and this prevents tissue damage after a stroke (Hines and Haydon 2013).

The time course of ATP release from single vesicles, studied by total internal reflection fluorescence microscopy and by monitoring the decline in intensity of quinacrine-positive vesicles, revealed an average delay between the stimulus and vesicle discharge of 3 min if stimulated by 1 mM glutamate or 5 μM ionomycin, both increasing $[Ca^{2+}]_i$ in astrocytes (Pangrsic 2007). This relatively long delay between the stimulus and secretory output, determined by monitoring single-vesicle discharge, is consistent with the whole-cell C_m measurements (Fig. 5.3b), indicating that the nature of fusion pore opening is an important barrier in determining the vesicle cargo to be released into the extracellular space.

Because ATP-laden vesicles overlap with vesicles containing peptides, such as the ANP peptide (Pangrsic et al. 2007), it can be concluded that the discharge of peptides, which have a much higher molecular weight than ATP, must be even slower. This may be associated with the fact that stimulation of astrocytes reduces the mobility of peptidergic vesicles as demonstrated in studies of ANP vesicles in the recycling pathway in rat astrocytes. Vesicle recycling has been considered for secretory granules, which are released by stimulated exocytosis (Taraska et al. 2003), but the granule remains intact, except for the loss of the contents and some of the membrane proteins. Recycling occurs when the fusion pore is rapidly resealed in the exocytotic process and the vesicle is retrieved into the cytoplasm without intermixing of membranes and without collapse of the vesicle membrane into the surface membrane (Jahn and Südhof 1999; Valtorta et al. 2001; Taraska et al. 2003). When recycling, ANP vesicles were studied, they exhibited one order of magnitude-slower mobility than secretory ANP vesicles (Potokar et al. 2013b). What is the physiological significance of these results? The mobility of vesicles retrieved from the plasma membrane after exocytotic fusion is likely related to the efficiency of vesicle cargo discharge. Although rare, swift complete peptide discharge from vesicles within 500 ms has been observed in astrocytes (Trkov et al. 2012), and if sufficient time is allowed (during attenuated vesicle mobility), the vesicle cargo can be completely discharged from the vesicle lumen more readily, especially if peptides in the vesicle are aggregated into dense matrices (detected as electron-dense material on electron microscopy); their discharge is inefficient unless the vesicles exhibit a fusion pore that permits prolonged discharge of the vesicle cargo.

ATP appears to be also localized in endolysosomes (Jaiswal et al. 2007; Zhang et al. 2007; Li et al. 2008; Verderio et al. 2012; Oya et al. 2013). The mobility properties of these organelles have been described in detail in mouse (Potokar et al. 2010; Vardjan et al. 2012) and rat astrocytes (Stenovec et al. 2011). These vesicles were labelled by LysoTracker dye and exhibited slow mobility compared with other vesicle types (Potokar et al. 2013b).

If ATP and peptides such as ANP are co-stored, a question arises whether there are mechanisms of differential release of these two chemical messengers? Perhaps more direct measurements of fusion pore properties (diameter) will resolve such questions in the future. Moreover, measurements of ATP release from a single vesicle would be instructive.

5.6 Time Course of Availability of Metabolites Associated with ATP and Glutamate

The concentration of ATP and glutamate in the cytoplasm is in the range of millimoles per liter, sufficiently high to support loading into vesicles via specific vesicle transporters, VNUT and VGLUTs, respectively (Danbolt 2001; Sawada et al. 2008; Parpura and Zorec 2010). Both ATP and glutamate are associated with the metabolism of glucose, which is linked to glycogen. The glycogen reservoir via glycogen shunt in astrocytes can provide fuel for energy production during hypoglycemia (Swanson and Choi 1993; Brown and Ransom 2007), as well as during normal brain metabolism (Fillenz et al. 1999). Incubation of astrocytes with diaminobenzidine (DAB), a glycogen phosphorylase a inhibitor (Henke and Sparks 2006), inhibits the glycogen shunt. Glycolysis and glycogenolysis seem to provide most of the energy required during an abrupt energy demand (Hertz et al. 2007). The glycogen serves as the source of lactate, which may be transferred to neurons (Wender et al. 2000) or converted into pyruvate, which enters the Krebs cycle.

Astrocytic networks can also effectively remove lactate from activated glycolytic domains, and the lactate can be dispersed throughout the syncytium to the end feet for release to blood (Gandhi et al. 2009). In addition to neurotransmission, glutamate also serves as an important potential fuel reserve (Dienel and Cruz 2006). Astrocytes respond to glutamate by enhancing both glucose use and lactate production (Magistretti and Pellerin 1999; Pellerin and Magistretti 2003). The neurotransmitter glutamate is also produced from glycogen under some conditions (Gibbs et al. 2007). On noradrenaline/adrenaline stimulation, glycogen breakdown may be stimulated in response to increased cyclic AMP (cAMP) levels, leading to an increase in cytosolic glucose. As shown in Fig. 5.6 the dynamics of the increase in noradrenaline in cAMP is more rapid than that of glucose, which arises from the breakdown of glycogen (Prebil et al. 2011; Vardjan et al. 2014). These rates may limit the loading of vesicles with gliotransmitters, ATP, and glutamate, and hence may also limit the time course of vesicular release of these two gliotransmitters (Hertz et al. 2014).

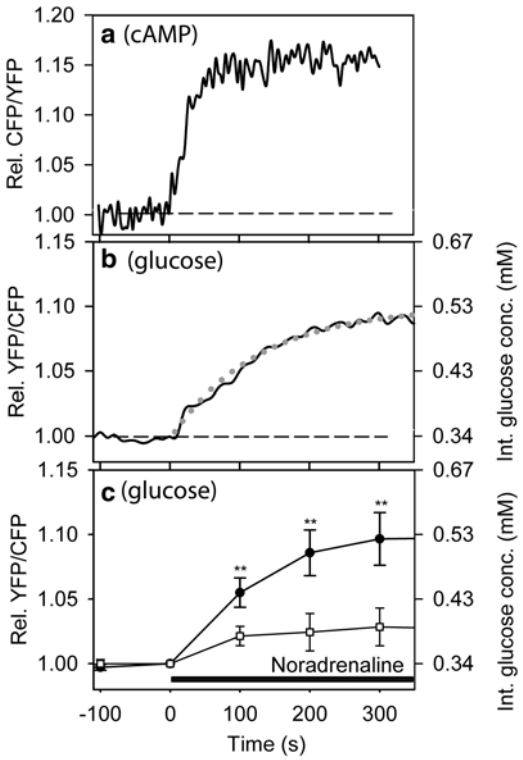

Fig. 5.6 Noradrenaline treatment increases cytosolic cAMP and glucose concentration in astrocytes. (**a**) Fluorescence resonance energy transfer (FRET) measurements of cAMP in living primary astrocytes transfected with Epac1-camps after 1 μM noradrenaline stimulation (*black bar* in (c)). (**b**) FRET measurements of intracellular glucose in astrocytes transfected with FLII12PGLU-700 μΔ6 FRET after 200 μM noradrenaline stimulation (*black bar* in *panel c*). (**c**) Mean response recorded in astrocytes in eight experiments without DAB preincubation (DAB is a glycogen phosphorylase a inhibitor, filled circles) and in 11 experiments with DAB preincubation (*empty squares*). Mean FRET ratio recorded with DAB preincubation after noradrenaline treatment was significantly lower ($P<0.05$) compared with recordings without DAB preincubation. The Student *t* test was used for paired data: $^{**}P<0.01$. The time constant for the increase in FRET ratio in glucose measurements after noradrenaline stimulation was 115.9 ± 8.2 s (*gray dotted lines* show the exponential fit to the *curve* in (**b**)). Note that the increase in cAMP was faster (Vardjan et al., 2014) than an increase in the concentration of intracellular glucose (Modified Prebil et al. 2011)

5.7 Conclusions and Perspectives

The progress in understanding astrocyte function in health and disease since the early 1990s has helped to shift the paradigm, so that these cells are no longer viewed as subservient entities to neurons, but are active partners in information processing in the brain. In this chapter, we addressed the role of vesicles, which also store

chemical messengers, and which get released into extracellular space, when vesicle membrane fuses with the plasma membrane on stimulation. This process, termed regulated exocytosis, was previously considered to be exclusively present in neurons. However, astrocytes also exhibit this process. An important distinction of this process in astrocytes is that it is extremely slow in comparison with that in neurons. This may indicate that astrocytes have signal integration roles in information processing in the brain. We focused on two gliotransmitter vesicles, containing glutamate or ATP, because these two molecules are also important metabolites in the CNS. The reason why regulated exocytosis in astrocytes is relatively slow is due to an amazingly complex regulation of vesicle traffic and by the processes determining the merger of the vesicle membrane with the plasma membrane. Furthermore, processes involved in the production/degradation of these two metabolites and their loading into vesicles may well be rate limiting as well. In the future, these processes will have to be studied by direct measurements of metabolites in the cytoplasm of astrocytes, as was done for glucose (Prebil et al. 2011). This will bring us closer to understanding how energy support is regulated in view of vesicle dynamics and the vesicular release of gliotransmitters, which will help us to understand the spatiotemporal coupling and interactions between neurons and glia.

Acknowledgements The authors' work is supported by grants from the Slovenian Research Agency (P3 310, J3 4051, J3 4146, L3 3654; J3 3236), CIPKEBIP, and COST Nanonet.

Conflict of interest The authors declare no conflict of interest.

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Chapter 6
Adenosine and Glutamate in Neuroglial Interaction: Implications for Circadian Disorders and Alcoholism

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Abstract Recent studies have demonstrated that the function of glia is not restricted to the support of neuronal function. In fact, astrocytes are essential for neuronal activity in the brain and play an important role in the regulation of complex behavior. Astrocytes actively participate in synapse formation and brain information processing by releasing and uptaking glutamate, D-serine, adenosine 5′-triphosphate (ATP), and adenosine. In the central nervous system, adenosine-mediated neuronal activity modulates the actions of other neurotransmitter systems. Adenosinergic fine-tuning of the glutamate system in particular has been shown to regulate circadian rhythmicity and sleep, as well as alcohol-related behavior and drinking. Adenosine gates both photic (light-induced) glutamatergic and nonphotic (alerting) input to the circadian

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_6

clock located in the suprachiasmatic nucleus of the hypothalamus. Astrocytic, SNARE-mediated ATP release provides the extracellular adenosine that drives homeostatic sleep. Acute ethanol increases extracellular adenosine, which mediates the ataxic and hypnotic/sedative effects of alcohol, while chronic ethanol leads to downregulated adenosine signaling that underlies insomnia, a major predictor of relapse. Studies using mice lacking the equilibrative nucleoside transporter 1 have illuminated how adenosine functions through neuroglial interactions involving glutamate uptake transporter GLT-1 [referred to as excitatory amino acid transporter 2 (EAAT2) in human] and possibly water channel aquaporin 4 to regulate ethanol sensitivity, reward-related motivational processes, and alcohol intake.

Keywords Adenosine • Glutamate • Astrocytes • Neuroglial interactions • Circadian rhythms • Sleep • Alcoholism • Alcohol

6.1 Introduction

Adenosine is an ubiquitous nucleoside which has various interrelated functions in the central nervous system (CNS) that are crucial to proper brain function (Latini and Pedata 2001; Burnstock 2006, 2008). As in all cells, central adenosine can be phosphorylated to produce the adenosine 5′-triphosphate (ATP) used as metabolic currency. Conversely, it is the product of ATP hydrolysis, and thus serves as an indicator of metabolic activity (Dunwiddie and Masino 2001). The physiological effects of adenosine are directly related to its metabolic function. For example, adenosine dilates blood vessels in the brain and periphery, thereby directing nutrients present in circulation to metabolically active cells. In addition to coupling the energy demands of neurons with blood flow necessary to maintain their activity, adenosine takes on a related role in the CNS as a modulator of neurotransmission. This role appears particularly important in the fine-tuning of excitatory glutamatergic signaling (Fig. 6.1; Ruby et al. 2010).

The extracellular adenosine concentration, normally ranging from 25 to 250 nM, is regulated to a great extent by production and transport (Burnstock 2006, 2008; Parkinson et al. 2006). This pattern of control allows adenosine levels to change rapidly, which is essential for fine-tuning the activity of neighboring neurons. Adenosine is thought to reach extracellular space primarily through two mechanisms: (1) it is produced extracellularly from ATP released by neurons or astrocytes, and (2) it is released by neurons (Lovatt et al. 2012; Wall and Dale 2013) or astrocytes (Parkinson et al. 2006; Asatryan et al. 2011) via equilibrative nucleoside transporters (ENTs). As such, both neurons and astrocytes appear to be significant sources of extracellular adenosine (Hamilton and Attwell 2010) and mounting evidence suggests that many of the known roles of adenosine in the CNS are a result of neuroglial interactions (Araque et al. 1999; Pascual et al. 2005; Halassa et al. 2009a).

Adenosine reduces neuronal excitability and regulates ion channel function through four subtypes of G protein-coupled receptors, A1, A2A, A2B, and A3, each

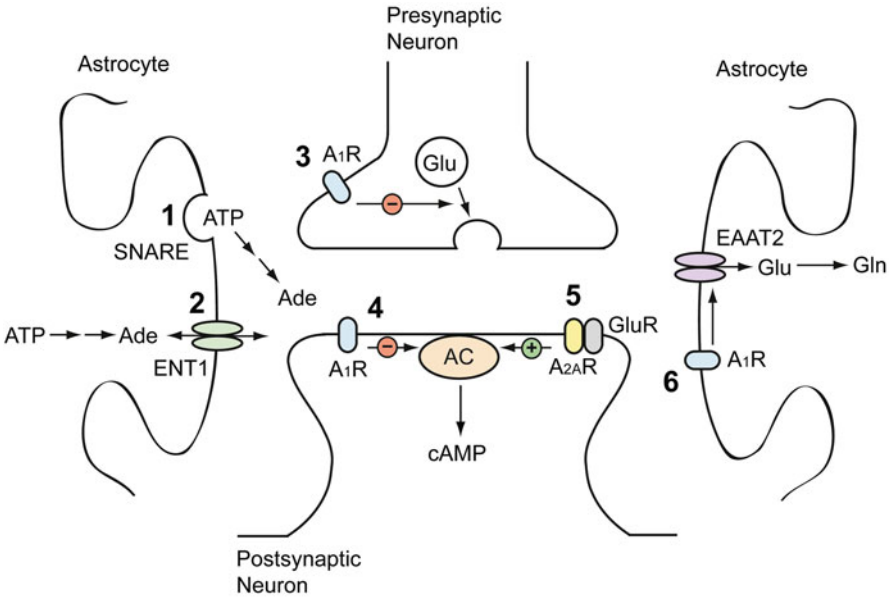

Fig. 6.1 Schematic representation of neuroglial adenosine-mediated fine-tuning of glutamatergic neurotransmission. (1) SNARE-mediated ATP release by astrocytes provides a major source of adenosine (Ade) in extracellular space. (2) Adenosine levels are also highly regulated by ethanol-sensitive equilibrative nucleoside transporter 1 (ENT1), a bidirectional nucleoside transporter. (3) Activation of presynaptic A1 receptors (A1R) inhibits glutamate release. (4) Activation of postsynaptic A1 receptors inhibits adenylate cyclase (AC) to reduce production of cyclic AMP (cAMP). (5) Activation of G_s-coupled A2A receptors stimulates the production of cAMP by AC. (6) A1 receptor activation on astrocytes regulates the expression of excitatory amino acid transporter 2 (EAAT2), which is responsible for the majority of glutamate (Glu) uptake in the central nervous system. Gln, glutamine

of which has a distinct affinity for adenosine (Fredholm 2010). Whereas the latter two subtypes have relatively low affinity for adenosine, A1 and A2A receptors have 10–100 nM binding affinity, making these the main receptors activated at physiological levels of adenosine. Adenosine A1 receptors are G_i coupled and expressed ubiquitously in the CNS, where they mediate the tonic inhibition of neuronal activity. Presynaptic A1 receptors are especially important in inhibiting the release of glutamate (Halassa et al. 2009a). On the other hand, adenosine A2A receptors are G_s coupled, increasing the level of cyclic adenosine 3′,5′ monophosphate (cAMP) production by adenylate cyclase and exerting excitatory influences on neurons. Although A2A receptors are excitatory at the cellular level, they are primarily expressed in the indirect (striatopallidal) circuit, resulting in the inhibition of motor activity (Aoyama et al. 2000). A2A receptors are also known to associate physically with other neurotransmitter receptors, including the adenosine A1, dopamine D2, and glutamate mGluR5 receptors (Ciruela et al. 2006; Ferre et al. 2010).

Adenosine has been shown to regulate several complex behaviors in both health and disease with well-established roles in homeostatic sleep and alcohol use

disorders (Cunha et al. 2008; Ruby et al. 2010; Asatryan et al. 2011; Nam et al. 2012). While adenosine itself may mediate sleep, adenosinergic fine-tuning of glutamate signaling appears responsible for its modulation of circadian timing (discussed below) and to a large extent, alcohol-related behavior and consumption. Dysregulated modulation of glutamatergic transmission by adenosine in the striatum has been strongly implicated in mediating the effects of and abuse potential for alcohol (Choi et al. 2004; Chen et al. 2010; Nam et al. 2010). Recent studies elucidating neuroglial mechanisms of adenosine signaling may lead to new therapeutic opportunities for the treatment of sleep, circadian, and alcohol use disorders.

6.2 Adenosinergic Modulation of Circadian Rhythms

Despite the well-established role for adenosine in sleep, surprisingly little is known about how adenosine interacts with the circadian system. Mammalian circadian rhythms in physiology and behavior are regulated primarily by the suprachiasmatic nucleus (SCN) of the hypothalamus. At the cellular level, circadian timing is mediated by expression of clock genes. Circadian rhythms are entrained to the external environment by two main inputs to the SCN: photic (light) input, and nonphotic input (typically alerting stimuli, such as novel environments, sleep deprivation, and exercise). Photic stimulation of the SCN arrives via a direct, glutamatergic projection from the retina (Pickard 1982; Moore 1983). Activation of adenosine A1 receptors located presynaptically on retinohypothalamic axon terminals attenuates photic phase-resetting of the SCN clock by inhibiting glutamate release (Hallworth et al. 2002; Sigworth and Rea 2003). Ethanol, which acutely increases the extracellular adenosine level by blocking adenosine transporter ENT1 (Nagy et al. 1990), also attenuates photic phase-resetting (Seggio et al. 2007; Ruby et al. 2009a, b) and shortens free-running circadian period (Seggio et al. 2009). Whether or not adenosine plays a role in these effects has yet to be determined, as ethanol is also well known to inhibit the glutamate *N*-methyl-D-aspartate (NMDA) receptor, whose activation is responsible for photic phase-shifts (Abe et al. 1991; Colwell et al. 1991; Ding et al. 1994; Mintz and Albers 1997; Mintz et al. 1999).

Adenosine has also been shown to modulate nonphotic phase-resetting of circadian behavioral rhythms. Antle et al. (2001) demonstrated that A1 receptor agonist N-CHA induced dose-dependent phase-shifts when administered during midday, mimicking the effect of a three-hour sleep deprivation procedure. In contrast, adenosine A1/A2A receptor antagonist caffeine, while not producing phase-shifts on its own, dose-dependently reduces sleep deprivation-induced phase-resetting in hamsters (Antle et al. 2001). Caffeine consumption also lengthens free-running circadian period in mice (Oike et al. 2011), although the mechanism by which this occurs is unknown. It is reasonable to speculate that delaying sleep phase may contribute to caffeine-induced period lengthening.

Interestingly, the relationship between circadian timing and adenosine signaling may be bidirectional. Adenosine has long been known to contribute to homeostatic

sleep regulation (discussed later in this section), so it is not entirely surprising that levels of extracellular adenosine vary in a circadian manner (Murillo-Rodriguez et al. 2004). Adenosine, as a product of ATP hydrolysis, accumulates in perisynaptic space in an activity-dependent manner (Pascual et al. 2005). Thus, a daily rise in central adenosine concentration is thought to reflect the homeostatic need for sleep. However, evidence for circadian variation in the expression of A1 receptors and ENT1 in wake-promoting areas of the brain (Virus et al. 1984; Alanko et al. 2003; Murillo-Rodriguez et al. 2004) may indicate that they are targets of cellular circadian timekeeping processes. Likewise, astrocytic release of ATP in the SCN, and presumably adenosine produced from its metabolism, follows a circadian pattern (Marpegan et al. 2011). The notion that a feedback loop may exist between the circadian and adenosinergic system merits future exploration.

6.3 Regulation of Homeostatic Sleep through Purinergic Mechanisms in Astrocytes

The role of astrocytes as mediators of synaptic activity is becoming increasingly recognized as an important contributor to both normal and pathological behavior (Araque et al. 1999; Halassa and Haydon 2010; Allaman et al. 2011). Moreover, it is clear that adenosinergic modulation of glutamate signaling is an essential mechanism by which astrocytes communicate with neurons. As members of the tripartite synapse, astrocytes are ideally situated to monitor and regulate synaptic transmission via release of chemical transmitters, including glutamate, D-serine, and ATP, the latter of which is rapidly hydrolyzed to adenosine in extracellular space (Nam et al. 2012). Much of our current understanding of this process in vivo, termed gliotransmission, can be credited to the generation of an inducible transgenic mouse line in which **s**oluble **N**-ethyl maleimide-sensitive fusion protein attachment protein receptor (SNARE)-mediated transmitter release is attenuated specifically in astrocytes (Pascual et al. 2005). Studies using dominant negative (dn)SNARE mice have revealed that astrocytes provide a tonic source of adenosine that acts on perisynaptic A1 receptors to modulate both basal and plastic glutamatergic synaptic activity (Pascual et al. 2005). More recent evidence points to a role for gliotransmission in postsynaptic A1 receptor-dependent reductions in the surface expression of glutamate NMDA receptor subunits GluN2A and GluN2B (Fellin et al. 2009; Deng et al. 2011). Furthermore, astrocyte-derived D-serine may be indirectly involved in purinergic mechanisms of synaptic modulation by decreasing surface expression of postsynaptic NMDA receptors (Fellin et al. 2012).

Adenosine has been identified as the factor that accumulates with wakefulness and promotes sleep (Porkka-Heiskanen et al. 1997). Specifically, the accumulation of adenosine activates A1 receptors to inhibit the wake-promoting neurons of the basal forebrain (BF; Radulovacki et al. 1984; Porkka-Heiskanen et al. 1997; Thakkar et al. 2003; Basheer et al. 2004), driving both sleep pressure and intensity as a function of prior time awake (Brown et al. 2012; Schmitt et al. 2012). The induction of

sleepiness and decreased alertness is mimicked by systemic or central administration of adenosine or A1 receptor agonists (Basheer et al. 2004; Blutstein and Haydon 2013; Frank 2013). It is noteworthy that dnSNARE mice exhibit a markedly attenuated response to sleep deprivation (Halassa et al. 2009b). During sleep deprivation, both extracellular adenosine and A1 receptor expression increase (Basheer et al. 2004; Brown et al. 2012) to further promote drowsiness and initiate compensatory rebound sleep. dnSNARE mice show reductions in the power of low frequency slow-wave sleep (0.5–1.5 Hz), a well-established index of sleep pressure directly related to the enhancement of adenosine tone that accompanies sleep deprivation as well as total recovery sleep time. Moreover, these effects are mimicked in wild-type mice with systemic administration of the A1 receptor antagonist cyclopentyltheophylline (Halassa et al. 2009b), suggesting that purinergic gliotransmission regulates sleep homeostasis. Interestingly, as dnSNARE mice do not exhibit an abnormal baseline sleep phenotype, it is possible that release of adenosine via ENTs, particularly ENT1, may be important for basal sleep pressure and that gliotransmission plays a greater role in recovery sleep after sleep deprivation.

Astrocytes also play a role in the promotion of wakefulness by clearing adenosine during sleep. Adenosine clearance is mainly mediated by the combined actions of ENTs facilitating the inward flow of adenosine and astrocytic adenosine kinase (AK), which phosphorylates intracellular adenosine to AMP. Reduction of AK within astrocytes causes an increase in inhibitory transmission through A1 receptor activation, while overexpression of AK results in the opposite effect (Diogenes et al. 2012), accelerating adenosine clearance and diminishing recovery sleep after sleep deprivation (Dias et al. 2013). The second pathway for adenosine clearance is extracellular breakdown to inosine by adenosine deaminase (AD). Bachmann et al. (2012) found that a polymorphism of AD that reduces the conversion of adenosine to inosine leads to significant increases in sleep pressure, waking alpha wave activity, and fatigue, while decreasing attention and vigor (Bachmann et al. 2012). Although much remains to be discovered regarding the roles of various adenosine signaling molecules, it is a clear that neuron-glial mechanisms in astrocytes are indispensable in maintaining sleep homeostasis. As ATP release by astrocytes appears to be regulated by cellular circadian timing, it is tempting to hypothesize that astrocytes may serve as an interface between the circadian and homeostatic sleep systems.

6.4 Low Adenosine Tone in Alcohol Withdrawal-Induced Insomnia

Alcohol and alcohol withdrawal have profound impacts on sleep, which has been extensively documented in the clinical literature (Colrain et al. 2009). The estimated cost of alcohol-related sleep disorders in the United States exceeds $18 billion per year (Brower et al. 2001). Acute ethanol intake in nonalcoholics decreases sleep latency (the amount of time to fall asleep) and increases non-rapid eye movement (NREM) sleep quantity and quality. In contrast, REM sleep is suppressed during the

first half of nocturnal sleep time and is followed by a "REM rebound" (increased REM sleep) during the second half. During alcohol withdrawal, recovering alcohol-dependent patients commonly experience severe and protracted sleep disruptions manifested as insomnia, reduced slow-wave sleep, and increased REM sleep along with excessive daytime drowsiness (Allen et al. 1971; Brower et al. 1998; Ehlers and Slawecki 2000; Roehrs and Roth 2001; Kubota et al. 2002; Veatch 2006; Mukherjee et al. 2008; Colrain et al. 2009; Mukherjee and Simasko 2009). These sleep impairments are so severe that they serve as a primary indicator of relapse (Brower and Perron 2010). Consistent with clinical studies, basic research also suggests that ethanol withdrawal is accompanied by insomnia-like symptoms, including increased wakefulness, reductions in total sleep time, and delta activity (Mendelson et al. 1978; Ehlers and Slawecki 2000; Kubota et al. 2002; Veatch 2006; Mukherjee et al. 2008; Mukherjee and Simasko 2009).

Recent studies indicate that the sleep-inducing effects of acute ethanol exposure may be mediated by adenosinergic mechanisms in the wake-promoting BF (Thakkar et al. 2010). Acute ethanol inhibits adenosine transporter ENT1 in astrocytes, leading to increased extracellular adenosine (Nagy et al. 1990), which may mediate ethanol-induced sedation. Likewise, alterations in adenosine signaling contribute to sleep disruptions during early withdrawal (Sharma et al. 2010). Ethanol-dependent rats displayed profound insomnia-like symptoms, manifested as markedly increased wakefulness coupled with significant reductions in both NREM and REM sleep. This sleep disruption is accompanied by greater numbers of c-Fos immunoreactive, wake-promoting cholinergic neurons in the BF (Thakkar et al. 2003). Ethanol-dependent rats also lack the normal rise in extracellular adenosine in the BF during sleep deprivation, and have lower BF expression of A1 receptors and ENT1 (Sharma et al. 2010). Consistent with these results, mice lacking ENT1 display a reduced central adenosine concentration (Nam et al. 2011), lower sensitivity to acute ethanol, and higher alcohol consumption compared to wild-types (Choi et al. 2004). Together, these studies support the idea that diminished adenosine tone in the BF resulting from decreased expression of ENT1 and A1 receptors may underlie insomnia during early withdrawal from alcohol. Given that astrocytes appear to be the main source of adenosine in recovery sleep, it is of great interest to determine whether gliotransmission may be compromised in alcohol-induced sleep disruption. It will also be important to characterize the impact of ethanol-induced dysregulation of circadian timekeeping processes on sleep homeostasis.

6.5 Regulation of Alcohol Drinking by Adenosine and Glutamate in Neuroglial Interaction

Similar to its role in other brain regions, one of the main functions of adenosine in the nucleus accumbens (NAc) is to inhibit glutamate release via activation of presynaptic A1 receptors (Harvey and Lacey 1997). As such, increased inhibition of glutamate release via the rise in adenosine during acute ethanol exposure partially

accounts for the intoxicating effects of ethanol (Dunwiddie and Masino 2001). Mice lacking ENT1 exhibit reduced ataxia and hypnosis in response to acute ethanol exposure and consume more ethanol than their wild-type littermates (Choi et al. 2004). Conversely, ENT1 overexpression in neurons increases ethanol intoxication in mice (Parkinson et al. 2009). Other recent studies lend further support to the inverse correlation of ENT1 gene expression and ethanol drinking (Short et al. 2006; Sharma et al. 2010). Several genetic variants of ENT1 (SLC29A1) are included among a 130 candidate gene-based array for clinical genomic studies of addiction (Hodgkinson et al. 2008). In addition, variants of ENT1 are associated with alcohol abuse and poor sleep in women (Gass et al. 2010) as well as alcohol dependency with a history of withdrawal seizures (Kim et al. 2011).

One of the key neural mechanisms underlying the ethanol-dependent phenotype of ENT1 null mice is attributed to altered glutamate neurotransmission in the NAc (Choi et al. 2004), which is highly implicated in driving addictive behavior (Kalivas 2009). Deletion of ENT1 appears to result in high extracellular glutamate level via two distinct mechanisms, both involving reduced extracellular adenosine concentration (Choi et al. 2004; Nam et al. 2011). First, the lower level of adenosine leads to reduced presynaptic A1 receptor-mediated inhibition of glutamate release (Choi et al. 2004). Second, decreased A1 receptor activation on astrocytes appears to cause decreased expression of astrocytic glutamate uptake transporter GLT-1/EAAT2 (Wu et al. 2010), reducing the amount of glutamate uptake. Resistance to acute ethanol intoxication is related to the increased glutamate signaling in ENT1 null mice (Chen et al. 2010). Consistent with this evidence, daily treatment with either glutamate NMDA receptor antagonist CGP37849 (Nam et al. 2011), antiglutamatergic medication acamprosate (Lee et al. 2011) or EAAT2 upregulator, ceftriaxone (Lee et al. 2013) was effective in reducing ethanol consumption and preference in ENT1 null mice. Thus, ENT1 null mice are considered a robust model of dysregulated adenosinergic modulation of glutamate transmission in alcohol dependence.

Recent studies continue to reveal the complex molecular and behavioral consequences of ENT1 deletion that influence alcohol drinking. Utilization of a proteomic technique called iTRAQ (Bantscheff et al. 2008; Han et al. 2008) combined with Western blot analysis and CRElacZ/ENT1 null double-mutant mice revealed that reductions in protein kinase C (PKC)γ-driven activation of neurogranin and Ca^{2+}/calmodulin-dependent protein kinase II leads to decreased cAMP response element-binding protein (CREB) activity in the NAc core, but not NAc shell region of ENT1 null mice (Nam et al. 2011). CREB is a well-established target of many drugs of abuse (Moonat et al. 2010) thought to be responsible for the synaptic plasticity-related changes occurring in addiction. The NAc core region regulates the motivational effects of conditioned stimuli (Everitt and Robbins 2005), suggesting that reduced CREB activity observed in ENT1 null mice might contribute to their deficient ethanol-induced conditioned place aversion (Chen et al. 2010) and alcohol overconsumption. More recently, reduced protein kinase A-mediated signaling through the A2A receptor has also been shown to contribute to alcohol drinking in ENT1 null mice by enhancing reward-related goal-directed behavior and the transition to habitual responding in operant paradigms (Nam et al. 2013).

6.6 EAAT2 as a Novel Target for the Treatment of Alcohol Use Disorders

Many lines of evidence support the notion that several key aspects of neuroplasticity associated with alcohol and drug addiction involve changes in glutamatergic neurotransmission. Increased brain extracellular glutamate levels and upregulated NMDA receptor expression have been demonstrated to be important in alcohol dependence in many studies. It is noteworthy that extracellular glutamate levels are increased in brain reward regions during ethanol intake (Moghaddam and Bolinao 1994; Selim and Bradberry 1996; Quertemont et al. 1998; Dahchour and De Witte 2000; Roberto et al. 2004; Melendez et al. 2005; Szumlinski et al. 2007; Kapasova and Szumlinski 2008). Of particular interest to the present discussion is the literature implicating neuroglial mechanisms in contributing to this hyperglutamatergic brain state. Ethanol exposure in cultured astrocytes increases glutamate uptake without affecting expression of glutamate transporters (Smith 1997; Othman et al. 2002). In vivo, however, the opposite effect is seen. As little as one week of moderate dose ethanol exposure induces a decrease in glutamate uptake in the rat NAc (Melendez et al. 2005), leading to an increase in extracellular glutamate. Similarly, twenty months of chronic ethanol self-administration downregulates glutamate transport cerebral cortex in ethanol-preferring (P) rats relative to ethanol-naïve P rats (Schreiber and Freund 2000). The differences between the in vitro and in vivo studies may reflect the differences in ethanol administration, which was continuous in the cultured cells but intermittent in the in vivo studies, the latter being more representative of clinical findings supporting hyperglutamate levels in alcoholism.

Extracellular glutamate is regulated by a number of glutamate transporters in neurons and glia (Gegelashvili and Schousboe 1997; Seal and Amara 1999; Anderson and Swanson 2000). Of these, sodium-dependent excitatory amino acid transporter 2 (EAAT2), expressed primarily by astrocytes (Rothstein et al. 1994; Anderson and Swanson 2000), is responsible for the removal of the great majority (~90 %) of extracellular glutamate from the synapse (Ginsberg et al. 1995; Rothstein et al. 1995; Danbolt 2001; Mitani and Tanaka 2003). Accordingly, several studies support activation of EAAT2 expression and/or function as a novel method to normalize hyperglutamatergic transmission. The murine EAAT2 (SLC1A2) gene is located in a central part of chromosome 2 (E2) (Kirschner et al. 1994), near quantitative trait loci that modulate neuroexcitability and seizure frequency in mouse models of alcohol withdrawal and epilepsy (Crabbe and Belknap 1993). EAAT2 is also implicated in preclinical models of morphine, methamphetamine, and cocaine addiction (Sari et al. 2009; Abulseoud et al. 2012). Moreover, it is noteworthy that genetic polymorphisms of EAAT2 have been linked to alcoholism in humans (Sander et al. 2000).

Interestingly, the β-lactam antibiotic ceftriaxone, which is known to cross the blood–brain barrier (Chandrasekar et al. 1984; Spector 1987; Nau et al. 1993; Lutsar and Friedland 2000) has been identified as a positive regulator of EAAT2 expression (Rothstein et al. 2005; Miller et al. 2008; Sari et al. 2009, 2010, 2011). As a hyperglutamatergic state has been repeatedly linked with alcohol dependence in clinical and

basic research, a role for ceftriaxone in reducing ethanol consumption through the activation of glutamate uptake has been investigated in several studies. One such study using alcohol-preferring P rats demonstrated a robust, dose-dependent, and relatively long-lasting suppression of ethanol intake by daily treatment with ceftriaxone for 5 days (Sari et al. 2011). This behavioral effect correlated with the upregulation of EAAT2 expression in the prefrontal cortex and NAc (Sari et al. 2011). In another recent study, ethanol intake was reduced by ceftriaxone treatment in ENT1 null mice, who have baseline deficits in EAAT2 expression (discussed above; Lee et al. 2013).

Also fascinating is the finding by Lee et al. (2013) that ceftriaxone treatment elevated not only EAAT2, but also the water channel aquaporin 4 (AQP4) in ENT1 null mice. AQP4 and EAAT2 are known to form macromolecular complexes (Hinson et al. 2008) and glutamate regulates AQP4 water permeability through group I metabotropic glutamate receptors (Gunnarson et al. 2008). Disruption in AQP4 expression and/or function has been implicated in brain injury resulting from acute and binge ethanol exposure (Sripathirathan et al. 2009; Katada et al. 2012) as well as other neurological disorders including ischemia (Taniguchi et al. 2000), edema (Vizuete et al. 1999), and neuromyelitis optica (Hinson et al. 2008), all of which involve disrupted brain water homeostasis and neuronal damage. A number of reports have suggested that expression of EAAT2 and AQP4 is regulated by PKC isoforms. PKC activators phorbol myristate acetate, 12-*O*-Tetradecanoylphorbol-13-acetate, and phorbol dibutyrate, which decrease cell surface expression and transporting activity of EAAT2 (Kalandadze et al. 2002; Gonzalez-Gonzalez et al. 2008) also downregulate AQP4 expression and reduce water permeability (Yamamoto et al. 2001). Interestingly, ENT1 null mice show reduced NMDA receptor-dependent PKCγ activity in the NAc (Nam et al. 2011), implying that reduced striatal EAAT2 and AQP4 expression in ENT1 null mice (Lee et al. 2013) may involve altered PKC signaling. Whether the mechanism by which ceftriaxone upregulates EAAT2 and AQP4 involves PKC signaling remains to be discovered. Likewise, a possible role for AQP4 in alcohol intake is not yet known. However, it is clear that enhancement of astrocytic function, particularly glutamate uptake by EAAT2, represents a promising therapeutic target for the treatment of alcohol dependence.

6.7 Conclusions

It is apparent that the multi-faceted modulatory effects of adenosine on glutamate neurotransmission are essential in the maintenance of circadian rhythms, sleep, and the regulation of alcohol intake. While the actions of adenosine on glutamate signaling culminate in neuronal regulation, astrocytes also play a fundamental role through the release of the gliotransmitter ATP and its by-product, adenosine, and via uptake of glutamate. As an endogenous sleep-promoting agent that may be regulated in a circadian as well as activity-dependent manner, adenosine may represent a bridge between the circadian and homeostatic sleep systems. Moreover, the implication of adenosine signaling in the soporific effect of acute ethanol and the

relapse-inducing insomnia of alcohol dependence attests to the utility of targeting adenosine signaling for the development of therapies for alcohol use disorders. Furthermore, the importance of adenosine signaling to astrocyte functions and alcohol intake is well-supported by studies in mice lacking adenosine transporter ENT1. Although much remains to be elucidated regarding the roles of EAAT2 and AQP4 in alcohol-related behavior, compromised astrocytic function may be a key contributor to alcohol use disorders, and the enhancement of astrocytic glutamate uptake by ceftriaxone appears to be central to its antidipsotropic effect.

Acknowledgement This project was funded by the Samuel Johnson Foundation for Genomics of Addiction Program at Mayo Clinic, by National Institutes of Health grant AA018779, and by the College of Natural Sciences and Mathematics, Indiana University of Pennsylvania.

Conflict of Interest The authors declare no financial interests or potential conflicts of interest.

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Chapter 7
Purinergic Receptor Stimulation Decreases Ischemic Brain Damage by Energizing Astrocyte Mitochondria

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Abstract As a leading cause of death in the world, cerebral ischemic stroke has limited treatment options. The lack of glucose and oxygen after stroke is particularly harmful in the brain because neuronal metabolism accounts for significantly more energy consumption per gram of body weight compared to other organs. Our laboratory has identified mitochondrial metabolism of astrocytes to be a key target for pharmacologic intervention, not only because astrocytes play a central role in regulating brain metabolism, but also because they are essential for neuronal health and support. Here we review current literature pertaining to the pathobiology of stroke, along with the role of astrocytes and metabolism in stroke. We also discuss our research, which has revealed that pharmacologic stimulation of metabotropic $P2Y_1$ receptor signaling in astrocytes can increase mitochondrial energy production and also reduce damage after stroke.

Keywords Astrocytes • Astroglia • ATP • ER Ca^{2+} release • Glucose • Ischemia • In vivo • Lactate • Metabolism • Microscopy • Neurons • Purinergic receptors • $P2Y_1R$ • Stroke • Photothrombosis • 2MeSADP • Mitochondrial membrane potential • Mitochondrial metabolism

Abbreviations

2MeSADP	2 Methylthioadenosine triphosphate
6-NBDG	6-Deoxy-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminoglucose
ANLS	Astrocyte-neuron lactate shuttle
ANT	Adenine nucleotide transporter

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_7

ApN	Aminopeptidase N
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CBF	Cerebral blood flow
DALY	Disability-adjusted life-years
ER	Endoplasmic reticulum
FAO	Fatty acid oxidation
GCL	Glutamate cysteine ligase
GFAP	Glial fibrillary acidic protein
GLUT	Glucose transporter
GPCR	G protein-coupled receptor
GS	Glutathione synthetase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione *S*-transferase
IP3	Inositol triphosphate
MCAO	Middle cerebral artery occlusion
MCT	Monocarboxylic acid transporter isoform
MICU	Mitochondrial EF hand Ca^{2+} uptake porter
MRP1	Multidrug resistance protein 1
NMDA	*N*-methyl-D-aspartate
NQO1	NAD(P)H dehydrogenase [quinone] 1
P2X	Ionotropic purinergic receptor
P2Y	Metabotropic purinergic receptor
PFKB3	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PIP2	Phosphatidylinositol 4,5-bisphosphate
PPP	Pentose phosphate pathway
RB	Rose Bengal
ROS	Reactive oxidative species
RSD	Recurrent spreading depolarizations
TCA	Tricarboxylic acid
Thy1	Tyrosine hydroxylase 1
TMRM	Tetra-methyl rhodamine methyl ester
tPA	Recombinant tissue plasminogen activator
VDCC	Voltage-dependent calcium channels

7.1 Introduction

Cerebral stroke is the second leading cause of mortality globally, estimated to have claimed approximately 5.7 million lives in 2005 and projected to cause between 7.7 and 8.4 million deaths annually by 2030. Stroke is predicted to be the fourth leading cause of morbidity by 2030 as determined by disability-adjusted life-years, or "DALYs"—an estimate of disease burden that reflects healthy years lost due to

premature death and chronic disability (Lopez et al. 2006). Globally, stroke's disease burden in 2005 was 50.8 million lost DALYs, and this is expected to increase to 60.9 million DALYs by 2030 (Lopez et al. 2006; Strong et al. 2007; Donnan et al. 2008).

Currently, few treatments for stroke exist, none of which target mechanisms of neuroprotection inside cells. As we discuss below, cell metabolism is primarily affected during ischemia because of a lack of glucose and oxygen perfusing within the tissue. Our laboratory has identified cell metabolism as an excellent candidate for stroke therapy, and our research over time has strengthened the rationale behind this initial approach. In particular, we have chosen to target astrocytes within the brain. Whereas neurons are responsible for the basic functioning of the nervous system, direct targeting of neurons for stroke therapies has yet to prove particularly efficacious. Astrocytes, on the other hand, are glial cells that play a significant role in supporting the health, metabolism, and function of neurons. We have discovered that acute stimulation of astrocyte mitochondrial metabolism via metabotropic purinergic stimulation can be an excellent strategy to reduce brain damage after stroke.

In this chapter, we begin by discussing stroke, with particular focus on the pathobiology. We subsequently discuss astrocytes, their role in brain metabolism, and their apparent role in stroke. We include an explanation of the $P2Y_1R$ purinergic pathway that we have targeted for stroke therapy. Finally, we review our findings showing $P2Y_1R$ stimulation increases astrocyte mitochondrial metabolism and reduces brain damage after stroke.

7.2 The Global Impact of Stroke

Advanced age is the top uncontrollable risk factor for stroke. The probability of stroke doubles every 10 years beyond age 55 irrespective of gender (Wolf et al. 1991), and nearly 86 % of stroke-related fatalities occur among those aged 60 or older (approximately 4.9 million of 5.7 million total deaths, according to 2005 estimates) (Lopez et al. 2006; Strong et al. 2007). Additionally, survivorship among stroke victims of all ages is predicted to increase from 62 million in 2005 to over 77 million by 2030, forecasting a greater number of individuals at risk for post-stroke disability (Strong et al. 2007). The extreme global health burden imposed by stroke is worsened by population aging (Strong et al. 2007; Donnan et al. 2008). Worldwide, the number of individuals aged 60 years or older grew from 600 million in the year 2000 to over 700 million in 2009 (Department of Economic and Social Affairs, Population Division 2002, 2010) Recent estimates by the United Nations have placed this figure at over 809 million in 2012, and by 2050 over two billion individuals will be over age 60 (Department of Economic and Social Affairs, Population Division 2012). Strikingly, in many industrialized countries this will translate to over one third of the overall population by the mid-twenty-first century (Lutz et al. 2008).

This projected increase in global health burden from stroke demands multifaceted strategies that reduce overall mortality rates as well as long-term disability among survivors. Lifestyle changes or pharmaceutical interventions that reduce

risk factors (such as obesity, type II diabetes, thrombosis, and hypertension) are effective in preventing first-time and recurrent strokes (Strong et al. 2007; Donnan et al. 2008). However, beyond access to intensive care, the acute treatment of stroke is limited by the availability of pharmaceutical treatments and the effective time frame of their administration (usually within 3 h of stroke onset). Currently, two pharmaceutical standards of care exist for management of acute stroke: recombinant tissue plasminogen factor (tPA) and 2-acetoxybenzoic (acetylsalicylic acid or aspirin). Unfortunately, both provide limited benefit. Although it is effective in reducing mortality rates when administered within the therapeutic time frame, tPA is only used in ~5 % of ischemic stroke cases (Donnan et al. 2008). Even less effective is aspirin therapy, which is associated with patient survival in only 1 out of every 90 patients receiving the treatment (Gilligan et al. 2005). In light of these statistics, significant efforts have been taken towards identifying novel targets and therapeutics for stroke management.

7.3 The Ischemic Cascade

Eighty-five percent of all clinical stroke cases are attributed to cerebral ischemia—an acute reduction in blood flow to the brain arising from occlusion of cerebral blood vessels, generally via thrombosis or embolism (Zorowitz et al. 2004; Donnan et al. 2008). Impaired cerebral circulation initially reduces tissue oxygenation near the site of ischemic infarct itself, triggering an outward spreading of pathological processes deemed the "ischemic cascade." As described in literature, the pathophysiology of the ischemic cascade may be grossly summarized as four complementary/semi-sequential processes (Dirnagl et al. 1999; Singhal et al. 2006; Doyle et al. 2008):

1. Anoxia/hypoxia-induced energy failure
2. Ion imbalance, excitotoxicity, and peri-infarct depolarization
3. Inflammation
4. Apoptosis

Vascular occlusion in the brain that reduces blood flow by 50–75 % results in a respective 5–95 % probability of cerebral infarction due to pathologically low tissue oxygenation (Heiss et al. 2001; Ginsberg 2003; Mergenthaler et al. 2004). The pathological potential of deoxygenation becomes clear when one considers that the brain consumes nearly ten times more oxygen (20 % of total bodily demand) than might be expected by its percentage of human body mass (2 %) Acute cerebral ischemia causes a rapid reduction in ATP production via oxidative phosphorylation because of limited O_2 concentrations. Evidence from animal models suggest that during ischemia cerebral ATP concentrations can decline by up to 80 % and require several hours to return to pre-ischemic levels (Edvinsson and Krause 2002). This overall reduction in ATP reflects both decreased synthesis and continued hydrolysis

(Paschen et al. 2000; Singhal et al. 2006). Additionally, ischemia elicits the release of both adenosine and ATP from cells, further depleting intracellular contents of ATP (Jurányi et al. 1999; Melani et al. 2005). ATP depletion slows or halts cellular processes dependent upon ATP hydrolysis, perhaps most notably in the numerous P-type ATPases responsible for maintaining the vital K^+, Ca^{2+}, Na^+, and H^+ gradients necessary for the brain's complex electrochemistry and metabolic function.

The regulation of cellular ion gradients is perturbed both in vitro by hypoxia and in vivo during cerebral ischemia. At rest, the internal K^+ concentration of a cell is 50- to 100-fold higher than external K^+. This steep concentration gradient is maintained by the activity of membrane Na^+/K^+ ATPases, which hydrolyze ATP to import two K^+ ions and export three Na^+ ions. During ischemia however, membrane Na^+/K^+ ATPase activity declines dramatically, and cells become unable to maintain their ion gradients, leading first to higher extracellular K^+ (Nedergaard 1996; Strong and Dardis 2005). At the same time, influx of Na^+ ions into cells followed by Cl^- ions increases osmotic pressure, thereby swelling cells and leading to lysis if ion homeostasis is not restored (Unterberg et al. 2004; Liang et al. 2007; Rosenblum 2007). Cell swelling is most prominent in astrocytes (Kimelberg 2005), but is also readily observed in neuronal processes as dendritic beading (Hasbani et al. 1998; Swann et al. 2000; Risher et al. 2010, 2011). Secondarily, depolarization of excitatory nerve terminals activates voltage-dependent calcium channels (VDCCs), Ca^{2+} entry, and excessive release of the neurotransmitter glutamate (Sonnewald et al. 1997; Li et al. 1999; Li and Stys 2000; Strong and Dardis 2005). In turn, glutamate activated *N*-methyl-D-aspartate (NMDA) receptors/channels allow excessive Ca^{2+} entry (and so excitotoxic) under conditions when neurons are depolarized for prolonged periods. Combined with the failure of plasmalemmal Ca^{2+} ATPase-dependent Ca^{2+} efflux, these processes together can increase global intracellular Ca^{2+} to toxic levels, beyond 50 μM—effectively saturating the most, if not all, cell's Ca^{2+}-dependent enzymes and cofactors. The activity of several lipases, DNAses, and proteases contribute to the necrotic degradation of cells experiencing the most severely restricted blood flow, forming the so-called necrotic core of an ischemic infarct (Choi 1988; Kristián and Siesjö 1998; Dirnagl et al. 1999; Schlaug et al. 1999; Edvinsson and Krause 2002; Mergenthaler et al. 2004; Doyle et al. 2008) (Fig. 7.1a, b). In general, excitotoxicity and cell death subsequent to disruption of ion homeostasis damage adjacent tissue, leading to the expansion of the infarct by secondary mechanisms (Heo et al. 2005; Nedergaard and Dirnagl 2005; Woitzik et al. 2008).

Immediately outside the necrotic core is an area referred to as the "penumbra" (Fig. 7.1b). Within the penumbra, limited nutrients are available from nearby unclotted/unobstructed vessels and tissue, however, viability is at risk. A distinct set of morphological changes can be observed of cells in the penumbra. Our laboratory has employed multiphoton imaging to visualize lesions in real time (Zheng et al. 2010, 2013) (Fig. 7.1). We found that astrocytes in the stroke penumbra became swollen within 3 h of the stroke (Zheng et al. 2010) (Fig. 7.1d). Similarly, neuronal dendrites exhibit the previously reported "beading" effect (Hasbani et al. 1998; Swann et al.

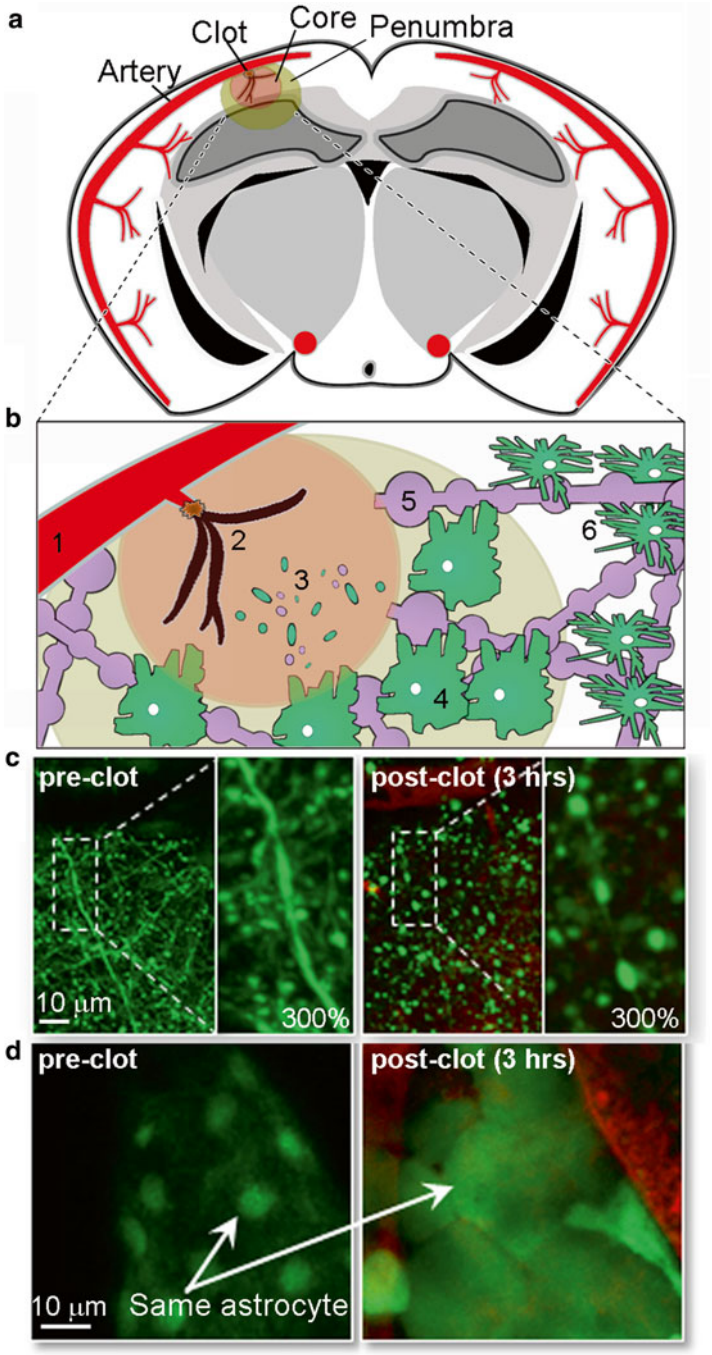

Fig. 7.1 The pathobiology of stroke. (**a**) A cartoon of a coronal section from a mouse brain with a focal lesion in the cortex. Blood can perfuse to the brain via the middle cerebral artery as well as

2000; Risher et al. 2010, 2011), in which dendrite contents become localized within discrete puncta (Zheng et al. 2013) (Fig. 7.1c). High extracellular K^+ at the peri-infarct (necrotic core) border initiates waves of recurrent spreading depolarizations (RSDs) into the surrounding penumbra (Nedergaard 1996; Strong and Dardis 2005). RSDs play a critical role in the expansion of cerebral infarcts (Mies et al. 1993; Dirnagl et al. 1999; Ohta et al. 2001; Hartings et al. 2003; Dreier et al. 2006; Dietz et al. 2009; Dreier 2011; Lauritzen et al. 2011). RSDs elevate metabolic stress in the penumbra primarily due to the increased energy demands required to recover ion homeostasis. Several studies suggest that the loss or dysfunction of astrocytes play a central role in stepwise expansion of the infarction. In particular, the astrocyte's role in maintaining extracellular K^+ levels is likely to be critical in reducing the occurrence and frequency of RSD. For example, inhibition of glial mitochondria increases astrocyte swelling and leads to necrotic cell death (Chu et al. 2007). Largo et al. reported that neurons are permanently injured by RSDs only if glial mitochondrial function fails. They also showed that glial mitochondria were required for the recovery of pathologically elevated extracellular K^+ (Largo et al. 1996a, b), which, as noted above, initiates RSDs. Astrocytes also play a major role in reducing high extracellular glutamate (Oliet et al. 2001). Glutamate is imported into astrocytes via plasma membrane Na^+-dependent transporters (Sonnewald et al. 1997; Chatton et al. 2000) (Fig. 7.2). Glutamate uptake into astrocytes is dependent on the maintenance of the Na^+ gradient via Na^+/K^+ ATPases. Loss of this gradient during ischemia severely impacts glutamate uptake and metabolism by astrocytes (Koyama et al. 1991; Bender et al. 1998). Finally, ischemia-induced astrocyte swelling further aggravates neuronal injury by release of excitatory amino acids (Kimelberg 2005; Leis et al. 2005), and astrocyte cell death may further contribute to neuronal death (Giffard and Swanson 2005). Taken together, these data suggest that pharmacological targeting of astrocytes can provide a unique approach to neuroprotection in preclinical models of stroke. In the next section, we review the physiology of astrocytes, along with their influence on the pathophysiology of ischemic stroke. Finally, we identify possible routes of intervention that promote neuroprotection via pharmacological targeting and manipulation of astroglia.

Fig. 7.1 (continued) peripheral arteries. In this example, a small penetrating artery has been occluded to generate a stroke lesion. (**b**) A cartoon of the morphological changes that are observed on the cellular level after artery occlusion: (1) Penetrating artery. (2) Occluded artery. (3) The necrotic core and debris cannot be rescued after a stroke. Unchecked, the core can spread. (4) Astrocytes become swollen due to a lack of energy needed to maintain osmotic gradients. (5) Neuronal dendrites exhibit pathological beading. (6) The beading dendrites extend past swollen astrocytes in the penumbra into areas where astrocyte morphology appears normal. (**c**) In vivo confocal microscopy of YFP expressing neuronal dendrites in a mouse before stroke (*left*) and 3 h after stroke (*right*). Note that the bright linear morphology has become punctate and unconnected after stroke. (**d**) In vivo confocal microscopy of GFP expressing astrocytes in a mouse before stroke (*left*) and 3 h after stroke (*right*). The cell body of the astrocyte is distinctly bright, and the astrocyte arborized processes can be visualized. After stroke, the arbor morphology is lost and the cell body swells. The *white arrows* point to the cell body of the same astrocyte for comparison. Images in (**c**) (Zheng et al. 2013) and (**d**) (Zheng et al. 2010) are reproduced with consent

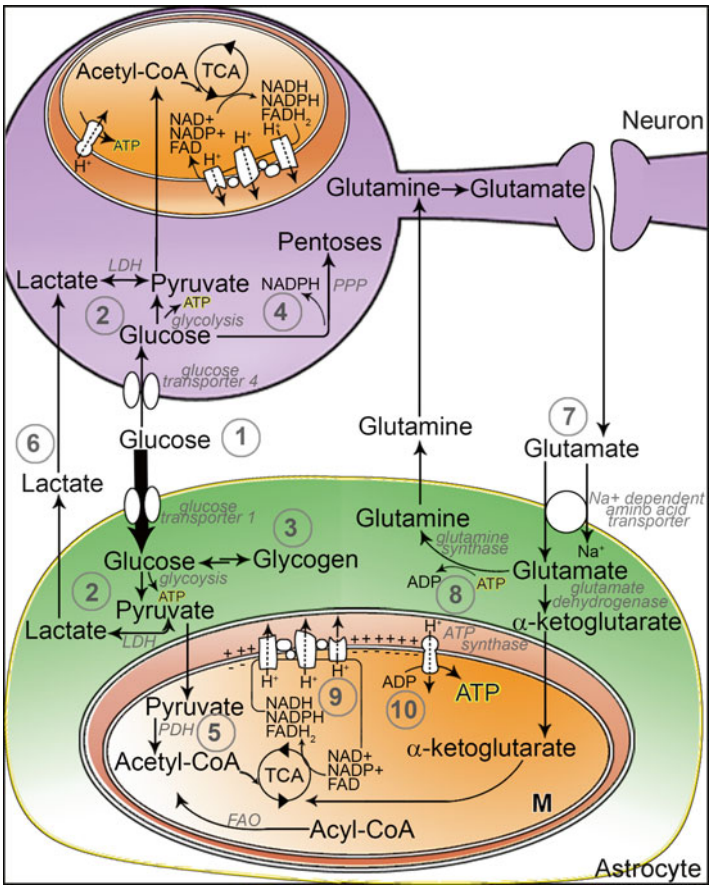

Fig. 7.2 Astrocyte and neuronal metabolic pathways. (1) Glucose is the primary source of energy in the brain. Both astrocytes and neurons transport glucose from the periphery into the cell, although astrocytes transport greater amounts of glucose. (2) Once inside, glucose is broken down into two separate pyruvate molecules during glycolysis, creating ATP and NADH. (3) Astrocytes can also convert glucose into glycogen for storage. (4) Neurons also utilize glucose in the pentose phosphate pathway to generate NADPH and pentose sugars that are important in other metabolic processes. (5) Pyruvate can enter the mitochondria and be decarboxylated into acetyl-CoA by PDH. Astrocytes also express pyruvate carboxylase (unlike neurons). This allows pyruvate to enter the TCA cycle after carboxylation into oxaloacetate via anaplerosis (not shown). (6) Alternatively, pyruvate can be converted into lactate via lactate dehydrogenase, which then is shuttled to neurons. Once in neurons, the lactate can be converted back to pyruvate, enter mitochondria, and then undergo decarboxylation and enter the TCA cycle as acetyl-CoA. (7) Glutamate that is released from neuronal synapses during excitatory neurotransmission is removed from extracellular spaces via an Na+-dependent amino acid transporter. (8) Glutamate can be converted into glutamine, consuming energy, or it can undergo dehydrogenation to make α-ketoglutarate, which can enter the TCA cycle in either astrocytes or neurons. (9) The TCA cycle is constantly replenished with cycle components, generating reducing equivalents NADH, NADPH, and FADH2. Those reducing equivalents donate electrons to the electron transport chain. The electrons enable chain components to transport protons against their concentration gradient. This sets up a mitochondrial membrane potential. Oxygen eventually accepts the protons and is converted to water. (10) ATP synthase utilizes the proton gradient as a motive force to phosphorylate ADP to create ATP. Note that the above description is a simplification. For instance, lactate can move either from astrocytes to neurons or from neurons to astrocytes. Moreover, the pentose phosphate shunt, shown only in neurons, is present in astrocytes as well. In either case the quantitative importance of this pathway relative to glycolysis is small

7.4 The Role of Astrocytes and Astrocyte Metabolism in the Brain

In the normal brain, astrocytes fulfill an array of roles necessary for maintaining an appropriate environment for neural activity. For decades, these versatile glial cells were regarded only as "brain glue" because of their inability to produce action potentials. In the 1980s, the discovery of astrocytic voltage-gated channels and neurotransmitter receptors attracted interest in astrocyte activity. Today, astrocyte involvement has been implicated in the functions of immune response, development, cerebral blood flow (CBF) regulation, neuronal signaling, and metabolism. Generally, astrocytes extend two types of processes: large, vascular processes that wrap almost entirely around intracerebral blood vessels and fine perisynaptic processes that envelope neuronal synapses (Harris et al. 1992; Nedergaard et al. 2003); not all astrocytes contact blood vessels, however. In response to neuronal activity, astrocyte processes show spontaneous morphological changes in a matter of minutes, indicating a highly plastic relationship with neurons (Hirrlinger et al. 2004).

7.5 Astrocytes Modulate Cerebral Blood Flow

To maintain a continuous supply of oxygen and glucose to the brain and prevent harmful fluctuations in arterial pressure, the CBF must be tightly controlled. These regulatory mechanisms are highly sensitive to changes in brain activity, as neural stimulation produces rapid increases in CBF to specific activated regions (Silva et al. 2000). Although the mechanisms governing this interaction are poorly understood, the close physical relationship between astrocytes and blood vessels suggests astrocytic involvement in neurovascular regulation. Astrocyte endfeet cover the majority of cerebral blood vessels (Simard et al. 2003; Iadecola and Nedergaard 2007). Electrical stimulation of afferent neuronal processes increases intracellular Ca^{2+} in astrocytic endfeet, corresponding to the dilation of cerebral arterioles (Zonta et al. 2003). Following stimulation, these neurons release glutamate, which interacts with the group I metabotropic glutamate receptors in astrocytes. These receptors are known to mediate the Ca^{2+} increase of astrocytes in response to synaptically released glutamate (Porter and McCarthy 1996), so the role these receptors play in arteriole dilation was tested using specific antagonists. Inhibiting these metabotropic glutamate receptors prevented the dilatory vascular response, demonstrating a relationship between neurotransmission and astrocyte-mediated vasodilation. Confirming this, direct activation of astrocytic glutamate receptors and direct electrical and chemical stimulation of individual astrocytes also succeeded in increasing intracellular astrocytic Ca^{2+} and recreated the arterial dilation (Zonta et al. 2003). However, in a study less than a year later, increased intracellular Ca^{2+} in astrocytes triggered vasoconstriction of arterioles via the phospholipase A2-arachidonic acid pathway (Mulligan and MacVicar 2004). Given that the same triggering event seemed to be

able to stimulate constriction or dilation in blood vessels, further investigation into a possible switching mechanism determining vessel response was required. A follow-up study revealed that altering the level of oxygen availability could differentiate between the two responses. Low oxygen supply coupled with high Ca^{2+} concentrations maximizes astrocyte glycolysis and lactate release, leading to an accumulation of prostaglandin E(2) (Chan et al. 2002) and resulting in vasodilation (Hein et al. 2006). This is complemented with the simultaneous increase in extracellular adenosine, which blocks astrocyte-induced vasoconstriction (Yamanishi et al. 2006; Gordon et al. 2008). During ischemia, the neurovascular unit dissociates and the astrocytic endfeet detach from the blood vessels, which can lead to catastrophic hemorrhagic transformation (Yamashita et al. 2009).

7.6 Astrocytes and Signaling

Although astrocytes do not generate action potentials, they do exhibit intracellular excitation in the form of elevated cytosolic Ca^{2+} levels, mainly due to mobilization of Ca^{2+} from inositol (1,4,5) tri-phosphate (IP3) ion channels at membrane of the endoplasmic reticulum (Cornell-Bell and Finkbeiner 1991; Lechleiter et al. 1991; Lechleiter and Clapham 1992; Berridge et al. 2003; Abbott et al. 2006). These Ca^{2+} elevations can spread from one astrocyte to another in waves after chemical and mechanical stimulation of IP_3 production (Cornell-Bell et al. 1990; Newman and Zahs 1997; Kuga et al. 2011). Astrocyte IP_3-mediated Ca^{2+} wave propagation can be stimulated by neurotransmitters and other chemical signals such as glutamate, GABA, acetylcholine, noradrenaline, dopamine, ATP, nitric oxide, and brain-derived neurotrophic factor (BDNF) (Khan et al. 2001; Matyash et al. 2001; Araque et al. 2002; Bowser and Khakh 2004). Glia express numerous G protein-coupled receptors (GPCRs) responsive to these transmitters including purinergic, bradykinin, histamine, cholinergic and glutamate receptors (Verkhratsky and Kettenmann 1996; Porter and McCarthy 1997; Franke et al. 2004; Colombo et al. 2012). It has been suggested that neurotransmitters interact directly with these receptors ectopically or indirectly from spill over from neuronal synapses (Matsui and Jahr 2004). Glial stimulation via non-synaptic release of neurotransmitters from axons has been demonstrated in the optic nerve, corpus callosum, and olfactory bulb, among other locations in the nervous system (Kuperman et al. 1964; Kriegler and Chiu 1993; Kukley et al. 2007; Rieger et al. 2007; Ziskin et al. 2007; Hamilton et al. 2008; Thyssen et al. 2010). Glutamate spillover from neuronal synapses has also been observed in the hippocampus and cerebellum (Kullmann and Asztely 1998; Mitchell et al. 2007; Stone et al. 2012; Coddington et al. 2013). This apparent sensitivity of astrocytes to neuronal synapses was termed the "tripartite synapse," which describes the information transmission between pre- and postsynaptic neurons and the astrocytes that interact with them (Araque et al. 1999). In addition to being excited by neuronal activity, astrocytes also actively regulate neuronal and synaptic physiology. Astrocytes themselves also release multiple signaling factors—such as glutamate,

D-serine, ATP, adenosine, GABA, tumor necrosis factor-α, prostaglandins, proteins, and peptides—through a process called gliotransmission (Perea et al. 2009). Through multiple mechanisms, gliotransmission can alter neuronal excitability, coordinate neuronal synchronization, facilitate non-synaptic neuronal communication, transiently control synaptic strength, and generate long-term potentiation (Angulo et al. 2004; Fellin and Carmignoto 2004; Fellin et al. 2004; Perea and Araque 2007; Mehta et al. 2008).

7.7 Astrocytes as Central Mediators of Brain Metabolism

As previously mentioned, the human brain consumes significantly higher amounts of energy per unit body weight than other organs, requiring about 20 % of the oxygen and 25 % of the glucose consumed by the body. Neuronal synaptic potentials, particularly in excitatory synaptic transmission, account for the vast majority of energy expenditure in gray matter. However, astrocytes take up the greatest quantities of glucose from the vasculature, more than is actually utilized within the cell for energy production. Although mathematical modeling had previously suggested that the catalytic capacity of neuronal plasmalemmal glucose transporter, GLUT3, greatly outmatched that of the astrocytic GLUT1 (Simpson et al. 2007; Mangia et al. 2009; DiNuzzo et al. 2010), research has shown that glucose intake in neurons is lower than in astrocytes (Lopes-Cardozo et al. 1986; Magistretti and Pellerin 1996; Chuquet et al. 2010). In hippocampal co-cultures of astrocytes and neurons, stimulation by glutamate actually decreases the uptake of glucose into neurons while increasing uptake in astrocytes (Porras et al. 2004). In a similar study in cerebellar rat brain slices, fluorescent glucose tracers, when applied in the extracellular space, were loaded into astrocytes at significantly higher concentrations than into Purkinje neurons (Barros et al. 2009). Likewise, whisker stimulation of immobilized rats triggered a rapid acceleration of the uptake of glucose analog 6-deoxy-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminoglucose (6-NBDG) in astrocytes, but not neurons (Chuquet et al. 2010). In support of these findings, a recent study of induced mouse GLUT3 haploinsufficiency did not diminish brain uptake of glucose and did not contribute to the development of neuropathies, indicating that GLUT3 may not be the main transporter utilized in brain glucose intake (Stuart et al. 2011). On the other hand, astrocytic GLUT1 haploinsufficiency decreased glucose uptake and resulted in severe neurological deficiencies, suggesting that after crossing the blood–brain barrier through endothelial cells, glucose is transported to the brain parenchyma by GLUT1 in astrocytes (Wang et al. 2006).

A simplified diagram of astrocyte and neuronal metabolic pathways can be found in Fig. 7.2. Once inside either neural cell-type, glucose undergoes glycolysis, wherein the 6-carbon glucose is converted via a series of ten reactions into the 3-carbon pyruvate (Fig. 7.2; 2). This pyruvate can undergo further catabolism via the pyruvate dehydrogenase (PDH) complex to form the 2-carbon acetyl-CoA. Acetyl-CoA can then enter the tricarboxylic acid (TCA) cycle (Fig. 7.2; 2–5),

in which reducing equivalents (NADH, NADPH, and FADH2) are made. As described in any biochemistry textbook, NADH, NADPH, and FADH2 donate electrons to the respiratory complexes, which, in turn, pump protons across the inner mitochondrial space and generate the membrane potential (Fig. 7.2; 2–9). This proton gradient is available for multiple processes, key of which is the oxygen-dependent production of ATP (Fig. 7.2; 10). Important specializations of neuronal and astrocytic metabolism are presented next.

Neurons display a high rate of oxidative metabolism in order to meet their significant energy needs (Martin et al. 1993; Itoh et al. 2003). However, neuronal glycolysis is impaired by the continual degradation of the important glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKB3) in a cellular mechanism that diverts glucose utilization for an alternative antioxidant pathway (described later) (Herrero-Mendez et al. 2009; Almeida 2012; Rodriguez-Rodriguez et al. 2013). The combination of lower glucose transport activity and low neuronal glycolytic activity raises the question of how a neuron meets its high-energy demands.

Unlike neurons, astrocytes demonstrate a high glycolytic rate. Rather than converting pyruvate into acetyl-CoA, a significant portion of pyruvate is converted into lactate via lactate dehydrogenase, which is then released into the extracellular space (Itoh et al. 2003; Bouzier-Sore et al. 2006) (Fig. 7.2; 2–6). This high lactate output prompted the conception of the astrocyte-neuron lactate shuttle (ANLS) model, where it was proposed that instead of utilizing glucose, neurons primarily accept lactate produced by astrocyte glycolysis, then convert it back to pyruvate to eventually use for oxidative metabolism (Pellerin and Magistretti 1994). Subsequent investigation has shown that neurons do take up lactate, and are able to use it as a substrate for oxidative phosphorylation (Schurr et al. 1997; Bouzier et al. 2000; Qu et al. 2000; Wyss et al. 2011), and more recent studies have indicated that neurons may even prefer to take up lactate rather than glucose (Bouzier-Sore et al. 2006; Wyss et al. 2011). As astrocytes account for only 5–15 % of the brain's energy expenditure and yet are responsible for about half of the brain's glucose uptake, this model perhaps reconciled the fact that astrocytes take up large amounts of glucose that far exceed their energetic requirements (Attwell et al. 2010; Chuquet et al. 2010). However, the relative importance of ANLS remains to be elucidated. Studies by Gandhi and colleagues suggest that astrocytes have higher lactate uptake activity than neurons, as much a 75 % under basal conditions. They further showed that astrocytes can shuttle glucose to neurons (Gandhi et al. 2009). Dienel has similarly pointed out that the metabolic fate of lactate has yet to be definitively proven (Dienel 2012). Moreover, others have found that glucose supports neuronal function more effectively than lactate (Dienel and Hertz 2005; Bak et al. 2006, 2012).

It's important to stress that in addition to glycolysis, astrocytes exhibit significant oxidative metabolism. In vitro, Silver and Erecinska (1997) estimated that ~76 % of ATP synthesis was due to oxidative metabolism as compared to 26 % that could be attributed to glycolysis (Silver and Erecinska 1997; Watts and Lechleiter 2008). In vivo, NMR studies have shown that metabolic fluxes through the astrocyte TCA cycle accounted for between 15 and 20 % of the total brain O_2 consumption

(Bluml et al. 2002; Lebon et al. 2002). This was directly measured by labeling the substrate acetate, which is almost exclusively metabolized by glia into acetyl-CoA (Muir et al. 1986; Badar-Goffer et al. 1990; Hassel et al. 1995; Waniewski and Martin 1998).

The inositol (1,4,5) triphosphate (IP_3) signaling pathway enables local extracellular signals to quickly cause an increase in intracellular ATP (Hajnoczky et al. 1995, 2000). IP_3 triggers Ca^{2+} release from the endoplasmic reticulum (ER), which in turn, increases mitochondrial Ca^{2+}, stimulates Ca^{2+}-sensitive dehydrogenases PDH, isocitrate dehydrogenase (ICD), and α-ketoglutarate dehydrogenase (αKGD), and consequently increases respiration and ATP production (Denton and McCormack 1985; McCormack et al. 1990; McCormack and Denton 1994; Hajnoczky et al. 1995, 2000) (see Fig. 7.5). IP_3-mediated Ca^{2+} release is stimulated by multiple GPCRs in astrocytes (Verkhratsky and Kettenmann 1996; James and Butt 2002), in particular, $P2Y_1$Rs as discussed below (Zheng et al. 2010; Watts et al. 2013).

Astrocyte energy production is not always directly linked to glucose uptake. Astrocytes are able to synthetize glycogen, a multibranched polysaccharide that serves as an energy reserve, which may provide a source of energetic substrates to buffer the damage caused by tissue injury or ischemia. Glycogen can be converted to glucose-1-P and eventually into lactate, which can then be passed on to neurons to temporarily enable oxidative phosphorylation to continue (Brown 2004) (Fig. 7.2; 3). In the case of glucose deprivation, astrocytes can also generate energy via the catabolism of fatty acids in a mechanism called fatty acid oxidation (FAO). FAO can produce significant quantities of acetyl-coenzyme A, which can be used for oxidative phosphorylation.

7.8 Astrocytes and Metabolism-Dependent Oxidant Defense

Astrocytes are known to play a crucial role in antioxidant defense, and the ability of astrocytes to synthesize antioxidants is highly dependent on their energy status. The most common mammalian thiol-containing antioxidant is glutathione (GSH). Glutathione disulfide (GSSG) is reduced to GSH by glutathione reductase in the presence of NADPH, which is maintained by the pentose phosphate shunt (Dringen et al. 2000; Dickinson and Forman 2002) (Fig. 7.2; 4). The cell's ability to maintain an appropriate NADPH/$NADP^+$ ratio ultimately affects the overall antioxidant cellular status (Dringen et al. 1999; Fernandez-Fernandez et al. 2012). Additionally, de novo synthesis of GSH by astrocytes is very important for maintenance of GSH levels. GSH synthesis itself depends on ATP-driven enzymatic activity (Suzuki and Kurata 1992; Papadopoulos et al. 1997). Glutamate cysteine ligase (GCL) is the rate limiting enzyme in GSH synthesis (Griffith and Mulcahy 1999). GCL activity increases during oxidative stress and produces γ-glutamyl cysteine (γ-glu-cys), a precursor of GSH (Ochi 1995). A second major ATP-driven enzyme is glutathione synthetase (GS), which utilizes γ-glu-cys and glycine to produce GSH. For neuronal synthesis of GSH, cysteine must be imported from other sources, both extracellular (Sagara et al. 1993, 1996; Wang and Cynader 2000) and intracellular (Bridges et al. 2012).

Extracellular cysteine levels themselves depend upon export of astrocyte GSH. GSH efflux from astrocytes is controlled, in large part, by the multidrug resistance protein type 1 (MRP1) (Dringen and Hirrlinger 2003). Once exported, GSH can be cleaved in a series of reactions: first, γ-glutamyl transpeptidase cleaved GSH to produce a glutamyl moiety and cysteinylglycine. Cysteinylglycine is then cleaved by aminopeptidase N (ApN) to produce cysteine (Wang and Cynader 2000). Intracellular cysteine is moved between cells via the System Xc-cystine/glutamate antiporter. Cystine is then cleaved into two molecules of cysteine, which then enter the GSH synthesis pathway (Bridges et al. 2012).

Astrocytes also express antioxidant scavengers and enzymes at a much higher concentration than neurons (Raps et al. 1989; Makar et al. 1994; Dringen et al. 2000). Under resting conditions, astrocytes express a large number of antioxidant enzymes, such as NAD(P)H dehydrogenase [quinone] 1 (NQO1) and glutathione *S*-transferase (GST) (Makar et al. 1994); in addition to GSH, they also contain other antioxidant compounds, e.g., vitamins C and E. Despite the fact that astrocytes are more capable of resisting oxidant stress, neurons are particularly susceptible to damage caused by oxidants such as reactive oxidative species (ROS). This may be due to their high-energy demands, as significant ROS can be generated during metabolism. Unchecked, ROS generation can lead to the disruption of the mitochondrial respiratory chain and cell death.

As previously stated, the $NADPH/NADP^+$ ratio affects the antioxidant status in the cell. In neurons, the $NADPH/NADP^+$ ratio is controlled by the pentose phosphate pathway (PPP), which oxidizes glucose to generate NADPH and pentoses (Bolanos and Almeida 2010; Hirrlinger and Dringen 2010) (Fig. 7.2; 4). Because the neuronal PPP and glycolytic pathway compete for glucose utilization, the activity of one causes a decrease in the activity of the other. As previously mentioned, in neurons the glycolytic pathways is significantly lower than in astrocytes (Herrero-Mendez et al. 2009; Bolanos et al. 2010); therefore, a significant competition with the PPP could only further reduce the glycolytic activities of neurons. Because the PPP cannot provide the neurons with energy, it is likely that astrocytes provide substrates other than glucose (possibly lactate), allowing neurons to meet their high-energy requirements while sustaining the PPP.

Through these multiple interactions, it is clear that astrocytes are critical mediators of neuronal survival, during normal brain function as well as under pathological circumstances, particularly stroke. In support of this, the presence of astrocytes in neuronal cultures enhances neuronal resistance to oxidative stress (Mattson and Rychlik 1990). Our laboratory has chosen to focus on promoting the survival and function of astrocytes to facilitate neuroprotection. As described earlier, both neurons and astrocytes undergo a dramatic loss of glucose and oxygen during ischemia. This causes rapid depletion of ATP in the ischemic core and initiates a cascade of events that lead to increased ROS, intracellular acidification, the loss of ionic gradients, and cell death. However, much of the penumbra experiences a less severe decrease in ATP, providing a window for treatment for these endangered cells that might prevent the lesion from expanding. The next section focuses on understanding

one method our laboratory has used to prevent expansion of the core and maintain viability of cells within the penumbra. We do this by targeting astrocyte metabolism via metabotropic purinergic stimulation.

7.9 Purinergic Receptors

Purinergic receptors bind the purine nucleotides ATP, ADP, UTP, and UDP. They fall into two classes: ionotropic P2X receptors and metabotropic P2Y receptors. P2X receptor activation results in opening of membrane ion channels, and is reviewed elsewhere (Coddou et al. 2011; Burnstock 2012; Kaczmarek-Hájek et al. 2012). P2Y receptor stimulation causes activation of its cognate heterotrimeric G protein. Eight genes encode different P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$), which have been grouped together based on functional and structural similarities (Abbracchio et al. 2006). P2Y receptors are expressed throughout the body, and function in several physiological processes, including immunity (Abbracchio et al. 2006; Bours et al. 2006; Mei et al. 2010), platelet activation (Born 1985; Gachet 2012), bone formation/resorption (Kumagai et al. 1991; Schofl et al. 1992; Rumney et al. 2012), kidney function (Pochynyuk et al. 2008; Toney et al. 2012), smooth muscle cell regulation (Lohman et al. 2012; Goyal et al. 2013), lung function (Burnstock et al. 2012), and others (Abbracchio et al. 2006; Burnstock 2012; McIntosh and Lasley 2012). P2Y receptors are widely expressed in the central nervous system and play a vital role in several important processes.

7.10 P2YR Stimulation Increases Astrocyte Mitochondrial Metabolism

In addition to purine nucleotides, a variety of structural analogues can bind and activate P2YRs (Abbracchio et al. 2006). Once activated, P2YRs interacts with GPCR. GPCRs are composed of three separate subunits: a Gα GTPase, a Gβ, and a Gγ. The Gα GTPase activity is activated when Gα is separated from the inhibitory β and γ subunits. P2YRs separate Gα from β and γ, thus enabling Gα hydrolysis of GTP. Activated Gα then signals via second messengers. Depending on the particular Gα, different second messengers are utilized. Several P2-coupled G proteins can modulate adenylyl cyclase activity, protein kinase activity, and phospholipase activity. One key function of purine signaling via P2Y receptors in astrocytes is to increase intracellular Ca^{2+} (Cotrina et al. 1998; Guthrie et al. 1999; Butt 2011). This is accomplished via phospholipase C (PLC), which can be activated by $P2Y_{1,2,4,6,11.}$ Active PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) on the plasma membrane into IP_3 and diacylglycerol. IP_3 then interacts with the IP_3R on the endoplasmic reticulum, thus inducing release of Ca^{2+} from this store into the cytosol.

Release of Ca^{2+} from the ER after purinergic signaling plays a variety of roles in the central nervous system, but is particularly important in the regulation of mitochondrial metabolism in astrocytes via stimulation of Ca^{2+}-sensitive dehydrogenases as discussed above. In fact, our laboratory originally reported that treating astrocytes with micromolar concentrations of extracellular ATP in vitro induced ER-dependent Ca^{2+} release which, in turn, elicited an increase in oxygen consumption, mitochondrial membrane potential, and intracellular ATP concentration (Wu et al. 2007). The neuroprotective benefits of stimulated astrocyte metabolism are discussed next.

7.11 $P2Y_1R$ Stimulation Decreases Damage After Ischemia

We have established that (1) disrupted energy metabolism is a precipitating event in stroke pathology; (2) astrocytes and their metabolism play a central role in maintaining neuronal integrity and function; and (3) $P2Y_1R$ signaling via astrocytes can stimulate astrocyte metabolism. As such, we hypothesized that stimulation of astrocyte mitochondrial metabolism via $P2Y_1R$ signaling could be protective after oxidative stress and cerebral ischemia.

We first tested this hypothesis in vitro by treating astrocytes with *tert*-butyl hydrogen peroxide to generate oxidative stress, and found that acute pretreatment (less than 15 min) with the purinergic agonist 2-methylthio adenosine diphosphate (2MeSADP) significantly enhanced astrocyte viability compared to untreated astrocytes (Wu et al. 2007). Importantly, the purinergic-stimulated protection against oxidative stress only functioned when astrocytes were present. Neuronal cultures by themselves exhibited decreased viability after oxidative stress when treated with 2MeSADP. When astrocytes were co-cultured with neurons, however, the pretreatment with 2MeSADP was able to increase viability of *both* astrocytes and neurons (Wu et al. 2007) (Fig. 7.3b). This indicates that stimulation of astrocyte metabolism not only rescues astrocytes, but enables astrocytes to rescue neurons.

To test our hypothesis in vivo, we developed a Rose Bengal (RB)-induced photothrombosis stroke model to generate either regional (multi-vessel) or focal (single-vessel) ischemia in the parietal cortex of mouse brains. RB-induced stroke was first pioneered by Watson et al. in 1985 using rats (Watson et al. 1985). In brief, when RB is injected into the vasculature of mice and exposed to green (560 nm) light, singlet oxygen is generated. This reactive oxygen species (ROS) is highly reactive, and will damage the endothelial walls of blood vessel, causing rapid coagulation of platelets that can lead to a blood clot. We successfully generated regional strokes by irradiating an ~300 μm^2 area of the cortex, which reliably developed into ~2 mm^2 infarcts 24 h later. We can also generate very precise single-vessel lesions that developed in ~0.1 mm^2 at 24 h. For both lesion types, the variance of the infarct areas was reduced by initially thinning the skull to optical clarity (i.e., a thickness around 50 μm). This model is noninvasive and allows specific targeting of any cortical regions of interest. The thinned-skull preparation is also particularly amenable for studying the cellular and molecular mechanisms underlying brain damage using in vivo optical imaging.

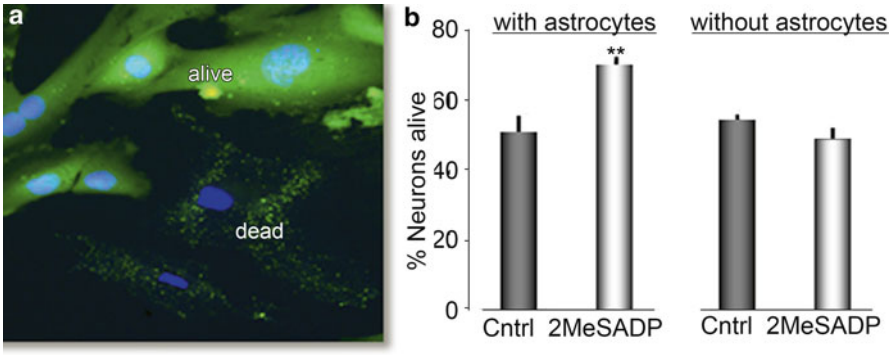

Fig. 7.3 Purinergic stimulation of astrocytes is protective of neurons during oxidative stress. Mouse primary neurons were co-cultured with astrocytes and subjected to oxidative stress by treatment with *tert*-butyl hydrogen peroxide, as described in (Wu et al. 2007). (**a**) Image showing a representative image of calcein-labeled primary astrocytes (*green*). Viable astrocytes retain the calcein label, whereas nonviable astrocytes do not. All astrocytes are labeled by Hoechst dye (*blue*). Counting the number of *green* viable cells as a percentage of total Hoechst-labeled cells was performed in (**b**). Here, neurons were co-cultured with astrocytes and subjected to killing via oxidative stress. Treatment with 2MeSADP resulted in significantly more viable neurons compared to untreated (*left*). Another group of neurons were co-cultured with astrocytes until just before the experiment. The astrocytes were then removed, and treated with 2MeSADP with oxidative stress. Here, neuron viability failed to be saved with 2MeSADP treatment (*right*), indicating that 2MeSADP-mediated neuronal protection requires astrocytes

After optimizing our models of focal ischemia, we were able to selectively stimulate mitochondrial metabolism in astrocytes by targeting $P2Y_1$Rs, which, in vivo, are primarily expressed on this glial subtype (Franke et al. 2004; Zheng et al. 2010). Initially, we used 2MeSADP, which has a tenfold greater affinity for $P2Y_1$ than ADP (Waldo and Harden 2004). The 2MeSADP also has some affinity for $P2Y_{12}$Rs and $P2Y_{13}$Rs, although evidence suggests that expression of the $P2Y_{12}$ receptor is not concentrated within the cortical astrocytes we studied (Franke et al. 2004), and it is not clear if $P2Y_{13}$ activity is present in cortical astrocytes. Regardless, the effect of 2MeSADP on $P2Y_{13}$ activity is much less potent than that of $P2Y_1$ (Fumagalli et al. 2004). Thus, 2MeSADP treatment in our hands was likely having its greatest effect upon astrocyte $P2Y_1$Rs in the cortex. As described below, we independently confirmed this inference in a separate series of experiments using the highly selective $P2Y_1$R agonist MRS2365 (Watts et al. 2013).

Our first series of experiments in vivo tested the efficacy of $P2Y_1$R stimulation after multi-vessel regional strokes (~2 mm^2 at 24 h). We found that when mice were given 2MeSADP at the same time as these strokes were induced, the lesion size was reduced to less than 1 mm^2 after 24 h (Zheng et al. 2010) (Fig. 7.4g, h). More remarkably, a delayed treatment of 2MeSADP, 24 h post-stroke, was able to significantly abrogate the expansion of the cerebral infarct at 48 h. This finding is particularly important because it demonstrates that treatment can be efficacious beyond 3–4.5 h, which we mentioned before is the current time limit at which human stroke victims can be treated with anything other than palliative measures.

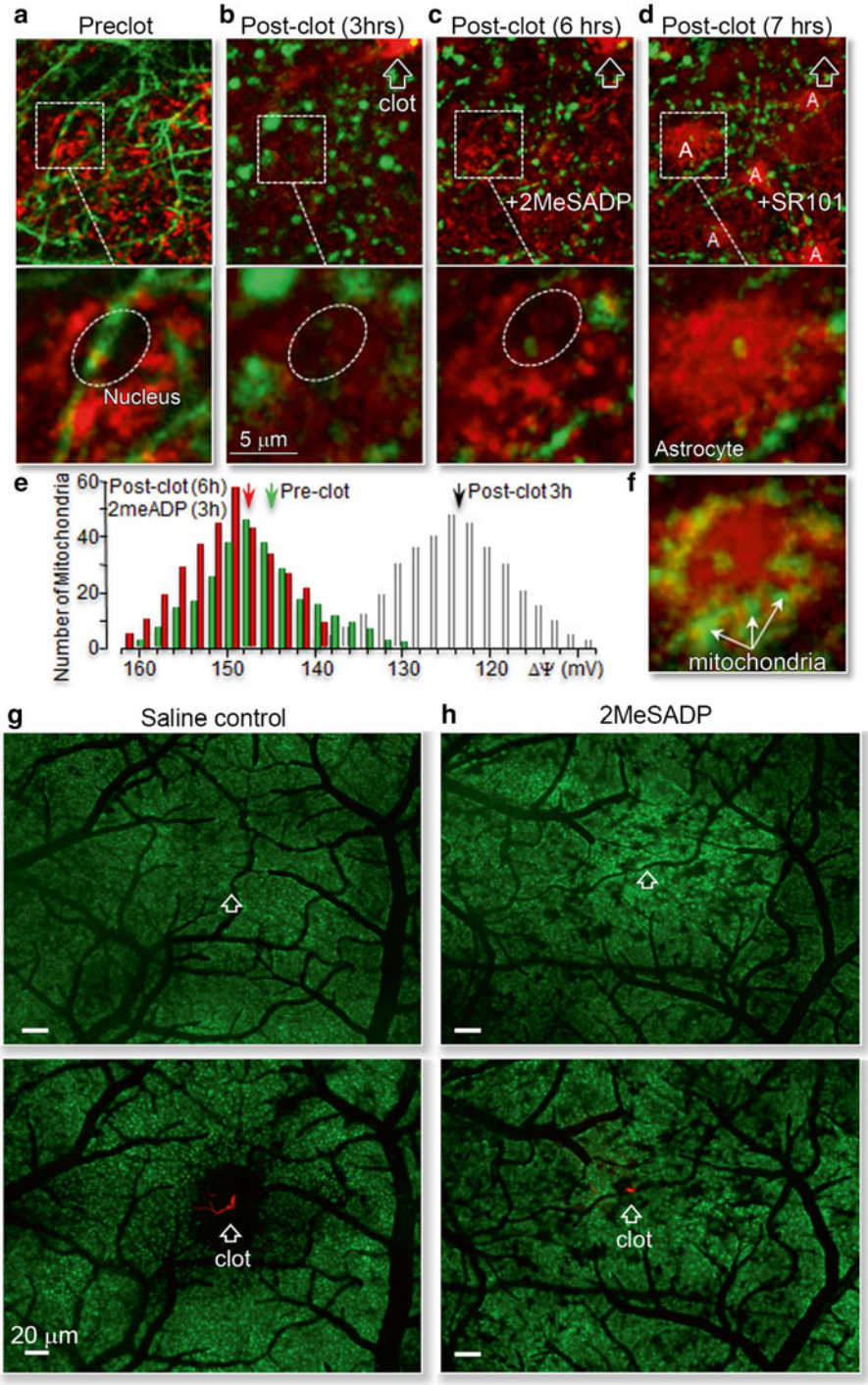

Fig. 7.4 Purinergic stimulation energizes astrocyte mitochondria and reduces stroke damage. Mice were subjected to RB-mediated focal ischemia in the parietal cortex of mice, and multiphoton

We subsequently tested the effect of 2MeSADP on single cells within and around lesions induced by single-vessel photothrombosis. Focal, single-vessel clots result in smaller ischemic lesions compared to the traditional middle cerebral artery occlusion (MCAO) model and are more similar to silent strokes (Leary and Saver 2003), which may be asymptomatic in isolation, but appear to accumulate with age and may lead to age-associated neurodegeneration. For our experiments, we utilized mouse lines that expressed fluorescent markers in astrocytes (*hGFAP*::GFP) or in neurons (*Thy1*::YFP). As shown in Fig. 7.1c, d, neuronal dendrites exhibited beading and astrocytes became markedly swollen with 1–3 h post-stroke. Treatment with 2MeSADP significantly reduced both dendrite beading and astrocyte swelling (Wu et al. 2007; Zheng et al. 2010). Moreover, delayed (3 h) 2MeSADP treatment reversed astrocyte swelling and dendrite beading in the penumbra after it was already manifest (Wu et al. 2007; Zheng et al. 2010), thereby slowing and potentially preventing the spread of the brain infarct.

To help elucidate the putative mechanism by which $P2Y_1R$ agonists were protective after stroke, we measured the energy status of astrocyte mitochondria before and after stroke. Previous research showed that 2MeSADP treatment increased primary astrocyte ATP content in vitro (Franke et al. 2004; Wu et al. 2007). In vivo, we measured the mitochondrial membrane potential of astrocytes using the fluorescent dye *tetra*-methyl rhodamine methyl ester (TMRM). TMRM has a diffuse positive charge and accumulates in the mitochondrial matrix in a Nernstian fashion (Farkas et al. 1989; Perry et al. 2011) (Fig. 7.4a–f). When the mitochondrial membrane potential is increased, TMRM intensity increases and indicates greater ATP production. Consistent with our working model of $P2Y_1R$-enhanced protection, single-vessel strokes caused a significant depolarization of mitochondria, which could be reversed (repolarized) by 2MeSADP treatment of mice 3 h post-stroke (Fig. 7.4b, c, e). Saline-injected control mice exhibited continued mitochondria depolarization. To test if it was increased energy production per se that enhanced protection, mitochondrial energy production was specifically disrupted in astrocytes using fluoroacetate.

Fig. 7.4 (continued) optical microscopy was utilized to visualize the effect on astrocytes in real time. Mice that express neuronal YFP were treated with TMRM to visualize astrocyte mitochondrial membrane potential (**a**) before stroke and (**b**) 3 h post-stroke. Note the bright red fluorescence of mitochondria, which disappears from the same cell 3 h post-stroke, indicating a decrease in mitochondrial membrane potential. After imaging at 3 h, mice were treated with 2MeSADP, then imaged at (**c**) 6 h post-stroke. Mice treated with saline control had unchanged mitochondrial membrane potential (not shown), but those treated with 2MeSADP had regained the mitochondrial membrane potentials. To confirm that the cells with TMRM labeling were astrocytes, mice were given SR101, a dye that specifically labels astrocytes. (**d**) Shows astrocytes (labeled with "A"). (**e**) Histogram plot of the numbers of mitochondria versus the membrane potential determined from TMRM intensity. (**f**) Merged image of TMRM label in an astrocyte (*green*) overlapping SR101 labeling (*red*), confirming that the mitochondrial used for quantification were from astrocytes. Image reproduced with permission from (Zheng et al. 2013). (**g**) Mice that express astrocyte GFP were subjected to multiphoton optical microscopy in vivo before (*top panel*) RB-mediated stroke and 24 h post-stroke (*bottom panel*). Shown are composite images collected of the infarct and peri-infarct region. A vessel of approximately 5 μm diameter was occluded (*white arrow*), and lesion size was and measured by determining the area in which astrocyte GFP fluorescence was lost. (**h**) Note that at 24 h, the loss of fluorescence in the 2MeSADP-treated mice is much smaller than saline-treated mice. Image reproduced with permission from (Zheng et al. 2013)

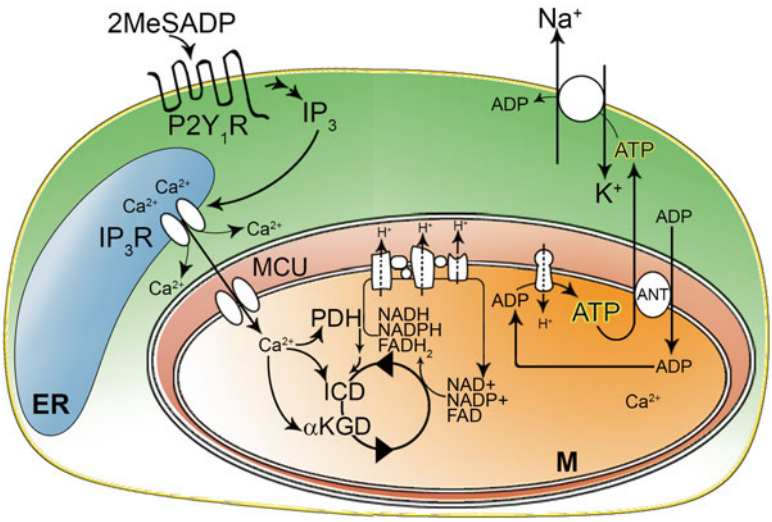

Fig. 7.5 Model of P2Y1 R-dependent increases in mitochondrial metabolism. The purinergic ligand binds and activates $P2Y_1R$ on the plasma membrane. $P2Y_1R$ activates G**α** causing hydrolysis of GTP and subsequent activation of phospholipase C (PLC). PLC cleaves PIP_2 on the plasma membrane to produce IP_3. IP_3 activates the IP_3R in the ER, which causes Ca^{2+} release. Due to proximity of the ER to the mitochondria, Ca^{2+} released from the ER can enter mitochondria through a mitochondrial Ca^{2+} uniporter (MCU) on the mitochondrial membrane. Ca^{2+} in the mitochondria then affects numerous processes. Importantly, Ca^{2+} activates Ca^{2+}-dependent dehydrogenases (*PDH* pyruvate dehydrogenase, *ICD* isocitrate dehydrogenase, *αKGD* **α**-ketoglutarate dehydrogenase), thus increasing TCA cycle enzymatic activity and therefore increasing overall ATP production. The ATP is trafficked out of the mitochondria via action of the adenine nucleotide transporter (ANT) into the cytosol. Once in the cytosol, ATP can be used to maintain a variety of important astrocyte functions, notable among them is that of the Na^+/K^+-dependent ATPase in ion homeostasis

Fluoroacetate is only transported into astrocytes due to the specificity of its monocarboxylic acid transporter isoforms (MCT1 and 4), where it is readily converted into fluoroacetyl-CoA. It then combines with oxaloacetate in the TCA cycle to form the toxin fluorocitrate, which irreversibly binds to aconitase, inhibiting its activity and disrupting energy production (Proudfoot et al. 2006). We found that pretreatment of mice with fluoroacetate abrogated the ability of 2MeSADP to protect astrocytes after stroke. We had similar findings when we genetically disrupted mitochondrial function in astrocytes using a doxycycline regulated, mitochondrially targeted, DNA restriction endonuclease (Zheng et al. 2010). Taken together, these data indicate that 2MeSADP exerts its protective effect after stroke by increasing astrocyte mitochondrial energy production.

Our in vivo work indicated that neuroprotection from transient cerebral ischemia could be enhanced by increasing astrocyte mitochondrial ATP production via $P2Y_1R$ stimulation. Our central mechanism of action of $P2Y_1Rs$ involves IP_3-mediated Ca^{2+} release from the ER, consistent with early reports in cultured astrocytes that Ca^{2+} release from the ER reduced edema (Duffy and MacVicar 1996; Quesada et al. 1999).

We further tested this working model of protection by utilizing a mouse knockout model of IP_3R type 2, which is the only IP_3R isoform expressed in astrocytes. We found that after RB-induced thrombosis, 2MeSADP stimulated Ca^{2+} release in wild-type cerebellar astrocytes, but failed to stimulate Ca^{2+} release in IP_3R type 2 knockout mice. We also measured the changes in mitochondrial membrane potential in IP_3R type 2 knockout mice and found that 3 h post-stroke, mitochondrial polarization failed to increase in response to 2MeSADP treatment (Zheng et al. 2013). In addition, the size of ischemic lesions was not reduced by $P2Y_1R$ stimulation in IP_3R type 2 knockout mice, nor was post-stroke dendritic beading reversed. We interpret these results as strong evidence that IP_3-mediated Ca^{2+} signaling is an essential component of $P2Y_1R$-enhanced neuroprotection (Wu et al. 2007; Zheng et al. 2010).

A simplified diagram of our working model of neuroprotection is presented in Fig. 7.5. Moving forward, it is not as clear which ATP-dependent processes in astrocytes are most critical for neuroprotection. Multiple energy-dependent processes are possible; for example, production of glutathione and/or neurotrophic factors (Barreto et al. 2011). Although trophic factors are clearly important over long time scales (days, weeks, and even months), it is likely that during the immediate stress of ischemia, the dominant role that astrocytes play in maintaining ion homeostasis is critical. As noted above, proper functioning of Na^+/K^+ ATPases and/or ATP-dependent K^+-inward rectifiers are likely critical players in preventing, reducing, and/or reversing cell edema as well as reducing and/or slowing the frequency of recurring spreading depolarizations and excitotoxicity (Wu et al. 2007; Zheng et al. 2010). Regardless of the precise intracellular mechanisms of action, our data show that astrocytes metabolism is critical in determining the severity of astrocyte edema and necrosis, dendrite damage and infarct sizes. Most importantly, our work demonstrates that it is possible to stimulate this energy-dependent mechanism during ischemic stress, making astrocyte mitochondria metabolism an attractive therapeutic target to improve the neurological outcome of brain damage.

Conflict of Interest The authors declare no conflict of interest.

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Chapter 8
Excitotoxicity and Mitochondrial Dysfunction Underlie Age-Dependent Ischemic White Matter Injury

Selva Baltan

Abstract The central nervous system white matter is damaged during an ischemic stroke and therapeutic strategies derived from experimental studies focused exclusively on young adults and gray matter have been unsuccessful in the more clinically relevant aging population. The risk for stroke increases with age and the white matter inherently becomes more susceptible to injury as a function of age. Age-related changes in the molecular architecture of white matter determine the principal injury mechanisms and the functional outcome. A prominent increase in the main plasma membrane Na^+-dependent glutamate transporter, GLT-1/EAAT2, together with increased extracellular glutamate levels may reflect an increased need for glutamate signaling in the aging white matter to maintain its function. Mitochondria exhibit intricate dynamics to efficiently buffer Ca^{2+}, to produce sufficient ATP, and to effectively scavenge reactive oxygen species (ROS) in response to excitotoxicity to sustain axon function. Aging exacerbates mitochondrial fusion, leading to progressive alterations in mitochondrial dynamics and function, presumably to effectively buffer increased Ca^{2+} load and ROS production. Interestingly, these adaptive adjustments become detrimental under ischemic conditions, leading to increased and early glutamate release and a rapid exhaustion of mitochondrial capacity to sustain energy status of axons. Consequently, protective interventions in young white matter become injurious or ineffective to promote recovery in aging white matter after an ischemic episode. An age-specific understanding of the mechanisms of injury processes in white matter is vital in order to design dynamic therapeutic approaches for stroke victims.

Keywords Compound action potential • Myelin • Ca^{2+} homeostasis • Oxidative injury • Astrocytes • Axon function • Electrophysiology • Optic nerve

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_8

8.1 Introduction

Human brain comprises equal percentages of gray and white matter by volume, which means that injuries sustained after a stroke in human predictably involve almost always both white and gray matter portions of the brain. The risk for ischemic stroke increases considerably with age. In addition to vascular factors, aging results in global changes, predominantly in white matter, such that the tissue becomes intrinsically more vulnerable to ischemic injury. Experimental stroke studies commonly use rodent brain, which constitutes little white matter by volume compared to gray matter. Subsequently, after an ischemic attack, the resultant injury in rodent brain is mainly due to neuronal loss with minimal or no contribution from white matter. Therefore it is plausible that the efficacy of neuroprotective drugs identified in experimental stroke models using young healthy male rodents is a poor predictor of clinical outcome and have so far failed to successfully translate to human trials (Del Zoppo 1995, 1998; Dirnagl et al. 1999)

An ideal stroke therapeutic must preserve neurons together with their axons and glial cells to gain optimal functional recovery. Neurons without functional axons simply cannot relay the information. Furthermore, the concept of neuroprotection must consider age, given that the mechanisms of ischemia change in such a way that those cytoprotective treatments in young brain may be ineffective or even become harmful for aging brain. Therefore, interventions to promote recovery following a stroke attack must target both portions of the brain, challenging the conventional idea that a single injury mechanism mediates the effects of ischemia in gray and white matter and that the injury mechanisms remain identical across life span in all age groups.

Over the last decade a number of molecular mechanisms by which glial cells and axons die or survive after an ischemic episode have been unraveled. In this chapter I focus on age-dependent structural and functional changes in white matter, which presumably evolved to protect the brain against the consequences of aging, but inadvertently made the tissue more vulnerable in the face of an ischemic attack. In particular, I concentrate on the age-dependent changes in energy status and glutamate homeostasis to test the hypothesis that increased excitotoxicity and impaired mitochondrial dynamics underlie the increased vulnerability of aging white matter to ischemia.

8.2 White Matter Structure and Function are Integrated

The white matter architecture is distinct from gray matter and is unexpectedly complex. Axons with variable amounts of myelin extend through oligodendrocytes, astrocytes, microglia, and precursor cells to establish a vital network between neurons. A variety of signaling mechanisms and molecules complement and contribute to white matter organization to ensure high fidelity communication between

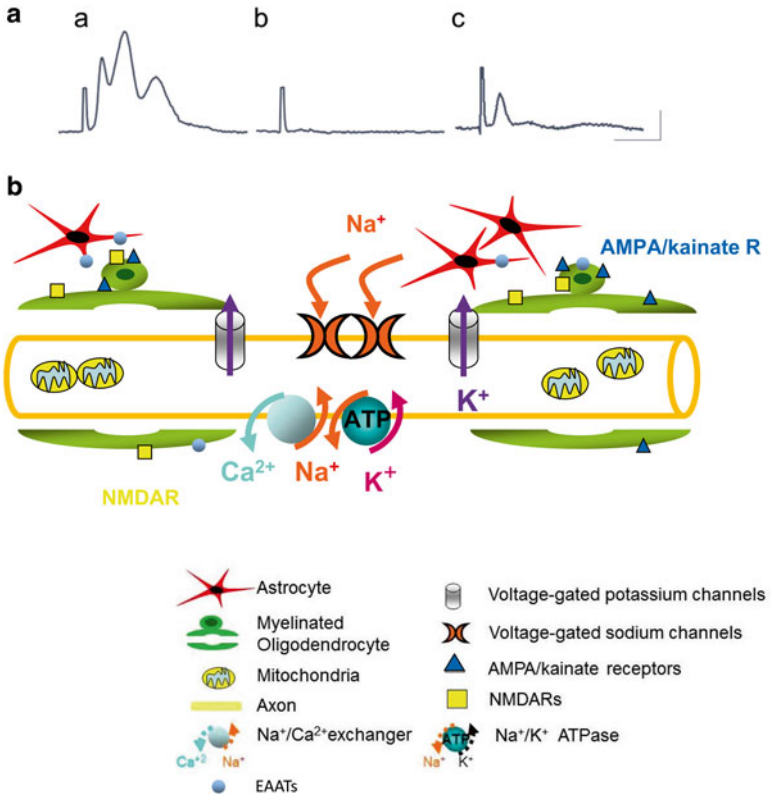

Fig. 8.1 White matter function and structure are integrated. (**a**) Evoked compound action potentials (CAP) recorded across young optic nerve illustrate (1) control, (2) after 60 min oxygen and glucose deprivation (OGD), and (3) recovery traces. Scale bar = 1 mV, 1 ms. (**b**) molecular and cellular architecture of white matter determines function and response to injury. See legend for symbols. AMPAR, α-amino-3-hydroxy-5-methyl-isoxazole propionate receptors; EAATs, excitatory amino acid transporters; NMDAR, N-methyl D-aspartate receptor

neurons. Among those voltage-gated Na^+ and K^+ channels, Na^+–Ca^{2+} exchangers (NCX), Na^+–K^+ ATPase pumps, and axonal mitochondria are essential to maintain axon excitability (Fig. 8.1). The cellular and molecular components of white matter are individually under attack during ischemia, while they maintain their intricate glia-to-glia and glia-to-axon interactions. Therefore, the structural composition of white matter, together with a concerted interaction among glial cells and axons, dictates pathophysiological mechanisms of ischemic white matter injury. Consequently, it is challenging to experimentally dissect injury mechanisms following a complex injury such as ischemia in white matter. Furthermore, there is a growing sense that the mechanisms of white matter injury vary from one white matter tract to another (Tekkök and Goldberg 2001; Tekkok et al. 2007), presumably due

to the fact that white matter structure conforms to its specific function. However, the reasons for regional differences in white matter injury are not yet understood at cellular levels. The existence of regionally diverse astrocytes and/or oligodendrocytes, the degree of expression or subunit composition of glutamate receptors that contribute to the injury process (Gallo and Russell 1995; Brand-Schieber and Werner 2003a, b; Tekkok et al. 2007), the extent of myelination (Olivares et al. 2001) and axonal specialization for the function and the type of injury such as ischemia vs. anoxia (Tekkok and Ransom 2004; Baltan 2009) and region- and cell-specific expression patterns of Class 1 histone deacetylases (HDACs) (Baltan et al. 2011a, 2013) may underlie these regional differences. Interestingly, among all, glutamate homeostasis of white matter involving glutamate receptors and glutamate transporters (Fig. 8.1), expressed primarily by astrocytes as well as other glial cells in a region-specific manner, attests to the sophisticated signaling mechanisms among white matter components to adapt to their regional function, but inevitably determines the vulnerability of different white matter tracts to ischemia. Therefore, to decipher axon-glia glutamate signaling and the mismatch of this signaling system, leading to excitotoxicity during ischemia, is key to prevent or restore young and aging white matter function.

8.3 Aging Alters the Mechanisms of Ischemic White Matter Injury

Although the immediate effect of oxygen and glucose deprivation (OGD) is deregulation of ionic homeostasis due to ATP depletion, this pathway is completely reversible (Fig. 8.2) (Tekkok et al. 2007). On the other hand the critical level of Na^+ overload that mediates the reversal of the Na^+-dependent glutamate transporters to accumulate glutamate dictates the irreversible injury (Fig. 8.2). Furthermore, convergence of the ionic pathway and the excitotoxic pathway concomitantly activates the oxidative injury by activating glutamate/cysteine system, (Oka et al. 1993) leading to mitochondrial dysfunction (Baltan et al. 2011b). Sequential merging of these three pathways is the basis for the demise of white matter during ischemia.

Naturally, white matter components that are vulnerable to excitotoxicity and oxidative injury are the principal targets of an ischemic attack. After induction of OGD, the earliest changes are observed on oligodendrocytes as swelling of the cell body that extends to myelin processes and subsequently axonal beading occurs (Fig. 8.3a). These morphological changes are irreversible such that even after resuming oxygen and glucose for prolonged periods of time, structural disruption progresses. Meanwhile, axon function gradually diminishes, resulting in complete loss of conduction (Fig. 8.3b). Oligodendrocyte death and axon loss are the ultimate outcome following ischemia (60 min), disabling white matter function. Oligodendrocytes express a wealth of glutamate receptors such as AMPA and NMDA receptors (Fig. 8.1), and although these are critical for glia-axon signaling, the presence of these receptors also sets oligodendrocytes as a target for excitotoxic injury. Axons

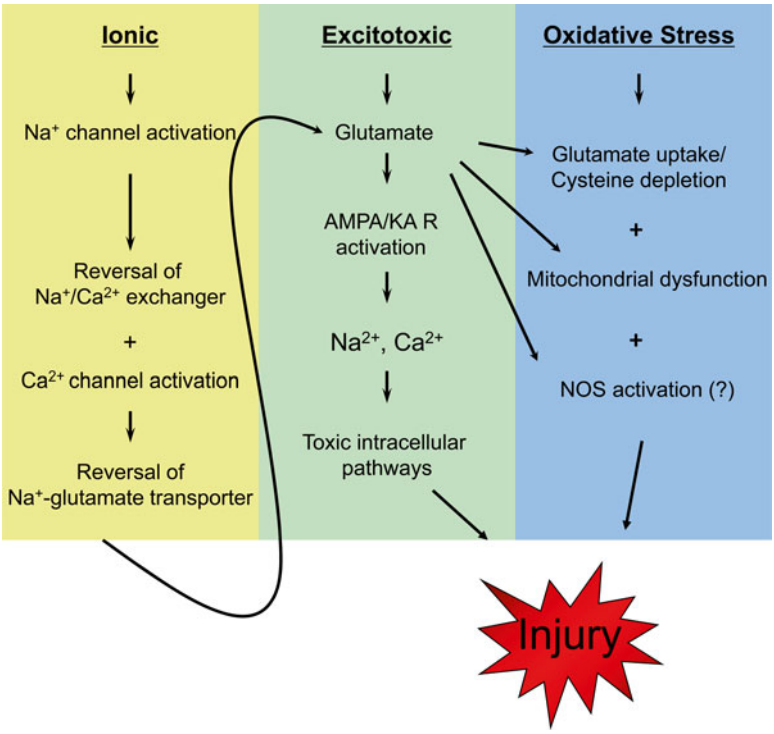

Fig. 8.2 Putative mechanisms of ischemic white matter injury. Ionic, excitotoxic, and oxidative stress converge in sequential order to cause irreversible injury. Glutamate release, due to reverse Na^+-dependent transport, dictates the irreversible nature of the injury (*curved arrow*). Note that there are multiple mechanisms that contribute to ischemic white matter injury. AMPA/KA R, AMPA/kainate receptors. Studies assessing nitric oxide synthase (NOS) activity are currently underway (Reproduced and Modified from Baltan 2009)

are disrupted secondary to oligodendrocyte death, although there is experimental evidence that axons are a potential source of glutamate (Li et al. 1999) and express glutamate receptors under myelin sheaths that are directly activated by glutamate (Ouardouz et al. 2005; Alberdi et al. 2006).

Aging is the most significant risk factor for an ischemic episode. Excitotoxicity and oxidative injury are essential steps in ensuing irreversible injury; therefore, the age-dependent changes in these two pathways are of the utmost importance to decipher the reasons underlying the increased vulnerability of aging white matter to ischemia. Exposing mouse optic nerves (MONs) obtained from 1- to 12-month-old animals to OGD confirms that aging axon function is more vulnerable to ischemia (rapid loss of axon function) and recovers less in a duration-dependent manner (Fig. 8.4a). On the other hand, keeping OGD duration constant, the axon function recovers less as age increases throughout the life span (Fig. 8.4b). Glutamate levels remain constant during the first 30 min of OGD (Tekkok et al. 2007—see also

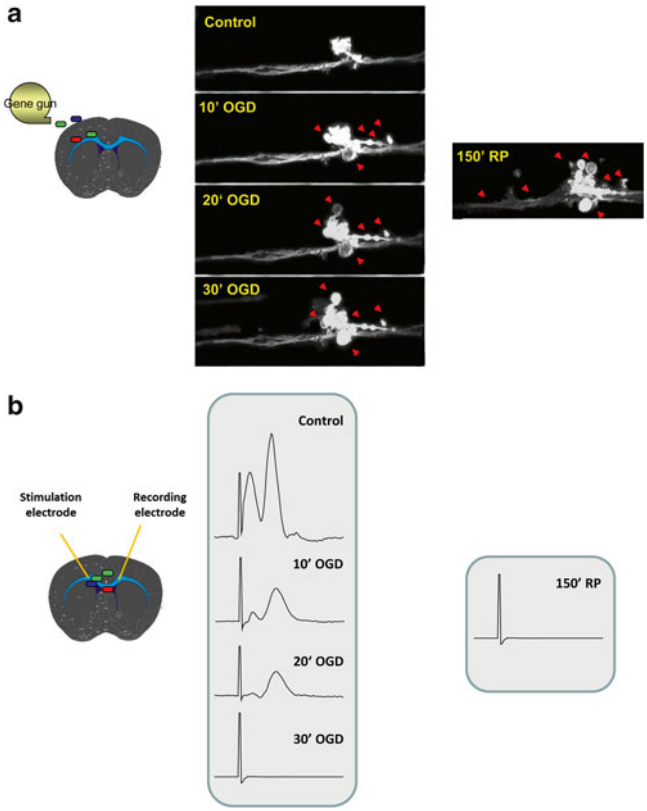

Fig. 8.3 Oligodendrocyte cell body, myelin sheaths, and axon function are under attack during ischemia. (**a**) Using multidiolistic technique and a "gene gun," gold pellets coated by a mixture of fluorescent dyes (DiI-*blue*, DiO-*green*, and DiA-*red*) is delivered to acute coronal slices to label white matter structures in corpus callosum. In some cases, an oligodendrocyte together with its cell body, myelin sheaths, and the myelinated axon are labeled (*top image*, control). Exposing slices to oxygen glucose deprivation (OGD) for 30 min causes swelling and blabbing/swelling (*red arrowheads*) of oligodendrocytes cell body (10 min). This swelling of the cell body that extends to myelin processes (20 min) and subsequently axonal beading occurs (30 min). These structural changes are irreversible even after prolonged periods of reperfusion (RP) (150′ RP). (**b**) Under these conditions, axon function, as per evoked compound action potentials recorded, progressively decreases over time and is completely lost after (30′ OGD). Axon function fails to recover after a prolonged period of RP

Fig. 8.7a), inferring that this period mostly involves ionic changes. In agreement, axon function shows comparable recovery until 18 months of age after 30 min of OGD while prolonging the OGD duration to 45 min degrades axon recovery at every age group, implying that switching from the ionic to the excitotoxic pathway unmasks the age-related vulnerability of axon function to ischemia. In accordance, two main steps in the ionic pathway, blockade of NCX (Fig. 8.5a) or removal of

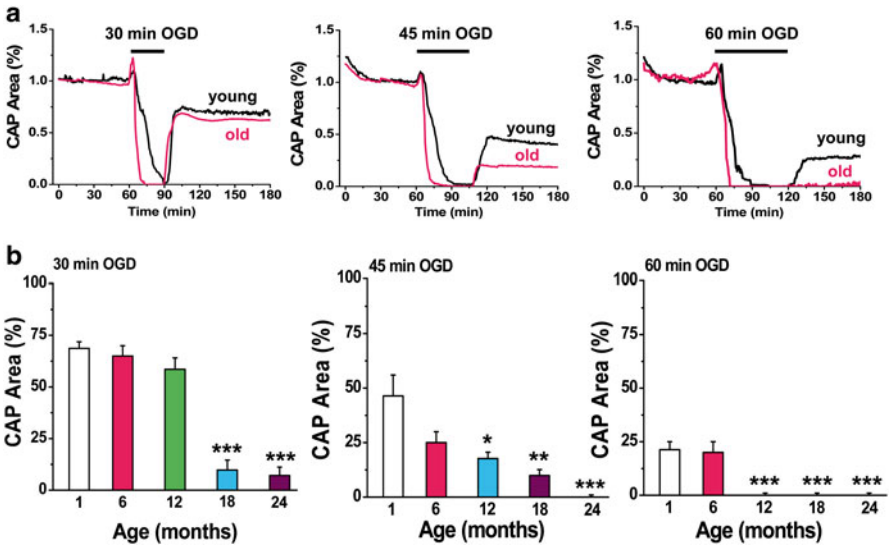

Fig. 8.4 Aging increases the vulnerability of axon function to ischemia as a function of age and injury duration. (**a**) Exposing optic nerves from 1- (*black*) and 12-month-(*magenta*) old mice to 30, 45, and 60 min OGD reveals that aging axons recover less as the duration of injury increases. (**b**) Exposing optic nerves from 1 (*white*)-, 6 (*magenta*)-, 12 (*green*)-, 18 (*blue*)-, and 24 (*purple*)-month-old mice to a fixed duration of injury shows that axons recover less with age over the same duration of injury. $*p<0.05$, $**p<0.01$, $***p<0.001$, one-way ANOVA

extracellular Ca^{2+} (Fig. 8.5b), fail to protect aging axon function against OGD. Indeed, the absence of Ca^{2+} hinders axon function recovery, pointing to an interesting dependence of aging axon excitability on Ca^{2+} entry. In contrast, blockade of the reversal of the Na^+-dependent glutamate transporters (Fig. 8.6a) or blockade of AMPA/kainate receptors (Fig. 8.6b) preserves aging axon function during OGD and promotes recovery comparable to young axons. Comparing spatiotemporal glutamate release patterns reveals an earlier and more robust glutamate release in aging white matter, despite efficient control of baseline glutamate levels (Fig. 8.7a). Moreover, in young white matter, glutamate levels return to baseline upon termination of OGD, while glutamate levels remain elevated after the end of OGD in aging MONs. The most prominent molecular structure leading to this enhanced excitotoxicity in aging white matter is the upregulation of GLT-1 (in human termed EAAT2) levels (red, Fig. 8.7b). The GLT-1 is mostly located on glial fibrillary acidic protein-positive (GFAP+) astrocytes in young white matter, but with aging GLT-1 is expressed on structures other than GFAP + astrocytes, presumably on oligodendrocytes based on their distinctive beads on a string appearance. Typically GLT-1 takes up glutamate with co-transport of Na^+ (Fig. 8.8). The Na^+–K^+ ATPase pump is crucial to sustain excitability of axons by expelling Na^+ for K^+ and

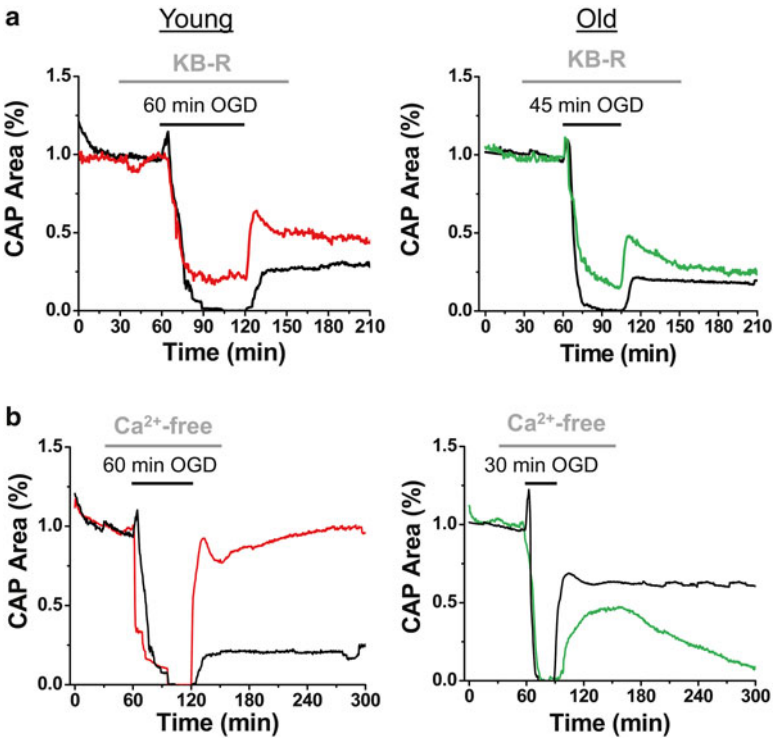

Fig. 8.5 Aging alters the mechanisms of ischemic white matter injury. (**a**) Blockade of reverse mode of Na^+–Ca^{2+} exchanger (NCX) operation with KB-R7943 (KB-R) promotes axon function recovery after OGD (60 min) in young axons but fails to benefit aging axons after a shorter OGD (45 min). (**b**) Removal of extracellular Ca^{2+} drastically ameliorates ischemic injury in young axons but hampers recovery in aging axons even after 30 min of OGD (Reproduced and modified from Baltan et al. 2008)

consuming ~50 % of ATP produced in the brain. During the ionic pathway of an ischemic attack, intracellular Na^+ levels increase following ATP depletion and failure of the Na^+–K^+ ATPase pump. With increased intracellular Na^+ and additional cell depolarization due to accumulation of extracellular K^+, GLT-1 reverses to release glutamate and, at the termination of OGD, takes up glutamate according to readjusted Na^+ levels. Therefore, the number of transporters determines the capacity of the tissue to transport glutamate but it is the ATP levels that indirectly dictate the direction of the GLT-1 to remove or to release glutamate. Therefore age-related increases in the number of GLT-1 that result in early and more glutamate release and sustained glutamate levels after OGD raise the question whether aging alters mitochondrial dynamics, thereby compromising white matter energy status.

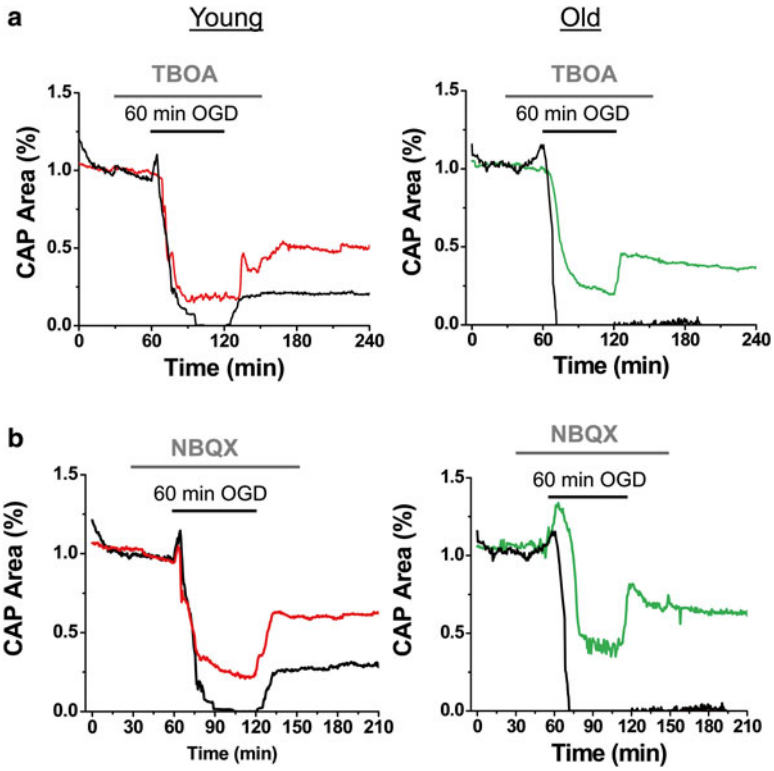

Fig. 8.6 Excitotoxicity underlies ischemic injury in young and old axons. (**a**) Blockade of the reversal of Na^+-dependent glutamate transporters with threo-beta-benzyloxyaspartate (TBOA) or (**b**) AMPA/kainate receptors with 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX) protects and promotes axon function recovery in young and old axons (Reproduced and modified from Baltan et al. 2008)

In addition to GLT-1, the critical members to conserve glutamate homeostasis are GLAST (a plasma membrane glutamate transporter in rodents; in human termed EAAT1), glutamate and glutamate synthetase (GS), an astrocyte-specific enzyme-converting glutamate to glutamine (Schousboe et al. 2014). White matter glutamate content increases considerably in correlation with increased GS levels with age (Baltan et al. 2013). Together with a twofold increase in GLT-1 levels in older white matter (Baltan et al. 2008), these adjustments may infer an age-related adaptive mechanism in white matter to remove and to convert excessive glutamate to glutamine in order to maintain glutamate homeostasis. These modifications raise the question whether changes in glutamate signaling underlie changes in aging white matter function.

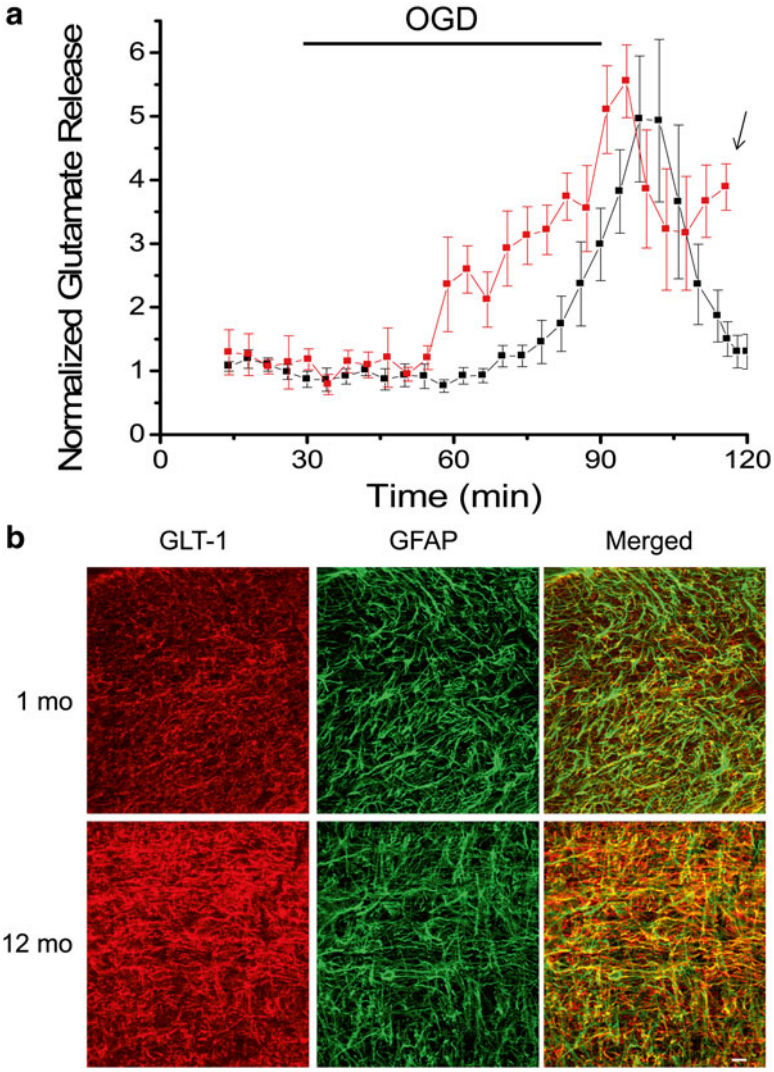

Fig. 8.7 Aging is correlated with early robust glutamate release due to upregulation of GLT-1. (**a**) Baseline glutamate levels are precisely controlled in young (*black squares*) and old axons (*red squares*). OGD causes early and robust glutamate release in older axons that remains sustained after the end of OGD (*arrow*). (**b**) The overlap of GLT-1 (*red*) and GFAP (*green*) labeling indicated that GLT-1 was mainly expressed in astrocytes (merged) in 1-month-old mouse optic nerves (MONs). There was a twofold increase in GLT-1 pixel intensity with age. The pattern of GFAP expression in MONs changed with age but without an increase in GFAP levels. $*p<0.05$, $** p<0.005$, one-way ANOVA (Reproduced from Baltan et al. 2008)

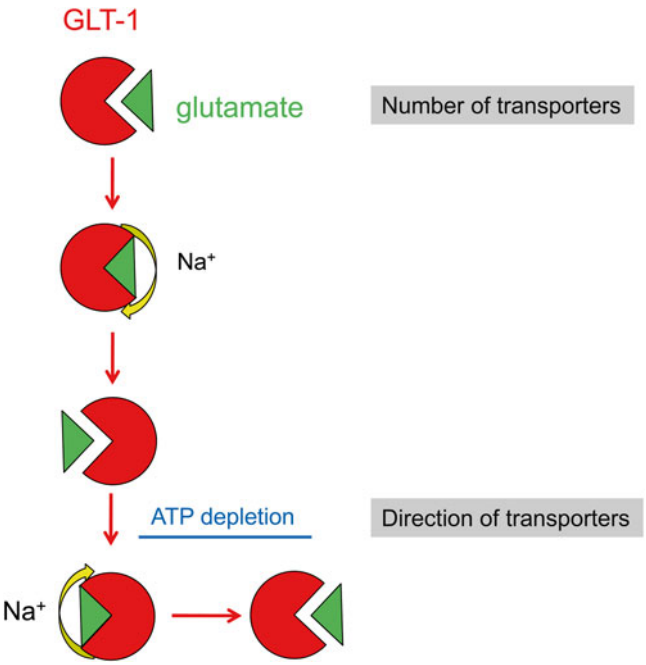

Fig. 8.8 Increased GLT-1 transporter numbers lead to enhanced excitotoxicity in aging white matter. The principal Na^+-dependent glutamate transporter, GLT-1, typically takes up glutamate with co-transport of Na^+. During ATP depletion, due to increased intracellular Na^+ levels, and with additional cell depolarization (not shown), the transporter reverses and releases glutamate. Therefore, the number of transporters determines the capacity of the system for the amount of glutamate that can be transported, but it is the ATP levels that determine the direction of the transporter (to take up or release glutamate). Reproduced and Modified from Baltan et al. 2013)

8.4 Synergistic Interaction of Excitotoxicity with Mitochondrial Dysfunction Mediates Ischemic White Matter Injury

The balanced delivery of mitochondria to cell body, dendrites, and axons helps them serve various functions, including energy generation, regulation of Ca^{2+} homeostasis, cell death, synaptic transmission, and plasticity (Chang and Reynolds 2006). The bioenergetics of mitochondria in neurons and their role in glutamate excitotoxicity are well defined in gray matter (Nicholls et al. 2007). Mitochondrial dysfunction and excitotoxicity share common features and are thought to act synergistically by potentiating each other (Albin and Greenamyre 1992; Jacquard et al. 2006; Silva-Adaya et al. 2008). Mitochondria are dynamic organelles that travel along microtubules, using axonal transport to reach peripheral locations (Hollenbeck

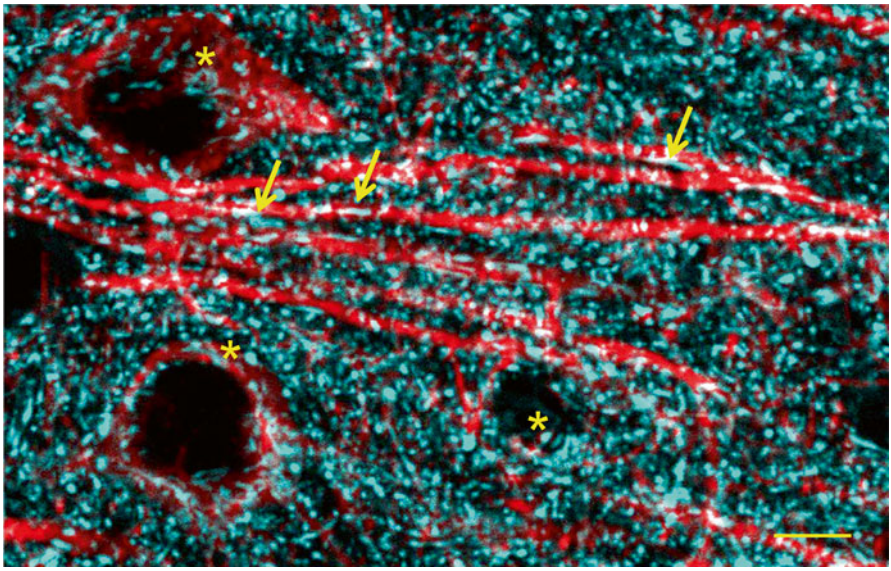

Fig. 8.9 CFP + somal and axonal mitochondria exhibit region-specific morphology. Neuronal mitochondria are readily observed as cyan fluorescent protein positive (CFP+) structures in a transgenic Mito-mouse. CFP + mitochondria are small and round in neuronal cells bodies (*yellow asterisk*) labeled with MAP2 (*red*), but are more linear and tubular in primary dendrites (*yellow arrows*). Scale bar = 5 μm (Reproduced from Baltan et al. 2013)

2005; Hollenbeck and Saxton 2005) (Fig. 8.9). They constantly undergo fission and fusion events (Karbowski et al. 2004), and the relative rates of mitochondrial fusion and fission have been implicated in the regulation of their number, size, and shape (Mozdy and Shaw 2003; Scott et al. 2003; Chen et al. 2007). As a result, mitochondria display a cell-specific morphology; neuronal mitochondria are small and round as opposed to the longer tubular mitochondria in dendrites and axons (Fig. 8.9, *arrows*).

The role of mitochondrial dynamics in white matter function, in particular the link between excitotoxicity and mitochondrial dysfunction, has been recently characterized (Baltan et al. 2011b; Baltan 2012). Functional studies correlated with advanced imaging techniques using MONs obtained from the so-called Mito-mice, i.e., a transgenic mouse [tg(Thy1-CFP/COX8A)] in which cyan fluorescent protein (CFP) is expressed in neuronal mitochondria (Misgeld et al. 2007), provided proof of principle that OGD causes a remarkable loss of CFP + mitochondria in white matter axons that can be completely reversed by blockade of AMPA/kainate receptors (Fig. 8.10a). Expectedly, an exciting correlation between CFP fluorescence and ATP levels connect excitotoxicity to impaired mitochondrial function in white matter (Fig. 8.10b). Interestingly, further support to verify the link between excitotoxicity and mitochondrial dysfunction in white matter comes from a series of experiments that tested the potential of HDAC inhibitors on white matter ischemic injury.

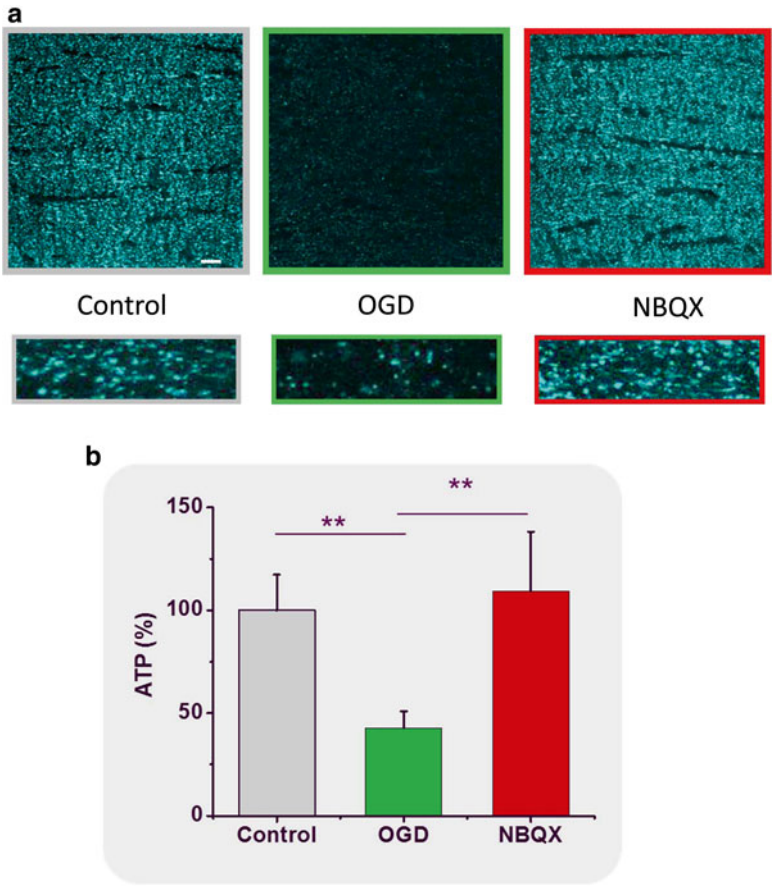

Fig. 8.10 Blockade of excitotoxicity preserves CFP + axonal mitochondria and ATP levels in response to OGD. (**a**) OGD drastically reduced CFP fluorescence in MONs from Mito-mice and pretreatment with NBQX (30 μM) protected against this loss. Note the change in mitochondrial morphology from small and tubular to tiny and punctuated with OGD. Scale bar = 10 μm (*insets* = 2 μM) (**b**) Consistent with the preservation of CFP pixel intensity, NBQX pretreatment conserved ATP levels in MONs. ***$p < 0.0001$, one-way ANOVA (Reproduced in part from Baltan et al. 2011b)

Administration of pan- or Class 1 HDAC inhibitors, before or after a period of OGD, promotes functional recovery of axons and preserves white matter cellular architecture. This protection correlates with the upregulation of GLT-1, delays and reduces glutamate accumulation during OGD, preserves axonal mitochondria and oligodendrocytes, and maintains ATP levels. Because significant protection is also observed when HDAC inhibitor is added after OGD (after maximal glutamate accumulation), HDAC inhibition must have at least two distinct sites of action during the sequential course of ischemic white matter injury; one site related to glutamate accumulation (GLT-1 expression) and the other involving post-excitotoxic mechanisms (Baltan et al. 2011b).

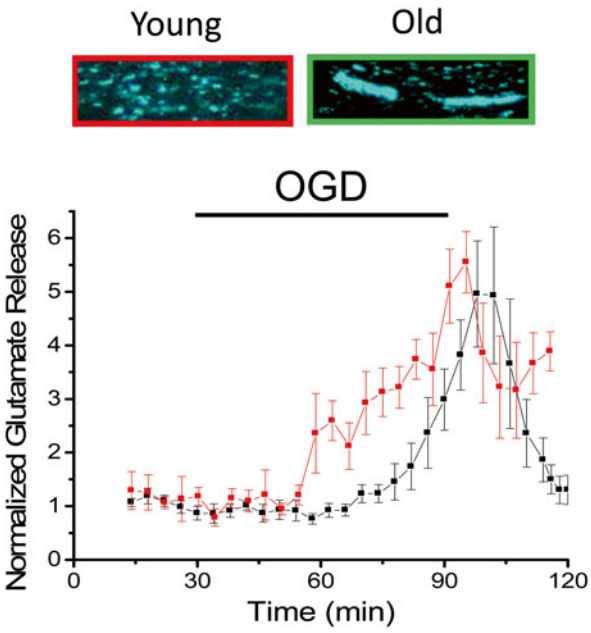

Fig. 8.11 Increased excitotoxicity and mitochondrial dysfunction underlie increased vulnerability of aging white matter to ischemia. Mitochondria in aging axons become longer and thicker compared to young axons (images), which may hinder ATP production and drive GLT-1 in reverse mode (see Fig. 8.8). Consistent with this, there is an early and robust glutamate release in aging white matter (*red*) compared to optic nerves from young mice (*black*). Note, that the glutamate levels return to baseline in young white matter, but remain elevated in aging white matter. $*p<0.05$, $**p<0.005$, one-way ANOVA (Reproduced in part from Baltan et al. 2008)

Due to the consistent protection, whether applied before or after injury, HDAC inhibitors target excitotoxic and oxidative pathways, so it is reasonable to expect that HDAC inhibition protects aging white matter function against ischemic injury. Indeed pan- and Class 1 HDAC inhibitors confer long-lasting benefits to aging axons following acute ischemic injury. Ironically, both aging and HDAC inhibition upregulate GLT-1 expression levels twofold above young and control white matter, respectively, raising the question how a similar modification of GLT-1 levels causes increased vulnerability to ischemia in aging while providing protection against ischemia with HDAC inhibition. The explanation for this perplexing issue lies in the principle of GLT-1 function. The axon protective effect of HDAC inhibition correlates with preservation of mitochondria in aging MONs comparable to young MONs (Fig. 8.11), suggesting that conservation of mitochondrial function and ATP levels keep the pump in the forward direction and switching the potential of GLT-1 function to attenuate glutamate accumulation. Therefore an increase in GLT-1 levels acts as an important molecular switch between protection and injury. It is therefore of great interest that HDAC inhibition offers a pharmacological strategy to upregulate

GLT-1, a target gene critically involved in various disease conditions including stroke (Baltan et al. 2011b), so as to effectively ameliorate excitotoxicity (Brevig et al. 2004; Wu et al. 2008; Allritz et al. 2009).

8.5 Aging Impairs White Matter Mitochondrial Dynamics

A variety of neurological diseases, as well as aging, is associated with defects in mitochondrial fusion and distribution (Karbowski and Youle 2003; Chen et al. 2007). Older neurons become more susceptible to glutamate excitotoxicity due to loss of mitochondrial membrane depolarization and increased reactive oxygen species (ROS) generation, leading to reduced energy supply (Parihar and Brewer 2007). Mitochondrial function appears to decline in older animals, presumably due to reduced ATP production. This has been demonstrated in cardiac (Lesnefsky et al. 2001), liver (Selzner et al. 2007), and brain (Toescu 2005) cells. Ion transport accounts for about 50 % of all ATP utilization and Na^+/K^+ ATPase activity alone is responsible for the majority of this consumption (Erecinska and Silver 1994). A loss of ATP reserve, diminishing the activity of this key enzyme with advanced age, is a plausible contributor to increased vulnerability of aging white matter to ischemia (Scavone et al. 2005).

Consistent with this axon function, when transiently challenged with OGD, it is slower to restore normal ion gradients (Fig. 8.4a), permitting pathological processes related to ion derangement to operate for longer periods; hence, reversing Na^+-dependent glutamate transporters earlier (Fig. 8.7a), and producing more injury in older MONs (Baltan et al. 2008). Because reversal of the Na^+-dependent transporter dictates when glutamate is released (Fig. 8.2), earlier release of glutamate implies accelerated Na^+ overload due to decreased tolerance to energy deprivation in aging white matter. The disadvantage of compromised ATP levels in older animals is further verified by better recovery of white matter function in older animals when OGD is induced at lower temperature (Baltan et al. 2008).

Excitotoxicity and elevated Ca^{2+} induce marked changes in mitochondrial structure, slowing their motion (Rintoul et al. 2003; Barsoum et al. 2006; Chang and Reynolds 2006) and generating ROS in neurons (Nicholls et al. 2007). In young white matter, excitotoxicity due to overactivation of AMPA/kainate receptors loads mitochondria with Ca^{2+} and fission is enhanced (Fig. 8.10-*insets*), associated with loss of fluorescence of mitochondria genetically tagged with CFP. A Ca^{2+} overload activates neuronal NOS to produce nitric oxide and ROS, which are proposed as diffusible second messengers linking oligodendrocyte excitotoxicity to axon injury (Matute et al. 2001; Ouardouz et al. 2006). Axon function directly correlates with tissue energy reserves, since Na^+–K^+ ATPase activity is intimately dependent on ATP levels. As a result, OGD causes a significant reduction in ATP levels and CFP + mitochondria, which could be prevented by AMPA/kainate receptor blockade (Fig. 8.10).

Expectedly, the compromised energy status of aging white matter is intimately related to mitochondrial dynamics. Imaging axonal mitochondria in aging MONs in

the mito-CFP mouse (Fig. 8.11-*insets*) revealed that mitochondrial shape, size, and location and response to OGD differ compared to young axons. Mitochondria in aging axons appear more abundant based on the increased levels of CFP fluorescence with longer and thicker mitochondria compared to young MONs (Fig. 8.11). The regulated process of mitochondrial fusion and fission controls the spatiotemporal properties of mitochondrial Ca^{2+} responses and the physiological and pathophysiological consequences of Ca^{2+} signals (Szabadkai and Rizzuto 2004; Szabadkai et al. 2004). By enhancing fusion or inhibiting fission, elongated mitochondria possibly absorb Ca^{2+}, efficiently preventing n-NOS activation and subsequent ROS production (Cheung et al. 2007). Age-specific mitochondrial fusion (Fig. 8.11) is accompanied by a modification in the levels of mitochondrial shaping proteins such as Mfn1, Mfn2, Opa1, and Drp-1 (unpublished data). However, this age-related adaptive reorganization of mitochondria becomes detrimental under ischemic conditions. Ischemia in aging white matter further exacerbates mitochondrial fusion, presumably due to age-dependent drop in Drp-1 and age-dependent arrest of mitochondrial motility with exposure to glutamate (Chang and Reynolds 2006; Parihar and Brewer 2007). Mitochondria fuse to collectively counteract the already increased excitotoxicity and Ca^{2+} load with aging, and this age-related change in mitochondrial dynamics hinders ATP production (unpublished data). Because basal ROS generation is already elevated with aging, further increases in ROS accumulation under ischemic conditions contribute to increased vulnerability to ischemia. Conditions that ameliorated excitotoxicity in older MONs, such as blockade of AMPA/kainate receptors with 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f] quinoxaline-7-sulfonamide (NBQX) (Baltan et al. 2008) significantly preserved CFP + mitochondria and improved axon function recovery in older MONs. Therefore if excitotoxicity and mitochondrial dysfunction act synergistically to mediate injury, amelioration of excitotoxicity must preserve mitochondrial function and ATP levels, which in turn maintains the function of GLT-1 in a forward direction, providing an energy-efficient cycle to minimize glutamate accumulation.

These results were further verified in experiments investigating the protective effects of Class I HDAC inhibitors in aging white matter (Baltan et al. 2011a, b—see above). Consequently, by delaying and reducing glutamate accumulation, HDAC inhibition interrupts the merging point of excitotoxicity to subsequent oxidative injury in aging axons such that the protective action associated with HDAC inhibition correlates with preservation of mitochondrial structure/function in axons and ATP levels (Baltan et al. 2013).

8.6 Conclusions

The main goal of this chapter is to establish the proof of principle that central nervous system white matter becomes inherently more susceptible to an ischemic attack with age and that enhanced excitotoxicity and impaired mitochondrial dynamics underlie the increased vulnerability of aging white matter to ischemia.

Predictably, age-related changes in the molecular architecture of white matter dictate the predominant injury mechanisms and determine the functional outcome. Consequently, protective interventions in young white matter, such as removal of extracellular Ca^{2+}, reduce functional recovery in aging axons (Fig. 8.5b). Together with the observation that blockade of reversal of NCX fails to protect function in older mice (Fig. 8.5a; Baltan et al. 2008), these results propose a diminished role for the ionic pathway with aging. On the other hand, aging causes a prominent increase in the expression pattern of glutamate, GS, and GLT-1 levels that extend to additional structures in white matter. Na^+-dependent and Ca^{2+}-dependent mechanisms, involving astrocytes, oligodendrocytes expressing EAAC1 plasma membrane glutamate transporter (Arranz et al. 2008), microglia expressing GLT-1 or perhaps axons with their vesicular glutamate transporters (Kukley et al. 2007; Ziskin et al. 2007), all may become additional sites of glutamate release with aging and contribute to increased excitotoxicity. These modifications may imply an age-related adaptive mechanism to maintain glutamate signaling and homeostasis. However, during an ischemic episode these adaptive changes act against the tissue and expedite and aggravate glutamate release (Fig. 8.7a) and expand the excitotoxic injury into the recovery period. Interestingly, AMPA/kainate receptors (Fig. 8.6b) mediate the ischemic injury across age groups, indicating that certain steps of injury are preserved irrespective of age. In young white matter, activation of either AMPA/kainate receptors loads mitochondria with Ca^{2+} and fission is enhanced due to abundant Drp-1 levels. Ca^{2+} overload activates NOS to produce NO and ROS, which are proposed as diffusible second messengers to link oligodendrocyte excitotoxicity to axon injury (Matute et al. 2001; Ouardouz et al. 2006). Mitochondria fuse to collectively counteract the already increased excitotoxicity and Ca^{2+} load with aging, and this age-related change in mitochondrial dynamics hinders ATP production and increases NO and ROS production. These challenge the Na^+/K^+ ATP pump to maintain axon excitability and the associated rise in Na^+ levels switches GLT-1 to function in the reverse direction to release glutamate. The isoforms of NOS that mediate ischemic injury and their cellular expression pattern in white matter as function of age remain unknown.

Moreover, aging modifies cell- and region-specific organization of white matter expression of Class 1 HDACs and these cellular adjustments may further contribute to increased vulnerability of aging white matter to ischemia. An age-specific understanding of the mechanisms of injury processes in white matter is essential to design dynamic therapeutic approaches for stroke victims. Therefore an age-dependent reduction in mitochondrial bioenergetics may underlie the increased vulnerability of aging axons to ischemia.

Acknowledgements I thank the past and present members of my laboratory for their many contributions. The author gratefully acknowledges the help with the multidiolistic technique by Jaime Grutzendler. Continuing studies are supported by a grant from NIA-NIH and by a gift from Rose Mary Kubik.

Conflict of Interest The author declares no conflicts of interest.

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Chapter 9
Role of Astrocytes in Delayed Neuronal Death: GLT-1 and its Novel Regulation by MicroRNAs

Yi-Bing Ouyang, Lijun Xu, Siwei Liu, and Rona G. Giffard

Abstract Astrocytes have been shown to protect neurons from delayed neuronal death and increase their survival in cerebral ischemia. One of the main mechanisms of astrocyte protection is rapid removal of excess glutamate from synaptic sites by astrocytic plasma membrane glutamate transporters such as GLT-1/EAAT-2, reducing excitotoxicity. Astrocytic mitochondrial function is essential for normal GLT-1 function. Manipulating astrocytic mitochondrial and GLT-1 function is thus an important strategy to enhance neuronal survival and improve outcome following cerebral ischemia. Increasing evidence supports the involvement of microRNAs (miRNA), some of them being astrocyte-enriched, in the regulation of cerebral ischemia. This chapter will first update the information about astrocytes, GLT-1, astrocytic mitochondria, and delayed neuronal death. Then we will focus on two recently reported astrocyte-enriched miRNAs (miR-181 and miR-29 families), their effects on astrocytic mitochondria and GLT-1 as well as on outcome after cerebral ischemia.

Keywords Astrocyte • Glutamate transporter • GLT-1 • MicroRNA • miR-181 • miR-29 • Cerebral ischemia • Delayed neuronal death

9.1 Introduction

Cerebral ischemia is a key pathological event in several disease states; stroke, cardiac arrest and resuscitation, and head trauma being the most common ones. Stroke is one of the leading causes of death worldwide and the leading cause of long-term neurological disability. Although many clinical stroke trials have been completed,

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_9

the only efficacious treatment identified to date is thrombolysis (Blakeley and Llinas 2007). Similarly, the cerebral injury resulting from cardiac arrest and resuscitation leads to death and neurological impairment, but clinically this has only been effectively treated with hypothermia (Bernard et al. 2002; THACAS 2002). Lack of consideration of the role of astrocytes, the importance of cell-cell interactions, the complex interplay among signaling pathways, and poorly defined treatment windows for specific targets are thought to be factors in the clinical failure of many potential neuroprotective strategies.

Astrocytes play many key roles both in normal and pathological central nervous system (CNS) functioning. Astrocytes are central to potassium homeostasis, neurotransmitter uptake, synapse formation and regulation of the blood brain barrier. Astrocytes are the most abundant brain cell type, and in addition to multiple important homeostatic roles, they are essential to CNS development, helping to organize the structural architecture of the brain and communication pathways, and modulating neuronal plasticity (for recent reviews see Sofroniew and Vinters (2010); Barreto et al. (2011); Clarke and Barres (2013)). The importance of astrocytes for neuroprotection after cerebral ischemia has been reviewed by our group and others recently (Sofroniew and Vinters 2010; Barreto et al. 2011).

MicroRNAs (miRNAs) are a novel and abundant class of 19- to 22-nucleotide noncoding RNAs that control gene expression primarily at the posttranscriptional level. The most common mechanism is for miRNAs to bind to messenger RNAs (mRNAs), known as their targets, based on sequence complementarity and direct the degradation or repression of translation of the targeting mRNAs. Recent evidence increasingly supports a role for miRNAs in response to cerebral ischemia, as we have reviewed recently (Ouyang et al. 2013a). The fast posttranscriptional effect of miRNAs, and their ability to simultaneously coordinate regulation of many target genes, suggests that miRNAs may have greater therapeutic potential as candidates for the treatment of stroke than therapies targeting a single gene by direct transcriptional control. Numerous miRNAs are expressed in a cell-specific manner and some are enriched in astrocytes (Smirnova et al. 2005; Ouyang et al. 2013b).

In this chapter we will summarize the central role of astrocytes in cerebral ischemia focusing on astrocytic glutamate transporters and then explore the novel regulation of astrocytes by astrocyte-enriched miRNAs.

9.2 Delayed Neuronal Death

The concept of “delayed neuronal death” was described with the development of forebrain ischemia (Kirino 1982). Brief global cerebral ischemia, as seen with cardiac arrest and resuscitation, and modeled in rodents as forebrain ischemia or four vessel occlusion, causes delayed loss of neurons in cornu ammonis 1 (CA1) pyramidal neurons, a slow process taking 4–7 days before the final morphologic outcome (see middle panel in Fig. 9.1a) is observed. Later the concept was extended to focal ischemia referring to neuronal death in the peri-ischemic (penumbral) area (Liu et al. 1999). In focal cerebral ischemia, modeled commonly in rodents as transient middle cerebral

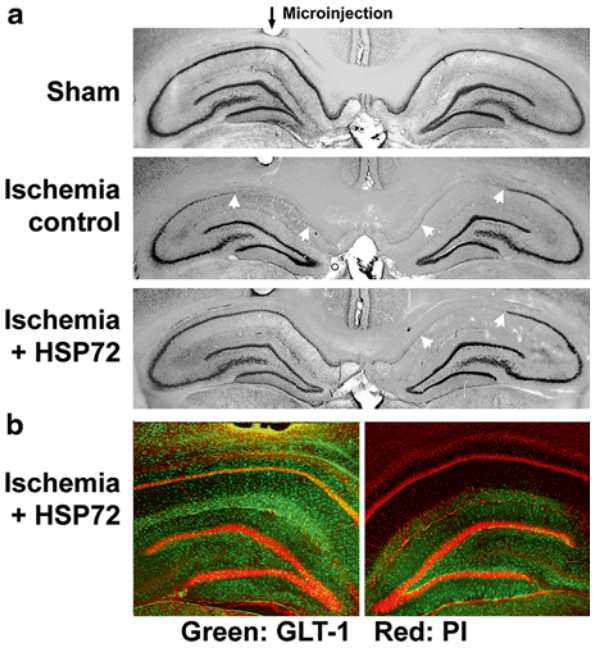

Fig. 9.1 Targeted overexpression of HSP72 in astrocytes reduces delayed CA1 neuronal death and preserves GLT-1 expression. (**a**) GFAPp-HSP72 or control DNA was injected stereotaxically just above CA1 on one side (*black arrow* for microinjection track) 2 days before forebrain ischemia. Selective loss of CA1 hippocampal neurons (between *white arrows* in *middle* and *lower* panels) was observed at 7 days reperfusion by cresyl violet staining. The loss of CA1 hippocampal neurons was significantly reduced on the HSP72 injected side compared with the noninjected side (*lower panel*) or the control DNA injected hippocampus (*middle panel*). (**b**) GLT-1 immunoreactivity was significantly greater after GFAPp-HSP72 injection compared with control after 10 min ischemia and 5 h reperfusion. Propidium iodide (PI) labels cell nuclei. Modified from Figs. 2 and 3 in Xu et al. (2010)

artery occlusion, the neurons die within hours in the center of the ischemic territory (ischemic core) forming the initial area of infarction while the neurons in the adjacent penumbra area may either survive via induction of pro-survival signaling pathways, or die at a later period of reperfusion (also called delayed neuronal death) via initiation of cell death pathways (Ferrer and Planas 2003).

The form of delayed neuronal death is generally believed to be programmed cell death or apoptosis (Nitatori et al. 1995; Kirino 2000). The mechanisms of delayed neuronal death are complex but one of the key mechanisms is prolonged excess release of glutamate, which activates neurotransmitter receptor ion channels and induces intracellular calcium overload (Choi 1996). Excessive increases in mitochondrial matrix calcium alter the function and permeability of mitochondria and finally lead to opening of the mitochondrial permeability transition pore (Ouyang et al. 1997), causing the release of cytochrome c (Ouyang et al. 1999) and other pro-apoptotic factors into the cytoplasm. The released cytochrome c activates caspase-3, one of the executioner caspases to initiate apoptotic cell death (for a recent review, see Ouyang and Giffard 2012).

9.3 Astrocytes and Their Vulnerability to Ischemia

For decades, astrocytes have been considered to be non-excitable support cells that are relatively resistant to ischemic injury. This view has changed radically during the past 20 years. Multiple essential functions are performed by astrocytes in normal brain. Astrocytes are dynamically involved in synaptic transmission, metabolic and ionic homeostasis, inflammatory response, antioxidant defense, trophic support of neurons, as well as in the establishment and maintenance of the blood brain barrier (Kettenmann and Ransom 2005). Advances in our understanding of astrocytes include new observations about their structure, organization, and function. Hippocampal astrocytes occupy exclusive, non-overlapping territories (Bushong et al. 2002). This pattern develops in the early postnatal period in parallel with neuronal and vascular territories; each astrocyte interfaces with the microvasculature and thousands of synapses (Bushong et al. 2004). That astrocytes both respond to signals from and signal actively to neurons, endothelium, and microglia is now well accepted (for reviews see Ransom et al. (2003); Volterra and Meldolesi (2005)).

It has often been said that neurons are the cells most vulnerable to ischemia. This view is based primarily on observations that astrocytes in culture show greater resistance than neurons to some ischemia-like insults (Goldberg and Choi 1993; Xu et al. 2001) and that brief forebrain ischemia apparently results in selective loss of neurons. However, vulnerability of cultured astrocytes is markedly increased by acidic conditions relevant to ischemic injury (Giffard et al. 1990; Bondarenko and Chesler 2001). Several laboratories have pointed out that glial cells may be injured more readily than previously thought, sometimes before neuronal damage is obvious (Petito et al. 1998; Liu et al. 1999; Zhao et al. 2003b). Astrocytic processes were fragmented and mitochondria inhibited after 15 min exposure to acidic conditions in hippocampal slice cultures (Hulse et al. 2001). Astrocytic demise was found to precede delayed neuronal death after focal ischemia and astrocytic glial fibrillary acidic protein (GFAP) mRNA declined more quickly than neuronal glucose transporter 3 (GLUT3) mRNA in the ischemic core (Liu et al. 1999). Astrocytic demise was also found early after traumatic brain injury (Zhao et al. 2003b). In intact brain astrocytes are highly metabolically active, while astrocytes in the absence of neurons have few energy demands. We demonstrated that providing astrocytes a transportable substrate, aspartate, markedly increased their vulnerability to ischemia (Volobouéva et al. 2013). In addition, loss of glutamate transport activity and immunoreactivity for the astrocyte-specific plasma membrane glutamate transporter-1 (GLT-1) in astrocytes occurs at early reperfusion times following forebrain ischemia, hours to days before the death of CA1 neurons (Ouyang et al. 2007).

9.4 Astrocytic Plasma Membrane Glutamate Transporters

Extracellular glutamate is maintained at low levels, and rapid removal of glutamate from the extracellular space is required for the survival and normal function of neurons. To date, five brain plasma membrane glutamate transporters have been cloned,

excitatory amino acid transporters 1–5 (EAAT1–5), of which GLT-1/EAAT2 and GLAST/EAAT1 are expressed in astrocytes. Although glutamate transporters are expressed by all CNS cell types, astrocytes are primarily responsible for glutamate uptake (Anderson and Swanson 2000). Neuronal vulnerability to glutamate is 100-fold greater in astrocyte-poor cultures than in cultures with abundant astrocytes (Rosenberg and Aizenman 1989), demonstrating that uptake by astrocytes in particular is key to neuronal survival. Reduction or loss of astroglial transporters EAAT2/GLT-1 or EAAT1/GLAST, but not the neuronal subtype EAAT3/EAAC1 (excitatory amino acid carrier 1), led to a tonic increase in extracellular glutamate concentration and subsequent neurodegeneration (Rothstein et al. 1996; Mitani and Tanaka 2003) and increased ischemic injury after focal cerebral ischemia in rats (Watase et al. 1998; Fukamachi et al. 2001; Rao et al. 2001). GLT-1 appears to be especially important in the hippocampus, because mice lacking GLT-1 showed spontaneous seizures, selective hippocampal neurodegeneration, and exacerbation of acute cortical injury (Tanaka et al. 1997), whereas mice deficient in GLAST were more susceptible to cerebellar injury (Watase et al. 1998). We will focus on GLT-1 in the rest of this chapter.

Ceftriaxone treatment, which induces GLT-1 expression, reduces CA1 delayed neuronal death in hippocampal slice culture and in transient forebrain ischemia (Ouyang et al. 2007). Selective overexpression of GLT-1 in astrocytes provided neuroprotection from moderate hypoxia-ischemia (Watase et al. 1998). Targeted overexpression of GLT-1 reduces ischemic brain injury in a rat model of focal ischemia (Harvey et al. 2011).

9.5 Oxidative Stress and GLT-1

Oxidative stress and inhibition of glutamate transport has been suggested to form a vicious circle in ischemia whereby increased oxidative stress impairs glutamate transport, which further exacerbates excitotoxicity which leads to more oxidative stress (Trotti et al. 1998). There is evidence that several different oxidants, including peroxide, superoxide, and treatment with amyloid β peptide, can reduce glutamate transport activity of GLT-1 (Harris et al. 1996; Trotti et al. 1998; Lauderback et al. 2001). Several glutamate transporters, including GLT-1, have redox sensitive sulfhydryl regulation of activity (Trotti et al. 1997), but strong oxidants such as peroxynitrite can inhibit glutamate transport in a way that is not fully reversed by dithiothreitol, suggesting reaction with other sites such as histidine or lysine may also occur.

Work by Weiss and colleagues (Rao et al. 2003) showed that motor neurons only died after glutamate transport activity was lost in the astrocytes surrounding the neurons. This work suggested that oxidative stress eventually compromised astrocyte function, which then precipitated neuronal death. Oxidative stress has also been shown to downregulate astrocytic glutamate uptake in ammonia-induced toxicity (Jayakumar et al. 2006). Oxidative stress is known to contribute to damage and misfolding of proteins, including glutamate transporters (Lauderback et al. 2001).

In CA1 after transient forebrain ischemia, increased generation of reactive oxygen species (ROS) compromises astrocyte function, which in turn compromises the ability of the CA1 pyramidal neurons to survive (Ouyang et al. 2007).

9.6 Mitochondria and GLT-1

Mitochondria play a central role in normal neuronal cell function by controlling cellular energy metabolism and producing ROS, but also as a central regulator of cell death via release of apoptotic factors into the cytosol (Murphy et al. 1999; Beal 2000; Kroemer and Reed 2000; Ravagnan et al. 2002). The mitochondrial respiratory chain, especially complexes I and III, is the major site of ROS production (Lin and Beal 2006). Glutamate uptake in the CNS by high-affinity sodium dependent transporters such as GLT-1 requires large amounts of energy, largely adenosine triphosphate (ATP) produced from mitochondrial respiration. It may be noted that glycolytically/glycogenolytically derived ATP may play an important role for maintenance of glutamate uptake capacity in astrocytes since glycolytic enzymes, the sodium-potassium ATPase and the glutamate transporters are tightly coupled (Volobоueva et al. 2007; Parpura et al. 2012). Oxygen deprivation or mitochondrial inhibition causes reductions of ATP in brain or cultured astrocytes, and produces reductions in glutamate transport capacity (Longuemare et al. 1994; Swanson et al. 1994; Ouyang et al. 2007). In the extreme case of ATP depletion, as occurs during severe ischemia, all of these membrane gradients collapse. This results not only in cessation of uptake, but also efflux of glutamate via uptake reversal (Szatkowski et al. 1990).

9.7 Astrocytic Mitochondria as Targets for Protection

Several studies in the literature suggest that neuronal mitochondria and astrocytic mitochondria respond differently to ischemic stress. Reichert et al. (2001) found that although co-cultured neurons and astrocytes subjected to combined oxygen glucose deprivation both showed marked loss of mitochondrial membrane potential, the astrocytes were able to recover over a period of more than an hour of reperfusion, while the neurons did not. Bambrick and coworkers have also pointed out differences between neuronal and astrocytic mitochondria and suggested that astrocyte mitochondria might be a potential target for treatment (Bambrick et al. 2004). Mitochondrial dysfunction has been implicated in the selective vulnerability of CA1 cells after ischemia, because cell death in this region after global ischemia is inhibited by cyclosporin A, a known inhibitor of the calcium-induced mitochondrial permeability transition in brain mitochondria (Uchino et al. 1995). The observation that cyclosporin A can protect mitochondria from calcium insult in astrocytes, but

not in neurons (Bambrick et al. 2006; Kahraman et al. 2011), suggests that astrocyte mitochondria are central to protection by this drug in vivo. Methods to protect astrocyte mitochondria are described next.

9.7.1 Molecular Chaperones

Originally molecular chaperones were defined as functionally related groups of proteins that assist in the folding or unfolding of proteins, the sequestration of denatured proteins, and the assembly or disassembly of multiprotein complexes. Recently, a more complex role of these chaperones has been recognized, that of functioning as a chaperone network, contributing to organelle interactions. Chaperones act within and between organelles to integrate and support essential cellular functions (Ouyang and Giffard 2012). The heat shock protein 70 kDa family (HSP70) is the most extensively studied ATP-dependent chaperone family, and includes a cytosolic form HSP73 (also HSC70), an inducible cytosolic form HSP72, a mitochondrial form HSP75/GRP75/mortalin, and an endoplasmic reticulum (ER) form, GRP78/BIP. HSP70 family members that are involved in ER-mitochondria crosstalk and calcium transfer during cerebral ischemia (Ouyang and Giffard 2012).

Work from our lab and several others has demonstrated neuroprotection from ischemic brain injury with overexpression of chaperones and co-chaperones, both in animal stroke models and in cultured brain cells (for a recent review, see Ouyang and Giffard (2013)). Our group has shown that selective overexpression of HSP72 in astrocytes reduces death of CA1 neurons (Fig. 9.1a). HSP72 was genetically targeted for expression in astrocytes using the astrocyte-specific human GFAP promoter (Xu et al. 2010). Protection was accompanied by preservation of astrocytic GLT-1 (Fig. 9.1b) and mitochondrial respiratory complexes I and IV activities, and reduced oxidative stress in the CA1 region (Xu et al. 2010).

9.7.2 Superoxide Dismutase 2

Superoxide dismutases (SODs) comprise a family of metal-containing proteins that catalyze dismutation of superoxide radicals. Among the SOD family members, SOD1/CuZn-SOD is a copper and zinc-containing homodimer, primarily localized in the cytoplasm; SOD2/Mn-SOD is a manganese-containing enzyme exclusively localized in mitochondria; and SOD3/EC-SOD is a copper- and zinc-containing tetramer, present largely in the extracellular space (Zelko et al. 2002). SOD overexpression provides direct neuroprotection, glioprotection, and protection of the blood brain barrier (Jung et al. 2009). Reduction of SOD2 activity was shown to increase neuronal death induced by transient cerebral ischemia (Chan 2005). We have shown that targeting SOD2 overexpression to hippocampal astrocytes improves the survival of CA1 neurons and reduces ROS production and GLT-1 loss after transient forebrain ischemia (Xu et al. 2010).

9.7.3 BCL2 Family

The BCL2 protein family, central regulator of life/death decisions in cells (Adams and Cory 2007) largely influences cell death through regulating mitochondrial membrane integrity and function. The BCL2 protein family is classified into three subgroups according to structural homology (BCL2 homology [BH] domains): the pro-survival proteins of the BCL2 family (BCL2, BCL-xL, BCL-w, MCL1, and A1), the multi-domain pro-apoptotic proteins BAX and BAK and the BH3-only pro-apoptotic proteins (BIM, PUMA, BID, BAD, BIK, BMF, HRK, NOXA). In response to diverse intracellular and extracellular signals, the cell's decision to undergo apoptosis is determined by interactions between these three groups within the BCL2 protein family.

We and others have reported that overexpressing pro-survival BCL2 family members protects against cerebral ischemia in vivo (Kitagawa et al. 1998; Zhao et al. 2003a) and in vitro (Xu et al. 1999). Neuroprotection involved maintaining mitochondrial function (for review see Ouyang and Giffard (2004)). Decreased BCL2 and increased BAX and BH3-only proteins were reported in CA1 neurons after global ischemia (Martinez et al. 2007). After global ischemia PUMA (p53-upregulated modulator of apoptosis) is upregulated in CA1 neurons, localizes to mitochondria, and binds BCL-xL and BAX (Niizuma et al. 2009). Selective CA1 injury induced by proteasomal inhibition was strongly reduced in PUMA knockout mice (Bonner et al. 2010; Tsuchiya et al. 2011). Interestingly anti-apoptotic protein BCL2 also exists in mitochondrial associated membranes (Ouyang et al. 2013a), a site of mitochondria and ER interaction, and affects ER and mitochondrial calcium homeostasis (Foyouzi-Youssefi et al. 2000).

In summary we propose a mechanism of selective astrocyte dysfunction as illustrated in Fig. 9.2. Differences in mitochondrial response lead to greater production of ROS in astrocytes, which leads to greater energetic compromise, oxidative damage, and loss of GLT-1 function. The impaired astrocytes may be unable to carry out many of their normal functions, such as glutamate uptake, antioxidant defense, and regulation of extracellular ions, which eventually leads to neurotoxicity and delayed neuronal death. Protecting astrocytic mitochondrial function by increasing expression of HSP70 family members, SOD2, or anti-apoptotic members of the BCL2 family protects mitochondria to better maintain energy production and reduces ROS, maintaining GLT-1 function. By decreasing extracellular glutamate GLT-1 keeps CA1 neurons alive.

Despite the importance of GLT-1 in both physiological (Lopez-Bayghen and Ortega 2011) and pathological (Kim et al. 2011) conditions, relatively little is known about the regulation of GLT-1 expression, especially by miRNAs. Global disruption of astroglial miRNA biogenesis through selective deletion of Dicer in cerebellar astrocytes significantly reduces GLT-1 expression (Tao et al. 2011). Recently a neuron-specific miRNA-mediated regulation of GLT-1 protein expression has been described. Neuronal exosomes containing miRNA 124a regulate astrocytic GLT-1

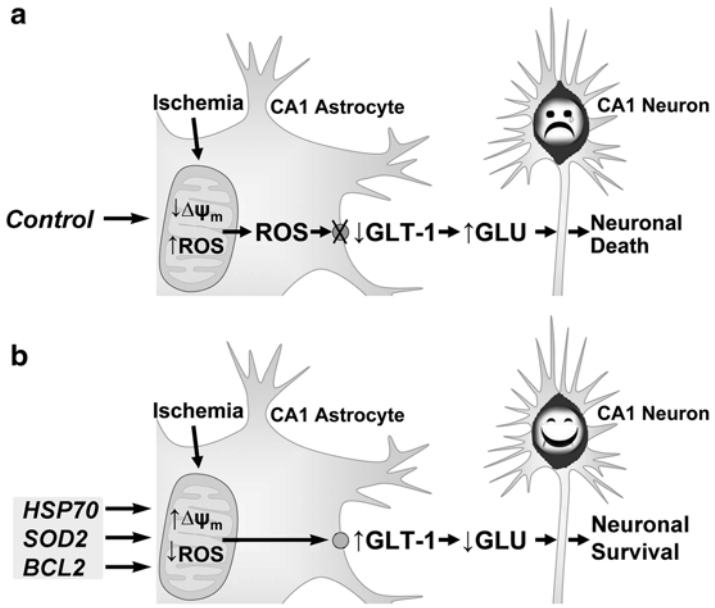

Fig. 9.2 Proposed mechanism of astrocyte contribution to delayed neuronal death. (**a**) Transient forebrain ischemia selectively decreases mitochondrial membrane potential ($\Delta\psi_m$) and increases ROS in CA1 astrocytes. The greater production of ROS leads to astrocyte impairment including oxidative damage of GLT-1 on the astrocyte membrane. The loss of GLT-1 contributes to the increase of extracellular glutamate and excitotoxicity of the pyramidal neurons. (**b**) By protection of astrocytic mitochondria using HSP72 or SOD2 or anti-apoptotic members of the BCL2 family, astrocytic GLT-1 is preserved and extracellular glutamate is decreased, which results in survival of CA1 neurons. Modified from Fig. 6 in Ouyang et al. (2007)

expression by transfer from neuron into astrocytes (Morel et al. 2013). However the results in the paper indicate that upregulation of GLT-1 protein expression by miR-124a is likely to be indirect. In the following section we will focus on astrocyte-enriched miRNAs and their possible role in regulating GLT-1.

9.8 Astrocyte-Enriched miRNAs as Potential Targets for Protection

We demonstrated recently that two brain-enriched miRNAs, miR-181a (Ouyang et al. 2012a) and miR-29a (Ouyang et al. 2013b), are involved in the regulation of outcome following cerebral ischemia. Interestingly, the literature and our experiments suggest that both the miR-181 and miR-29 families are astrocyte-enriched (Hutchison et al. 2013; Ouyang et al. 2013b).

9.8.1 miR-181 Family

The miR-181 family consists of four mature members (miR-181a, miR-181b, miR-181c, and miR-181d) from three polycistronic miRNA genes—miR-181a-1/b-1, miR-181a-2/b-2, and miR-181c/d. The miR-181 family was reported first as an important regulator of immune cell development (Chen et al. 2004). These earlier studies found that the miR-181 family, especially miR-181a and miR-181b, are enriched in brain (Chen et al. 2004; Miska et al. 2004) and their aberrant expression has been associated with brain diseases. miR-181a and miR-181b are reduced in human gliomas and glioma cell lines and expression is negatively correlated with tumor grade (Shi et al. 2008). miR-181a sensitizes human malignant glioma cells to radiation by targeting anti-apoptotic protein BCL2 (Chen et al. 2010). It was found recently that expression of the miR-181 family was strongly enriched in cultured astrocytes compared to neurons derived from neural stem cells (Hutchison et al. 2013). miR-181 family members were expressed at significantly higher levels in adult cortex relative to embryonic telencephalon, an early developmental stage prior to astrogenesis.

It was demonstrated by our group that miR-181a increased in vulnerable regions such as the ischemic core in focal ischemia (Ouyang et al. 2012b) or the hippocampal CA1 region after global ischemia (Moon et al. 2013), and decreased in the ischemia-resistant areas, the penumbra and hippocampal dentate gyrus, respectively. Antagomir to miR-181a reduced miR-181a levels in the brain and reduced infarct size in focal ischemia (Ouyang et al. 2012b) and CA1 neuronal loss in global cerebral ischemia (Moon et al. 2013). Anti-miR-181a reduced miR-181a levels in the brain and reduced infarct size in focal (Ouyang et al. 2012b) and CA1 neuronal loss in global cerebral ischemia (Moon et al. 2013). Furthermore, transfecting primary cultures with miR-181a inhibitor led to protection of astrocytes, but not neurons from ischemia-like stresses (Ouyang et al. 2012a; Moon et al. 2013).

Recently, miR-181a was shown to directly target molecular chaperone GRP78 (Ouyang et al. 2012b), anti-apoptotic members of the BCL2 family BCL2 and myeloid cell leukemia (MCL) 1 (Ouyang et al. 2012a), and X-linked inhibitor of apoptosis (XIAP) (Hutchison et al. 2013) as well as some target proteins involved in controlling mitochondrial function, redox state, and inflammatory pathways (for recent reviews see Ouyang and Giffard (2013); Ouyang et al. (2013a)). Although miR-181a antagomir reduces miR-181a levels and significantly inhibits the decrease of GLT-1 after forebrain ischemia, miR-181a does not directly target GLT-1 (Moon et al. 2013). One interesting observation is the difference in effect of reducing miR-181a in different cell types. Reducing miR-181a increased BCL2 as one of the targets and increased survival of primary astrocytes (Ouyang et al. 2012b) while it failed to significantly change levels of BCL2 and did not change outcome in primary neurons after ischemia-like injury in vivo (Moon et al. 2013).

Some evidence supports the concept that not only mature miRNA but also pri-/pre-miRNA functions in target recognition and repression (Chen 2013). One relevant observation is that although miR-181a-2/b-2 and miR-181a-1/b-1 produce identical mature miR-181a and miR-181b, deletion of miR-181a-1/b-1, but

not miR-181a-2/b-2, selectively inhibits tumor transformation induced by Notch oncogenes (Fragoso et al. 2012). Because of the different effects of miR-181a it is interesting to further assess which miR-181a gene(s) (miR-181a-1/ or a-2) is expressed in brain cells and if pri/pre-miR-181a are also involved in cell type specific response in astrocytes compared to neurons.

9.8.2 miR-29 Family

The miR-29 family consists of three members (a, b, and c) that map to two distinct genomic loci in clusters (Ouyang et al. (2013b)): miR-29 a/b-1 on chromosome 6 on mouse and 7 in human, and miR-29c/b-2 on chromosome 1 in both mouse and human. It has been demonstrated that miR-29a/b-1 is developmentally regulated in mouse brain with the highest expression observed in adults (Hebert et al. 2008; Kole et al. 2011). During brain development miR-29 was found more strongly expressed in astrocytes than neurons using primary cultures (Smirnova et al. 2005). The investigators used an equimolar mixture of the miR-29b and miR-29c isoforms as a probe for Northern blot analysis of miRNA expression in mouse brain development. Recently, using qPCR we compared postnatal brain, primary neuron, and astrocyte cultures, and showed the strongly astrocytic expression of miR-29a (Ouyang et al. 2013b). While miR-29a increased in brain, astrocytes, and neurons with development, at each time point levels of miR-29 in cultured astrocytes were 20–40 times higher than in cultured neurons, and levels in brain tissue were about 1/2 that seen in cultured astrocytes. This suggests that the increase of miR-29a in the brain with development may largely reflect increases in astrocyte miR-29a.

Interestingly miR-29a changed in the opposite direction compared to miR-181a after transient forebrain ischemia, showing a decrease in CA1 and increase in dentate gyrus area (Ouyang et al. 2013b). The protective effect of miR-29a on CA1 delayed neuronal death was demonstrated after forebrain ischemia by overexpressing miR-29a (Ouyang et al. 2013b).

The study of miR-29 began primarily in cancer research and focused on its role in regulation of apoptotic pathways. However, a question that has stirred controversy for several years is whether miR-29 is pro-survival or pro-apoptotic (Pekarsky et al. 2006). While miR-29 expression is elevated in some cancers where it appears to function as an oncogene (Gebeshuber et al. 2009; Han et al. 2010), others have found miR-29 to have tumor suppressor functions (Pekarsky et al. 2006; Wang et al. 2008). This question is not only relevant in cancer research but is also important in ischemia research. While downregulation of miR-29 protected hearts against ischemia-reperfusion injury (Ye et al. 2011), upregulation of miR-29 protected neurons from apoptosis during neuronal maturation (Kole et al. 2011) and forebrain cerebral ischemia (Ouyang et al. 2013b). Luciferase target assays conducted in our lab indicate that the miR-29 targets both pro- and anti-apoptotic BCL2 family members (Ouyang et al. 2014b). The results strongly suggest that the reported pro-apoptotic and anti-apoptotic effects of miR-29 result from different targets of

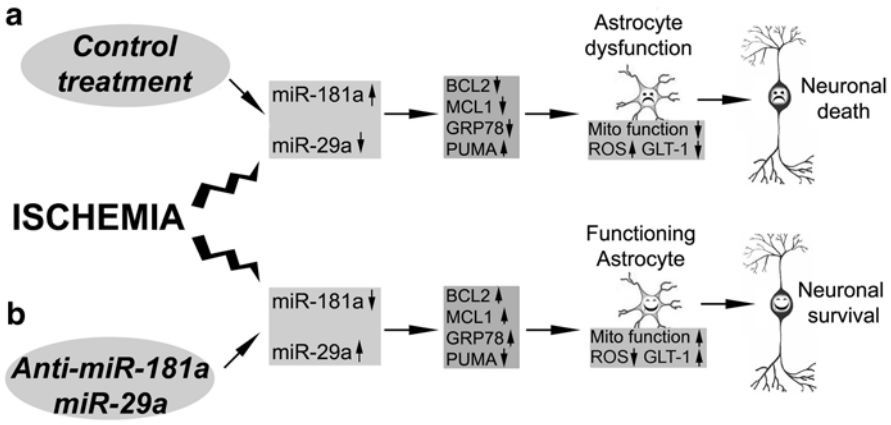

Fig. 9.3 Protecting astrocytes using astrocyte-enriched miRNAs or their antagomirs improves neuronal survival after cerebral ischemia. (**a**) Cerebral ischemia induces changes in astrocyte-specific miRNAs (increase in miR-181a and decrease in miR-29a) in vulnerable regions of the brain. They target specific proteins that disturb astrocyte mitochondrial function, increase production of ROS and damage GLT-1 in astrocytes, producing "unhappy" astrocytes and leading to delayed neuronal death. (**b**) Treatment with miR-181a antagomir or miR-29a protects astrocyte mitochondria, reduces ROS and protects GLT-1, supporting neuronal survival. Modified from Fig. 3 in Ouyang et al. 2014b

miR-29 being inhibited in different cells or under different physiological or pathological settings. A group has reported that miR-29b is activated during neuronal maturation and targets several pro-apoptotic genes, *BIM*, *BMF*, *HRK*, *PUMA*, and *BAK* in the BCL2 family (Kole et al. 2011). We found that miR-29a targets BH3-only protein PUMA and reduces neuronal vulnerability to forebrain ischemia (Ouyang et al. 2013b). In contrast, increasing miR-29b had the effect of promoting neuronal cell death in focal ischemia by inhibiting BCL-w, an anti-apoptotic member of the BCL2 protein family (Shi et al. 2012).

Knowing that the targets of miR-181a and miR-29a validated to date (GRP78, BCL2 family members) are ER and mitochondria related (Ouyang and Giffard 2012, 2013; Ouyang et al. 2013a), a common mechanism for these two miRNAs in regulating cerebral ischemia may involve manipulating mitochondrial function. Indeed, using primary cultured astrocytes we have found that miR-181a inhibitor and miR-29a mimic both preserve mitochondrial function, reduce ROS production and protect astrocytes from ischemia-like stress (Ouyang et al. 2012a, 2013b) (Fig. 9.3).

9.9 Future Directions

Strategies to improve the neuron-supportive functions of astrocytes have been used successfully in animal and in vitro studies. We speculate that miRNAs may have greater therapeutic potential as candidates for the treatment of stroke than therapies

targeting induction of a single gene because of their faster posttranscriptional effect and their ability to simultaneously regulate many target genes. Several astrocyte-enriched miRNAs such as miR-181a and miR-29a have been demonstrated to regulate focal and global cerebral ischemia in animal models by targeting several important cell death/survival pathways through specific mRNA targets (Fig. 9.3). While pretreatment has been tested in these early studies, investigating the effect of manipulating these astrocyte-enriched miRNAs after the onset of ischemia is an essential next step for translating these ideas toward clinical use. An increasing body of literature suggests that individual miRNAs may have modest effects on their target mRNAs, and several miRNAs may be required for larger effects. This raises the possibility that combination therapy with astrocyte-enriched miRNAs may be even more neuroprotective than a single miRNA. Several miRNAs are already in clinical trials in liver diseases, suggesting that formulation and administration will be possible in a new disease setting or for a new miRNA target. Work remains to be done to target different organs.

Acknowledgments Grant sponsor: NIH; Grant numbers: NS084396, NS053898, NS080177. The authors thank William Magruder for help preparing the manuscript.

Conflict of interest The authors have no conflicting financial interests.

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Chapter 10
Ca^{2+} Signaling in Astrocytes and its Role in Ischemic Stroke

Shinghua Ding

Abstract Astrocytes have been found to play important roles in physiology being fundamental for ionic homeostasis and glutamate clearance from the synaptic cleft by their plasma membrane glutamate transporters. Astrocytes are electrically non-excitable, but they exhibit Ca^{2+} signaling, which now has been demonstrated to serve as an indirect mediator of neuron-glia bidirectional interactions through gliotransmission via tripartite synapses and to modulate synaptic function and plasticity. Spontaneous astrocytic Ca^{2+} signaling was observed in vivo. Intercellular Ca^{2+} waves in astrocytes can be evoked by a variety of stimulations. Astrocytes are critically involved in many pathological conditions including ischemic stroke. For example, it is well known that astrocytes become reactive and form glial scar after stroke. In animal models of some brain disorders, astrocytes have been shown to exhibit enhanced Ca^{2+} excitability featured as regenerative intercellular Ca^{2+} waves. This chapter briefly summarizes astrocytic Ca^{2+} signaling pathways under normal conditions and in experimental in vitro and in vivo ischemic models. It discusses the possible mechanisms and therapeutic implication underlying the enhanced astrocytic Ca^{2+} excitability in stroke.

Keywords Astrocytes • Ca^{2+} signaling • Ischemic stroke • Hypoxia

10.1 Astrocytes in the Central Nervous System

Astrocytes are specialized and the most numerous glial cell type in the central nervous system (CNS) (Agulhon et al. 2008; Barres and Barres 2008; Haydon 2001). There are two major types of astrocytes: protoplasmic astrocytes in grey matter and fibrous astrocytes in white matter (Fig. 10.1). Morphologically, protoplasmic astrocytes are complex and highly branched with numerous fine processes and their

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_10

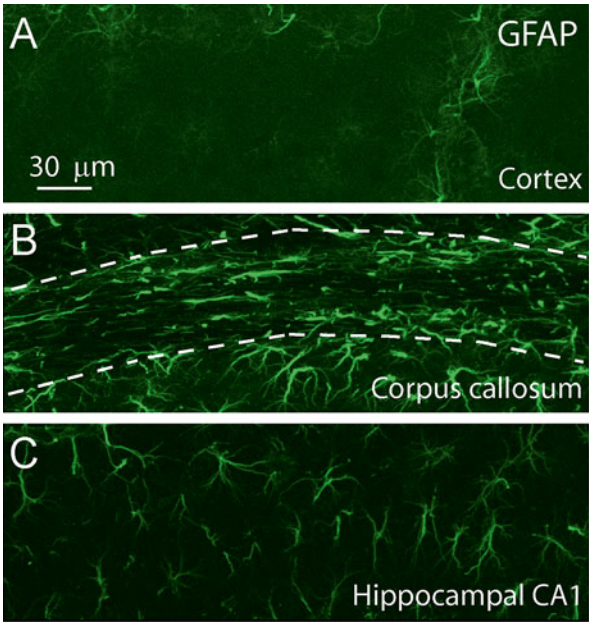

Fig. 10.1 Heterogeneity of morphology and GFAP expression of astrocytes in the mouse brain under normal conditions. (**a**) GFAP expression in protoplasmic astrocytes in the cortex layer 2–3. (**b**) GFAP expression in fibrous astrocytes in the corpus callosum (*dashed outline*, *middle*). (**c**) GFAP expression in protoplasmic astrocytes in the CA1 region of the hippocampus. Notice the thick process and high express levels of GFAP in the corpus callosum and highly branched astrocytes in the hippocampal CA1 region. All the mages were taken by confocal microscopy and are the maximum projection images. Adapted from Ding (2013)

endfeet wrap around blood vessels. Fibrous astrocytes are less complex and have thicker and less branched processes. It is well known that astrocytes control CNS homeostasis by regulating ionic concentration (e.g., astrocytes act as a K^+ sink to maintain extracellular K^+ homeostasis) (Djukic et al. 2007) and removing glutamate from the synaptic cleft by their plasma membrane glutamate transporters to avoid glutamate toxicity (Huang et al. 2004; Bergles et al. 1999; Danbolt 2001). Astrocytes provide nutritional and structural support for neurons. Some astrocytes express the unique intermediate filament protein, glial fibrillary acidic protein (GFAP), which is used to distinguish them from other cell types in the CNS; however, its expression levels in astrocytes are different in different regions. For example, under normal conditions, protoplasmic astrocytes in the mouse cortex express much lower levels of GFAP than do protoplasmic astrocytes in the hippocampus (Fig. 10.1), although the densities of astrocytes in these two regions are similar (Ding 2013). It is also clear now that not all astrocytes express GFAP and vice versa, not all cells that express GFAP are astrocytes (Oberheim et al. 2012). Electrophysiological recordings also showed that even in the same region astrocytes have differential patterns of current-voltage relationship; for instance, one type of astrocytes termed outward

rectifying astrocytes and the other termed variably rectifying astrocytes (Zhou and Kimelberg 2000). In vivo Ca^{2+} imaging also demonstrates that astrocytes in the cortical layer 1 (L1) exhibited distinct Ca^{2+} dynamics in vivo from astrocytes in the cortical layer 2/3 (L2/3) in rats anesthetized with urethane (Takata and Hirase 2008). Astrocytes in L1 nearly doubled the Ca^{2+} activity of astrocytes in L2/3. Furthermore, Ca^{2+} fluctuations of processes within an astrocyte were independent in L1, while those in L2/3 were more synchronous (Takata and Hirase 2008). Thus, astrocytes in the CNS are heterogeneous in function, morphology and molecular expression.

10.2 Ca^{2+} Signaling in Astrocytes

10.2.1 GPCR-Mediated Ca^{2+} Signaling

More than two decades ago, it was discovered that astrocytes could mediate Ca^{2+} signaling (i.e., transient Ca^{2+} increase) (Cornell-Bell et al. 1990), which suggested that they can play more active roles in the CNS than previously delineated. Astrocytes express a variety of G-protein coupled receptors (GPCRs), e.g., for glutamate, γ-aminobutyric acid (GABA), ATP, serotonin, norepinephrine, and dopamine. These receptors can all mediate astrocytic Ca^{2+} signaling and intercellular waves in vivo by the activation of metabotropic glutamate receptors (mGluRs) (Ding et al. 2007; Fellin et al. 2004; Sun et al. 2013), P2Y receptors (Ding et al. 2009; Thrane et al. 2012; Sun et al. 2013; Wang et al. 2006; Nizar et al. 2013), $GABA_B$ receptors ($GABA_B$Rs) (Ding et al. 2009; Meier et al. 2008), noradrenergic receptors (Bekar et al. 2008), and dopamine receptors (Haydon and Carmignoto 2006; Ni et al. 2007). mGluRs are classified into three groups (Pin and Duvoisin 1995; Schoepp et al. 1999; Niswender and Conn 2010). Group I mGluRs includes mGluR1 and 5 which are coupled to phospholipase-C/inositol 1,4,5-triphosphate (PLC/IP_3) pathway to mobilize Ca^{2+} from the internal store. Group II (mGluR2 and 3) and III (mGluR4-6 and 7-8) are negatively coupled to adenylyl cyclase. Cortical and hippocampal astrocytes predominantly express mGluR5 and mGluR3 (Schools and Kimelberg 1999). $GABA_B$Rs were also reported to be expressed in the cortical and hippocampal astrocytes (Meier et al. 2008; Nilsson et al. 1993; Oka et al. 2006; Charles et al. 2003), $GABA_B$Rs can mediate Ca^{2+} elevations in astrocytes in response to interneuron activation in brain slices (Meier et al. 2008; Kang et al. 1998). Similarly, $GABA_B$R agonist baclofen can stimulate Ca^{2+} elevations in astrocytes in brain slices (Meier et al. 2008) and in vivo (Ding et al. 2009). It is likely that $GABA_B$R-stimulated Ca^{2+} signaling in astrocyte is mediated through the release from the internal Ca^{2+} store although the mechanism is not fully studied (Doengi et al. 2009). ATP has been extensively used to stimulate Ca^{2+} release in vivo through P2Y receptors (Ding et al. 2007, 2009; Sun et al. 2013; Wang et al. 2006; Thrane et al. 2012; Nizar et al. 2013). Ca^{2+} signaling is now considered as a primary form of cellular excitability in astrocytes that can be determined by fluorescent imaging using different Ca^{2+} indicators.

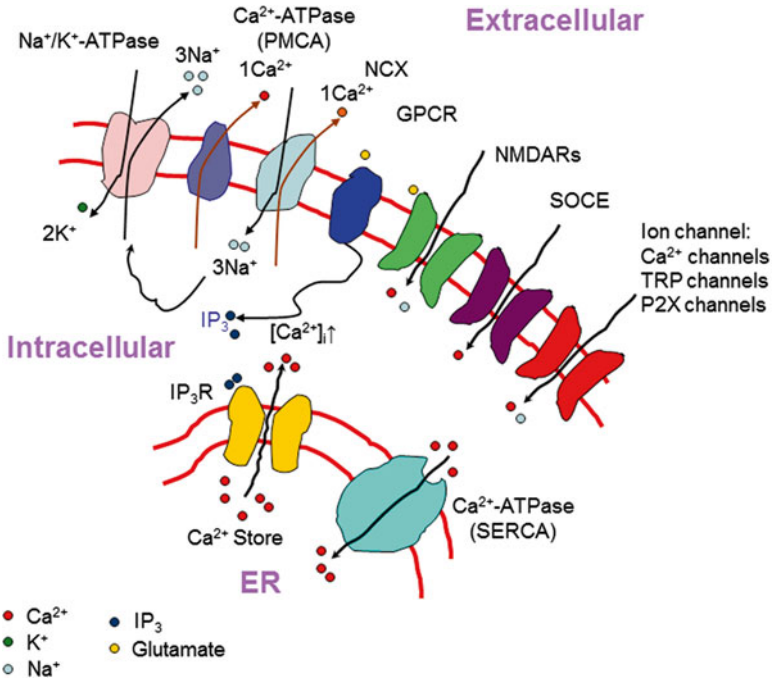

Fig. 10.2 Ca^{2+} signaling pathway and routes of Ca^{2+} entry. *ER* endoplasmic reticulum; *SOCE* store-operated Ca^{2+} entry. *NCX* sodium-calcium exchanger, forward mode operation; *PMCA* plasma membrane Ca^{2+} ATPase; *SERCA* sarco/endoplasmic reticulum Ca^{2+}-ATPase; *NMDARs* *N*-methyl-D-aspartate receptors; *TRP* transient receptor potential channel

GPCR stimulation activates PLC with subsequent IP_3-mediated Ca^{2+} release (Agulhon et al. 2008; Haydon 2001; Petravicz et al. 2008) (Fig. 10.2). Among the three types of IP_3R (IP_3R1-3), IP_3R2 seems to be the predominant type in astrocytes in the rodent brain (Hertle et al. 2007; Holtzclaw et al. 2002; Sharp et al. 1999). IP_3R2 knock-out (IP_3R2 KO) mice do not exhibit GPCR agonists-evoked Ca^{2+} increase in astrocytes in brain slice and in vivo, demonstrating that IP_3R2 is a key mediator of intracellular Ca^{2+} release in astrocytes, but IP_3R2 has no effect on long-term plasticity and vascular tone (Nizar et al. 2013; Petravicz et al. 2008).

10.2.2 Ca^{2+} Influx Through the Plasma Membrane

A number of plasma membrane proteins can also regulate Ca^{2+} homeostasis by controlling Ca^{2+} influx from the extracellular space. Those proteins include Na^+/Ca^{2+} exchanger (Reyes et al. 2012; Takuma et al. 2013; Kirischuk et al. 2012), plasma membrane Ca^{2+} ATPase (Reyes et al. 2012), store-operated channels (Linde et al. 2011), P2X purinoceptors (Illes et al. 2012; Palygin et al. 2010), transient receptor

potential A (TRPA) channels (Shigetomi et al. 2012), C (TRPC) channels (Linde et al. 2011; Shirakawa et al. 2010; Malarkey et al. 2008; Reyes et al. 2013), and *N*-methyl-D-aspartate (NMDA) receptors (Palygin et al. 2010) (Fig. 10.2).

10.2.3 Ca^{2+}-Dependent Gliotransmission and the Tripartite Synapse

Studies using cultured astrocytes (Parpura et al. 1994; Parpura and Haydon 2000) and brain slice preparations (Ding et al. 2007; Fellin et al. 2004; D'Ascenzo et al. 2007; Angulo et al. 2004) indicate that an increase in astrocytic Ca^{2+} leads to the release of chemical transmitters, the process referred to as *gliotransmission*. Thus, Ca^{2+} signaling in astrocytes serves as a mediator of bidirectional interactions between neurons and astrocytes which are the integral part of a *tripartite synapse*. A tripartite synapse is composed of presynaptic nerve terminal, postsynaptic terminal, and astrocytic processes (Haydon 2001). *Gliotransmission* is predominantly mediated by mGluRs (Fellin et al. 2004; D'Ascenzo et al. 2007; Pasti et al. 1997; Porter and McCarthy 1996). Glutamate (Ding et al. 2007; Fellin et al. 2004; D'Ascenzo et al. 2007; Angulo et al. 2004; Jourdain et al. 2007; Parri et al. 2001; Parpura and Haydon 2000; Parpura et al. 1994), ATP (Cotrina et al. 2000; Pascual et al. 2005; Newman 2001), and D-serine (Mothet et al. 2005; Henneberger et al. 2010; Martineau et al. 2013; Stobart et al. 2013; Takata et al. 2011) can all be released from astrocytes. Thus, astrocytes can send signal back to neurons through gliotransmission to modulate neuronal activity (Ding et al. 2007; Fellin et al. 2004; D'Ascenzo et al. 2007; Angulo et al. 2004) and synaptic plasticity in brain slice and in vivo (Pascual et al. 2005; Takata et al. 2011; Chen et al. 2012; Navarrete et al. 2012). Under pathological conditions, enhanced astrocytes Ca^{2+} signaling may consequently increase excitotoxicity through gliotransmission.

10.2.4 Imaging Intracellular Ca^{2+} in Astrocytes

Astrocytic Ca^{2+} signaling has been extensively studied in cultured astrocytes using fluorescence imaging. Organic acetoxymethyl (AM) ester form of Ca^{2+} indicators such as fluo-4, Oregon green BAPTA-1 (OGB), Rhod-2, or x-Rhod-1 have been largely used for in vitro and in vivo Ca^{2+} imaging due to their high sensitivity and speed (Wang et al. 2006; Takano et al. 2006; Ding et al. 2007, 2009; Ding 2012; Hirase et al. 2004; Takata and Hirase 2008; Takata et al. 2011; Thrane et al. 2012; Sun et al. 2013; Nimmerjahn et al. 2004; Aguado et al. 2002; Porter and McCarthy 1996). The selective labeling of astrocytes by organic Ca^{2+} indicators in vivo can be confirmed by an astrocyte selective dye sulforhodamine 101 (SR101), (Nimmerjahn et al. 2004). Recently developed genetically encoded Ca^{2+} indicators (GECIs) including FRET-based GECIs such as YC3.6 and single-fluorophore GECIs such as

GCaMP provide an alternative way to label astrocytes in vivo. The major advantage for using GECIs is that they can be expressed in astrocytes for long-term imaging. The GECIs can be expressed in astrocytes using viral transduction, in utero electroporation or transgenic mice (Tian et al. 2009; Zariwala et al. 2012; Akerboom et al. 2012; Shigetomi et al. 2013). Astrocyte-specific expression of GECIs using adeno-associated viral vectors can be achieved by astrocyte-specific promoter (Xie et al. 2010; Tong et al. 2013). Transgenic mice expressing floxed GCaMP3 can cross with Cre mice with specific promoters for neuronal and astrocytic Ca^{2+} imaging (Zariwala et al. 2012). For astrocytic specific expression of GCaMP3, GFAP-Cre mice are commercially available. Since GECIs can be expressed in astrocytes for prolonged times, it is feasible to perform long-term and repeated in vivo Ca^{2+} imaging in astrocytes, inducing that in ischemic conditions. Usually, for in vivo imaging, a cranial window in the cortex must be prepared for loading organic Ca^{2+} dyes. For detailed procedures and protocols of the surgery procedures and dye loading for in vivo Ca^{2+} imaging in astrocytes, readers can consult detailed reviews (Ding 2012; Tian et al. 2006).

10.3 In Vivo Ca^{2+} Signaling in Astrocytes Under Normal Conditions

In the past decade, the application of two-photon (2-P) laser-scanning fluorescence microscopy in in vivo imaging provided a valuable tool to understand the behavior of astrocytic Ca^{2+} signaling in live animals. Before I discuss astrocytic Ca^{2+} signaling in stroke, I briefly summarize the current studies on in vivo Ca^{2+} signaling in astrocytes in rodents under normal conditions. Detailed discussion about this topic can be found in a recent review (Ding 2013). Ca^{2+} signaling in cultured astrocytes will not be discussed as the morphology and gene profiles of cultured astrocytes are generally, but not always, altered as compared with those of astrocytes in vivo.

10.3.1 In Vivo Spontaneous Ca^{2+} Signaling in Astrocytes

Spontaneous Ca^{2+} signaling of astrocytes in the cortex and hippocampus in rodents has been observed in vivo using 2-P microscopy. Data from published studies show that Ca^{2+} signaling properties are affected when different preparations are used.

Cortical astrocytes in adult mice (more than 2-month old) usually exhibit low frequency spontaneous Ca^{2+} oscillations in cell body and processes when anesthetized with urethane (Ding et al. 2007, 2009), ketamine/xylazine (Wang et al. 2006), or isoflurane (Thrane et al. 2012). The duration of Ca^{2+} signal in the cell body and processes is short (somewhat between 10 and 25 s). Importantly, Ca^{2+} signals in the cell bodies of different astrocytes and in the different processes are independent. Thus, spontaneous Ca^{2+} signaling in cortical astrocytes in anesthetized adult mice do not exhibit intercellular wave-like behavior, and Ca^{2+} signals in the processes are limited within microdomains of the processes.

Astrocytes exhibit regional heterogeneity in spontaneous Ca^{2+} signaling. Astrocytes in the cortical layer 1 (L1) exhibited distinct Ca^{2+} dynamics in vivo from astrocytes in the cortical layer 2/3 (L2/3) in rats anesthetized with urethane; astrocytes in L1 nearly doubled the Ca^{2+} activity of astrocytes in L2/3 (Takata and Hirase 2008). Furthermore, Ca^{2+} fluctuations in the processes within an astrocyte were independent in L1, while those in L2/3 were more synchronous (Takata and Hirase 2008). On the other hand, in urethane-anesthetized young mice (P9-25), hippocampal astrocytes in the network exhibited synchronized Ca^{2+} oscillations and intercellular waves (Kuga et al. 2011; Sasaki et al. 2011). The difference in Ca^{2+} activities in astrocytes between the cortex and the hippocampus in mice might reflect the functional heterogeneity, which possibly resulted from the different neuronal activities, and microenvironment and/or different properties of astrocytes per se. The fact that GFAP levels are higher in hippocampal astrocytes than in the cortical astrocytes supports the latter reason (see Fig. 10.1). However, since the mice used in the study were young (P9-25), it is not clear whether astrocytes in adult mice would exhibit similar Ca^{2+} signaling.

In awake mice, cortical astrocytes exhibit much higher frequency of spontaneous Ca^{2+} signaling in the cell body and processes than in mice anesthetized by isoflurane, ketamine, and urethane (Thrane et al. 2012). In addition, somatic Ca^{2+} signals in astrocytes are highly synchronized, suggesting that synchronization of cortical astrocytic Ca^{2+} activity is a hallmark of wakefulness of an animal.

In summary, the properties of spontaneous Ca^{2+} signaling in astrocytes could be affected by animal species (e.g., mice, rats or other rodents), animal ages (e.g., young or adult animals), locations of astrocytes (e.g., in the cortex, hippocampus or other regions), anesthesia (e.g., isoflurane, ketamine/xylazine or urethane), and wakefulness of animals.

10.3.2 In Vivo Stimulated Ca^{2+} Signaling in Astrocytes

Astrocytic Ca^{2+} signals can be stimulated by a variety of approaches including GPCR agonists, sensory stimulations, mechanical stimulations, and photolysis of caged compounds (Ding et al. 2007; Sun et al. 2013; Wang et al. 2006; Takano et al. 2006; Tian 2005). ATP is a powerful agonist that can induce intercellular Ca^{2+} waves in a large number of astrocytes (Ding et al. 2009; Sun et al. 2013; Wang et al. 2006; Thrane et al. 2012; Nizar et al. 2013). Regenerative intercellular Ca^{2+} waves with high frequency can be elicited with continuous presence of ATP in the cortex (Ding et al. 2007, 2009; Ding 2012), while the group I mGluR agonists (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) and (RS)3,5-dihydroxyphenylglycine (DHPG) stimulate intercellular Ca^{2+} waves with low frequency (Ding et al. 2007). Sensory stimulations, including whisker deflection (Wang et al. 2006), locomotion (Nimmerjahn et al. 2009; Dombeck et al. 2007), limb stimulation (Bekar et al. 2008; Winship et al. 2007; Ghosh et al. 2013), light illumination of visual cortex (Chen et al. 2012; Schummers et al. 2008), and odor stimulation of olfactory glomeruli (Petzold et al. 2008), can also induce intercellular Ca^{2+} waves in astrocytes in vivo

in anesthetized animals. Ca^{2+} signals of Bergmann glial (BG) cells were associated with motor behavior and neuronal activity in awake and behaving mice (Nimmerjahn et al. 2009). Interestingly, BG cells exhibit three different forms of Ca^{2+} excitation: flares, bursts, and sparkles. Bursts and sparkles are ongoing in awake mice at rest, whereas flares are initiated during locomotor behavior. Locomotor performance initiates synchronized Ca^{2+} signals arising within the network of BG cells. The macroscopic flare events are correlated with blood perfusion levels, suggesting that they are sufficient to potentially regulate blood flow. Thus, the specific animal behaviors dictate Ca^{2+} excitability.

10.3.3 Effect of Ca^{2+} Signaling in Astrocytes on Neuronal Function and Cerebral Blood Flow In Vivo

From in vivo studies, Takata et al. reported that combined stimulation of mouse whisker and the nucleus basalis of Meynert (NBM), the principle source of cholinergic innervation to the cortex, enhances whisker-evoked local field potential (LFP) (Takata et al. 2011). This plasticity is dependent on Ca^{2+} increase in astrocytes and Ca^{2+} dependent D-Serine release from astrocytes as IP_3R2 KO mice abolish the plasticity. In visual cortex, paired visual and electrical stimulation of the nucleus basalis (NB) induces significant potentiation of visual responses in the primary visual cortex in mice (Chen et al. 2012). By removing the cortical tissue, Navarrete et al. studied astrocyte-dependent plasticity in hippocampal CA3-CA1 synapses in vivo (Navarrete et al. 2012). Their results show that cholinergic-induced long-term potentiation (LTP) requires astrocyte Ca^{2+} elevation and Ca^{2+}-dependent release of glutamate from astrocytes to act on mGluR5 presynaptic terminals. Thus, in addition to the wealth of information from in vitro studies, astrocytes are actively involved in synaptic plasticity through Ca^{2+} signaling in in vivo setting.

Astrocytic Ca^{2+} signaling has been shown to affect neuronal function and vasculature tone in brain slice preparations. Hence, Ca^{2+} elevation in astrocytes by neuronal afferent stimulation and photolysis of caged Ca^{2+} compound induced vasodilation (Stobart et al. 2013; Zonta et al. 2003), while other studies suggested that the polarity of astrocytic Ca^{2+}-dependent regulation of blood flow was dictated by tissue metabolism of brain and retina slices (Gordon et al. 2008; Mishra et al. 2011). These studies suggest complex mechanisms of blood flow regulation by astrocytic Ca^{2+} signals (Attwell et al. 2010). In in vivo studies, photolysis of caged Ca^{2+} induced rapid dilation of the artery in the cortex (Takano et al. 2006). Motor behavior induced astrocytic Ca^{2+} flares were correlated with blood flow increase (Nimmerjahn et al. 2009). Odor stimulation induces mGluR5-dependent Ca^{2+} transients in astrocyte endfeet and an associated dilation of upstream arterioles. These processes are also dependent on cyclooxygenase activation (Petzold et al. 2008). Electrical stimulation of a forepaw induced vasodilation and astrocytic Ca^{2+} increase in the somatosensory cortex (Nizar et al. 2013). However, the stimulus-induced vasodilation is independent of IP_3R-mediated Ca^{2+} increase since IP_3R2 KO mice exhibit normal

functional hyperemia. In addition, the onset of vasodilation precedes astrocytic Ca^{2+} increase. The results suggest that functional hyperemia induced by forepaw stimulation is mainly neuron-dependent, and the astrocytic Ca^{2+} increase may not be sufficient enough to exert an additional effect on the onset of vasodilation. Similarly, inhibition of group I mGluRs does not affect transient hemodynamic responses following a brief whisker stimulation in rats under isoflurane anesthesia (Calcinaghi et al. 2011). The results suggest that group I mGluRs, regardless of their expression in neurons and/or astrocytes, do not play a role in early hemodynamic responses following sensory stimulation in rats.

10.4 Astrocytic Ca^{2+} Signaling in Ischemic Stroke and Hypoxia

10.4.1 Astrocytes in Stroke

Stroke causes severe brain damage and is the leading cause of human death and long-term disability. It has a major impact on public health. Cerebral ischemia accounts for approximately 80 % of all human strokes. Cerebral ischemia initiates from vasculature dysfunction (disease) and begins with the mechanical occlusion of blood vessels by thrombus or embolus that reduces or blocks blood flow. The degree to which blood flow is reduced largely determines the damage to the brain. Due to the loss of glucose and oxygen, synthesis of ATP through glycolysis and oxidative phosphorylation is impaired in the ischemic region. Thus, ischemia results in energy depletion, which leads to loss of ionic gradients and membrane potential depolarization in neurons and astrocytes. Consequently, this induces the release of glutamate and other neurotransmitters from presynaptic terminals to the extracellular space. The energy depletion further contributes to the malfunction of astrocytic and neuronal glutamate transporters, which are critical in clearing the glutamate released into the synaptic cleft. A variety of potential mechanisms by which ischemia leads to neuronal death and brain damage have been proposed from experimental studies of stroke. These mechanisms include glutamate and Ca^{2+} toxicity, oxidative stress, acidosis, inflammation, and mitochondrial dysfunction. Glutamate excitotoxicity, largely resulting from influx and intracellular overloading of Ca^{2+} through overstimulation of glutamate receptors (especially NMDARs), is the primary mediator of acute neuronal death (Choi 1988). The excessive accumulation of glutamate in the extracellular space leads to the overstimulation of ionotropic and metabotropic glutamate receptors, especially ionotropic NMDARs, and results in pathological overloading of intracellular Ca^{2+} in neurons after a short latency period (Medvedeva et al. 2009; Tanaka et al. 1997). The overloading of intracellular Ca^{2+} during ischemia is linked (along with glutamate excitotoxicity) to the triggering of activations of downstream phospholipases and proteases that cause degradation of membranes and proteins and eventually neuronal death (Lo et al. 2003; Dirnagl et al. 1999). Thus glutamate excitotoxicity is the primary mediator of neuronal death; it does not

only cause acute neuronal death, but also initiates molecular events that lead to delayed neuronal death and brain damage (Dirnagl et al. 1999).

After ischemia, the brain experiences temporal- and spatial-dependent changes of pathological processes. In the ischemic core region, cells die rapidly from the onset of ischemia due to severely impaired energy production and the ensuing breakdown of ionic homeostasis. In the penumbra, the region surrounding the core of a focal ischemic locus, the brain tissue is hypoperfused with collateral blood flow and energy metabolism is partially preserved. The partial energy reduction does not cause severe and terminal disruption of ionic gradients and immediate cell death. This region can completely or partially recovered after reperfusion, but can progress to infarction without treatment due to the ongoing excitotoxicity (Rossi et al. 2007). Astrocytes play an important role in reducing glutamate excitotoxicity by glutamate uptake through glutamate transporters in the acute phase of ischemia. However, in severe ischemia, reversed glutamate uptake could contribute to glutamate elevation (Rossi et al. 2000).

Compared with neurons, the role of astrocytes in ischemia is less understood. After the onset of ischemia, astrocytes undergo numerous pathological processes (Nedergaard and Dirnagl 2005; Barber and Demchuk 2003; Swanson et al. 2004). It is well known that glutamate transporters in astrocytes, which normally function to remove glutamate from the synaptic cleft to avoid glutamate toxicity, are impeded due to energy depletion. Glutamate can be even released from astrocytes by transporter reversal during in vivo and in vitro ischemia (Rossi et al. 2000; Phillis et al. 2000). This leads to extracellular glutamate elevations and aggravates glutamate excitotoxicity. Astrocytes rapidly swell after ischemia, to which a number of factors contribute (Kimelberg 2005; Zheng et al. 2010; Risher et al. 2012). Swelling gradually spreads outward from the ischemic core to the adjacent tissue, and eventually many of swollen astrocytes lyse, while astrocytes in the penumbral region exhibit reversible swelling (Zheng et al. 2010). In general, astrocytes are more resistant to ischemia than neurons (Lo et al. 2003) largely because they can use glycogen stores as an alternative energy source (Kasischke et al. 2004; Brown 2004; Rossi et al. 2007; Gurer et al. 2009). Most glycogen in adult brain is found in astrocytes, not in neurons (Brown 2004). Astrocytes can convert glycogen to lactate and pass lactate to neurons to supplement neuronal energy requirement during the pathological shortage of glucose (Brown and Ransom 2007). Astrocytes can also convert glycogen to glucose, thus delay energy depletion after ischemia through producing ATP by glycolysis (Brown 2004). Astrocytes may also provide glucose to neurons because both astrocytes and neurons express glucose transporters, thereby allowing neurons to delay their own ATP depletion by glycogen-derived glucose (Brown and Ransom 2007). Astrocytes are known to become reactive over time after ischemia, as characterized by an excessive expression of GFAP (i.e., astrogliosis) and eventually form a glial scar around the area of the injury (Nedergaard and Dirnagl 2005; Panickar and Norenberg 2005; Li et al. 2013; Barreto et al. 2011; Pekny and Nilsson 2005). Reactive astrocytes in glial scar could result from the upregulation of GFAP in existing astrocytes or newly generated astrocytes with a common feature of high GFAP expression levels (Barreto et al. 2011; Li et al. 2013). Glial scar formation is associated with substantial tissue shrink and morphological changes of reactive

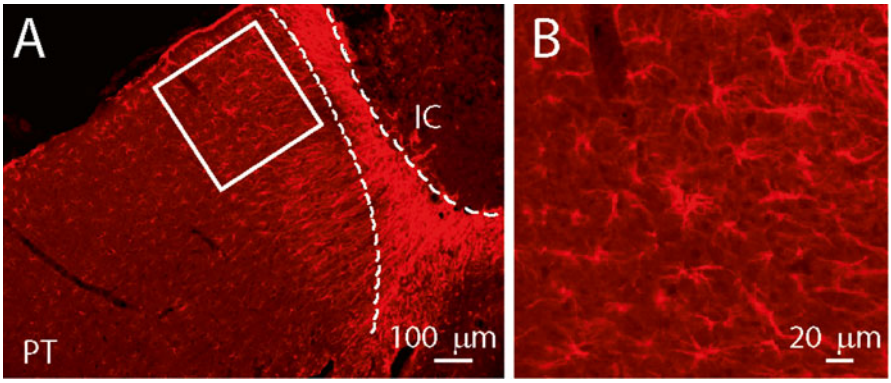

Fig. 10.3 Reactive astrocytes and the glial scar formation after ischemia. (**a**) GFAP staining images in the ipsilateral side of the brain 2 weeks after photothrombosis (PT). (**b**) is the high resolution image of the *boxed region* in (**a**). The *dashed outline* in (**a**) indicates the boundary of glial scar. *IC* ischemic core. Adapted from Li et al. (2013)

astrocytes (Fig. 10.3) (Li et al. 2013). Spontaneous recovery of brain injury in the chronic phase of ischemia may involve astrocyte reactivation (Cramer 2008).

Current strategies to treat ischemic stroke are aimed at restoring blood supply by administration of thrombolytic drugs, which is effective within a narrow window of about 3 h following ischemic stroke (Stapf and Mohr 2002; Huang and McNamara 2004). Thus, identifying novel therapeutic targets and elucidating cellular and molecular mechanisms by which ischemia induces neuronal death and brain damage are of great importance to provide effective therapeutic avenues. Although neuronal glutamate and Ca^{2+} excitotoxicity is widely acknowledged (Szydlowska and Tymianski 2010; Choi 1988), whether or how astrocytic Ca^{2+} is altered after ischemia has not been studied well. Since astrocytic Ca^{2+} signaling is the major feature of astrocytic excitability and due to the tripartite nature of synapse, Ca^{2+}-dependent gliotransmitter release will expectedly affect neuronal excitability and toxicity in stroke.

10.4.2 Ca^{2+} Signaling in Astrocytes in In Vitro Stroke

Astrocytic Ca^{2+} signaling was studied after in vitro ischemia using cultured astrocytes, acutely isolated astrocytes and brain slices using an oxygen/glucose deprivation (OGD), a common in vitro ischemia model. Using live cell imaging, it was reported that 2 h OGD did not cause changes in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) increase in cultured astrocytes, but led to a significant increase in endoplasmic reticulum Ca^{2+} ($[Ca^{2+}]_{ER}$) (Liu et al. 2010). However, cultured astrocytes exhibit $[Ca^{2+}]_{cyt}$ increase about 2 h after reoxygenation (REOX) following 2 h OGD. Inhibition of IP_3R with its specific blocker xestospongin largely blocked the delayed rise of $[Ca^{2+}]_{cyt}$, suggesting that the ER Ca^{2+} store is the source for post-OGD Ca^{2+} elevation in the cytosol. The study further showed that the delayed Ca^{2+} increase can be suppressed by the inhibition of Na^+/Ca^{2+} exchanger (NCX) and Na^+–K^+–Cl^- cotransporter isoform

1 (NKCC1), suggesting that the Ca^{2+} is provided from the extracellular space through the delivery by these transporters. Mitochondrial Ca^{2+} ($Ca^{2+}{}_m$) content was increased significantly within 15 min REOX followed by a secondary rise (~4.5-fold) and a release of mitochondrial cytochrome *c* (Cyt *c*). Cell death assay showed that the majority of cell death occurred during the 125–180 min of reoxygenation when the cytosolic Ca^{2+} dysregulation was triggered. These results illustrate that OGD/REOX triggers a time-dependent loss of Ca^{2+} homeostasis in the cytosol and organelles (ER and mitochondria) in astrocytes. Collective stimulation of NKCC1 and the reverse mode function of NCX contribute to these changes. These findings suggest that IP_3R-mediated Ca^{2+} depletion in astrocytes may occur in ischemic brain, especially during the brain reperfusion, and exacerbate astrocyte damage.

In isolated astrocytes, OGD induces a small and slowly developing Ca^{2+} increase in the presence of extracellular Ca^{2+}; this increase is completely reversible by the removal of extracellular Ca^{2+} (Duffy and MacVicar 1996). Thus, the results from isolated astrocytes can be interpreted by Ca^{2+} influx through functional Ca^{2+} channels.

Cultured and acutely isolated astrocytes cannot mimic tissue environment after ischemia with release of extracellular messengers including neurotransmitters. Acute brain slices provide a better preparation for studying Ca^{2+} signaling in astrocytes in ischemia. Duffy and MacVicar (1996) studied the presence of Ca^{2+} signals in hippocampal astrocytes in response to in vitro ischemia. To study Ca^{2+} signaling in astrocytes, astrocytes were loaded with a Ca^{2+} indicator using iontophoretic loading techniques. Using rat brain slices, they found that a short episode (5 min) of simultaneous hypoxia and hypoglycemia can induce intracellular Ca^{2+} increase within an average of 7.5 min and it took 2.5 min to reach a peak. After reoxygenation, astrocytic Ca^{2+} remained elevated for a highly variable period of time ranging from several minutes to 1 h. In the absence of extracellular Ca^{2+}, Ca^{2+} increase in astrocytes could be still observed with a relative consistent duration. Electrophysiological recordings show that hypoxia and hypoglycemia also depolarize astrocytes (Duffy and MacVicar 1996). The data from brain slice experiments suggest that astrocytes can mediate Ca^{2+} increase from the internal store release and influx of voltage-dependent Ca^{2+} influx. Alternatively, internal Na^+ accumulation could lead to Ca^{2+} influx through reversal operation of the NCX.

Another study found that OGD induced slow inward currents (SICs) mediated by extrasynaptic NMDA receptors in rat CA1 pyramidal neurons brain slices (Dong et al. 2013). Moreover, dialysis of the Ca^{2+} chelator BAPTA into astrocytic network decreased the frequency of OGD induced SICs, indicating that the activation of extrasynaptic NMDA receptors depended on astrocytic Ca^{2+} activity. Using two-photon Ca^{2+} imaging, it was found that astrocytes exhibited rare spontaneous Ca^{2+} elevation under control conditions, but frequent astrocytic Ca^{2+} elevations were observed during OGD. Strikingly, over 60 % of astrocytes displayed detectable Ca^{2+} elevations within the 10 min of OGD. In addition, most astrocytes displayed more than two transients. To further demonstrate the importance of astrocytic Ca^{2+} activity, hippocampal slices from IP_3R2 knock-out mice were used. Astrocytes exhibited rare Ca^{2+} elevations during OGD and OGD induced lower frequency of SICs in hippocampal CA1 neurons from IP_3R2 knock-out mice than that from wild-type mice.

10.4.3 In Vivo Ca^{2+} Signaling in Astrocytes in Acute Stroke

Although in vitro studies on astrocytic Ca^{2+} signaling using cultured astrocytes and brain slice preparations with OGD model can derive insights into the role of astrocytes in neuronal injury in ischemia, in vivo study using live animals is essential to elucidate the mechanism of astrocytic Ca^{2+} signaling in ischemia. Photothrombosis (PT)-induced ischemia model has been used for in vivo imaging of neuronal and astrocytic responses because of the following advantages (Zhang et al. 2005; Winship and Murphy 2008; Ding et al. 2009). First, the surgical procedures of PT are relatively easy and noninvasive. Second, ischemia can be induced using light illumination through a microscopy objective and 2-P in vivo Ca^{2+} imaging can be performed conveniently after ischemia. Third, the location of ischemic core and penumbral region can be controlled and determined easily (Ding et al. 2009; Risher et al. 2010, 2012; Winship and Murphy 2008; Zhang et al. 2005). Using this model, Ding et al. studied in vivo astrocytic Ca^{2+} signaling in urethane-anesthetized adult mice using 2-P microscopy (Ding et al. 2009). In the study, they found that astrocytes exhibit enhanced Ca^{2+} signaling characterized as intercellular Ca^{2+} waves starting ~20 min after PT and Ca^{2+} signals reach the plateau 60 min after PT (Fig. 10.4). Both amplitude and frequency of PT-induced astrocytic Ca^{2+} signals were significantly increased as compared to relative quiescent Ca^{2+} signaling prior to ischemia. An important feature of the Ca^{2+} signals was that most of them initiated and returned to the basal level at the same time among astrocytes in the imaging field, i.e., they were highly synchronized (Fig. 10.4d). This was confirmed by a large cross correlation coefficient between adjacent astrocyte under ischemic condition (Ding et al. 2009). To further determine the nature of astrocytic Ca^{2+} signals in ischemia, antagonists for GPCRs including mGluR5, $GABA_BR$ and P2Y receptors were applied after emergence of Ca^{2+} signals. 2-methyl 6 (phenylethynyl)pyridine hydrochloride (MPEP), an antagonist of mGluR5, significantly reduced Ca^{2+} signals (~62 %), while CGP54626, the antagonist of $GABA_B$ receptor, also significantly reduced Ca^{2+} signals (~55 %). When MPEP and CGP54626 were co-administered, the astrocytic Ca^{2+} signals were reduced. However, suramin, a non-specific inhibitor for P2Y receptors, did not reduce the PT-induced Ca^{2+} signals. Pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), a general P2 receptor antagonist, did not attenuate the Ca^{2+} signal either. Similarly, inhibition of adenosine receptor A1 by its antagonist 8-cyclopentyltheophylline (CPT) did not inhibit PT-induced astrocytic Ca^{2+} signals. The pharmacological study suggests that glutamate and GABA are likely to be released to the extracellular space following PT to stimulate intercellular Ca^{2+} waves in astrocytic network. It is surprising that neither P2Y nor A1 receptor contributes to the enhanced Ca^{2+} signaling after PT, presumably due to the rapid degradation of ATP and adenosine by enzymes such as ectonucleotidases after ischemia (Dunwiddie and Masino 2001). The results represent the first direct study of astrocytic Ca^{2+} response after ischemia in in vivo setting. In this study, overloading of astrocytic Ca^{2+} was not observed; this is probably because the recording time was too short, so that Ca^{2+} homeostasis was not disrupted. In future study, it will be

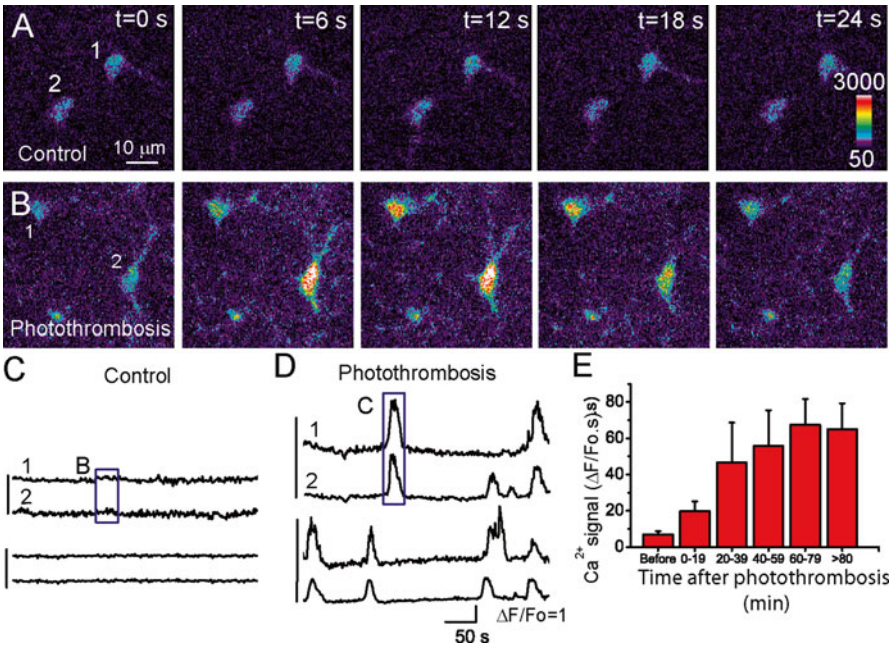

Fig. 10.4 Enhanced Ca^{2+} signaling in astrocytes in the mouse cortex in vivo after photothrombosis. (**a**, **b**) Represent images of astrocytes loaded with fluo-4 before (**a**) and after (**b**) photothrombosis. (**c**, **d**) Time courses of somatic Ca^{2+} oscillation of individual astrocytes expressed as $\Delta F/F_o$ before (**c**) and after photothrombosis (**d**). The *box regions* correspond to the images in (**a**) and (**b**) as indicated. (**e**) Time course of somatic Ca^{2+} signal in astrocytes developed after photothrombosis. Adapted from Ding et al. (2009)

interesting to compare the time course of neuronal and astrocytic Ca^{2+} signaling and overloading after ischemia as neuronal Ca^{2+} overloading is one of the major events that causes neuronal excitotoxicity followed by neuronal death.

10.4.4 In Vivo Ca^{2+} Signaling in Astrocytes in Chronic Stroke

In addition to the acute changes in astrocytic Ca^{2+} signaling, the responses of astrocytes are also altered in the chronic phase after ischemia. The functional recovery starts 2 weeks after ischemia in both ipsilateral and contralateral hemispheres. Winship et al. studied the neuronal and astrocytic Ca^{2+} signaling and functional rewiring in somatosensory neurons in ipsilateral hemisphere after stroke (Winship and Murphy 2008). Astrocytic Ca^{2+} responses were examined in anesthetized mice to study whether the prevalence of these responses was altered in peri-infarct cortex half, one, and 2 months after PT. Increased astrocyte responses to both preferred and nonpreferred limbs after stroke were observed. Data analysis revealed that there was

a significant increase in the prevalence of responses to preferred limb stimulation in the primary somatosensory cortex. The selectivity of astrocyte responses between regions of overlap between contralateral hindlimb- and contralateral forelimb-evoked intrinsic optical signals and regions without intrinsic optical signals overlap were also significantly different. These results suggest that mechanisms for rapid neuron–astrocyte communication are preserved, or even enhanced, after stroke in the penumbra.

It is known that ischemia also causes changes at molecular level in contralateral hemisphere. To study the role of astrocytes in the functional recovery in contralateral hemisphere, Takatsuru et al. performed in vivo Ca^{2+} imaging to examine the neuronal and astrocytic Ca^{2+} responses in the region contralateral to the site of stroke at different times following PT (Takatsuru et al. 2013). The results showed that the number of astrocytes with a response to limb stimulation was small in the sham group, but in the stroke groups the number was significantly increased in the contralateral somatosensory cortex responding to ipsilateral limb stimulation at the first and second week after infarction. A significantly larger number of astrocytes responded only to the single-limb stimulation in the sham group as compared with stroke groups, but a smaller number of astrocytes responded to multiple-limb stimulation in the sham group as compared to the stroke groups. Interestingly, unlike neurons, astrocytes showed no preference in response to contralateral and ipsilateral limb stimulation (Takatsuru et al. 2013). The peak amplitude of Ca^{2+} response was also increased in stroke groups. Microdialysis study showed a large increase in glutamate concentration 2 weeks after the stroke compared with the sham and 1-week group; however, glutamine concentration was much higher in the contralateral side in the 1-week group than in the sham and the 2-week group. Further study suggested that astrocytic plasma membrane glutamate transporter 1 (GLT-1) may contribute to the uptake of glutamate in the 1-week groups. Together, these findings demonstrate that activated astrocytes increase the uptake of glutamate by glutamate transporters during the first week, and indicate that astrocytes play an important role in functional recovery and cortical remodeling in the area contralateral to ischemic lesion in the post-ischemic period.

10.4.5 Ca^{2+} Signaling in Astrocytes in Hypoxia

Hypoxia is a pathological condition in which the body or a region of the body has reduced oxygen supply that is insufficient to maintain cellular functions (Howard et al. 2011). The brain is the most hypoxia-sensitive organ because of its need for high oxygen supply. Hypoxia does not cause severe brain injury provided the system circulation is preserved, but it can cause malfunction of neurons and astrocytes. BG cells are a special type of astrocytes in the cerebellum and they exhibit radially transglial Ca^{2+} waves mediated by P2Y receptors (Hoogland et al. 2009). A recent study found that ATP-induced Ca^{2+} increase in BG cells was correlated with the reduction of local tissue O_2 tension in the cerebellum (Mathiesen et al. 2013).

The incidence and frequency of spontaneous Ca^{2+} waves were significantly higher in aging mice than those in adult mice. Furthermore, the aging mice had lower basal O_2 tension than adult mice, but they had comparable blood oxygen saturation. The results suggest that aging-increased incidence and frequency of Ca^{2+} waves are correlated with decreased tissue O_2 tension. Whether the increase in the frequency of Ca^{2+} waves could be mimicked by hypoxia was further studied. Indeed, acute hypoxia of the reduction of O_2 tension by 20 % for as short as 20 min could increase spontaneous Ca^{2+} wave in BG. The study suggests that the cortical O_2 tension may play a role in inducing glial Ca^{2+} waves in aging mice, and ATP levels in brain tissue might be higher in aging brain. Whether the increased Ca^{2+} waves in BG is beneficial or detrimental to neurons is unclear and the mechanisms of hypoxia-induced increase in Ca^{2+} signal in BG cells need to be further studied.

10.4.6 The Mechanisms and Implications of Ca^{2+} Signaling in Astrocytes in Stroke

All the aforementioned in vivo studies on astrocytic Ca^{2+} signaling in ischemia used the PT model. Usually, the infarction by PT was much smaller than that using middle cerebral artery occlusion (MCAO) model. Given the different severities of brain injury, it is likely that the responses of astrocytes and neurons to ischemic insult will differ between the two models. Due to the complicated surgical procedure, it is difficult to perform in vivo imaging on mice using MCAO model to study astrocyte and neuronal Ca^{2+} signaling before, during, and after ischemia although it is still feasible to study their structural changes because of its low temporal resolution (Li and Murphy 2008).

The mechanisms of increased Ca^{2+} waves in ischemia and hypoxia might be different. Ischemia can cause the release of a large amount of glutamate or GABA and immediate cell death in the brain; hence, mGluR5 and $GABA_BR$ can play a major role in astrocytic Ca^{2+} signaling after ischemia (Ding et al. 2009). Compared with ischemia, hypoxia does not cause immediate neuronal death. The study from BG cells suggests that hypoxia can release ATP to induce Ca^{2+} hyperexcitability in astrocytes. On the other hand, glial Ca^{2+} can cause a reduction of O_2 tension in the brain, which means that the glial Ca^{2+} waves are closely related to brain energy metabolism in hypoxia. In chronic phase of ischemia, the altered response of astrocytic Ca^{2+} signaling to limbic stimulation in contralateral hemisphere suggests the change of astrocytic Ca^{2+} plasticity after ischemia and may contribute to brain remodeling in the recovery process. In summary, elucidation of the mechanisms of Ca^{2+} signaling in astrocytes may provide insights for potential therapeutic targets in stroke.

Synchronized and intercellular Ca^{2+} waves in astrocytes were also observed in other experimental disease models in the CNS. Using pilocarpine-induced SE model and 2-P in vivo imaging, Ding et al. found that 3 days following SE a significant increase in astrocytic Ca^{2+} oscillation was observed in mice that had entered SE compared to the control mice which either received a saline injection or sub-threshold

injection of pilocarpine (Ding et al. 2007). This prolonged enhancement of Ca^{2+} excitability was detected at day 2 and 3 following SE, then returned to control levels thereafter (Ding et al. 2007). They further found that mGluR5 antagonist MPEP (1 mg/kg weight) injected through the tail vein significantly reduced the astrocytic Ca^{2+} oscillations 3 days following SE.

A recent study by Choo et al. showed that traumatic brain injury (TBI) using a micromechanical impact of exposed cortex to a depth of 150–200 μm immediately elicits Ca^{2+} waves in astrocytes in vivo (Choo et al. 2013). The Ca^{2+} increase can be inhibited by apyrase that hydrolyzes ATP to AMP and inorganic phosphate, but not by flufenamic acid, a gap junction inhibitor. The results demonstrated that mechanical impact to astrocytes in vivo triggers ATP-mediated Ca^{2+} waves, which propagated beyond the initial epicenter of mechanical trauma.

Astrocytes also exhibit altered Ca^{2+} signaling in in vitro and in vivo Alzheimer's disease models. In neuronal-glial co-culture, application of Aβ triggers Ca^{2+} elevation in astrocytes that is initiated from a focal rise (Abramov et al. 2003). The Ca^{2+} elevation can maintain for a long period in the presence of Aβ. In transgenic APP/PS1 mice which express mutant human Aβ precursor protein (APP) and mutant presenilin (PS1) in neurons, but not in astrocytes, it was found that the resting astrocytic Ca^{2+} in mice with plaques is higher than in wild-type mice; nevertheless, the resting Ca^{2+} concentration is independent of the proximity of astrocytes to the plaques (Kuchibhotla et al. 2009). Remarkably, the frequency of spontaneous astrocytic Ca^{2+} activity in mice with cortical Aβ deposition is much higher than in younger mice before developing senile plaques or wild-type mice. The increased spontaneous Ca^{2+} activity is also independent on the plaque proximity.

The common observation in these disease models is that the cortical astrocytes exhibit increased frequency of Ca^{2+} elevation characterized with synchronized intercellular Ca^{2+} waves in astrocytic network. Such dramatic changes may represent the micro-environmental changes in brain tissue. Thus, astrocytes exhibit distinct Ca^{2+} signaling in health versus diseases. Although the patterns of Ca^{2+} responses in the discussed disease models are similar, the mediators of the synchronized Ca^{2+} waves might be different. Precipitating invasive brain injury after stroke, epilepsy, and TBI could cause release of large amount of glutamate and other released neurotransmitters to stimulate GPCR in astrocytes and induce enhanced Ca^{2+} hyperexcitability in astrocytes. This was supported by results where mGluR5 antagonist was shown to inhibit Ca^{2+} excitability and reduce neuronal deaths after SE (Ding et al. 2007) and PT (Ding et al. 2009), and P2Y1 antagonist was shown to reduce neuronal loss in the CA3 subregion of the hippocampus after TBI (Choo et al. 2013). In a less severe condition such as hypoxia, released ATP from brain tissue may induce astrocytic Ca^{2+} waves (Mathiesen et al. 2013). In chronic neurodegenerative diseases, the proinflammatory factors released from microglia and astrocytes might be potential candidates. In AD mice, Aβ-plaques or plaque-induced or -associated bioactive species might induce intercellular Ca^{2+} waves in astrocytes (Kuchibhotla et al. 2009); on the other hand, the modification of the proteins that maintain Ca^{2+} homeostasis might change the resting Ca^{2+} levels in astrocytes. Candidate proteins for this change include NCX, P2X receptors, and TRP channels (Reyes et al. 2012; Shigetomi et al. 2012; Shirakawa et al. 2010).

Although enhanced astrocytic Ca^{2+} excitability is found in different disease models, the observation of this phenomenon can be dependent on various factors. For example, the time from disease onset and the distance from disease core will likely affect the magnitude and frequency of Ca^{2+} increase in astrocytes. The enhanced intercellular Ca^{2+} waves in astrocytes could cause the release of vast amounts of gliotransmitter in disease; hence, Ca^{2+}-dependent gliotransmission might impose a detrimental effect on neuronal excitotoxicity, and inhibition of astrocytic Ca^{2+} signals could represent a potential target for therapeutic intervention in the treatment of brain disorders.

Conflict of interest The author declares no conflict of interest.

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Chapter 11
Pathological Potential of Astroglial Purinergic Receptors

Heike Franke and Peter Illes

Abstract Acute brain injury and neurodegenerative disorders may result in astroglial activation. Astrocytes are able to determine the progression and outcome of these neuropathologies in a beneficial or detrimental way. Nucleotides, e.g. adenosine 5′-triphosphate (ATP), released after acute or chronic neuronal injury, are important mediators of glial activation and astrogliosis.

Acute injury may cause significant changes in ATP balance, resulting in (1) a decline of intracellular ATP levels and (2) an increase in extracellular ATP concentrations via efflux from the intracellular space. The released ATP may have trophic effects, but can also act as a proinflammatory mediator or cytotoxic factor, inducing necrosis/apoptosis as a universal "danger" signal. Furthermore, ATP, primarily released from astrocytes, is a means of communication between neurons, glial cells, and intracerebral blood vessels.

Astrocytes express a heterogeneous battery of purinergic ionotropic and metabotropic receptors (P2XRs and P2YRs, respectively) to respond to extracellular nucleotides.

In this chapter, we summarize the contemporary knowledge on the pathological potential of P2Rs in relation to changes of astrocytic functions, determined by distinct molecular signaling cascades, in a variety of diseases. We discuss specific aspects of reactive astrogliosis, with respect to the involvement of prominent receptor subtypes, such as the P2X7 and $P2Y_{1/2}$Rs. Examples of purinergic signaling of microglia, oligodendrocytes, and blood vessels under pathophysiological conditions will also be presented.

The understanding of the pathological potential of purinergic signaling in "controlling and fine-tuning" of astrocytic responses is important for identifying possible therapeutic principles to treat acute and chronic central nervous system diseases.

Keywords Astroglia • Astrogliosis • Microglia • Oligodendrocytes • Purinergic signaling • P2X/Y receptors • Pathophysiology

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5_11

Abbreviations

AD	Alzheimer's disease
AKT	Serine-threonine kinase AKT
ALS	Amyotrophic lateral sclerosis
AP-1	Activator protein-1
AQP-4	Aquaporin-4
ATP	Adenosine 5′-triphosphate
BBB	Blood-brain barrier
$[Ca^{2+}]_i$	Intracellular free calcium concentration
CNS	Central nervous system
COX	Cyclooxygenase
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular signal-regulated protein kinase
GFAP	Glial fibrillary acidic protein
GSK-3	Glycogen synthase kinase-3
IL	Interleukin
$InsP_3$	Inositol-(1,4,5)-trisphosphate
IR	Immunoreactivity
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
NF-κB	Nuclear factor-κB
NG2	Chondroitin sulfate proteoglycan
OPC	Oligodendrocyte precursor cells
P2R	Purinergic receptor
PD	Parkinson's disease
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
SAPK	Stress-activated protein kinase
SCI	Spinal cord injury
STAT3	Signal transducer and activator of transcription 3
TBI	Traumatic brain injury
TNF	Tumor necrosis factor

11.1 Introduction

Astrocytes, the most abundant cell type of neuroglia in human brain, are ideally situated to integrate glial and neuronal functions as well as neurovascular coupling by way of their multiple contacts with neurons, glia, and the vascular wall of capillaries (Butt 2011). Neuroglia, the sizable cellular population in the brain, is

generally classified in astrocytes, oligodendrocytes, microglia and recently also chondroitin sulfate proteoglycan glia (NG2+ or oligodendrocyte precursor cells, OPC) (Butt et al. 2005; Butt 2011).

Astrocytes, acting alone and in cooperation with other glial cells, are broadly defined as homeostasis-maintaining cells because of their main function providing central nervous system (CNS) homoeostasis (Verkhratsky et al. 2012a). They organize the structural architecture of the brain, control the development of the nervous system, and are fundamental for the regulation of synaptogenesis and synaptic transmission. Astroglial reactions determine progression and outcome of many neuropathologies, but they are also critical for regeneration and remodeling of neural circuits following trauma, ischemia, or neurodegenerative disorders (Nedergaard et al. 2003; Verkhratsky et al. 2012a).

Remarkably, despite the neuroglia diversity, an apparently universal property of all cell types is their responsiveness to purines and pyrimidines via multiple purinoceptors (P2) (James and Butt 2002; Butt 2011). The purinergic system is a pivotal factor in neuronal-glial (regulating synaptic plasticity) and glial-glial (initiating propagating Ca^{2+} waves) communication (Verkhratsky et al. 2009; Hamilton and Attwell 2010; Lalo et al. 2011). The ubiquitous signaling molecule adenosine 5′-triphosphate (ATP) is released from both neurons and glial cells. ATP in astrocytes triggers a rise in intracellular calcium ($[Ca^{2+}]_i$), which is the substrate for glial excitability and intercellular communication in many ways. Based on its specific role in controlling astrocyte functions and cell-to-cell communication, ATP has been defined as "gliotransmitter" in the brain (Butt 2011; Lecca et al. 2012). ATP, as an executer of normal brain functions, serves as a fast excitatory neurotransmitter and has pronounced trophic roles in neural growth and development. ATP has also pathophysiological significance; it is released from damaged cells and acts as proinflammatory mediator and cytotoxic factor (an universal "danger signal") finally inducing apoptosis and necrosis (Amadio et al. 2002; Burnstock and Verkhratsky 2010; Lecca et al. 2012).

CNS insults can be regarded as a homeostatic failure, either inherited, or lost as a consequence of acute (trauma or ischemia) or chronic (neurodegeneration) deleterious processes; this makes astrocytes to become the main targets in neuropathological events (Verkhratsky et al. 2009, 2012a, b; Sofroniew and Vinters 2010; Robel et al. 2011). They react promptly leading to cellular activation and development of "astrogliosis," the conserved and ubiquitous glial defensive reaction to CNS pathologies, with subsequent beneficial and/or detrimental effects (Zhang et al. 2010; Verkhratsky et al. 2012a). New data indicate that adult reactive astrocytes may undergo a dedifferentiation process, revealing characteristics of progenitor or stem cells (Buffo et al. 2010).

In conclusion, experimental evidence highlights the purinergic system in "controlling and fine-tuning" astrocyte responses to acute and chronic events. In this chapter we focus on ATP and the pathological potential of astroglial P2Rs under certain pathophysiological conditions and partly with respect to cell-to-cell-communication.

11.2 Astrocytes

11.2.1 Physiology: Nucleotide-Induced Modulation of Astroglial Functions

Astroglia is important for maintaining molecular homeostasis; in the CNS, they regulate neurotransmitter functions, like those of ATP and glutamate, as well as ion concentrations and neurohormone effects (for review see Verkhratsky et al. 2012a).

11.2.1.1 ATP Storage and Release

Astrocytes are the main source of physiologically released ATP in the CNS (Coco et al. 2003; Anderson et al. 2004). This signaling molecule is stored in ATP-containing vesicles or vesicles co-storing ATP with other neurotransmitters (e.g., glutamate or γ-aminobutyric acid) (North and Verkhratsky 2006; Pankratov et al. 2007; Araque and Navarrete 2010). The release of ATP from astrocytes may occur by (1) Ca^{2+}-dependent exocytosis, (2) lysosomal release, (3) through hemichannels formed by connexins and pannexins (connexin-43, pannexin-1 channels), and (4) efflux through $P2X_7Rs$ and through plasmalemmal voltage-dependent ion channels (Araque et al. 1999; Cotrina et al. 2000; Pankratov et al. 2006; Zhang et al. 2007).

Further, ATP-induced short-term communication among astrocytes can modulate exocytotic glutamate release (Araque et al. 1998; Jourdain et al. 2007; Parpura and Haydon 2000). Astroglia also regulate the uptake of glutamate and the release of glutamine ("glutamate-glutamine cycle") maintaining both glutamatergic and GABAergic synaptic transmissions (Benarroch 2005; Kimelberg and Nedergaard 2010). For more details the reader is referred to the chapters of Schousboe et al. (2014) and Vardjan et al. (2014).

11.2.1.2 Calcium Signaling

Astrocytes respond to a wide range of physiological and pathophysiological stimuli with an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$). Astroglial ATP, released by various mechanisms, activates purinergic receptors (mainly astroglial $P2Y_1Rs$), which initiate subsequent release of Ca^{2+} from intracellular stores through inositol 1,4,5-trisphosphate ($InsP_3$) receptors initiating "calcium waves" in the astroglial networks. Finally, astroglial Ca^{2+} signals induce the secretion of ATP, which may feed back to neighboring cells (Guthrie et al. 1999; Scemes and Giaume 2006; Bowser and Khakh 2007; Hamilton and Attwell 2010; Di Castro et al. 2011).

11.2.1.3 Degradation

After being released, extracellular ATP undergoes rapid degradation by ectonucleotidases into adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine. Adenosine can often exert protective effects which may counteract

the deleterious influence of ATP (Zimmermann and Braun 1999; Zimmermann 2006; Verkhratsky et al. 2009). The actions of ATP and its breakdown products produce responses that last from milliseconds to minutes, or even longer, through changes in gene regulation via second messengers (Bowser and Khakh 2007; Khakh and North 2012).

11.2.2 Pathophysiology: Complexity in Purinergic Modulation of Reactive Astrogliosis

Astrocytes are able to respond to all forms of CNS injury and disease with the development of "reactive astrogliosis," regulated by specific molecular signaling events in context-dependent manner. These changes range from reversible alterations in gene expression, to cell hypertrophy with preservation of cellular domains and long lasting scar formation with rearrangement of tissue structure (Sofroniew 2009; Sofroniew and Vinters 2010; Verkhratsky et al. 2012a). A considerable amount of data indicates a key role of purines and purinergic signaling in the initiation and maintenance of reactive gliosis.

11.2.2.1 History

Within the first few hours after damage, in dependence on the grade of severity of the injury, neuronal and glial cells undergo cell death at the damaged site. As the first line of defense, microglia responds immediately to danger signals, e.g. ATP released by astrocytes, and converges to the site of damage to form a barrier between healthy and injured tissues. There is ample evidence, that proinflammatory factors, cytokines or other soluble factors, released by microglia play an important role as triggers and modulators of astrogliosis, under the influence of released ATP (for references see Davalos et al. 2005; Zhang et al. 2010).

It has been shown that after acute injury (e.g., spinal cord injury; SCI) low ATP concentrations were detected within the lesioned area, that was found to be surrounded by a peritraumatic area, characterized by sustained ATP release (e.g., for 1–10 min) (Wang et al. 2004). The elevated concentrations immediately after acute injury are not only due to the lack of efficient metabolism by degrading enzymes, but are also a consequence of active release from surviving cells, or nucleic acid degradation upon cell death during persistent or stronger insults (Wang et al. 2004; Melani et al. 2005; Franke et al. 2006; Robson et al. 2006; Choo et al. 2013).

Astrocytes migrate from the adjacent undamaged parenchyma towards the injured core region and start reparation, for example by protruding processes into this region of the CNS (Hatten et al. 1991; Norenberg 1994). In the early 1990s the groups of J. Neary and M. Abbracchio have shown under in vitro conditions that extracellular ATP promotes astrocyte hypertrophy, elongation of glial processes and proliferation, through the activation of the extracellular signal-regulated protein kinase (ERK)1/2 and of the proinflammatory cyclooxygenase (COX)-2 signaling pathways that ultimately result in full-blown astrogliotic phenotype with subsequent formation of the glial scar (for review see Neary et al. 1996a; Abbracchio

et al. 1999). Astrocytes react to extracellular ATP and its structural analogues with upregulation of glial fibrillary acidic protein (GFAP) and increased DNA synthesis (Neary and Norenberg 1992; Neary et al. 1994b). Morphological remodeling in vitro ("stellation", elongation of GFAP-positive cellular processes) has repeatedly been described (Neary and Norenberg 1992; Neary et al. 1994a; Abbracchio et al. 1995; Brambilla et al. 2000).

Within 3–5 days after injury, hypertrophic and elongated astrocytes at the injury border express high levels of vimentin and brain lipid-binding protein (reviewed by Robel et al. 2011). Additionally, the proliferation of astrocytes, as indicated by bromodeoxyuridine (BrdU) incorporation or the expression of Ki67, is stimulated by ATP and P2R agonists (e.g., Abbracchio et al. 1994; Neary et al. 1998; Rathbone et al. 1999; Franke et al. 2001b, 2004b).

In 1999 we showed in a rat model of traumatic brain injury (TBI) for the first time that the in vitro findings are also relevant under in vivo conditions (Franke et al. 1999), suggesting that effects induced by extracellular nucleotides do have a functional significance for in vivo reactive astrogliosis. It is also worth mentioning, that synergistic effects of purinergic ligands and classical growth factors acting in combination to stimulate astrocytic proliferation, contribute to the process of reactive astrogliosis, in a receptor subtype dependent manner (Abbracchio et al. 1995; Lenz et al. 2001; Neary et al. 2006, 2008).

11.2.2.2 Consequences

Activated astrocytes may form a glial scar, which acts as a neuroprotective barrier to infiltration of inflammatory cells and infectious agents. Many studies suggest the benefit of this glial scar, and give credence to astrocytic functions such as the release of neurotrophic factors and adenosine, glutamate uptake, free radical elimination, or the reduction of vasogenic edema after trauma or stroke (for details see Sofroniew 2009; Sofroniew and Vinters 2010; Kimelberg and Nedergaard 2010). Astroglial scar may also become detrimental by delaying or restricting axonal regeneration, hindering functional recovery, and the excessive secretion of neurotoxic substances by activated astrocytes (Zhang et al. 2010; Verkhratsky et al. 2012a). Therefore, astrocytes may favor either beneficial or detrimental outcomes in the pathogenesis and progression of diverse neuropathological conditions.

It is an important new observation that parenchymal reactive astrocytes possess stem cell-like properties (Buffo et al. 2010; Fumagalli et al. 2011b; Lecca et al. 2012). It remains an open issue by which intrinsic and extrinsic mechanisms mature astrocytes contribute to the formation of precursor cells in the process of reactive gliosis (McGraw et al. 2001; Robel et al. 2011). Highly relevant is that NG2+ cells react to pathological conditions by active proliferation and contribution to glial scar formation. NG2+ cells generate oligodendrocytes in the developing and mature CNS and serve as the primary source of remyelinating cells in demyelinated lesions (for review see (Fumagalli et al. 2011b)). It is of special interest that following a lesion, at least a portion of the astrocytes and oligodendrocytes that constitute the glial scar can be generated from NG2+ progenitors (Alonso 2005; Zhao et al. 2009).

11.3 P2 Nucleotide Receptors in Astrocytes and Their Signaling Cascades

11.3.1 The Family of P2 Receptors

Physiological effects of purines and pyrimidines are mediated through an extended family of purinoceptors activated by adenosine, classified as P1 receptors (A1, A2A, A2B, A3), or by ATP/ADP, classified as P2 receptors (P2X, P2Y) (Ralevic and Burnstock 1998; North 2002; Köles et al. 2011). P2Rs are further divided into ionotropic P2XRs and metabotropic P2YRs, both of which mediate raised $[Ca^{2+}]_i$ and have identified roles in glial cell physiology and pathology (James and Butt 2002; Abbracchio et al. 2009).

11.3.1.1 P2XRs

The ionotropic P2XR family with seven mammalian subtypes (P2X1-7) consists of trimeric ligand-gated ion channels and can be distinguished by their relative ion permeabilities, gating kinetics, and their sensitivity to ATP (most of them are activated by low concentrations of ATP) and a range of selective/preferential agonists and antagonists. P2XRs are involved in fast synaptic transmission and synaptic plasticity (Verkhratsky et al. 2012b). The P2X7R subtype is rather unusual among the P2XR family; it is activated only by high concentrations of ATP (0.1–1 mM range) and is capable of membrane pore formation (Surprenant et al. 1996; North 2002).

11.3.1.2 P2YRs

Eight subtypes of metabotropic G protein-coupled P2YRs have been cloned in mammals. They exhibit differential sensitivities to nucleotides: the adenine nucleotides ATP/ADP act at $P2Y_{1,11,12,13}$Rs, the uracil nucleotides UTP/UDP at $P2Y_{4,6}$Rs, and both adenine and uracil nucleotides at the $P2Y_2$R. The $P2Y_{14}$R is sensitive to UDP-glucose and -galactose. P2YRs mediate long-term effects including trophic responses through a variety of intracellular pathways involving gene activation (Burnstock and Verkhratsky 2009, 2010; Zimmermann 2011). A recently deorphanized P2Y-like receptor GPR17 responds to both uracil nucleotides and cysteinyl-leukotrienes (Fumagalli et al. 2011b). For a more detailed review on these topics the reader is referred to the chapter by Verkhratsky and Burnstock (2014).

11.3.2 P2XR (P2X7R) – Mediated Signaling Cascades

On the transcriptional level, all seven P2XR subunits were identified in astrocytes in vitro and in situ, although these cells normally express only low levels of P2XR proteins (Verkhratsky et al. 2009; Illes et al. 2012).

11.3.2.1 Characteristics

Astroglial P2XRs are activated by synaptic transmission and mediate fast local signaling through the elevation of cytoplasmic Ca^{2+}/Na^{+} concentrations. As mentioned above, astroglial ATP (by P2X7R) stimulate L-glutamate, D-aspartate (Anderson and Swanson 2000; Duan et al. 2003) and GABA release (Wang et al. 2002). Furthermore, astroglial $P2X_7Rs$ decrease glutamate uptake, glutamine synthetase activity and alter the plasmalemmal glutamate aspartate transporter (GLAST) (Lo et al. 2008; Liu et al. 2010). P2XRs are able to interact with cell-adhesion molecules, physically cross-talk with other receptor channels (e.g., nicotinic acetylcholine and $GABA_A$), and interact with G protein-coupled receptors (e.g., P2X7R and $P2Y_2R$ in primary astrocytes (Carrasquero et al. 2010; Weisman et al. 2012b)).

11.3.2.2 P2X7R Subtype

The P2X7R was first cloned from rat brain (Surprenant et al. 1996), and subsequently has been found to be expressed in microglia, neurons, and astrocytes (reviewed by Sperlagh et al. 2006; Domercq et al. 2013). Several splice variants of the P2X7R may account for the differential outcome of P2X7R activation in various experimental settings (Adinolfi et al. 2010).

The expression of $P2X_7Rs$ in astrocytes has been described, also defining a functional role for this receptor in these cells (e.g., Kukley et al. 2001; Panenka et al. 2001; Duan et al. 2003; Gendron et al. 2003a; Nobile et al. 2003), but the exact contribution of P2XRs to reactive astrogliosis is still unclear. Functional roles of astroglial $P2X_7Rs$ might be related to Ca^{2+} signaling and secretory activities (examples are given in Table 11.1).

$P2X_7Rs$ have the ability to modulate the cytoskeleton and regulate the processing and release of proinflammatory cytokines, e.g. interleukin-1β (IL-1β; the key mediator in neurodegeneration, neuroinflammation, and chronic pain) as well as trigger necrotic/apoptotic cell death of neurons and glia (e.g., Surprenant et al. 1996; Ferrari et al. 2006; Skaper et al. 2010; Lenertz et al. 2011). The stimulation of the P2X7R regulates the gating of nonselective cation channels, mitochondrial and plasma membrane depolarization, the formation of plasma membrane pores and the production of reactive oxygen species (ROS), responses ultimately leading to cell death (e.g., Schulze-Lohoff et al. 1998; Morelli et al. 2003; Adinolfi et al. 2005; Wang and Sluyter 2013). The stimulation of $P2X_7Rs$ may convey in a species-specific manner a cell death signal to cerebellar astrocytes (Salas et al. 2013). Furthermore, prolonged activation of $P2X_7Rs$ kills all CNS cells (Domercq et al. 2013).

The expression of the P2X7R subtype in astrocytes can be detected following traumatic or ischemic events, or when cells are activated in vitro by the culturing procedure. P2X7R-mediated currents were identified in cultured rodent cortical astrocytes (Duan et al. 2003; Nörenberg et al. 2010) and in astrocytes patch-clamped in acute rodent brain slices (Oliveira et al. 2011) (Fig. 11.1).

Table 11.1 Examples of P2X7R-mediated signaling in astrocytes

Effects/transduction signals	References
p38/MAPK and SAPK/JNK; FGF2-induced proliferation	Neary et al. 2008
p38 (MAPK) induction (cortex), caspase-1 activation, IL-1β release	Bianco et al. 2009
↑Ca^{2+} levels, Lucifer yellow permeation, [^{3}H]purine release	Ballerini et al. 1996
PKC regulated PLD activation	Sun et al. 1999
ERK1/2- and p38-activation, ↑MCP-1 expression	Panenka et al. 2001
PKC-γ, Ca^{2+} signaling	Hung et al. 2005
↑iNOS–NO expression	Murakami et al. 2003
TNF-α increase	Kucher and Neary 2005
PI3K/Akt-pathway, Src family kinase, [Ca^{2+}]i	Jacques-Silva et al. 2004
ERK1/2 activation, phosphorylation of Pyk2, c-Src, PI3K, PKC delta	Gendron et al. 2003a
PLD activation (PKC-dependent/-independent pathways)	Hung and Sun 2002
TGF-β1 mRNA expression; ERK1/2 activation; PKC/MAPK	Wang et al. 2003
ATP-mediated sAPPα release, ERK1/2, JNK, α-secretase activity	Delarasse et al. 2011
IL-1-induced NOS, ↑NO	Narcisse et al. 2005
MAPK/ERK1/2, JNK and p38 activation, effects of AA	Barbieri et al. 2008
PKD(1/2) activation, PI-PLC, PLD	Carrasquero et al. 2010
IL-1β-signaling, expression of IL-8, NF-κB-, and AP-1 activation	John et al. 2001
AQP4 protein expression	Lee et al. 2008
↓ in glutamate uptake and glutamine synthetase activity	Lo et al. 2008
Ca^{2+}-dependent reduction of GLAST expression, PI3K signaling	Liu et al. 2010
IL-1β-stimulated NO expression, IL-6; activation of NF-κB, AP-1, TNF-α	Liu et al. 2000
Release of L-glutamate and D-aspartate	Duan et al. 2003
Px-1 hemichannels, negative-feedback regulation of ATP release	Iwabuchi and Kawahara 2011
P2X7R currents, membrane blebbing, calmodulin	Roger et al. 2008
2-arachidonoylglycerol release	Walter et al. 2004
SOD1G93A astrocytes, P2X7R-initiated motor neuron death: increased ATP-dependent proliferation, implications for amyotrophic lateral sclerosis	Gandelman et al. 2010
(Mechanical strain: astrogliosis, GSK3	Neary and Kang 2006

AP-1 activator protein-1, *AA* arachidonic acid, *SOD1G93A* astrocytes expressing superoxide dismutase 1, *GLAST* glutamate/aspartate plasmalemmal transporter, *GSK-3* glycogen synthase kinase-3, *MCP-1* monocyte chemoattractant protein-1, *NO* nitric oxide, *Px-1* pannexin-1 channels, *PI3K* phosphoinositide 3-kinase, *sAPPα* soluble amyloid precursor protein (APP) ectodomain, *NF-κB* transcription factors nuclear factor-kappa B, *TNF-α* tumor necrosis factor alpha

11.3.2.3 Signaling Pathways

The P2X7R activates a particularly wide range of signal cues (Table 11.1; Fig. 11.2). Mitogen-activated protein (MAP) kinases are a large protein family consisting of ERKs, the stress-activated protein kinases (SAPKs), the Jun N-terminal kinases (JNKs), and p38/MAPKs, which are activated consecutively. The role of MAPK/

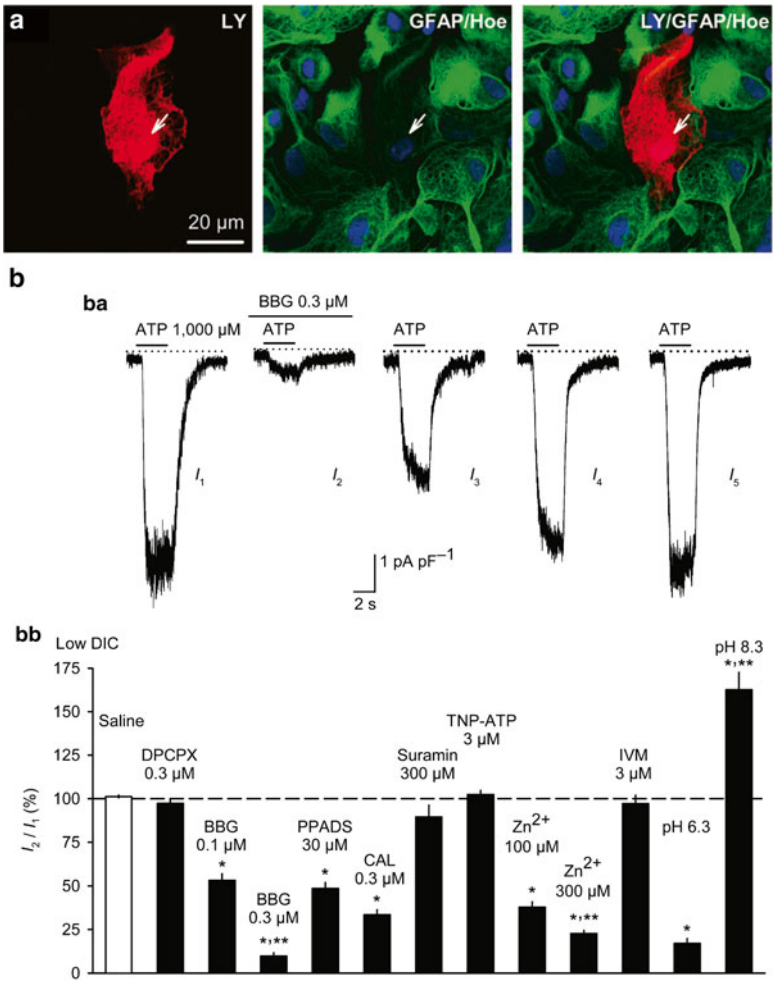

Fig. 11.1 Electrophysiological and immunocytochemical phenotyping of rat cultured cortical astrocytes. (**a**) Example confocal microscopy images from astrocytes of which one (*arrow*) is filled during whole-cell patch-clamp recordings with Lucifer yellow (LY), showing (from *left* to *right*) the *red* carbocyanine (Cy)3 immunofluorescence for LY and the Cy5 *green* color-coded immunofluorescence for the astrocyte marker glial fibrillary acidic protein (GFAP) together with the ultraviolet light excited blue fluorescence for the nucleic acid probe Hoechst 33342 (Hoe), as well as the co-localization of LY with GFAP and Hoe. (**ba**) Whole-cell currents in response to five subsequent applications (I_1–I_5) of ATP (1,000 µM for 3 s at −80 mV) spaced by 4 min intervals. The P2X7 receptor antagonist Brilliant Blue G (BBG; 0.3 mM) was present in the superfusion medium for the 4 min before and during I_2. A Cs^+-based pipette solution was used, and the bath solution contained a low Ca^{2+} medium with no added Mg^{2+} (low divalent cation-containing medium; low DIC) to augment the otherwise rather small nucleotide responses. (**bb**) The histogram summarizes the data derived from similar interaction experiments. Shown are the effects of the drug-free bath solution (saline), of the adenosine receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DCPCX; 0.3 mM), of the P2X receptor antagonists BBG (0.1 and 0.3 mM), pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 30 mM), calmidazolium (CAL; 0.3 mM),

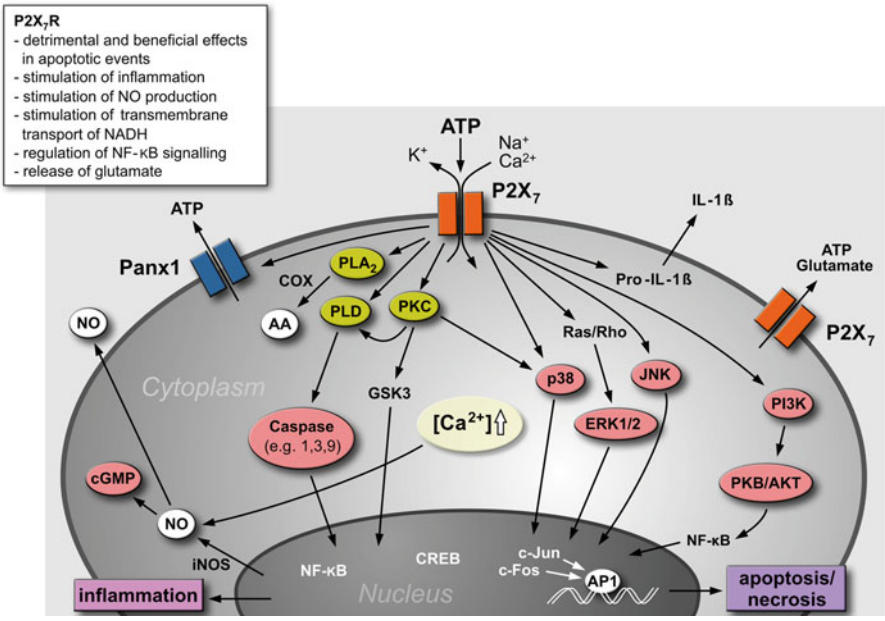

Fig. 11.2 Schematic illustration of examples of signal transduction pathways in astroglial cells following $P2X_7R$ activation. After channel opening, the $P2X_7R$ is permeable for Na^+, K^+, and Ca^{2+}. Activation of the $P2X_7R$ triggers the efflux of K^+ from cells and activates IL-1 converting enzyme, leading to cleavage of pro-IL-1β to mature IL-1β and release from the cell. Many events downstream of $P2X_7R$ activation are dependent on extracellular Ca^{2+} influx. Stimulation of ionotropic $P2X_7Rs$ leads to activation of phospholipases A_2 and D (PLA_2, PLD) and protein kinase C (PKC), e.g., resulting in the activation of glycogen synthase kinase 3 (GSK3) or the activation of caspase cascades. Furthermore, the induction of second messenger- and enzyme-cascades promotes, e.g., the activation of mitogen-activated protein kinase (MAPK) pathway proteins (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK) as well as PI3K/Akt activation. The activity of transcription factors, such as nuclear factor κB (NF-κB), cAMP response element-binding protein (CREB), and activator protein (AP-1) are also upregulated, leading to the expression of proinflammatory genes, such as cyclooxygenase-2 (COX-2) or inducible nitric oxide oxidase (iNOS); this in turn causes the production of arachidonic acid (AA) or nitric oxide (NO), respectively. Finally, the release of ATP via pannexin-1 (Panx1) hemichannels as well as of ATP and glutamate via $P2X_7Rs$ was also found to take place. The present data suggest that astroglial $P2X_7R$ stimulation is associated with neurological disorders leading to neuroinflammation, and apoptosis (artwork by courtesy of Dr. Jens Grosche; taken from Franke et al. [2012] by permission)

Fig. 11.1 (continued) suramin (300 mM), and 2′-3′-*O*-(2,4,6-trinitrophenyl) ATP (TNP-ATP; 3 mM), and of the P2X receptor modulators Zn^{2+} (100, 300 mM) and ivermectin (IVM, 3 mM), as well as those induced by acidification (pH 6.3) or alkalinization (pH 8.3) of the bath. The columns represent the ratio of I_2 with respect to I_1 expressed as percentage of I_1. $*P<0.05$, significant differences from the effects of drug-free bath solution (saline); $**P<0.05$, significant differences from the effects of BBG 0.1 mM, Zn^{2+} 100 mM or pH 6.3 respectively. For further experimental details see Nörenberg et al. (2010) from which this Fig. was modified with permission

ERKs is implicated in cell growth and differentiation, whereas SAPKs and p38 appear to regulate the cell death machinery. In astrocytes, binding of BzATP, a prototypic P2X7 agonist, has been linked to cell-signaling pathways including ERK1/2, p38 MAP kinase, as well as transcriptional complexes of the activator protein (AP)-1 (Panenka et al. 2001; Gendron et al. 2003a; Duan and Neary 2006; Skaper et al. 2010). $P2X_7Rs$ differentially modulate hippocampal and cortical astrocytes, e.g., with respect to pore opening, p38 MAPK activation, and caspase-1 activation (Bianco et al. 2009).

The role of the $P2X_7R$ in proinflammatory gene expression implicates this receptor in a wide range of pathologic conditions of inflammatory nature (Narcisse et al. 2005). $P2X_7Rs$ have been linked to the upregulation of chemokines (e.g., monocyte chemotactic protein-1; MCP-1); as well as IL-1β and IL-8 in human and rat astrocytes, respectively (John et al. 2001; Panenka et al. 2001; Bianco et al. 2009). Autocrine/paracrine signaling via P2Rs significantly modulates both IL-1β- and tumor necrosis factor (TNF)-α-mediated activation of NF-κB and AP-1 in human fetal astrocytes, supporting a regulatory role for these receptors in inflammatory processes (Liu et al. 2000).

Moreover, P2X/YRs are involved in the activation of NO signaling and in the ATP/NO cross talk because of the presence of the Ca^{2+}-dependent NO synthase (NOS) isoforms in astrocytes. ATP triggered the formation of NO by P2X2R-mediated $[Ca^{2+}]_i$ elevation (Florenzano et al. 2008; Latini et al. 2010) and the upregulation of IL-1β-induced NOS expression via the P2X7R (Liu et al. 2000; Narcisse et al. 2005). The injury-induced P2X7R activation is likely to play a role in the protective downregulation of aquaporin-4 (AQP4), which might inhibit water influx to the cells and attenuate acute cytotoxic brain edema (Lee et al. 2008).

In conclusion, the reported data suggest versatility and plasticity of the P2X7R, and its ability to adopt different dispositions and conformations to elaborate particular actions when expressed in different cell types and tissues (Ortega et al. 2009).

11.3.3 P2YR ($P2Y_{1,2\ or\ 12}R$) – Mediated Signaling Cascades

All eight P2YR subtypes are expressed in astrocytoma cells or rat/mice astrocytes depending on species, age, and brain regions (Verkhratsky et al. 2009).

Characteristics: Four main heterotrimeric G protein subfamilies have been characterized (G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13,}$). As previously reported (Franke et al. 2012), the individual P2YR subtypes may be linked to one or more of them: (1) $P2Y_{1,2,4,6}$ and $P2Y_{11}Rs$ couple to G_q proteins stimulating membrane-bound phospholipase C (PLC) which then cleaves phosphatidylinositol-bisphosphate (PIP_2) in the membrane into two second messengers $InsP_3$ and diacylglycerol (DAG). $InsP_3$ mobilizes $[Ca^{2+}]_i$, while DAG activates protein kinase C (PKC), leading to various other intracellular events. (2) $P2Y_{12,13,14}Rs$ bind preferentially to the $G_{i/o}$ subunit. G_i activation is associated with the inhibition of adenylate cyclase (AC) and reduces intracellular levels of cyclic adenosine 5′ monophosphate (cAMP) (Abbracchio et al. 2006; Erb et al. 2006; Fischer and Krügel 2007; von Kügelgen and Harden 2011).

11.3.3.1 Signaling Pathways

P2YRs in astrocytes are linked to a variety of signal transduction mechanisms (Table 11.2, Fig. 11.3). This receptor family can activate (1) the MAP kinase pathway, which is an obligatory step for the triggering and/or persistence of reactive astrogliosis; (2) the phosphoinositide 3-kinase (PI3K)/Akt-pathway, associated with cell growth, fiber regeneration, and inhibition of apoptosis; (3) the phosphatidylinositol-specific phospholipase C (PI-PLC) pathway, and (4) phospholipase A_2 (PLA_2) and arachidonic acid (AA) release.

MAPKs are activated by phosphorylation at threonine and tyrosine residues and phosphorylate themselves transcription factors, which control early gene expression (e.g., factors such as c-Fos, c-Jun, Elk1, ATF2 or the signal transducer and activator of transcription 3, STAT3) (Neary et al. 1996b, 1999; Neary and Kang 2005). Phosphorylation of c-Fos and c-Jun is involved in increased astrocyte proliferation and GFAP expression; ATP-induced upregulation of c-Fos protein and the formation of AP-1 transcriptional complexes have been shown in vitro (Neary et al. 1996b; Bolego et al. 1997) and in vivo (Franke et al. 2012).

P2YR-mediated MAPK/ERK activation has been reported to participate in astrogliosis (Brambilla et al. 2002; Neary et al. 2003); caspase-3 activation and subsequent apoptosis have been shown to be a consequence of P2YR-stimulation (Sellers et al. 2001; Neary et al. 2005). In vivo studies with P2YR antagonists indicated a role of P2X2Rs and $P2Y_1$Rs in injury-induced ERK activation (Neary et al. 2003), $P2Y_2$Rs and $P2Y_4$Rs (partly $P2Y_1$R) regulate astrogliosis via ERK1/2 and STAT3 signaling cascades (Washburn and Neary 2006; Robel et al. 2011); $P2Y_2$Rs are involved in PI3-K- and ERK1/2-mediated proliferation (Gendron et al. 2003b; Weisman et al. 2005). Pharmacological modulation of TBI in rats indicated the involvement of MAPK/ERK1/2, mediated via $P2Y_1$Rs, in astroglial proliferation (Franke et al. 2009). Moreover, after retinal detachment, the P2YR-mediated ERK1/2- and PI3-K activation are necessary for mitogenic effects during proliferative vitreoretinopathy (Milenkovic et al. 2004).

The PI3K/Akt-pathway is associated with controlling the balance between survival and cell death. Purinergic stimulation (via $P2X_{1,2,3}$Rs and $P2Y_2$Rs) of Akt phosphorylation in astrogliosis was first demonstrated in vitro (e.g., Jacques-Silva et al. 2004; Neary and Kang 2005; Neary et al. 2005; Burgos et al. 2007). Later $P2Y_1$R-mediated Akt activation was observed also under in vivo conditions. The obtained data suggest that the PI3K/Akt cascade stimulates astroglial proliferation and may prevent apoptosis (Franke et al. 2009).

P2YRs in cultured astrocytes may be coupled to PI-PLC via G_q, which triggers a PI-PLC/calcium cascade (Neary et al. 1999). Glycogen synthase kinase (GSK)-3 is a signaling molecule involved in cell survival, cell cycle regulation, and proliferation. $P2Y_{1,2,4}$Rs and $P2X_7$Rs are able to couple to GSK3β by a PKC-dependent pathway that is independent of Akt, p70 S6 kinase and ERK1/2, indicating that the regulation of GSK-3 functions may be one of the responses of astrocytes to injury and ATP release (Neary and Kang 2006).

Upregulation and activation of PLA_2 and COX, leading to prostaglandin E_2 (PGE_2) production, have been implicated in a number of neurodegenerative diseases.

Table 11.2 Examples of P2Y ($P2Y_1$)R-mediated signaling in astrocytes

Effects/transduction signals	References
Astrogliosis, proliferation, BrdU	Abbracchio et al. 1994
$[Ca^{2+}]_i$, MAPK activation	King et al. 1996
ATP released from astrocytes mediates glial calcium waves	Guthrie et al. 1999; Fumagalli et al. 2003; Gallagher and Salter 2003; Fam et al. 2000
ATP induced rapid and transient $[Ca^{2+}]_i$ increases, Ca^{2+} wave propagation	
Proliferation, ERK, MAPK, cRaf-1	Lenz et al. 2001
PLA_2 activation, AA release, c-Fos, c-Jun	Bolego et al. 1997
PLC activation, Ca^{2+} release	Centemeri et al. 1997
PI-PLC/Ca^{2+} and ERK pathways, Ca^{2+}-independent PKC isoform, PLD	Neary et al. 1999
Astrogliosis, COX-2 activation, PGE_2 activation, ERK1/2-mediated COX-2 induction	Brambilla et al. 1999; Brambilla et al. 2000; Brambilla et al. 2002
Induction of c-Fos, c-Jun, junB, TIS11mRNA; cAMP accumulation, PKC	Priller et al. 1998
AA release, PKC, MAPK, $cPLA_2$	Chen and Chen 1998
ERK cascade, MAP/ERK kinase activation, cRaf-1	Lenz et al. 2000
IL-1β-induced NF-κB and AP-1 activation	John et al. 2001
TNF-α-induced apoptosis, ERK1/2-, Akt-, JNK phosphorylation	Mamedova et al. 2006
↓Cx43 mRNA expression after IL-1β treatment, Ca^{2+} wave propagation	John et al. 1999
Ras-ERK cascade activation, PI3K, Src, PKC; SAPK, apoptosis	Sellers et al. 2001
Proliferation, activation of PLC-PKC-ERK1/2 signaling pathway; PKA	Quintas et al. 2011
Ca^{2+} dependent glutamate release, TNF-α signaling, TNFR1, prostaglandins	Domercq et al. 2006
P2Y ($P2Y_{2,4}$Rs, partial role of $P2Y_1$R), STAT3 signaling, proliferation, astrogliosis	Washburn and Neary 2006
Mechanical strain: ERK activation; Akt phosphorylation; astrogliosis, ↓GSK3 activity; p38 phosphorylation, ERK1/2-, Akt/PKB signaling	Neary et al. 2003; Neary et al. 2005; Neary and Kang 2006; Burgos et al. 2007
H_2O_2-evoked oxidative injury: release of IL-6	Fujita et al. 2009
Stab wound injury: PI3K/Akt; MEK/ERK cascade, astrogliosis, proliferation, active caspase 3, injury-induced ATP-/glutamate release	Franke et al. 2001b; Franke et al. 2004b; Franke et al. 2006; Franke et al. 2009
Transient MCAO: p-RelA-mediated NF-κB-pathway; chemokine/cytokine release (e.g. IL-6, TNF-α); cerebral infarct volume	Kuboyama et al. 2011
Transient MCAO: JAK2/STAT3, PI3K/Akt/CREB, GDNF	Sun et al. 2008
Photothrombosis: mitochondrial metabolism, ↑$InsP_3$-dependent Ca^{2+} release	Zheng et al. 2010; Zheng et al. 2013
Traumatic brain injury: $P2Y_1$R stimulation decreases cerebral edema; $InsP_3$-signaling pathway; neuronal swelling, gliosis, AQ4 expression	Talley et al. 2013
Traumatic brain injury: reduction in $[Ca^{2+}]_i$ waves, antagonism of purinergic signaling, histological and cognitive effects	Choo et al. 2013

CREB cAMP response element-binding protein, *GDNF* glial cell-line-derived neurotrophic factor, *ERK* extracellular signal-regulated kinase, *MCAO* middle cerebral artery occlusion, *JAK2* phosphorylated Janus kinase 2, *p-RelA* phosphorylated-RelA, *PI3K* phosphatidylinositol 3-kinase, *STAT3* signal transducer and activator of transcription 3, *TNF-α* tumor necrosis factor alpha

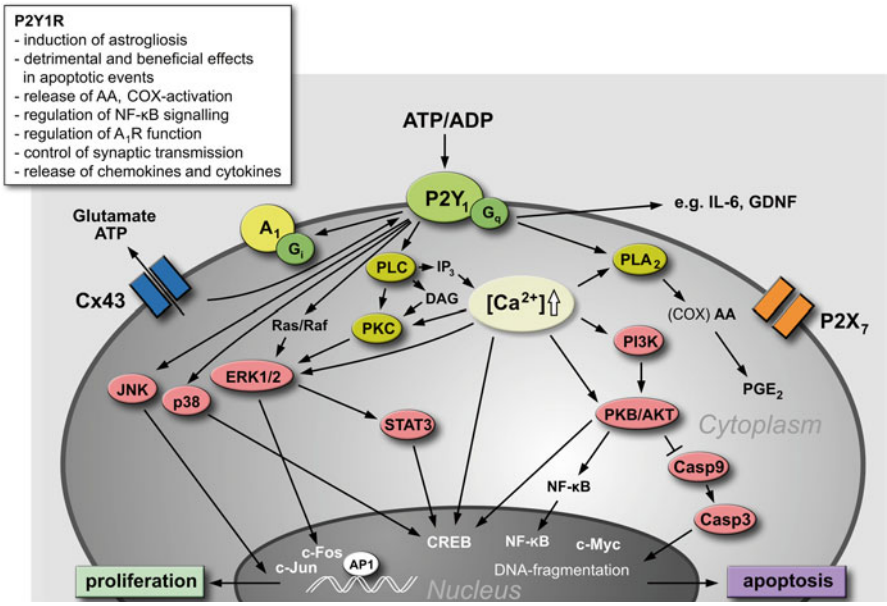

Fig. 11.3 Schematic illustration of examples of signal transduction pathways in astroglial cells following $P2Y_1R$ activation. Stimulation of $P2Y_1Rs$ leads to the activation of phospholipases A_2 and C (PLA_2, PLC) and protein kinase C (PKC), as well as an increase in intracellular calcium ($[Ca^{2+}]_i$). The activation of $P2Y_1Rs$ results in the induction of second messenger and enzyme cascades, e.g. activation of the mitogen-activated protein kinase (MAPK) pathway proteins (ERK1/2), p38 MAPK, c-Jun N-terminal kinase (JNK), and PI3K/Akt activation. $P2Y_1R$-mediated signal transducer and activator of transcription 3 (STAT3) signaling may play a role in astrocyte proliferation and reactive astrogliosis. $P2Y_1R$ activation appeared to be involved in the activation of caspase (Casp) cascades and the release of arachidonic acid (AA) and increase in prostaglandin E_2 (PGE_2) levels. In addition, $P2Y_1R$ activation induces the activity of transcription factors such as nuclear factor κB (NF-κB), cAMP response element-binding protein (CREB), activator protein (AP-1) (which up regulates the expression of proinflammatory genes, e.g. c-Fos, c-Jun, c-Myc). Interaction between adenosine A_1 and $P2Y_1Rs$ may alter the nucleotide signaling cascades. Modulation of astrocytic $P2Y_1Rs$ by the C-terminal domain of the gap junction protein connexin43 (Cx43) appears to be involved in the release of ATP and glutamate. The present data suggest that astroglial $P2Y_1R$ stimulation is associated with neurological disorders leading to neuroinflammation, and apoptosis (artwork by courtesy of Dr. Jens Grosche; taken from Franke et al. [2012] by permission)

Transductional signaling to COX-2 induction involves an early activation of PLA_2 and AA release; this may act as an inducer of the COX-2 gene via the PKC/MAPK pathway. ATP triggers reactive astrogliosis via activation of a P2YR-linked induction of COX-2 and P2YR antagonists might counteract excessive COX-2 activation in acute and chronic neurological diseases (Brambilla et al. 1999, 2000).

Astroglial $P2Y_2R$ upregulation in injury processes represents a cellular response to tissue damage and to proinflammatory conditions (Peterson et al. 2010; Weisman et al. 2012b). $P2Y_2R$ stimulation regulates a variety of signal transduction pathways, such as PLC, which activates different second messenger cascades, e.g., PKC, PLA_2, and ERK1/2 (for review see Peterson et al. 2010; Weisman et al. 2012a). Astroglial $P2Y_2Rs$ are involved in $\alpha_v\beta_3/\beta_5$ integrin signaling, subsequently controlling cytoskeletal

remodeling and motility (Weisman et al. 2005). Activation of $P2Y_2Rs$ induces phosphorylation of the epidermal growth factor receptor (EGFR), a response dependent upon the presence of a SH3 binding domain in the intracellular C terminus of the $P2Y_2R$ that promotes Src binding and transactivation of EGFR, regulating the proliferation of cortical astrocytes (Peterson et al. 2010). Human 1321N1 astrocytoma cells, expressing a recombinant $P2Y_2R$, have been used to assess the role of this receptor in the regulation of anti-apoptotic (bcl-2 and bcl-xl) and proapoptotic (bax) gene expression (Chorna et al. 2004). Furthermore, P2X7R activation increases the expression of $P2Y_2Rs$ in rat astrocytes (D'Alimonte et al. 2007).

11.3.3.2 $P2Y_1R/P2Y_{12}R$ Pharmacology

A number of studies indicate that simultaneous activation of both $P2Y_1Rs$ and $P2Y_{12}Rs$ might play a role in balancing cell growth and cell death.

In vitro and in vivo data support a role for $P2Y_1Rs$ in astroglial proliferation (e.g., Franke et al. 2001b, 2004b; Neary et al. 2003; Quintas et al. 2011). The ADPβS ($P2Y_{1,12,13}R$ agonist)- and ATP (0.01–1 mM)-induced proliferation has been shown to be mediated by all three types of P2YRs (Quintas et al. 2011). The activation of PLC-PKC-ERK1/2 signaling pathways and the additional involvement of PKA (supporting the contribution of the adenosine A_2Rs) have also been described. An opposite effect was shown, using 2-MeSADP ($P2Y_{1,12,13}Rs$ agonist, more potent at the $P2Y_1R$ than 2-MeSATP). 2-MeSADP at lower concentrations (0.001–10 μM) had no effect on astroglial proliferation, but at higher concentrations (0.1–1 mM) inhibited it by mechanisms independent of $P2Y_{1,12,13}R$ activation (Quintas et al. 2011). It was discussed that 2-MeSADP inhibition of astroglial proliferation depended on its conversion into 2-MeS-adenosine, which activated A_3Rs, leading to blockade of [^{3}H]-thymidine uptake by astrocytes and eventually to cell death.

Moreover, 2-MeSADP can induce apoptosis by activating the human (h) $P2Y_1R$ heterologously expressed in astrocytoma cells (Sellers et al. 2001). ADP and also 2-MeSADP induced concentration-dependent phosphorylation changes of the same MAPK family members. 2-MeSADP stimulated ERK1/2 and several isoforms of the SAPK family. It is concluded that adenosine di- and triphosphate stimulation of the $hP2Y_1R$ can transiently activate the Ras-ERK cascade via the cooperative effects of PI3K, Src, and PKC. The sustained ERK stimulation, via a Ras-insensitive pathway, culminates in Elk-1 activation without inducing proliferation. The transient SAPK activity did not evoke transcription factor phosphorylation but was required for the $P2Y_1R$-mediated apoptotic function (Sellers et al. 2001). Using 1321N1 astrocytoma cells stably expressing the $hP2Y_1R$ or $P2Y_{12}R$, it was demonstrated that the activation of $P2Y_1Rs$ induced apoptosis, whereas that of $P2Y_{12}Rs$ did not (Mamedova et al. 2006).

It has been shown that $hP2Y_{12}R$ stimulation by 2-MeSADP (10 nM) activates ERK1/2, Akt, and JNK by phosphorylation. However, at a still lower protective concentration of 2-MeSADP (100 pM), activation of the $hP2Y_{12}R$ phosphorylated only ERK1/2, but not Akt or JNK. The authors discussed that different signaling

pathways are activated in response to ADP in systems which express both $P2Y_1Rs$ and $P2Y_{12}Rs$, such as platelets and astrocytes.

In conclusion, 2-MeSADP has dual effects on cell death and survival, inducing apoptosis via activation of the $P2Y_1R$ and protecting cells against induced apoptosis, through the activation of the $P2Y_{12}R$ (Mamedova et al. 2006).

11.4 Pathological Potential of Astroglial P2Rs in Injury and Disease

The pathophysiology of acute and chronic disorders may include release of excitatory neurotransmitters, oxidative stress, cerebral ischemia, edema, inflammation, or apoptosis (Sofroniew and Vinters 2010; Feeser and Loria 2011). Here we focus on purinergic signaling during different kinds of pathophysiological events. Examples of the pathophysiological potential of P2X/YRs are presented for the human brain (Table 11.3) and that of laboratory animals (Tables 11.4 and 11.5).

11.4.1 Acute Injury

11.4.1.1 Traumatic Brain Injury

TBI is a multi-faceted injury resulting in a range of symptoms and disabilities and includes reactive gliosis surrounding the lesion that occurs in a graded fashion in relation to the severity of the injury in a region- and time-dependent manner.

Role of ATP. Following acute injury, cells at the trauma site rapidly lose their functions or invariably die. Cells beyond the trauma site, initially spared by the lesion, are vulnerable to secondary degeneration (by, e.g., Ca^{2+} release, glutamate-excitotoxicity, and free radical formation) (Savigni et al. 2013). TBI causes breakdown of the blood-brain barrier (BBB), which is followed by vasogenic edema formation, increased intracranial pressure, decreased cerebral blood flow, and subsequent ischemia. Cytotoxic edema forms, because of loss of intracellular ATP and disruption of ion homeostasis (Talley et al. 2013). As mentioned above, Wang and co-worker (2004) have shown after SCI (1) ATP decrease in the center of the injury and (2) high ATP release in the peritraumatic zones reflecting a combination of continued production of energy metabolites and a persistent inability to regulate efflux pathways. This agrees very well with data of other groups, demonstrating ATP release after mechanical strain of cultured rat cortical astrocytes (Neary et al. 2005) or after mechanical lesion of the in vivo rat brain (Franke et al. 2006).

Purinergic signaling and TBI. The direct role of ATP and its breakdown products in the development of astrogliosis after TBI is well analyzed. First experiments indicated astrogliosis in vivo after direct infusion of ATP or ADP into one of the

Table 11.3 Human brain–examples of the involvement of astroglial (glial) cells in different kinds of pathophysiological events

Disease	Receptor subtype	Characteristics	References
Trauma	$P2Y_1R$ (astrocytes, neurons)	autoptic TBI cases, ↑$P2Y_1R$-IR, ↑reactive astrocytes, astrogliosis	Franke et al. 2012
	P2Y-like GPR17 (OPC, microglia, astrocytes, neurons)	TBI patients; autoptic TBI cases; ↑GPR17-IR; ↑activation/proliferation of multipotent progenitors (reactive astrocytes, OPC)	Franke et al. 2013
Epilepsy	P2X/YR	patients with epileptic episodes; ATP, ADP, and AMP hydrolysis rates; soluble nucleotide phosphodiesterase (PDEase) activity	Grosso et al. 2009
Temporal lobe epilepsy (TLE)	P2X7R (microglia, neurons)	↑protein levels in human resected TLE cortex	Jimenez-Pacheco et al. 2013
Alzheimer's disease (AD)	$P2Y_1R$	localization: neurofibrillary tangles, neuritic plaques, and neuropil threads	Moore et al. 2000
	$P2Y_2R$ no change of $P2Y_{4,6}R$	postmortem neocortex AD patients: ↓$P2Y_2R$-IR in parietal cortex; reduction is correlated with neuropathologic score and markers of synapse loss	Lai et al. 2008
	P2X7R (microglia)	↑P2X7R-expression on microglia, in association with Aβ plaques; ↑P2X7 mRNA in microglia (AD patients) and in fetal human microglia exposed to Aβ(1-42); BzATP-induced increase in Ca^{2+} responses	McLarnon et al. 2006
Parkinson's disease (PD)	$A_{2A}R$	postmortem brain (PD patients with dyskinesias), ↑$A_{2A}R$ mRNA; in situ hybridization, receptor binding autoradiography	Calon et al. 2004
Multiple sclerosis (MS)	P2X7R	↑P2X7R-IR in reactive astrocytes (autopsy brain tissue)	Narcisse et al. 2005
	P2X7R (oligodendrocytes)	↑P2X7R-expression in normal-appearing axon tracts in MS patients	Matute et al. 2007
MS, ALS	P2X7R (microglia)	↑COX-2, CB2-, P2X7-IR in activated microglial cells/macrophages; ATP contributes via P2X7R activation to release IL-1β which in turn induces COX-2 and downstream pathogenic mediators	Yiangou et al. 2006
	$P2Y_{12}R$	postmortem MS (cerebral cortex); $P2Y_{12}R$-expression in myelin and interlaminar astrocytes; ↓$P2Y_{12}R$-IR in proximity to the lesions (correlated with the extent of demyelination)	Amadio et al. 2010
MS, EAE	P2Y-like GPR17	↑GPR17 in demyelinating lesions in the CNS induced by EAE and in MS; ↑GPR17mRNA expression in human MS plaques	Chen et al. 2009

ALS amyotrophic lateral sclerosis, *Aβ* β-amyloid plaques, *EAE* experimental autoimmune encephalomyelitis, *IR* immunoreactivity, *MS* multiple sclerosis, *OPC* oligodendrocyte precursor cells, *RT-PCR* reverse transcriptase polymerase chain reaction

Table 11.4 Examples of P2R-mediated astroglial-CNS pathology: Trauma/Mechanical injury

Disease	Receptor subtype	Model	Mechanism/Effect	References
Trauma in vitro	P2X2R, $P2Y_1R$	Mechanical strain	injury-induced astroglial ERK activation	Neary et al. 2005
	$P2Y_2R$	Mechanical strain	activation of ERK1/2 and PKB/Akt through PI3K-dependent mechanisms enhance cell survival	Burgos et al. 2007
	$P2Y_4R$	Mechanical strain	synthesis and release of TSP-1 (promote synaptogenesis)	Tran and Neary 2006
			regulation of TSP-1 via ERK and p38/MAPK	Tran et al. 2012
Trauma in vivo	P2X7R	Corticectomy (rat)	p38, ERK pathways, involved in MCP-1 expression	Panenka et al. 2001
	$P2Y_1R$	Stab wound injury (rat)	agonist-induced stimulation of astrogliosis, PI3K/Akt signaling, astroglial proliferation, anti-apoptosis; antagonist-mediated inhibition of gliosis	Franke et al. 2001b; Franke et al. 2004b; Franke et al. 2006; Franke et al. 2009
	P2X1-4,7Rs	Stab wound injury (rat)	astrogliosis; expression on activated astrocytes	Franke et al. 2001a
	$P2Y_1R$	Cell stretch injury (in vitro; ex vivo); cortical brain injury (mice)	$P2Y_1R$ antagonist (MRS 2179): improves histological and cognitive outcomes; reduction in the intercellular calcium restores neural activity	Choo et al. 2013
	$P2Y_1R$	controlled closed skull injury model (mice); $InsP_3R2^{-/-}$ mice	$P2Y_1R$ agonists (2-MeSADP; MRS2365): ↓oedema, neuronal swelling, reactive gliosis, AQP4 expression; $InsP_3$-calcium signaling pathways	Talley et al. 2013
	P2X7R	controlled cortical impact (moderate TBI) (mice)	P2X7R antagonist (BBG): ↓GFAP expression, attenuated AQP4 expression	Kimbler et al. 2012
SCI	P2X7R	thoracic SCI (rats)	P2X7R antagonist (BBG): ↓SC anatomic damage, improved motor recovery; BBG reduced activation of astrocytes and microglia, neutrophil infiltration	Peng et al. 2009
SCI	P2X7R	weight-drop device at New York University (rat)	ATP release, high-frequency spiking, irreversible increases in cytosolic calcium, cell death;	Wang et al. 2004
			antagonists (oxATP, PPADS): significantly improved functional recovery, diminished cell death in the peritraumatic zone	
SCI	$P2Y_1R$	spinal cord injury (NYU impactor device; rat)	antagonist (PPADS, suramin): reduced gliotic response and spared tissue	Rodriguez-Zayas et al. 2012

AQP4 aquaporin-4, *BBG* Brilliant blue G, *ERK* extracellular signal regulated, *MAPK* mitogen-activated kinase, *SCI* spinal cord injury, *TSP-1* thrombospondin-1, *MCP-1* monocyte chemoattractant protein-1

Table 11.5 Examples of P2R-mediated astroglial-CNS pathology: Ischemia

Disease	Receptor subtype	Effect/model	Mechanism/effect	References
Stroke	P2X7R	MCAO (rat)	increased receptor expression after MCAO	Franke et al. 2004a
	P2X7R	4-VO (four vessel occlusion, rat)	P2X7R antagonists (BBG, A-0348079, oxATP): ↑survival rates, ↓learning memory deficit, ↓neuronal death, DNA cleavage; ↓astro-/microglial activation, inflammatory cytokine expression	Chu et al. 2012
	$P2Y_1R$	tMCAO (rat)	$P2Y_1R$ agonist (MRS 2365): ↑infarct volume; antagonist (MRS 2179) - ↓infarct volume, recovered motor coordination; regulation of cytokine/chemokine response through a p-RelA-mediated NF-κB pathway	Kuboyama et al. 2011
	P2X/YRs	MCAO (rat)	P2X/YR antagonist (PPADS): functional recovery of motor and cognitive deficits: reduction of paresis-induced sideslips; ↓infarct volume; improvement of electrophysiological, functional, and morphological alterations	Lämmer et al. 2006 Lämmer et al. 2011
	$P2Y_1R$	tMCAO	$P2Y_1R$ mediated GFAP- and GDNF-production; JAK2/STAT3- and PI3-K/Akt/CREB- signaling	Sun et al. 2008
	$P2Y_1R$	Bengal Rose photothrombosis (mouse)	$P2Y_1R$ agonist (2-MeSADP): reduces cytotoxic edema, continued growth of brain infarction; $InsP_3$-dependent calcium release	Zheng et al. 2010
	$P2Y_1R$	Bengal Rose photothrombosis (mouse); transgenic animals; $InsP_3R2^{-/-}$ mice	$P2Y_1R$ agonist (2-MeSADP): ↓neuronal damage; $InsP_3R2^{-/-}$ mice: no reduction or reverse of neuronal damage; $InsP_3$-calcium signaling pathways	Zheng et al. 2013

oxATP adenosine 5′-triphosphate-2′,3′-dialdehyde, *BBG* Brilliant blue G, *4VO* four vessel occlusion, *GDNF* glial cell-line-derived neurotrophic factor, *GFAP* glial fibrillary acidic protein, *p-RelA* phosphorylated-RelA, *PPADS* pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid, *tMCAO* transient middle cerebral artery occlusion

cerebral hemispheres (Hindley et al. 1994). Intrastriatal microinjection of ATP caused cell death within 24 h in the striatum of rats (Ryu et al. 2002). Finally, a single intracerebrally injected dose of the P2X/YR antagonist suramin reduced inflammation, as judged by decreased cellular proliferation, GFAP levels, and tenascin C-immunoreactivity (IR) (Di Prospero et al. 1998).

Neary and co-workers developed an in vitro model for brain trauma using astrocytes cultured on deformable Silastic® membranes, which were subjected to the rapid, reversible strain (stretch)-induced injury (Neary and Kang 2005). Important findings using this model were, e.g., the lesion-induced release of ATP, the characterization of involved P2Rs (e.g. P2X2R, $P2Y_1R$), the calcium-dependent ERK signaling and the activation of the PKB/Akt cascade (Neary et al. 2003, 2005). Mechanical strain also activates $P2Y_2Rs$, promoting the activation of ERK1/2 and PKB/Akt through PI3K-dependent mechanisms to enhance cell survival (Burgos et al. 2007) as well as $P2Y_4Rs$, stimulating the synthesis and release of thrombospondin-1, an extracellular matrix molecule, inducing synaptogenesis (Tran and Neary 2006; Tran et al. 2012).

Under in vivo conditions, activation of $P2X_7Rs$ promotes cerebral edema and neurological injury after TBI in mice (Kimbler et al. 2012). The authors described $P2X_7Rs$ localized within astrocyte endfeet and that administration of Brilliant Blue G (BBG), a P2X7R antagonist, decreased the GFAP-expression and attenuated AQP4-expression.

We have demonstrated that endogenous ATP participates in the in vivo astrogliosis caused by stab wound injury in the nucleus accumbens (NAc) and cortex of rats (Franke et al. 1999). We found an increase in GFAP and astroglial proliferation following microinjection of ATP analogues into the NAc (Franke et al. 1999, 2004b) as well as changes in the astroglial P2X/YR expression (upregulation of P2X1,7Rs and $P2Y_{1,2,6}Rs$) (Franke et al. 2001a, 2004b). Pharmacological characterization indicated a role of $P2Y_1Rs$ in the mediation of astroglial activation and proliferation (Franke et al. 2001b, 2004b, 2009). The in vivo data perfectly agreed with substance treatment studies in cultured astrocytes, where the involvement of $P2Y_{12}R$ was also shown (Quintas et al. 2011). Finally, we have shown that TBI in vivo is accompanied by a $P2Y_1R$-mediated modulation of PI3-K/Akt- and MAPK/ERK-signaling pathways, resulting in astroglial proliferation and anti-apoptotic processes (Franke et al. 2009). Furthermore, recent data indicate a role of astroglial $P2Y_1Rs$ in astrogliosis after TBI in human brain (Franke et al. 2012). In prefrontal cortical samples of patients with TBI, we observed GPR17/GFAP-positive reactive astrocytes (Franke et al. 2013). These findings correlate with the above discussed new role of mature astrocytes contributing to the formation of precursor cells during astrogliosis (Fig. 11.4). GPR17 has also been reported to be expressed on OPCs and modulate proliferation of oligodendrocytes (Fumagalli et al. 2011b; Lecca et al. 2012).

Pharmacology. In vitro screening shows that astrocyte signaling through the "mechanical penumbra" affects the activity of neural circuits distant from the injury epicenter, and a reduction in the intercellular calcium waves within astrocytes restores neural activity after injury (Choo et al. 2013). In a preclinical model of TBI, the selective $P2Y_1R$ antagonist MRS 2179 improved histological and cognitive

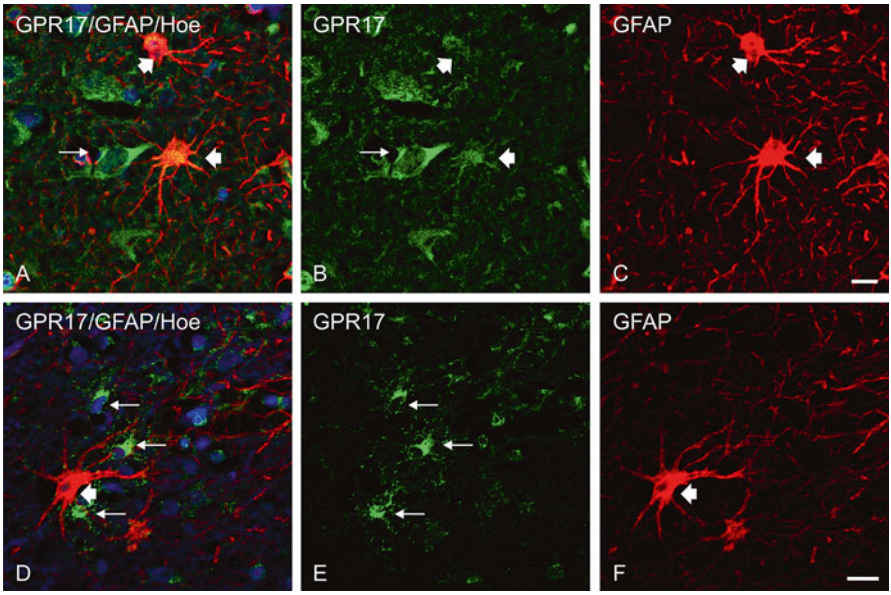

Fig. 11.4 Changes in receptor expression in human brain after traumatic brain (TBI) injury. GPR17 expression in the pericontusion area after TBI in tissue collected from human autoptic samples. (**A**–**C**) Examples of confocal microscopy images indicating the co-expression of GPR17 (*yellow green* Cy2 immunofluorescence) on reactive GFAP-positive astrocytes (*thick arrows*; *red* Cy3 immunofluorescence). (**D**–**F**) Examples without co-expression on activated astrocytes (*thick arrows*); (**A**,**B**) GPR17-positive neurons (*thin arrow*; *yellow green* Cy2 immunofluorescence); (**D**,**E**) GPR17-positive OPC without GFAP (*thin arrows*; yellow green Cy2 immunofluorescence). (**A**,**D**) Merge image of the same cells after GPR17 and GFAP labeling, together with the ultraviolet light excited blue fluorescence for the nucleic acid probe Hoechst 33342 (Hoe, blue). (scale bars: 10 μm, H. Franke and M. Weber unpublished)

outcomes. This "*antagonism of purinergic signaling*" indicated neuroprotective effects in the penumbra of the injury rather than at the lesion epicenter and has two components: (1) the reduction of glutamate release from astrocytes, and (2) the restoration of pro-survival synaptic signaling (Choo et al. 2013).

Characterizing the role of "*agonism in purinergic signaling*" the used $P2Y_1R$ agonists (2-MeSADP, MRS2365) significantly reduced the extent of cellular damage in the primary injury phase after TBI, thereby also diminishing neurotoxic effects observed during the secondary injury phase (Talley et al. 2013). $P2Y_1R$ stimulation (30 min after TBI) decreased post-injury symptoms, including cerebral edema, reactive gliosis, and AQP4-expression, mediated by the $InsP_3$-signaling pathway. The authors discussed an early critical need for energy utilization after TBI for the maintenance of ion homeostasis and an enhanced astrocyte mitochondrial metabolism via $P2Y_1R$ stimulation.

In conclusion, the pathophysiological processes strongly correlate with energy balance after injury, but the different studies indicate that it remains hitherto unclear as to what the optimal level of synaptic signaling is that supports neuronal survival without triggering deleterious excitotoxic pathways (Choo et al. 2013).

It should be also mentioned, that after SCI the excessive ATP release from peritraumatic regions contributes to the inflammatory response by activation of low-affinity $P2X_7Rs$ (Wang et al. 2004). P2X7R blockade (oxATP, PPADS) significantly improved functional recovery and diminished cell death in the peritraumatic zone. In a weight-drop model of thoracic SCI the P2X7R antagonist BBG directly reduced local activation of astrocytes, as well as microglia, and neutrophil infiltration (Peng et al. 2009). Somewhat unexpectedly, astrogliosis has been described as an important event after SCI that limits tissue damage and lesion spreading (Rodriguez-Zayas et al. 2012).

11.4.2 Ischemia

Ischemic/hypoxic events have been attributed to membrane depolarization and transmitter release (e.g., ATP, glutamate) that induce cytosolic calcium overload, loss of mitochondrial potential, and the activation of intracellular enzymes with deleterious consequences for cell function and viability. Cell death is partially related to glutamate-excitotoxicity, and *N*-methyl D-aspartate receptors are considered as the main targets responsible for Ca^{2+} overload in the ischemic brain. Additionally, neuroinflammatory processes play an important role in cerebral ischemia/reperfusion (for references see Chu et al. 2012).

11.4.2.1 Characteristics

Also under ischemic conditions, the intracellular levels of ATP rapidly fall, whereas extracellular purine nucleotides are elevated due to membrane damage, cell death, and release from nonneuronal cells (Phillis et al. 1993; Braun et al. 1998; Juranyi et al. 1999; Melani et al. 2005). In hippocampal slices, a delayed release of ATP occurred after anoxic depolarization, whereas adenosine release (being protective during hypoxia/ischemia) was apparent almost immediately after the onset of ischemia (Frenguelli et al. 2007; Burnstock et al. 2011a). Normally, ATP is hydrolyzed by ATP diphosphohydrolase and 5′-nucleotidases; an upregulation of these enzymes after ischemia might modify the nucleotide concentrations and alter the metabolic limitation (Braun et al. 1998). For more details see the chapter of Ding (2014).

Depending on the degree of the ischemic event as well as on the length of the post-ischemic period, region-dependent changes in the expression of P2XRs (e.g., $P2X_{2,4,7}$) and P2YRs (e.g., $P2Y_{1,2}$) were found.

11.4.2.2 Purinergic Signaling - P2X7R

ATP can be released from astrocytes by mechanisms involving ion channels (such as the P2X7R itself) and through pannexin-1 (Px1) hemichannels under ischemic stress. The opening of Px1 channels is regulated by the activity of $P2X_7Rs$; the blockade of $P2X_7Rs$ during simulated ischemia resulted in the opening of Px1

hemichannels, leading to enhanced release of ATP (Iwabuchi and Kawahara 2011; Matute and Cavaliere 2011).

Ischemia in organotypic brain slices upregulated $P2X_7Rs$ (Cavaliere et al. 2004b). Middle cerebral artery occlusion (MCAO) in rat brain led to an increase of P2X7R-IR in the penumbra surrounding the necrotic region especially on microglial cells, but later also on astrocytes and eventually on neurons (Collo et al. 1997; Franke et al. 2004a). Immunoelectron microscopy revealed the expression of P2X7Rs on presynaptic elements of neurons and perisynaptic processes of glial cells (Franke et al. 2004a).

Overactivation of $P2X_7Rs$ on can induce excitotoxic neuronal death by calcium overload. Under sustained application of ATP (and P2X7R agonists) P2X7R action switches from a "typical" ion channel, selective for small cations, to a mode consistent with a large pore that allows passage of molecules up to 900 Da. This may result in increased membrane permeability that promotes actin disaggregation and rapid cytoskeletal rearrangements such as membrane blebbing, cell lysis, cytokine release, and apoptosis (for review see Arbeloa et al. 2012).

11.4.2.3 Pharmacology

$P2X_7R$ blockade prevents ATP excitotoxicity in neurons, is protective in white matter injury as well as reduces infarct volume and brain damage after ischemic injury (Melani et al. 2006; Domercq et al. 2010; Arbeloa et al. 2012). An anti-inflammatory therapy by blockade of $P2X_7Rs$ might be beneficial for the treatment of cerebral ischemia. In fact, P2X7R antagonists are protective in a dosage-dependent manner: a high dosage of BBG (10 μg) and a low dosage of oxATP (1 μg) significantly reduced ischemia-induced learning memory deficit, neuronal death, and ischemia-induced astro-/micoglial activation and inflammatory cytokine (IL1β, TNF-α, IL-6) overexpression (Chu et al. 2012). After MCAO in rats, BBG treatment produced a 60 % reduction in the extent of brain damage (Arbeloa et al. 2012).

It is noteworthy that some studies failed to show any protective effect of P2X7R antagonists in MCAO models (Le Feuvre et al. 2003; Yanagisawa et al. 2008). In addition the toxicity of some antagonists at a high dose range (e.g., oxATP may be fatal to neurons and macrophages under such conditions) as well as apparent species differences in the kinetic properties of the $P2X_7Rs$ (Hibell et al. 2000) may limit the therapeutic use of P2X7R antagonists.

11.4.2.4 Purinergic Signaling - $P2Y_1R$

$P2Y_1Rs$ are expressed in ischemia-sensitive areas of the hippocampus and cerebral cortex (Moran-Jimenez and Matute 2000). Intensive accumulation of $P2Y_1Rs$ was observed in the penumbra around the necrotic tissue in these areas of the brain (Kuboyama et al. 2011). Using the MCAO model, we could observe a

PPADS-sensitive upregulation of $P2Y_1Rs$ in the peri-infarct area (H. Franke, U. Krügel, A. Lämmer, unpublished data) and the inhibition by PPADS improved morphological (infarct volume, cell death) and functional alterations (recovery of electrophysiological and motor functions) (Lämmer et al. 2006, 2011).

After transient MCAO, the intracerebroventricular administration of a $P2Y_1R$ agonist (MRS 2365) rather than the antagonistic PPADS (see above; Lämmer et al. 2006, 2011) increased cerebral infarct volume whereas $P2Y_1R$ antagonists (MRS2279, MRS2179) significantly reduced the infarct volume and led to recovered motor coordination (Kuboyama et al. 2011). $P2Y_1R$ inhibition also resulted in cytokine/chemokine transcriptional suppression and cerebroprotection via a p-RelA-mediated NF-κB pathway (Kuboyama et al. 2011). $P2Y_1Rs$ in cultured astrocytes have been reported to be involved in the protection against hydrogen peroxide (a ROS, generated in ischemic stroke)-induced damage (Fujita et al. 2009).

Furthermore, under ischemic conditions, stimulation of $P2Y_1Rs$ also reduced both astrocytic swelling and brain infarct volume (Zheng et al. 2010, 2013). In a Rose Bengal-induced photothrombotic model in mice, treatment with 2-MeSADP resulted in improved ischemic and neurological outcomes. The authors discussed, that astrocyte mitochondria are a key energy source in the post-ischemic penumbra and $P2Y_1R$-initiated, $InsP_3R$-dependent stimulation of the mitochondrial metabolism reduces and partially reverses neuronal damage suggesting that $InsP_3$-Ca^{2+}-signaling in astrocytes is critical for $P2Y_1R$-enhanced neuroprotection (Zheng et al. 2013). For further discussion see the chapter of Sayre et al. (2014).

Purine and pyrimidine nucleotides are equally released during brain ischemia and in consequence UTP/UDP-sensitive P2Y receptors might also be involved in neuronal injury/protection; the UTP-induced activation of $P2Y_4Rs$ causes cell death in human neuroblastoma cells (Cavaliere et al. 2004a). Endothelial $P2Y_2R$-mediated dilatations of rat MCA in response to UTP were potentiated after ischemia-reperfusion, while $P2Y_1R$-mediated dilatation was simultaneously attenuated (Marrelli et al. 1999; Pelligrino et al. 2011).

11.4.3 Chronic Injury

11.4.3.1 Epilepsy

Several brain foci are associated with seizure generation and also glial alterations may contribute to the generation and spread of seizure activity (Verkhratsky et al. 2012a; Jimenez-Pacheco et al. 2013).

Characteristics. Seizures result from recurrent firing of excitatory neurons as a consequence of an imbalance favoring hyperexcitability, which leads to excessive release of glutamate (Matute and Cavaliere 2011). Additionally, ATP has emerged as a potential contributor to prolonged seizures and possible roles for ATP in the

initiation and spread of epileptiform discharges have been described (Jourdain et al. 2007; Burnstock 2008; Kumaria et al. 2008; Engel et al. 2012b).

Purinergic signaling. Microinjection of ATP analogues into the prepiriform cortex induces generalized motor seizures (Burnstock et al. 2011b). Adenosine, an endogenous inhibitor of excitatory synaptic transmission with potent anticonvulsive properties, accumulates from the hydrolysis of ATP and is believed to inhibit distant synapses by acting on adenosine P1Rs (Kumaria et al. 2008; Burnstock et al. 2011a; Matute and Cavaliere 2011).

Kainic acid-induced status epilepticus (SE) increased the expression of P2X7R and $P2Y_{6,12}R$ mRNAs in mouse hippocampal microglia (Avignone et al. 2008). In addition, microglial membrane currents mediated by these P2 receptors were strongly potentiated. After pilocarpine- or kainic acid-induced SE in rodents, P2X7R upregulation has been described in the hippocampus and cerebral cortex (Vianna et al. 2002; Engel et al. 2012a; Jimenez-Pacheco et al. 2013). In the resected neocortex from patients with pharmacoresistent temporal lobe epilepsy, a similar change in P2X7R expression was observed (Burnstock et al. 2011a). In studies using a P2X7R reporter mouse, the increased P2X7R transcription after SE and in the state of chronic epilepsy was limited to hippocampal neurons and microglia in distinction to astrocytes (Engel et al. 2012a; Jimenez-Pacheco et al. 2013). It has to be noted, that a loss of GFAP-positive astrocytes after SE has been described, indicating the disruption of astrocytes network and accompanied by upregulation of $P2X_7Rs$ in activated microglia (Kim et al. 2009).

Pharmacology. Astrocyte intercellular calcium waves have been shown to underlie seizures, and conventional antiepileptic drugs attenuated these calcium waves (Kumaria et al. 2008; Burnstock et al. 2011b). Pharmacological modelling also indicated that $P2X_7Rs$ differentially modulate SE-induced astroglial loss in distinct brain regions (Kim et al. 2011).

P2X7R agonists and antagonists confirmed the recruitment of the P2X7R during seizure generation and seizure-induced cell death; e.g., pretreatment with the P2X7R antagonist A-438079 reduced electroencephalographic and clinical seizure severity and seizure-induced neuronal death in mice (Jimenez-Pacheco et al. 2013). UTP, also released during epileptic fits, acts in the 4-aminopyridine-induced epilepsy model as an inhibitory neuromodulator by stimulating P2YRs (Slezia et al. 2004).

11.4.4 Neurodegenerative Disease

Chronic neuroinflammation participates in the progressive nature of diverse neurological diseases, including Parkinson's disease, Alzheimer's disease, and multiple sclerosis, and occurs when glial cells (i.e. astrocytes, microglia) undergo prolonged activation (Peterson et al. 2010; Gao et al. 2011). Reactive astrocytes contribute and reinforce inflammatory cascades (Fuller et al. 2009).

11.4.4.1 Alzheimer's Disease

In Alzheimer's disease (AD), astrocytes become activated and there is a degree of correlation between the severity of astrogliosis and cognitive decline (Fuller et al. 2009; Heneka et al. 2010; Verkhratsky et al. 2012a).

Characteristics. The amyloid precursor protein (APP) is proteolytically processed by β- and γ-secretases to generate β-amyloid peptide (Aβ), the main component found in senile plaques of AD patient brains. Alternatively, APP can be cleaved within the Aβ sequence by α-secretase, thus precluding the generation of Aβ (Leon-Otegui et al. 2011). In astrocytes of triple transgenic AD mice, the production, accumulation and degradation of Aβ were reported to occur (Verkhratsky et al. 2012a).

Purinergic signaling. In studies characterizing brains of AD patients and in animal models, alterations in the density of P2X7 and $P2Y_{1,2}$Rs were observed. P2X7R-IR and mRNA were upregulated around Aβ plaques in animal models of AD and in microglia obtained from AD patients (Parvathenani et al. 2003; McLarnon et al. 2006). The $P2Y_1$R was localized in characteristic AD structures such as neurofibrillary tangles, neuritic plaques, and neuropil threads (Moore et al. 2000). The $P2Y_2$R-IR, studied in postmortem materials from AD patients, was selectively reduced in the parietal cortex correlating with neuritic plaque and neurofibrillary tangle scores as well as synapse loss (Lai et al. 2008).

$P2X_7$Rs. In different neuroblastoma cell lines, primary astrocytes and neural progenitor cells, selective activation of P2X7R induces the release of the soluble APPα ectodomain (sAPPα; with neuroprotective properties), mediated by a metalloprotease (Delarasse et al. 2011). ERK1/2 and JNK are involved in P2X7R-dependent α-secretase activity. The capacity of the P2X7R, expressed in hippocampal neurons and glial cells (especially microglia), to trigger non-amyloidogenic APP processing suggests a neuroprotective effect in AD (Delarasse et al. 2011). Furthermore, extracellular ATP, acting via $P2X_7$Rs, can alter Aβ-induced cytokine release from macrophages and microglia (Rampe et al. 2004; Sanz et al. 2009) and P2X7R mediates superoxide production in primary microglial cultures (Parvathenani et al. 2003). P2X7R inhibition, possibly by acting to inhibit inflammatory reactivity, confers neuroprotection in an animal model of AD; e.g., attenuated gliosis, diminished leakiness of the BBB and neuroprotection in the Aβ (1-42)-injected rat brain (Ryu and McLarnon 2008).

P2Y2Rs. Astrocytes are a potential source of APP and studies in rat primary cultures of cortical astrocytes indicated that $P2Y_{2,4}$R activation can increase the APP production and secretion via MAPKs (but not Akt) (Tran 2011). The $P2Y_2$R has been shown to stimulate α-secretases that mediate the proteolytic processing of APP to generate the non-amyloidogenic sAPPα peptide in human 1321N1 astrocytoma cells (Camden et al. 2005) and Neuro-2a cells (Leon-Otegui et al. 2011). The opposite effect of P2X7 and $P2Y_2$Rs on α-secretase-dependent APP processing in Neuro-2a cells was also described (Leon-Otegui et al. 2011). It has been discussed, that $P2Y_2$Rs in glial

cells contribute to the phagocytosis and degradation of neurotoxic forms of Aβ in vivo, under conditions where elevated levels of ATP release and IL-1β generation occur (for review see Weisman et al. 2012b).

11.4.4.2 Multiple Sclerosis

Hallmarks of multiple sclerosis (MS) are oligodendrocyte death, demyelination, and axonal damage. It has been hypothesized that released ATP from astrocytes, infiltrating cells of the monocyte/macrophage lineage, and activated microglia contribute to purinergic activation and cell death of oligodendrocytes (Matute et al. 2007; Matute and Cavaliere 2011).

Purinergic signaling. P2X7R-IR was observed on reactive astrocytes in MS lesions in autopsy brain tissues (Narcisse et al. 2005) and was elevated in normal-appearing axon tracts in MS patients (Matute et al. 2007). Sustained P2X7R activation in vivo causes lesions that are reminiscent of the major features of MS plaques (Matute et al. 2007).

P2X7R-mediated increase in IL-1 levels caused nitric oxide synthase activity in cultured astrocytes suggesting that $P2X_7Rs$ may modulate the astrocytic response to inflammation (Narcisse et al. 2005). Furthermore, the application of P2X7R antagonists in a model of MS (chronic experimental autoimmune encephalomyelitis; EAE) reduced demyelination and ameliorated the associated neurological symptoms. It has been discussed that ATP can kill oligodendrocytes via P2X7R activation and that this cell death process contributes to EAE (Matute et al. 2007; Domercq et al. 2013).

The analysis of cerebral cortex sections from postmortem MS brain indicated that $P2Y_{12}R$ protein is present in myelin and interlaminar astrocytes, is highly enriched in oligodendrocytes, but is absent from microglia/macrophages (Amadio et al. 2010). Activating $P2Y_{12}Rs$ on oligodendrocytes and astrocytes might perform a direct and/or indirect role in the promotion of myelination. ATP induced the secretion of the regulatory protein leukemia inhibitory factor that promotes the myelinating activity of oligodendrocytes and is released by astrocytes in response to ATP liberated from axons firing action potentials (Ishibashi et al. 2006). GPR17 has also been identified as a modulator of oligodendrocytes, e.g., by participating in the differentiation during maturation and in myelinating disorders (Chen et al. 2009; Ceruti et al. 2011; Fumagalli et al. 2011a). qRT-PCR analysis of human MS tissues indicated a significantly higher GPR17 expression level in MS plaques, associated with demyelinating lesions induced by EAE and in MS, than in the intact brain (Chen et al. 2009).

Additionally, in amyotrophic lateral sclerosis (ALS) the decline in the number of motor neurons in spinal cord ventral horn correlated well with an increase in P2X4 IR structures (Casanovas et al. 2008). In mSOD1 mice (a transgenic animal model for familial ALS), the treatment with a P2X4R modulator (ivermectin) resulted in 10 % life span extension possibly by boosting favorable ATP effects (Andries et al. 2007). Finally, it appears that during the progression of ALS, microglia, astrocytes, and motor neurons might cross-talk via ATP release/degradation and also P2X7R activation, generating a feedback loop that drives a sustained pro-inflammatory and detrimental response (Yiangou et al. 2006; D'Ambrosi et al. 2009; Gandelman et al. 2010; Amadio et al. 2011; Volonte et al. 2011).

11.5 Examples of Purinergic Signaling of the Neuroglia

Here, we focus on examples of purinergic signaling in microglia, oligodendrocytes, and blood vessels interacting with astrocytes under physiological and pathological conditions (also illustrated in Fig. 11.5).

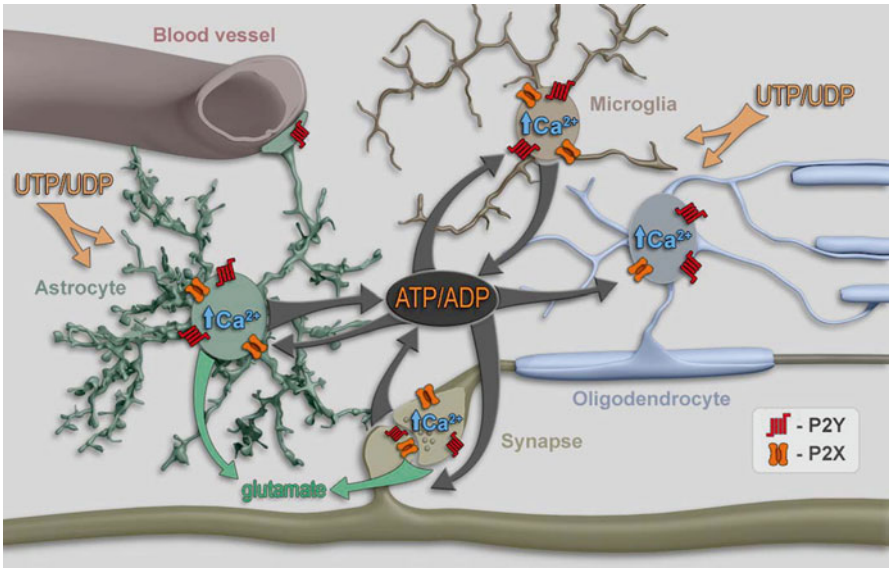

Fig. 11.5 Schematic illustration of ATP (and glutamate) release and P2X/YR activation under pathological conditions and the resulting cell-to-cell communication between glial cells, blood vessel, and neurons. Release of ATP may occur by exocytosis from neurons, astrocytes, or microglia. The released ATP acts postsynaptically on P2X and P2YR subtypes, also activated by ADP, UTP, and UDP. ATP is broken down by ectonucleotidases to ADP and adenosine. ADP also contributes to P2YR stimulation and adenosine can often exert protective actions which may counteract the toxic cascade initiated by ATP. Excessive ATP and glutamate release prolongs activation of $P2X_7Rs$ which leads to calcium overload of the cytosol and to activation of enzymes resulting in mitochondrial failure and cell death. Astrocytes release ATP through $P2X_7Rs$, to amplify Ca^{2+} signaling and lead to propagation and intercellular Ca^{2+} waves. This expression of multiple P2R subtypes (e.g. $P2X_7Rs$, $P2Y_{1,2}Rs$) by astrocytes provides a multiphasic signaling mechanism, which couples astroglial Ca^{2+} excitability to neuronal activity. Additionally, ATP stimulates astrocytes (via $P2Y_1Rs$) to release glutamate. ATP controls microglial activation by acting at P2X4 and $P2Y_{12}Rs$ to induce motility and chemotaxis, by $P2Y_6Rs$ to induce phagocytosis or by $P2X_7Rs$ driving activation and proliferation. Activation of microglial $P2X_7Rs$, serves as an important component of a neuroinflammatory response in a broad range of CNS pathologies. ATP induces a rise in cytosolic Ca^{2+} in oligodendrocytes by activating P2X7R. $P2Y_{1,12}Rs$ contribute to the migration and adhesion of glial cell processes to axons during pre-myelination. Altered ATP and glutamate homeostasis, mediated via $P2X_7Rs$ causes oligodendrocyte death by $P2X_7Rs$. ATP is an important signaling substance at the gliovascular interface and $P2Y_2$ and $P2Y_4Rs$, were strongly expressed in the glial endfeet in proximity to blood vessel walls. Artwork by courtesy of Dr. Jens Grosche; H. Franke, unpublished

11.5.1 Microglial Cells

Microglial cells, the resident immune cells of the CNS, express several P2X/YRs which act as sensors of astrocyte activity and trigger cytokine release (Färber and Kettenmann 2006). In the activated state, they contribute to alterations in neurotransmitter homeostasis via different mechanisms, e.g., (1) release of glutamate and ATP; (2) interfering with glutamate uptake (mainly carried out by astrocytes) leading to extracellular glutamate accumulation; and (3) altering astrocyte gliotransmitter release (e.g., glutamate) (Domercq et al. 2013). For instance, after activation, microglial cells rapidly release small amounts of ATP, and astrocytes, in turn, amplify this release. This ATP stimulates astrocytes (via $P2Y_1Rs$) to release glutamate, which modulates neuronal activity through metabotropic glutamate receptors. As shown above, such a mechanism has important relevance during most brain diseases (Pascual et al. 2012).

After local damage, the release of ATP induces microgliosis, activated microglia migrate to the side of injury, proliferate, and phagocytose cellular debris (Kettenmann et al. 2011; Domercq et al. 2013). ATP controls microglial activation by acting at P2X4Rs and $P2Y_{12}Rs$ to induce microglial motility and microglial chemotaxis (Davalos et al. 2005; Haynes et al. 2006; Ohsawa et al. 2007; De Simone et al. 2010), by $P2Y_6Rs$, important for phagocytosis (Koizumi et al. 2007), or $P2X_7Rs$, driving microglial activation and proliferation (Monif et al. 2009). Activation of microglial $P2X_7Rs$, serves as an important component of a neuroinflammatory response in a broad range of CNS pathologies (Di Virgilio et al. 2009). Often $P2X_7Rs$ are overexpressed in microglial cells during their neuroinflammatory response, coupled to the capacity to release bioactive substances, e.g., IL-1β or TNF-α.

Chemokines from microglia may induce surrounding tissues to react to danger signals, moreover, astrocytes produce cytokines that can act on microglia, thus creating a paracrine and autocrine feedback loop whereby microglia-derived factors and astroglia-derived factors regulate each other (Hanisch and Kettenmann 2007; Zhang et al. 2010).

TNFα is an important constituent of glial interaction. The proinflammatory cytokine TNF has been implicated in the acceleration of pathological events of the brain, but emerging evidence suggests that TNF may also serve a protective role (Bezzi et al. 2001; Suzuki et al. 2004). ATP stimulates the *de novo* synthesis and release of TNF-α from microglia mediated via $P2X_7Rs$ (Hide et al. 2000). In neuron-microglia co-cultures, it has been shown that P2X7R-activated microglia protect neurons against glutamate neurotoxicity, primarily because they are able to release TNF (Suzuki et al. 2004). Furthermore, LPS caused in microglia a massive release of TNF-α, and at these high concentrations, TNF-α changed its mode of action, not only gating, but also directly causing glutamate release from astrocytes (Bezzi et al. 2001; Domercq et al. 2013).

Constitutive TNF-α was reported to control astrocytic gliotransmission under physiological conditions (Stellwagen and Malenka 2006; Santello et al. 2011). As shown above, $P2Y_1R$ stimulation is coupled to glutamate exocytosis and the

"*stimulus-secretion coupling mechanism*" involves Ca^{2+} release from internal stores and is controlled by additional transductive events, e.g., mediated by TNF-α, operating selectively in astrocytes (Domercq et al. 2006; Santello et al. 2011; Pascual et al. 2012). Moreover, microglia may initiate astrogliosis by acting on cytokine receptors; e.g., TNF-α has been shown to induce, enhance, or accompany astrogliosis and induce directly astrocyte proliferation and increase in GFAP expression (for review see Zhang et al. 2010).

11.5.2 Oligodendrocytes

Cells of the oligodendrocyte lineage are endowed with P2X/YRs, which can act as mediators of axo-oligodendroglial communication involved in myelination control (Matute 2011) as well as in the development of precursors (Agresti et al. 2005).

ATP induces a rise in cytosolic Ca^{2+} in oligodendrocytes by activating ionotropic $P2X_7Rs$ (James and Butt 2002; Matute et al. 2007). $P2Y_1Rs$ (Moran-Jimenez and Matute 2000) and $P2Y_{12}Rs$, contribute to the migration and adhesion of glial cell processes to axons during pre-myelination (Amadio et al. 2006, 2010). Oligodendrocytes display a great vulnerability to overactivation and excessive signaling, inducing altered glutamate and ATP homeostasis and resulting in oligodendrocyte death (via P2X7R-induced mitochondrial damage, initiating an apoptotic cascade), which mediates, e.g., ischemic damage to white matter (Butt 2011; Matute 2011; Domercq et al. 2013).

ATP (adenosine) released from axons during electrical impulse activity regulates the migration, proliferation, and differentiation of OPC (NG2+ glia) via $P2Y_1$ and $P2X_7Rs$ (Agresti et al. 2005). Perinatal ischemia triggers lethal activation of $P2X_7Rs$ in OPC (Wang et al. 2009). Such ATP-mediated neuron- and astrocyte-OPC-signaling provide mechanisms for regulating the regeneration of oligodendrocytes (Hamilton and Attwell 2010).

11.5.3 Blood Vessels

Contacts with blood vessels are established via highly specialized terminal expansions, or perivascular endfeet, which express high densities of proteins required for calcium signaling. ATP is a potent vasoconstrictor, whilst its metabolite adenosine is a potent vasodilator, and so their release by astrocytes (and extracellular conversion of ATP to adenosine) at the gliovascular interface could provide a biphasic mechanism or neurovascular coupling by astrocytes (Butt 2011). $P2Y_2Rs$ and $P2Y_4Rs$, were strongly expressed at the gliovascular interface, in the glial endfeet in proximity to blood vessel walls. It is speculated that calcium signaling may play a role in astrocytic functions related to the BBB, including blood flow regulation, metabolic trafficking, and water homeostasis (Simard et al. 2003).

11.6 Concluding Remarks

In conclusion, the present data emphasize a specific role of extracellular nucleotides and nucleosides in controlling glial cell functions. ATP is central to glial signaling and has biphasic effects on glia, evoking calcium excitability via P2Y and P2XRs and these calcium waves regulate astroglial release of neurotransmitters which modulate neuronal synaptic activity (Butt 2011).

Acknowledgements This work was supported by the Deutsche Forschungsgemeinschaft (FR 1253/3-1; IL 20/18-2).

Conflict of interest The authors declare no conflict of interest.

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V. Parpura et al. (eds.), *Glutamate and ATP at the Interface of Metabolism and Signaling in the Brain*, Advances in Neurobiology 11,
DOI 10.1007/978-3-319-08894-5