Vladimir Parpura · Alexei Verkhratsky
Editors

Pathological Potential of Neuroglia

Possible New Targets for Medical Intervention

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To our Families

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Prof. Alexei Verkhratsky, MD, PhD, DSc 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 PhD (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.

Vladimir Parpura, MD, PhD 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 (MEA) in 2012. His current research includes: (i) studying the modulation of calcium-dependent glutamate release from astrocytes in health and disease; (ii) assessing the role of the enteric glia in gut functions; (iii) visualization of vesicular/receptor trafficking; (iv) examination of the nature and energetics of interactions between exocytotic proteins using single molecule detection approaches; (v) development of scaffolds and dispersible materials, most notably modified carbon nanotubes, which can be used in repair after brain injury; (vi) development of biosensors (e.g. botulinum toxin and nanofabricated carbon-based detectors), and (vii) bio-mimetic micro-robotics. He has been interfacing neuroscience with nanoscience/nanotechnology, synthetic biology and biomedical engineering.

Chapter 1
General Pathophysiology of Neuroglia: Neurological and Psychiatric Disorders as Gliopathies

Alexei Verkhratsky and Vladimir Parpura

Abstract Astrocytes are responsible for the structural organization of the neural tissue, for neurogenesis and development of the central nervous system, for generation and maintenance of the blood- and cerebrospinal fluid-brain barriers, for homeostatic maintenance of neurotransmitters and ions, for synaptogenesis and metabolic support, for scavenging of reactive oxygen species and for regulation of local blood flow. Oligodendrocytes provide for the maintenance of interneuronal connectivity. Microglial cells exert multiple trophic effects and are actively involved in shaping the synaptic connections by removing silent or redundant synapses. NG2 cells receive synaptic inputs which might be fundamental for life-long control over myelinating capabilities of the brain tissue. In the periphery, satellite glia, enteric glia and olfactory ensheathing cells oversee various homeostatic functions, whereas Schwann cells support nerve impulse propagation. Homeostatic functions of neuroglia also extend to forming the defense of the nervous system. Every insult to the nerve tissue triggers glial homeostatic response and initiates specific glial defensive reaction. The homeostatic response is primarily neuroprotective. Neurological and psychiatric disorders are, conceptually, failures of such homeostatic responses in which neuroglia display a suboptimal function. Thus, neuroglia are ultimately involved in pathogenesis of many (if not all) brain disorders.

Keywords Astrocyte · Microglia · Neurodegeneration · Neuroglia · Neurological diseases · NG2 cells · Oligodendrocyte · Psychiatric diseases · Stroke

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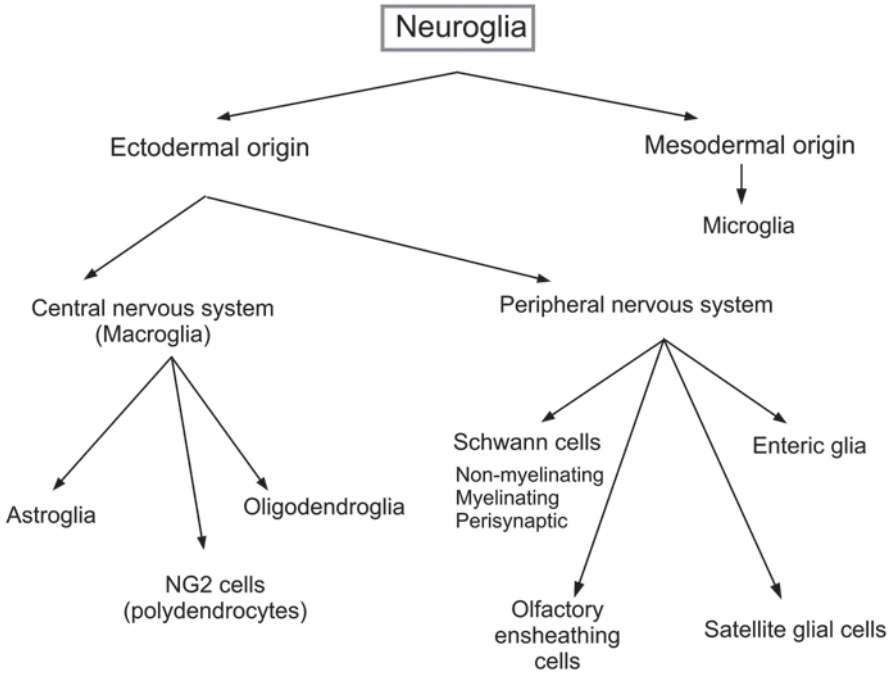

Fig. 1.1 Classification of neuroglia

1.1 Neuroglia Are the Cells Responsible for the Homeostasis of the Brain

Neuroglia are electrically non-excitable cells of the nervous system with variety of functions. Neuroglial cells are highly heterogeneous, being represented by cells of neural (ectodermal) and mesodermal (myeloid) origins. Neuroglia of neural origin are broadly subdivided into peripheral glia and glia of the central nervous system (CNS), these two big groups being further sub-classified into several classes (Fig. 1.1). The peripheral glia include satellite glial cells cells that are present in sensory and sympathetic ganglia, the highly diverse enteric glia, the olfactory ensheathing cells and Schwann cells that support and myelinate peripheral axons, and cover neuromuscular junctions. The CNS macroglia includes astrocytes, lineage related neuron-glial antigen 2 (NG2) glia and oligodendroglia that are fundamental for CNS myelination. NG2 glia draw their name for their specific expression of the named proteoglycan, also known as chondroitin sulfate proteoglycan 4 or as melanoma-associated chondroitin sulfate proteoglycan, that in humans is encoded by the *CSPG4* gene. The brain and the spinal cord also contain cells of myeloid origin known as microglia. These microglial cells are the scions of fetal macrophages entering the brain during embryogenesis; after invasion, these myeloid cells undergo remarkable metamorphosis and acquire specific “neural” phenotype that allows

microglial cells to be a part of neural networks being essential for many aspects of CNS physiology (for references and detailed narration of neuroglial physiology see (Parpura and Haydon 2008; Kettenmann et al. 2011; Kettenmann et al. 2013; Verkhratsky and Butt 2013).

Despite a remarkable heterogeneity of neuroglial cells they have one feature in common: the main function of neuroglia is the preservation of nervous system homeostasis. This function is accomplished differently by different cells and they naturally have their specializations. Yet they are indispensable for housekeeping of the nerve tissue: astrocytes assume responsibility for multiple homeostatic matters in the CNS, oligodendrocytes provide for the maintenance of interneuronal connectivity (or connectome, as it is often referred to today), microglial cells are responsible for defensive homeostasis; similarly in the periphery satellite glia, enteric glia and olfactory ensheathing cells oversee various homeostatic functions, whereas Schwann cells support nerve impulse propagation.

The range of homeostatic functions of neuroglial cells is extremely wide. Astrocytes are responsible for the structural organization of the nervous tissue, for neurogenesis and development of the CNS, for generation and maintenance of the blood- and cerebrospinal fluid (CSF)-brain barriers, for homeostatic maintenance of neurotransmitters and ions, for synaptogenesis and metabolic support, for scavenging of reactive oxygen species and for regulation of local blood flow (Table 1.1). Astrocytes are also central for various systemic functions such as central chemoception (Shimizu et al. 2007; Gourine et al. 2010; Gourine and Kasparov 2011) and regulation of sleep (Blutstein and Haydon 2013) (Table 1.1). Microglial cells exert multiple trophic effects and are actively involved in shaping the synaptic connections by removing silent or redundant synapses. NG2 cells receive synaptic inputs which might be fundamental for life-long control over myelinating capabilities of the brain tissue. Finally, neuroglial cells are secreting elements of the nervous tissue which release multiple factors that are indispensable for the survival and plasticity of neural networks.

1.2 Neuroglial Cells Are the Central Elements of Brain Defense

Homeostatic functions of neuroglia also extend to forming the defense of the nervous system. Every insult to the nerve tissue triggers glial homeostatic response and initiates a specific glial defensive reaction. The homeostatic response is primarily neuroprotective. For instance, astrocytes, using multiple molecules contributing to maintaining normal brain environment, contain the damage and sustain neuronal survival by maintaining CNS homeostasis. In conditions such as brain ischemia/hypoglycemia, astroglial cells sustain brain metabolism by mobilizing glycogen stores; breaking glycogen to lactate that is transferred to adjacent neural elements where it is used aerobically as fuel (Brown and Ransom 2007; Suh et al. 2007). Similarly, microglia respond to damage by releasing multiple factors that have neuroprotective and trophic effects.

Table 1.1 Functions of astrocytes. (From Verkhratsky and Butt (2013))

Function	Involvement/tasks
Development of the CNS	Neurogenesis
	Neural cell migration and formation of the layered grey matter
	Synaptogenesis
Structural support	Parcellation of the grey matter through the process of tiling
	Delineation of *pia matter* and the vessels by perivascular glia
	Formation of neuro-vascular unit
Barrier function	Regulation of formation and permeability of blood-brain and CSF-brain barriers
	Formation of glial-vascular interface
Homeostatic function (*sensu stricto*)	Control over extracellular K^+ homeostasis through local and spatial buffering
	Control over extracellular pH
	Regulation of water transport
	Removal of neurotransmitters from the extracellular space
Metabolic support	Uptake of glucose; deposition of glycogen
	Providing energy substrate lactate to neurons in activity dependent manner
Synaptic transmission	Regulation of synapses maintenance and assisting in synaptic pruning
	Providing glutamate for glutamatergic transmission (through de novo synthesis and glutamate-glutamine shuttle)
	Regulating synaptic plasticity
	Integrating synaptic fields
	Providing humoral regulation of neuronal networks through secretion of neurotransmitters and neuromodulators
Regulation of blood flow	Regulate local blood supply through secretion of vasocontrictors or vasodilators
Higher brain functions	Chemoception—regulation of body Na^+ homeostasis
	Chemoception—regulation of CO_2 and ventilatory behavior
	Sleep
	Memory and learning
Brain defense, neuroprotection and post injury remodeling	Isomorphic and anisomorphic reactive astrogliosis
	Scar formation
	Catabolizing ammonia in the brain
	Immune responses and secretion of pro-inflammatory factors (cytokines, chemokines and immune modulators)

In addition, neuroglial cells are in possession of evolutionarily conserved defensive program that is generally referred to as reactive gliosis (Parpura et al. 2012). Reactive changes in response to pathology are characteristic for all types of glial cells, and can be sub-classified as reactive astrogliosis, reactive activation of NG2 cells and activation of microglia. Oligodendroglia and Schwann cells are also activated in response to axonal injury by participating in Wallerian degeneration. Conceptually, reactive gliosis is a defensive reaction that is aimed at protecting stressed neurons, isolating injured area, removing pathogens, dying cells and cellular debris, and remodeling the nerve tissue after the resolution of pathology.

Reactive astrogliosis is activated in many (if not all) types of CNS pathology. Reactive astrogliosis is often associated with hypertrophy and proliferation of astrocytes along with an up-regulation of cytoskeletal components such as glial fibrillary acidic protein (GFAP), vimentin or nestin (Pekny and Nilsson 2005; Sofroniew 2009; Sofroniew and Vinters 2010); an increase in the immunoreactivity of GFAP is generally considered as a specific marker for astrogliotic response. Astrogliosis is commonly regarded as a pathological glial reaction with a negative outcome; often astrogliosis is regarded as a sign of neuroinflammation. In contrast to this general belief, astrogliosis is primarily a defensive, survivalist response which develops in a multi-stage graded and controlled fashion (Sofroniew 2009; Sofroniew and Vinters 2010; Verkhratsky et al. 2012). Astrogliosis results in remodeling of astroglial properties and functions, which are specific for various pathological contexts and resolve in different ways. Conceptually, reactive astrocytes increase neuroprotection and trophic support of neurons under stress, form an astroglial scar that isolates the damaged area from the rest of the CNS, promote post-lesion regeneration of the nervous tissue, and are instrumental in the reconstruction of the compromised blood–brain barrier. All these events are beneficial for nervous tissue and suppression of astrogliotic response exacerbates CNS damage (Sofroniew and Vinters 2010; Robel et al. 2011).

Reactive astrogliosis can be isomorphic (i.e. preserving morphology) or anisomorphic (i.e. changing the morphology), and these two types of reactive changes are associated with different functional responses and outcomes. In isomorphic gliosis, astrocytes undergo hypertrophy associated with changes in physiology, biochemistry and immunological presence, all of these, however, proceeding without altering normal astroglial domain organization. Isomorphic astrogliosis facilitates neurite growth and synaptogenesis, thus being permissive for the regeneration of neuronal networks. Importantly, isomorphic gliosis is fully reversible, and astrocytes return to a healthy state after the resolution of pathology. In anisomorphic gliosis, astroglial hypertrophy is associated with proliferative response, which results in the disruption of astroglial domains and in the formation of a permanent glial scar; this scar prevents any axonal growth due to the presence of chondroitin and keratin, which are secreted by the anisomorphic reactive astrocytes (Sofroniew 2009; Sofroniew and Vinters 2010; Verkhratsky et al. 2012).

Similar to astrocytes, insults to CNS induce reactive response of NG2 glia, characterized by an increase in proliferation and morphological remodeling, the latter being manifested by shortening and thickening of the cellular processes and a

strong increase in the expression of NG2. In the spinal cord, activated NG2 cells generate new oligodendrocytes that remyelinate the demyelinated lesions (Tripathi et al. 2010; Zawadzka et al. 2010). Being a source of chondroitin sulphate proteoglycan, NG2 cells may also contribute to scar formation.

Microglial cells represent another important component of gliotic response. It has become increasingly clear that the microglial activation is primarily a defensive reaction, which develops in many stages and results in multiple phenotypes, many of which are neuroprotective (Hanisch and Kettenmann 2007; Ransohoff and Perry 2009; Kettenmann et al. 2011). Activation of microglia progresses between different stages and phenotypes with a variety of morphological, biochemical, functional and immunological changes. Local insults induce rapid converging of microglial processes to the injured locus (Davalos et al. 2005; Nimmerjahn et al. 2005). Stronger insults, however, induce morphological remodeling where microglial somata enlarges and processes retract to become fewer and thicker; this parallels with changes in the expression of various enzymes and receptors, and with increased production and release of immune response molecules. Some of the activated microglia enter into a proliferative mode. With persisting insult, microglial cells become motile and using amoeboid-like movements they gather around the sites of damage; the ultimate climax of this activation is the transformation of microglial cells into phagocytes (Hanisch and Kettenmann 2007; Ransohoff and Perry 2009; Boche et al. 2013).

Lesions to peripheral nerves and to the white matter also set in motion reactive changes in myelinating cells, Schwann cells and oligodendrocytes, respectively, that determine the functional preservation of neural connectome. Activation of myelinating cells also take a part in Wallerian degeneration, the coordinated program, which progressively results in the disintegration of the distal axonal segment, the removal of myelin and remodeling of myelinating cells, and finally in the nerve fiber regeneration, by the axonal growth from the nerve stump proximal to the site of insult (Vargas and Barres 2007; Rotshenker 2011). In the peripheral nervous system, severance of the axon triggers proliferation of Schwann cells, which eventually form the pathway/scaffold for axonal regeneration. In the CNS the balance between de- and remyelination (which involves both oligodendroglia and NG2 cells) defines the course of demyelinating pathology (Franklin and Ffrench-Constant 2008; Miron et al. 2011; Kipp et al. 2012).

Neuroglial contribution to the pathology can, however, exert not only neuroprotection, but can be also neurotoxic. This represents an intrinsic dichotomy of every homeostatic system, where in conditions of extreme stress the system acquire a deleterious scope. The very same molecular cascades that protect brain environment can also contribute to neuronal death. Overstimulation of astrocytes can induce the release of glutamate through various existing release pathways and this release can add to the excitotoxicity. Abnormal water transport through astroglial aquaporins can contribute to edema, and astroglial failure of K^+ buffering is contributing to spreading depression. Similarly, over-activation of microglia results in the release of neurotoxic factors and phagocytic activity can further assist neuronal death.

1.3 Neuropathology as Homeostatic Failure: Central Role of Glia in Neurological and Psychiatric Disorders

Neurological diseases are, conceptually, failures of tissue homeostasis. The systemic homeostatic function of neuroglia makes these cells fundamental for evolution and outcome of neuropathology. The neuron-centric view still dominates the thinking of neuropathologists, neurologists and psychiatrists, which is reflected by fundamental and clinical research that is mainly concentrated on reactions of neurons in the contexts of various types of neurological and psychiatric disorders. Nonetheless, evolution of the nervous system went through specialization that resulted in the appearance of two interdependent, yet interacting, cellular networks, the executive system represented by neurons which perfected their ability for rapid signaling, and the housekeeping systems represented by neuroglia, which assume full responsibility for keeping neurons alive and ascertain their proper functioning. As such neurons have little capability to counterbalance environmental stress and pathological invasions, and it is indeed the neuroglia that protects and maintains the nervous system operation. When and if the neuroglial homeostatic and defensive mechanisms are exhausted, the neural tissue dies. While these views of central role for neuroglia in neuropathology began to be explored only recently (Giaume et al. 2007; Rajkowska and Miguel-Hidalgo 2007; De Keyser et al. 2008; Heneka et al. 2010; Cheng et al. 2012; Coulter and Eid 2012; Goldman et al. 2012; Molofsky et al. 2012; Verkhratsky et al. 2012; Benedetto and Rupprecht 2013; Parpura and Verkhratsky 2013; Rajkowska and Stockmeier 2013; Seifert and Steinhauser 2013), it is, however, becoming increasingly clear that brain pathology is, to a very great extent, a pathology of glia, and it is glial defensive capabilities that define the outcome and the scale of neurological deficit.

Gliopathology can be primary or secondary. Primary gliopathologies are represented, for instance, by the thus far only known genetic glial disease, the Alexander disease, in which an expression of mutant GFAP results in the aberration of astrocyte function and severe failure in white matter development (Messing et al. 2012). Similarly, astroglial cells are the primary targets in various toxic encephalopathies such as Wernicke-Korsakoff syndrome, hepatic encephalopathy or poisoning by heavy metals; the common denominator of all these disorders is the astroglial failure to contain extracellular glutamate (because of specific down-regulation of astroglial glutamate transporters) with ensuing excitotoxicity and neuronal death (Hazell 2009; Hazell et al. 2009; Brusilow et al. 2010; Butterworth 2010; Ni et al. 2012; Sidoryk-Wegrzynowicz and Aschner 2013). Secondary gliopathologies contribute to a variety of acute neurological disorders such as trauma, stroke or infection; they involve astrocytes, which define spreading of ischemic insults through the penumbra, and microglia, which act as a common target for many viruses. The nature of glial contribution in chronic diseases such as neurodegenerative disorders, neurodevelopmental disorders and neuropsychiatric disorders could be either secondary (when glia reacts to a neuronal disease specific lesion) or primary when impairment of glial function can assume etiopathological role; this matter yet requires detailed investigation.

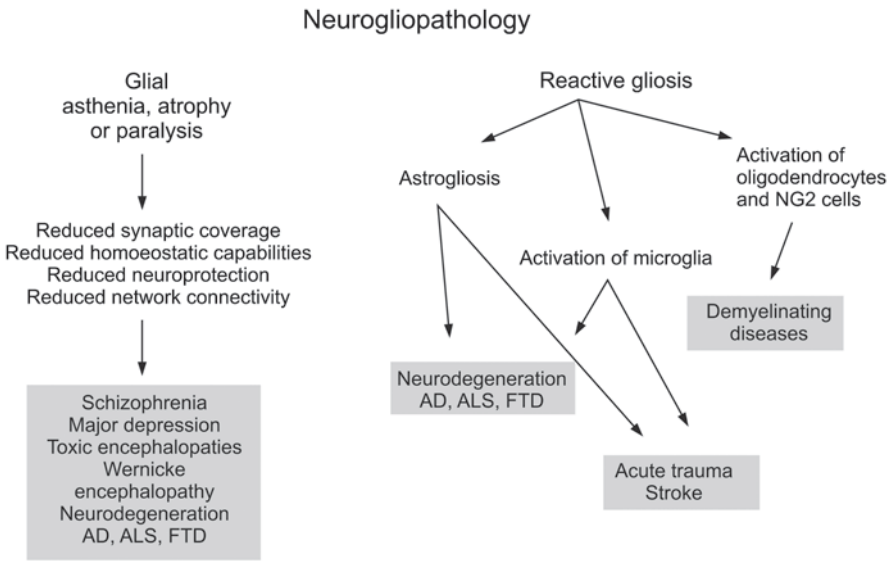

Fig. 1.2 Diversity of neuroglial pathological reactions

1.4 Glial Asthenia and Paralysis Define Neuropathological Progression

A relatively new aspect in gliopathology associated with glial asthenia, atrophy and functional "paralysis" began to be unfolding in recent years. It turned out that various neuropathologies are associated not only with reactive glial responses, but include gliodegenerative changes that may underlie diminution of glial homeostatic reserve with negative consequences for neuroprotection and normal functioning of the neural networks (Fig. 1.2). Atrophic changes in astroglia are seen, for example, at the early stages of amyotrophic lateral sclerosis (ALS), when functional atrophy and apoptosis of astrocytes precede the death of motoneurons. In ALS, astrocytes reduce their glutamate uptake, which contributes to increased excitotoxicity (Rossi and Volterra 2009; Staats and Van Den Bosch 2009). Selective silencing of the ALS related mutant superoxide dismutase 1 expression in astrocytes delays the progression of ALS in transgenic mice (Wang et al. 2011). Early atrophic changes in astroglia were also observed in transgenic animal models of the Alzheimer's disease (AD) (Verkhratsky et al. 2010; Rodriguez and Verkhratsky 2011). In these animals, substantial functional heterogeneity of astroglial responses to pathology is also found. In particular, it appeared that astrocytes in entorhinal cortex were unable to mount astrogliotic reaction in response to tissue deposition of β-amyloid; this failure in defensive function may underlie high vulnerability of certain brain regions to AD-like pathology (Yeh et al. 2012). Functional paralysis of microglia that fails to clear β-amyloid contributes to AD progression. Inhibition of microglial inflamma-

some shifted the microglial activation towards the neuroprotective phenotype and reduced β-amyloid load (Heneka et al. 2013). Astroglial asthenia and atrophy are manifested in major neuropsychiatric disorders such as schizophrenia and major depressive disorders; changes in astrocytes are substantially more prominent than those of neurons and may indicate a primary role for gliopathology in these diseases (Rajkowska et al. 2002; Hercher et al. 2009; Rajkowska and Stockmeier 2013; Williams et al. 2013). Loss of function of microglia in neurodevelopment is implicated in the pathogenesis of certain types of autistic spectrum disorders such as trichotillomania (Chen et al. 2010).

1.5 Envoi: Glial Cells as Therapeutic Targets

This chapter was meant to introduce a glio-centric view to the pathology of the brain in an attempt to complement the more prevailing neuron-centric view. As it is abundantly clear from the above general considerations of neuroglia in the pathophysiology of the brain, these cells are ultimately involved in the pathogenesis of many (if not all) disorders, in which they display a suboptimal, either reactive or suppressed, function. Naturally, both cell types, neurons and glia, along with the vasculature, and their homo- and heterocellular interactions and signaling pathways, contribute to the brain pathological states. In the following chapters of this edited volume many aspects of glial multifaceted roles in the neural pathology are discussed. Moreover, the emphasis has been put on glial cells as novel therapeutic targets for medical intervention. In this respect, we explore not only the basic research discoveries/components, but also the translational, from bench to bed, approaches, which are perhaps, at present, the most evident in the treatment strategies of gliomas. We also have obtained a valuable input from industrial partners, to gain an insight into the whereabouts of how a purely scientific finding can be translated into clinical use.

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Chapter 2
Ionic Signaling in Physiology and Pathophysiology of Astroglia

Alexei Verkhratsky and Vladimir Parpura

Abstract Excitability of astrocytes is based on spatio-temporally organized fluctuations of intracellular concentrations of two ions, Ca^{2+} and Na^{+}. This is dictated by ionic movements between intracellular compartments, and between the cytosol and the extracellular space, achieved by concentration-driven diffusion through membrane channels or transport by pumps and exchangers. Neuronal activity triggers transient elevation of Ca^{2+} and Na^{+} in astrocytes; changes in cytosolic levels of these ions translate into functional responses through multiple molecular cascades. Aberrant ionic signaling contributes to pathological reactions of astroglia in various forms of neurological diseases, such as stroke, epilepsy, and various neurodegenerative and neuropsychiatric disorders.

Keywords Astroglia · Calcium signaling · Sodium signaling · Endoplasmic reticulum · Mitochondria · Ca^{2+} channels · Ionotropic receptors · Metabotropic receptors · TRP channels · Na^{+}- Ca^{2+} exchanger

2.1 Cytoplasmic Ionic Signaling as a Substrate for Glial Excitability

Astrocytes, the "star-like cells", were named by Michael von Lenhossék (Lenhossek 1895) at the end of the nineteenth century. In reality, however, astrocytes rarely have a star-like appearance. Rather, their morphology is extremely heterogeneous

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and if anything, many of them have a spongiform appearance with myriads of very thin distal processes. Incidentally, von Lenhossék was acutely aware of this issue as he wrote: "I would suggest that all supporting cells be named spongiocytes. And the most common form in vertebrates be named spider cells or astrocytes, and use the term neuroglia only *cum grano salis* (with a grain of salt), at least until we have a clearer view." (Lenhossek 1895). At present, the term astroglia is commonly used to define all non-myelinating macroglial cells in the central nervous system (CNS), and these cells are responsible for all conceivable aspects of the brain homeostasis, thus heterogeneous not only in form, but also in function (Verkhratsky et al. 2012; Verkhratsky and Butt 2013). There is no unifying marker that would specifically label all the astroglial cells. Hence, the glial acidic fibrillary protein (GFAP), that is commonly regarded as an astrocytic marker, is not expressed by all the astroglial cells; just to the contrary many of the astrocytes in the mature brain do not express GFAP at identifiable levels. Also, the proportion of astrocytes that express GFAP varies substantially between brain regions, from ~80% in the hippocampus down to only ~10–15% in the neocortex (Kimelberg 2004). Thus, it would be advisable to attain a classification of astrocytes based on multiple markers/antigens as it has been the practice in the field of immunology for a variety of cells. Nonetheless, astroglia, as a class of neural cells, cover classical protoplasmic and fibrous astrocytes, the radial glia, radial Müller retinal glial cells, pseudo-radial cerebellar Bergmann glial cells, laminar and polarized astrocytes of the primate brain, velate astrocytes of the cerebellum, tanycytes that connect ventricular walls with parts of hypothalamus and spinal cord, pituicytes in the neuro-hypophysis, and perivascular and marginal astrocytes (Kimelberg 2010; Kimelberg and Nedergaard 2010; Zhang and Barres 2010; Verkhratsky and Butt 2013). In addition, cells that line the ventricles or the subretinal space represented by ependymocytes, choroid plexus cells and retinal pigment epithelial cells also belong to astroglia.

Homeostatic function of astrocytes is executed on many levels, and once more there is a remarkable heterogeneity in astroglial specialization in various parts of the CNS (Matyash and Kettenmann 2010; Verkhratsky 2010; Zhang and Barres 2010; Verkhratsky et al. 2011). To fulfil such a function, astroglial cells use sophisticated ion signaling systems allowing them to rapidly perceive changes in their immediate neighborhood and rapidly react to them. Although astrocytes are electrically non-excitable, i.e., they cannot generate and propagate action potentials, they possess a form of excitability in which the same ions that mediate electrical signals, by moving charges, act as signaling molecules through binding to multiple effector molecules responsible for astroglial functions. Two main ion signaling systems operative in astroglia are represented by calcium and sodium signaling. These two ions, being transported to and from the cytosol in response to various stimuli, regulate multiple molecular pathways and thus control astroglial function.

2.2 Glial Calcium Signaling

Calcium signaling, which is mediated by dynamic spatio-temporally coordinated changes in free Ca^{2+} concentration in the cellular compartments, has ancient evolutionary roots (Case et al. 2007; Plattner and Verkhratsky 2013) and is universal for most of the life forms on the Earth (Petersen et al. 2005). Changes in free Ca^{2+} concentrations in the cytosol ($[Ca^{2+}]_i$) of astrocytes result from Ca^{2+} fluxes across cellular membranes mediated either by Ca^{2+} diffusion through numerous ion channels down the concentration gradients or by energy-dependent Ca^{2+} transport associated with the activity of Ca^{2+} pumps and exchangers (Kostyuk and Verkhratsky 1995; Verkhratsky et al. 1998; Berridge et al. 2000, 2003; Carafoli 2002; Bregestovski and Spitzer 2005). Importantly, all molecular pathways involved in Ca^{2+} fluxes are regulated by Ca^{2+} ions themselves that constitute a robust feedback control preventing cellular Ca^{2+} overload (Burdakov et al. 2005).

It is generally believed that the chief source of astroglial Ca^{2+} signaling is associated with Ca^{2+} release from the endoplasmic reticulum (ER) Ca^{2+} stores; recent experiments, however, indicated an important role for plasmalemmal Ca^{2+} entry, which, in particular, can assume leading role in shaping Ca^{2+} signals in astroglial perisynaptic processes. Below we shall briefly overview the main pathways for astroglial Ca^{2+} signaling concentrating on the ER, on the plasmalemmal Ca^{2+} entry and on mitochondria.

2.2.1 ER in Astroglial Ca^{2+} Signaling

Astroglial Ca^{2+} signals were discovered and characterized at the beginning of the 1990s (see (Finkbeiner 1993; Verkhratsky and Kettenmann 1996) for overview of early experimental works) in cells in primary cultures (*in vitro*). These experiments identified the astroglial expression of a surprisingly wide array of G-protein coupled receptors, i.e. metabotropic receptors linked to phospholipase C, production of inositol 1,4,5-trisphosphate ($InsP_3$) and subsequent $InsP_3$-induced Ca^{2+} release from the ER (Fig. 2.1). It turned out that almost every neurotransmitter and neuromodulator administered to astrocytes in culture triggers ER Ca^{2+} release. Functional importance of ER in Ca^{2+} signaling in astroglia was subsequently confirmed in experiments *in situ* and *in vivo* (see (Verkhratsky et al. 2012) and references therein), although astrocytes in the brain tissue had much more restricted expression of metabotropic receptor subtypes when compared with *in vitro* conditions. The most common receptors, found in astrocytes in most regions of the brain are represented by metabotropic glutamate receptors of mGluR1 and mGluR5 types, purinoceptors of $P2Y_{1,2,4\,6}$ varieties and α- and β-adrenoreceptors (Kirischuk et al. 1995; Zonta et al. 2003a; Hamilton et al. 2008; Verkhratsky et al. 2009; Hertz et al. 2010; Di Castro et al. 2011), although their patterns can display regional specificity and can change with aging (Sun et al. 2013).

Activation of metabotropic receptors with subsequent $InsP_3$-induced Ca^{2+} release from the ER represents the main mechanism of propagating intra- and intercellular

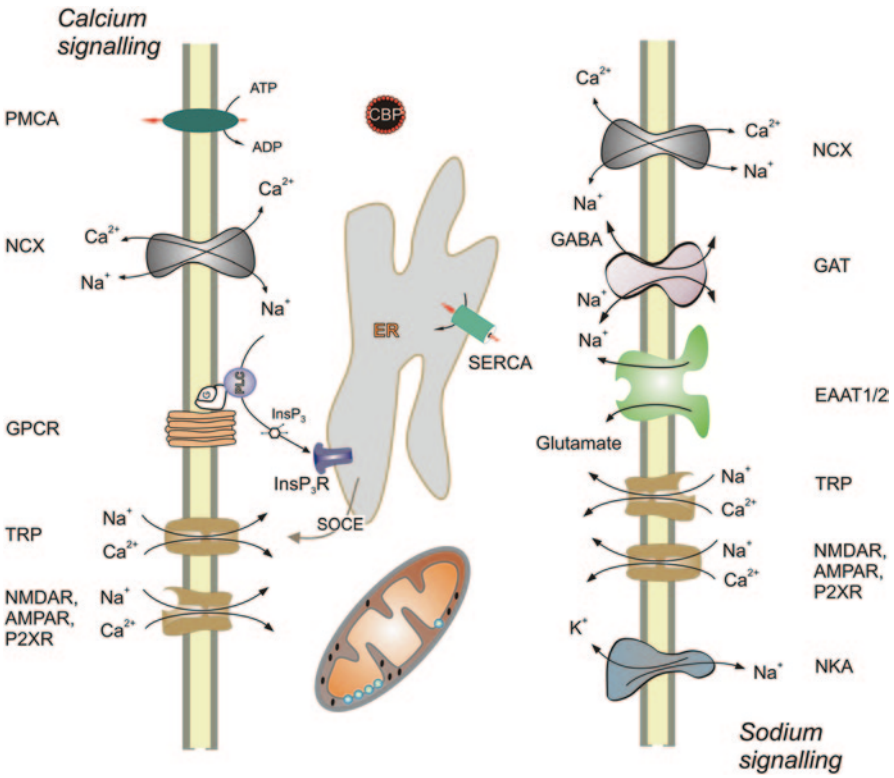

Fig. 2.1 Molecular cascades of calcium and sodium signaling in astroglia. Abbreviations: *AMPAR* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, *CBP* Ca^{2+} binding protein, *EAAT* excitatory amino acid transporter, *ER* endoplasmic reticulum, *GABA* γ-aminobutyric acid, *GAT* GABA transporter, *GPCR* G-protein coupled receptor, *InsP$_3$R* inositol 1,4,5 trisphosphate ($InsP_3$)-gated Ca^{2+} channel/receptor, *NCX* Na^+/Ca^{2+} exchanger, *NKA* Na^+/K^+ ATP-ase, *NMDAR* N-methyl D-aspartate receptor, *PMCA* plasmalemmal Ca^{2+}-ATPase, *P2XR* purinergic 2X receptor, *SERCA* sarco(endoplasmic) reticulum Ca^{2+} ATPase, *SOCE* store-operated Ca^{2+} entry, *TRP* transient receptor potential. (Modified from Parpura and Verkhratsky 2012)

Ca^{2+} waves, the latter commonly considered as a main mechanism for long-range communication in glial syncytia. Propagation of these calcium waves can involve direct diffusion of $InsP_3$ through gap junctions, or astroglial release of neurotransmitters (usually ATP), or combination of both (Giaume and Venance 1998; Scemes and Giaume 2006). At the same time, the role for another ER Ca^{2+} channel, the ryanodine receptor (RyR), in glial calcium signaling remains debatable. Astrocytes express RyRs both at the mRNA and protein level (Matyash et al. 2002; Parpura et al. 2011). Caffeine-induced RyR-mediated Ca^{2+} release was observed in astrocytes in the thalamus (Parri and Crunelli 2003), and inhibition of RyRs was shown to reduce ER stress in astrocytes in culture and in organotypic slices (Alberdi et al. 2013), and yet the physiological role for RyRs in astroglial Ca^{2+} signaling remains questionable (Beck et al. 2004).

What are the consequences and the physiological role of ER Ca^{2+} signaling in astroglia? Several lines of evidence showed that ER Ca^{2+} release is instrumental for initiating exocytotic neurotransmitter release from astrocytes *in vitro* (Reyes and Parpura 2009; Parpura et al. 2011). First, it was demonstrated that the inhibition of Sarco(Endo)plasmic Reticulum Ca^{2+} ATPases (SERCA) by thapsigargin (that causes depletion of the ER from releasable Ca^{2+} due to an unopposed leakage) (Fig. 2.1) almost completely blocked the Ca^{2+}-dependent release of glutamate from astrocytes (Innocenti et al. 2000; Jeremic et al. 2001). Similarly, thapsigargin blocked the mechanically-induced glutamate release from cultured astroglia (Hua et al. 2004). The same effect was observed after treating astrocytes with diphenylboric acid 2-aminoethyl ester that is known to inhibit $InsP_3$ receptors and store-operated Ca^{2+} entry (Hua et al. 2004). Calcium signals originated from the ER initiate the release of vasoactive substances (for example derivatives of arachidonic acid or carbon monoxide) that affect the tone of cerebral arterioles and hence contribute to astroglia-dependent regulation of local blood flow (Zonta et al. 2003b; Mulligan and MacVicar 2004). There are data that Ca^{2+} release from the ER regulates astroglial apoptosis through transactivation of pro-apoptotic factor Bax (Morales et al. 2011). Dynamic changes in ER Ca^{2+} that accompany Ca^{2+} release are also involved in the regulation of multiple ER functions, most notably in controlling the activity of chaperones and protein folding; long-lasting decrease in ER Ca^{2+} level can bring upon ER stress, which contribute to various pathologies (Alberdi et al. 2013). Finally, global astroglial Ca^{2+} signals associated with ER Ca^{2+} release dynamically affect mitochondrial metabolism thus regulating ATP synthesis and providing for activity-metabolic coupling.

At the same time, the role of ER Ca^{2+} release in astroglial physiology *in situ* remains debatable. For example, experiments on genetically modified mice in which ER Ca^{2+} release in astrocytes was specifically affected showed that neither amplification nor occlusion of astroglial metabotropic Ca^{2+} signaling affects synaptic transmission/plasticity in hippocampus (Fiacco et al. 2007; Petravicz et al. 2008; Agulhon et al. 2010). Similarly, ultrastructural study has shown that perisynaptic astroglial processes in hippocampus do not contain ER structures, which are localized mainly in the more proximal processes (Partushev et al. 2013).

These observations revived interest to plasmalemmal Ca^{2+} fluxes that, in particular, can underlie rapid and highly localized Ca^{2+} signals in perisynaptic astroglial processes; these Ca^{2+} signals are critical for the homeostatic control of synaptic cleft and for the regulation of synaptic plasticity.

2.2.2 Plasmalemmal Ca^{2+} Entry in Astrocytes

Astrocytes have several molecular pathways responsible for the generation of transmembrane Ca^{2+} fluxes, which include ionotropic receptors, transient receptor potential (TRP) channels, sodium-calcium exchangers (NCX), and possibly voltage-gated Ca^{2+} channels (Fig. 2.1). The plasma membrane Ca^{2+}-ATPase (PMCA) is the

major Ca^{2+} extruder in resting astrocytes, but it plays a less important role during times of Ca^{2+}cytosolic loads caused by mechanical stimulation (Reyes et al. 2012).

Ionotropic Receptors Astrocytes, both *in vitro* and *in situ*, are endowed with several types of Ca^{2+} permeable ligand-gated channels. The first class of these channels is represented by ionotropic glutamate receptors of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) types; functional expression of kainate receptors in astrocytes has not yet been reported (Parpura and Verkhratsky 2013). The Ca^{2+} permeable AMPA receptors (that lack GluA2 subunit) have been routinely found in cultured astrocytes and their expression is confirmed in Bergmann glial cells in the cerebellum (Steinhauser and Gallo 1996). However, the extent to which Ca^{2+} permeable receptors are present in other brain regions needs further characterization. Nonetheless, Ca^{2+} permeability of GluA2-devoid AMPA receptors is relatively low ($P_{Ca}/P_{monovalent} \sim 1$, (Burnashev et al. 1992)) which, together with rapid desensitization of AMPA receptors in physiological context, very much limits Ca^{2+} entry. In contrast, NMDA receptors, that have been found in mouse cortical astrocytes and also identified in human astroglia (Lalo et al. 2006; Verkhratsky and Kirchhoff 2007; Lee et al. 2010; Lalo et al. 2011), are characterized by much larger Ca^{2+} permeability ($P_{Ca}/P_{monovalent} \sim 3$) and slow desensitization that allows Ca^{2+} influx, resulting in substantial $[Ca^{2+}]_i$ rise in astrocytes studied in acute isolation and in acute brain slices (Palygin et al. 2010). In addition, astroglial NMDA receptors have a weak Mg^{2+} block at physiological resting potential that permits receptor activation by glutamate release during on-going synaptic activity (Lalo et al. 2011). Astroglial Ca^{2+} signaling is also mediated by ionotropic P2X purinoceptors; specific heteromeric P2X receptors are operative in neocortical astroglia (Lalo et al. 2008). These receptors have very high sensitivity to ATP ($EC_{50} \sim 50$ nM) and do not desensitize in the presence of an agonist. They are Ca^{2+} permeable ($P_{Ca}/P_{monovalent} \sim 2$) and, similar to NMDA receptors, mediate $[Ca^{2+}]_i$ signals in astrocytes in isolation and *in situ* (Palygin et al. 2010). Astrocytes in the neocortex were also reported to express $P2X_7$ receptors which, when activated, may provide large Ca^{2+} influx (Norenberg et al. 2010; Oliveira et al. 2011), although these receptors, most likely, mediate pathological responses associated with massive release of ATP (Franke et al. 2012; Illes et al. 2012). Besides ionotropic glutamate receptors and purinoceptors mediated Ca^{2+} entry into astrocytes, these ions can enter through α7 Ca^{2+} permeable nicotinic cholinoreceptors, identified in cultured astroglia (Sharma and Vijayaraghavan 2001; Oikawa et al. 2005).

TRP Channels Astroglia express TRPA1, TRPV4, and TRPC1, 4 and 5 channels, of which TRPC channels are directly involved in Ca^{2+} signaling, being a substrate for store-operated Ca^{2+} entry (Verkhratsky and Parpura 2013; Verkhratsky et al. 2014).

Similarly, Orai channels and their respective currents have been recently recorded in primary cultured astrocytes and astroglial cell lines, which also expressed stromal interacting molecule1 (STIM1), the molecular sensor that monitors the intra-ER Ca^{2+} concentration (Moreno et al. 2012; Motiani et al. 2013).

Astrocytes express TRPC1,4,5 subunits at both mRNA and protein levels (Pizzo et al. 2001; Grimaldi et al. 2003; Golovina 2005; Malarkey et al. 2008). In TRPC

heteromultimers the TRPC1 channel is obligatory subunit, whereas TRPC4 and TRPC5 proteins have an auxiliary role (Strubing et al. 2001; Hofmann et al. 2002). Specific inhibition of TRPC1 channels by either expression down-regulation with an antisense or by exposing cells to a blocking antibody directed at an epitope in the pore forming region of the TRPC1 protein substantially reduced SOCE following metabotropic or mechanical stimulation in cultured astrocytes (Golovina 2005; Malarkey et al. 2008).

Hippocampal astrocytes seem to express TRPA1 mediating "spotty", localized near-membrane, $[Ca^{2+}]_i$ changes (Shigetomi et al. 2012). In cultured astrocytes, these $[Ca^{2+}]_i$ transient were inhibited by the broad spectrum TRP channel antagonist HC 030031 and by anti-TRP silencing RNA, while the TRPA1 agonist allyl isothiocyanate increased frequency of these events. Activity of TRPA1 channels contributed to setting the resting $[Ca^{2+}]_i$ in hippocampal astrocytes (both in culture and *in situ*), as inhibition of these channels resulted in a significant (from ~120 nM to ~50 nM) decrease in basal $[Ca^{2+}]_i$ (Shigetomi et al. 2012).

Sodium/Calcium Exchanger Important molecular pathway regulating Ca^{2+} entry especially in astroglial perisynaptic processes is represented by sodium-calcium exchange mechanism. Astrocytes are in possession of all three types of mammalian Na^+/Ca^{2+} exchangers, the NCX1, NCX2 and NCX3. The NCX molecules are concentrated in the perisynaptic processes and are often co-localized with plasmalemmal glutamate transporters and NMDA receptors (Minelli et al. 2007). The NCX dependent Ca^{2+} transport in astrocytes operates in both forward (Ca^{2+} extrusion) and reverse (Ca^{2+} entry) modes; because of the relatively high cytosolic concentration of Na^+ ions (see below), the reversal potential of NCX is set rather close to astroglial resting membrane potential and therefore even a moderate depolarization or an increase in the intracellular Na^+ readily reverses the NCX and favors Ca^{2+} influx (Kirischuk et al. 2012). The NCX-mediated Ca^{2+} fluxes in both forward and reverse modes are documented for primary cultured astrocytes and astroglial cells *in situ* (Goldman et al. 1994; Kirischuk et al. 1997). Influx of Ca^{2+} through NCX can be activated by cytosolic Na^+ increase following the activation of ionotropic receptors (Kirischuk et al. 1997) or glutamate transporters (Kirischuk et al. 2007); in cultured astrocytes the reverse mode of NCX can be induced by a moderate depolarization (Paluzzi et al. 2007).

Voltage-gated Ca^{2+} Channels (VGCCs) Although there are numerous reports indicating the expression of VGCCs in astrocytes *in vitro* (see (Parpura et al. 2011; Verkhratsky et al. 2012) for details and references), the role for these channels in physiologically relevant Ca^{2+} signaling in astroglia in the brain tissue remains questionable. There are some indications for VGCC-dependent Ca^{2+} dynamics in astrocytes from the neurogenic subventricular zone (Young et al. 2010) and the ventrobasal thalamus (Parri et al. 2001; Parri and Crunelli 2003) and yet these reports remain sporadic and unconfirmed. It might be argued that VGCCs may become important for Ca^{2+} signals in reactively remodeled astroglia. In particular, reactive astrocytes in the hippocampi of young mice, which experienced pilocarpine-induced status epilepticus, showed an increased expression of L- and P/Q- type channels at 1 week and 2 months following an insult (Xu et al. 2007).

2.2.3 Mitochondria in Astroglial Ca^{2+} Signaling

Mitochondria are able to accumulate Ca^{2+} ions from the cytosol through Ca^{2+} ion channels localized in the outer and in the inner membrane; these channels are represented, respectively, by the voltage-dependent anion channels with considerable Ca^{2+} permeability and by the highly selective Ca^{2+} uniporter (composed of the channel protein of mitochondrial calcium uniporter, and auxiliary EF-hand-containing protein that regulates the uniporter, MICU1/CBARA1 (De Stefani et al. 2011)). Mitochondrial Ca^{2+} sequestration has been well documented in astrocytes (Reyes and Parpura 2008). In addition, mitochondria may release Ca^{2+} via mitochondrial Na^+/Ca^{2+} exchanger NCLX (Parnis et al. 2013) and through the mitochondrial permeability transition pore (Basso et al. 2005; Reyes and Parpura 2008).

2.3 Glial Sodium Signaling

2.3.1 Molecular Physiology of Na^+ Signaling

Many astroglial functions are regulated by the transmembrane gradient for Na^+ ions (Verkhratsky et al. 2013c), which in turn is subject to dynamic variations induced by the physiological stimulation of astrocytes. Astroglial sodium homeostasis is somewhat different from that of neurons at least in one parameter: the resting cytosolic Na^+ concentration ($[Na^+]_i$) in astrocytes is about two times higher (Rose and Ransom 1996; Reyes et al. 2012; Unichenko et al. 2012). High levels of $[Na^+]_i$ are important because they set reversal potentials for numerous Na^+-dependent solute transporters expressed in astroglial membrane (Parpura and Verkhratsky 2012). Physiological stimulation of astrocytes *in vitro* or *in situ* by exogenous neurotransmitters, by synaptic inputs, or by mechanical indentation trigger transient elevation of $[Na^+]_i$ by up to 20–25 mM from the resting level. Furthermore, these $[Na^+]_i$ increases may propagate through astroglial syncytia using gap junctions, thus, generating propagating Na^+ waves. These observations led to a concept of astroglial Na^+ signaling (see (Kirischuk et al. 2012; Langer et al. 2012; Parpura and Verkhratsky 2012; Rose and Karus 2013; Verkhratsky et al. 2013c) and references therein).

Increases in $[Na^+]_i$ are mediated through several molecular pathways (Fig. 2.1). Sodium fluxes are generated by all ionotropic receptors present in astroglial membranes, these receptors being, in essence, cationic channels provide for a large Na^+ entry upon their activation (Kirischuk et al. 1997; Langer and Rose 2009). Another important route is associated with the activation of TRPC channels (Reyes et al. 2013), which (through store-operated mechanism) provide a link between ER Ca^{2+} release and plasmalemmal Na^+ flux (Verkhratsky et al. 2014). In astrocytes from subfornical organ Na^+ influx is mediated by Na_x channels sensitive to extracellular Na^+ concentration. Opening of these channels with subsequent Na^+ entry is instrumental for astroglial chemosensing and systemic regulation of Na^+ homeostasis (Shimizu et al. 2007).

Another physiologically important Na^+ influx pathway is associated with the activation of plasmalemmal neurotransmitter transporters for glutamate and GABA, the astroglial specific excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2) and the GABA transporters of GAT1 and GAT3 types. The stoichiometry of EAAT1/2 is 1 Glu^-:3 Na^+:1K^+:1H^+, of which Na^+, proton and glutamate enter the cell in exchange for K^+ efflux, whereas GAT1 and GAT3 exchange 1 GABA molecule for 2 Na^+ ions and 1 Cl^- anion. Accordingly, in physiological conditions, astroglial uptake of neurotransmitters is accompanied with net Na^+ influx that underlie electrogenicity of transporters and can increase $[Na^+]_i$ by ~20 mM following the activation of EAAT1/2 or by ~7 mM following the activation of GAT1/3 (Kirischuk et al. 2007; Unichenko et al. 2012).

Astrocytes possess Na^+/K^+-ATPase (NKA), a pump which is the major Na^+ extruder in resting astrocytes. However, NKA seems to be less important during times of Na^+ cytosolic loads caused by mechanical stimulation. Instead, NCX operating in reverse mode appeared as a major contributor to the overall Na^+ homeostasis in astrocytes, both at rest and when these glial cells were mechanically stimulated (Reyes et al. 2012).

2.3.2 *Functional Role of Na^+ Signaling*

Increases in $[Na^+]_i$ regulate multiple molecular cascades responsible for astroglial homeostatic function (Fig. 2.2). Elevation in $[Na^+]_i$ activates astroglial NKA, which (i) affects K^+ buffering (Wang et al. 2012) and (ii) triggers lactate synthesis and therefore mobilize astrocyte-neuron lactate shuttle (Pellerin and Magistretti 2012); lactate can be released (or taken up) from astrocytes through the proton-coupled transporter MCT-1. In this way astroglial metabolic support is tailored to the neuronal activity and hence local neuronal energy demands. Changes in $[Na^+]_i$ also directly affect $K_{ir}4.1$ channels that are critical for astroglial K^+ buffering (Kucheryavykh et al. 2012), and regulate other K^+ transporters such as, for example, $Na^+/K^+/Cl^-$ co-transporter NKCC1, which also contributes to K^+ buffering (Kirischuk et al. 2012).

Changes in $[Na^+]_i$ have profound effects on many astroglial solute carriers that are controlling molecular homeostasis of the CNS environment. First and foremost, $[Na^+]_i$ regulates neurotransmitter homeostasis. Both glutamate and GABA plasmalemmal transporters are directly affected by $[Na^+]_i$, albeit with different functional consequences. Physiological increases in $[Na^+]_i$ may slow down, but never reverse glutamate transporters, which have reversal potential ~+60 mV (Kirischuk et al. 2007, 2012). Reversal of glutamate transporter, that has been observed in experiments (Szatkowski et al. 1990), may only occur in pathology when massive $[Na^+]_i$ increase coincides with an increase in cytosolic glutamate concentration and membrane depolarization. In contrast, reversal potential of astroglial GABA transport (~−80 mV) is quite close to the resting membrane potential, and therefore even minute increases in $[Na^+]_i$ (~7 mM) can switch GABA transport into the reverse mode and, thus, mediate GABA release from astroglia (Unichenko et al. 2012).

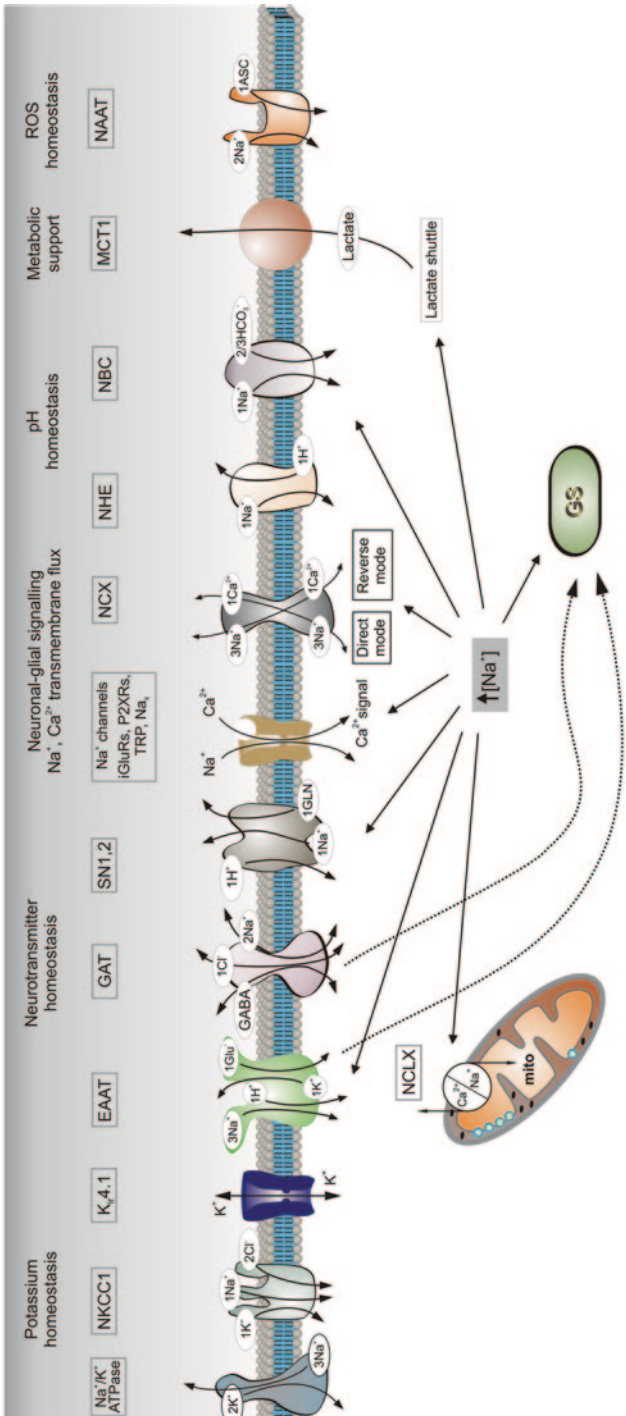

Fig. 2.2 Functional targets of Na^+ signaling in astroglia. Schematic diagram showing receptors and transporters involved in and sensitive to changes in $[Na^+]_i$ and their relations to main homeostatic functions of astroglia. Abbreviations: *EAAT* excitatory amino acid transporters, *GAT* GABA transporters, *GS* glutamine synthetase, *iGluRs* ionotropic glutamate receptors, *mito* mitochondrion, *Kir4.1* inwardly rectifying K^+ channels involved in K^+ buffering, *MCT1* monocarboxylate transporter 1, Na_x Na^+ channels activated by extracellular Na^+, *NAAT* Na^+-dependent ascorbic acid transporter, *NBC* Na^+/HCO_3^- (sodium-bicarbonate) cotransporter monocarboxylate, *NCX* Na^+/Ca^{2+} exchanger, *NCLX* mitochondrial Na^+/Ca^{2+} exchanger, *NHE* Na^+/H^+ exchanger, *NKCC1* $Na^+/K^+/Cl^-$ cotransporter, *P2XRs* ionotropic purinoceptors, *SN1,2* sodium-coupled neutral amino acid transporters 1 and 2 which underlie the extrusion of glutamine, *TRP* transient receptor potential channels. (Modified and updated from Kirischuk et al. 2012)

Cytosolic Na^+ levels also regulate glutamine synthesis and glutamine release by directly affecting glutamine synthetase and the plasmalemmal sodium-dependent neutral amino acid transporter SNAT3/SLC38A3, respectively (Mackenzie and Erickson 2004). In this way astroglial Na^+ signaling regulates both excitatory and inhibitory neurotransmission, because both are critically dependent on continuous supply of glutamine acting as a precursor for glutamate and GABA.

Astroglial $[Na^+]_i$ increases favor reversal of NCX, which, in astrocytes, dynamically fluctuates between forward/reverse modes and thus mediates local Ca^{2+} signals. Similarly $[Na^+]_i$ levels regulate mitochondrial exchanger NCLX, thus, controlling mitochondrial Ca^{2+} release (Parnis et al. 2013). Astroglial Na^+ signals also modulate several other homeostatic pathways, such as Na^+/H^+ exchanger (NHE), which regulate pH homeostasis, and sodium-bicarbonate co-transporter (NBC). Intracellular Na^+ also controls the release of radical oxygen species (ROS) scavenger ascorbic acid through dedicated Na^+-dependent transporter NAAT (see (Kirischuk et al. 2012; Parpura and Verkhratsky 2012; Verkhratsky et al. 2013c) for details).

2.4 Glial Ca^{2+} Signaling in Pathology

Astroglial contribution to neurological diseases is fundamental for the progression and outcome of pathology; this contribution includes neuroprotection through the activation of astroglial homeostatic cascades and through the induction of reactive astrogliosis (Verkhratsky et al. 2013a).

Focal Ischemia and Stroke Astrocytes are intimately involved in the pathogenesis of ischemic insults to the CNS. Cessation of blood flow triggers necrotic process in the infarction core, where all cells, including astroglia, die; this process results from a failure of ion homeostasis and overloading the cells with Ca^{2+} and Na^+. In the ischemic penumbra, surrounding the core region, the cellular fate can be different, and indeed the progression of cell death through the penumbra that may take days after the initial insult, is to a large extend, defined by astrocytes (Nedergaard and Dirnagl 2005; Giaume et al. 2007). In the penumbra region, astrocytes display higher resistance compared to all other cell types; they not only outlive neurons but can support them through the release of neurotrophic factors and ROS scavengers, as well as through containing glutamate excitotoxicity, K^+ buffering and metabolic support (Tanaka et al. 1999; Pekny and Nilsson 2005; Verkhratsky et al. 2013a). Most of these neuroprotective functions are regulated by astroglial ionic signaling. At the same time astrocytes may contribute to the spread of death signals through the penumbra; this spread can be associated with aberrant astroglial Ca^{2+} waves that can produce glutamate release distal to the infarction core and, thus, induce distant cell death (Nedergaard and Dirnagl 2005; Takano et al. 2009). These aberrant glial Ca^{2+} signaling can be further exacerbated by the ATP released from the damaged cells; high concentration of ATP activates both metabotropic and $P2X_7$ ionotropic astroglial receptors that results in pathological Ca^{2+} signaling (Franke et al. 2012).

Epilepsy Astroglial contribution to the pathogenesis of epilepsy received increasing attention in recent years. Profound astrogliosis is regularly observed in both animal models of the disease and in human brain tissue (de Lanerolle and Lee 2005; Seifert et al. 2006); these changes develop in parallel with functional astroglial deficiency that includes impaired K^+ buffering, neurotransmitter uptake and water transport (Seifert et al. 2006). Pathological ionic signaling in astrocytes can even contribute to epileptogenesis. Aberrant Ca^{2+} signaling manifested by spontaneous Ca^{2+} oscillations was observed in astrocytes isolated from the brains of patients suffering from Rasmussen's encephalitis, the autoimmune form of child epilepsy. These abnormal Ca^{2+} signals are arguably connected with pathological modification of AMPA receptors that results in their reduced desensitization and increased Ca^{2+} influx (Manning and Sontheimer 1997; Seifert et al. 2004). Astroglial Ca^{2+} signaling can also be affected through an increase in the expression of metabotropic glutamate receptors and $InsP_3$-induced Ca^{2+} release. Pathologically, increased astroglial Ca^{2+} signals may contribute to the formation of a recurrent excitatory loop with neurons, thus, initiating a focal seizures (Carmignoto and Haydon 2012). Finally, aberrant astroglial Ca^{2+} signals may induce a massive release of glutamate that could trigger paroxysmal depolarization shifts in neurons, thus, provoking seizure (Tian et al. 2005). In addition, the epileptic tissue is characterized by impaired inhibitory transmission and it appeared that the GABA-ergic transmission is particularly sensitive to astroglial glutamine supply; epilepsy is accompanied with an inhibition of glutamine synthetase that suppresses GABA-ergic inhibitory transmission (Ortinski et al. 2010).

Psychiatric Disorders Modern views on the major psychiatric diseases, such as schizophrenia, bipolar disorder and major depression, consider neurotransmitter imbalance and failures in connectivity in neural circuitries as the main pathogenetic factor. In particular, glutamate-NMDA theory of schizophrenia regards the hypofunction of NMDA receptors as the key element of the disease (Kondziella et al. 2007). The NMDA transmission is, in turn, under the control of astroglia through positive modulation by D-serine and the inhibition by astroglial specific neuromodulator, kynurenic acid. Schizophrenia is associated with remarkable hypotrophy of astrocytes, which may be an etiopathological factor. Astroglial deficiency in proper regulation of glutamatergic transmission may be the underlying mechanism. Both D-serine and glycine, which, at least in part, are released from astrocytes through Ca^{2+}-dependent mechanism (Henneberger et al. 2010), have been shown to alleviate the negative symptoms of schizophrenia (Heresco-Levy et al. 2004, 2005). Thus, abnormal Ca^{2+} signaling in astroglia may contribute to schizophrenic evolution.

Neurodegenerative Diseases Astrocytes are involved in majority of neurodegenerative diseases through both astrogliotic response and homeostatic failure associated with astroglial atrophy. Not much is known about astroglial Ca^{2+} signaling in neurodegeneration, and yet there seems to be some specific alterations. In astrocytes expressing mutant human superoxide dismutase hSOD1(G93A), linked to amyotrophic lateral sclerosis, the activation of metabotropic glutamate receptors triggered persistent $[Ca^{2+}]_i$ increases, instead of Ca^{2+} oscillations observed in healthy cells. Treatment of these cells with the protein transduction domain of

the HIV-1 TAT protein, which interacts with the anti-apoptotic protein Bcl-xL, restored Ca^{2+} signaling and counteracted astrodegeneration (Martorana et al. 2012). In Alzheimer's disease (AD) animal models, abnormal astroglial Ca^{2}+ signaling triggered by β-amyloid, arguably interacting with α7 nicotinic cholinoreceptors, was linked to increased glutamate release that caused neuronal death (Talantova et al. 2013). Exposure of astrocytes to β-amyloid was also shown to modify the expression of Ca^{2+} signaling related molecules, such as mGluR5 and $InsP_3$ receptors, and increased Ca^{2+} signals in cells from the hippocampus, but not from the entorhinal cortex (Grolla et al. 2013). Aberrant Ca^{2+} signals were also observed in astrocytes associated with senile plaques *in vivo* in AD mice model; these pathological changes were reflected in the global elevation in resting $[Ca^{2+}]_i$ levels and substantially enhanced spontaneous $[Ca^{2+}]_i$ oscillations and Ca^{2+} waves (Kuchibhotla et al. 2009).

2.5 Pathological Potential of Astroglial Sodium Signaling

Several forms of neuropathology were reported to disturb astroglial Na^+ homeostasis, which, in turn, can initiate astroglial neurotoxicity. In particular, ischemic stress can induce Na^+ overload resulted from Na^+ entry via plasmalemmal glutamate transporters, Na^+/H^+ exchanger and $Na^+/K^+/Cl^-$ co-transporter. Pathological increases in $[Na^+]_i$ generate secondary Ca^{2+} influx mostly through reversed NCX which induces astroglial death (Lenart et al. 2004; Bondarenko et al. 2005). Simultaneous $[Na^+]_i$ overload with depolarization, following pathological increase in the extracellular potassium concentration to ~60 mM, may contribute to the reversal of glutamate transporters (Attwell et al. 1993; Phillis et al. 2000), thereby exacerbating excitotoxicity associated with ischemic insults (Gemba et al. 1994). Failure in sodium homeostasis also impacts on volume regulation and can be considered as one of the causes of pathological edema. Finally, several disorders, such as cerebrovascular pathology, tumors and multiple sclerosis, result in an astroglial expression of voltage-gated Na^+ channel which can further compromise sodium homeostatic machinery with pathological consequences (Black et al. 2010).

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Chapter 3
Pathophysiology of Vesicle Dynamics in Astrocytes

Nina Vardjan, Maja Potokar, Matjaž Stenovec, Jernej Jorgačevski, Saša Trkov, Marko Kreft and Robert Zorec

Abstract In astrocytes, as in other eukaryotic cells, vesicles have key cellular functions including constitutive housekeeping of the plasma membrane structure and cell-to-cell communication. On the one hand, vesicle traffic is associated with cell surface morphology exhibiting distinct glial microdomains. These determine the signaling potential and metabolic support for neighboring cells. On the other hand, vesicles are used in astrocytes for the release of vesicle-laden chemical messengers. This chapter addresses the properties of membrane-bound vesicles that store gliotransmitters (glutamate, adenosine 5'-triphosphate, peptides), other recycling vesicles, and endocytotic vesicles that are involved in the traffic of plasma membrane receptors such as the class II major histocompatibility molecules and membrane transporters (aquaporin 4 and excitatory amino acid transporter 2). Vesicle dynamics are also considered in view of diseases such as amyotrophic lateral sclerosis, multiple sclerosis, autistic disorders, trauma, edema, and states in which astrocytes contribute to neuroinflammation. In multiple sclerosis, for example,

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fingolimod, a recently introduced drug, apparently also affects vesicle traffic and gliotransmitter release from astrocytes, indicating that these processes may well be used as a target for the development of new therapies.

Keywords Astrocyte · Glia · Vesicle · Trafficking · Reactive astrogliosis · Morphology · Gliotransmitter · Antigen presentation · Neuroinflammation · Autism · Amyotrophic lateral sclerosis · Multiple sclerosis

3.1 Introduction

Astrocytes, the most abundant glial cells in the brain, have been considered subservient to neurons for almost a century. However, in the last two decades, experimental evidence has shown that astrocytes actively contribute to information processing in the central nervous system (CNS). Many new functions have been described, including the regulation of synaptogenesis, synaptic transmission, brain microcirculation, roles in the formation and maintenance of the blood–brain barrier (BBB), contributions to the formation and resolution of brain edema, metabolic support for neurons, and participation in pathologic immune responses (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). All these functions depend to a large extent on the mechanisms by which astrocytes communicate with the surrounding cells. These include plasma membrane channels, receptors, transporters, and mechanisms that mediate the exchange of molecules by exo- and endocytotic processes (Kreft et al. 2004; Osborne et al. 2009; Parpura and Zorec 2010; Guček et al. 2012; Parpura et al. 2012; Zorec et al. 2012). In exo- and endocytotic processes, signaling molecules are released from or are internalized into vesicles, respectively. In the cytoplasm, both exo- and endocytotic vesicles reach their cytoplasmic destination by trafficking, which involves the cytoskeleton (Potokar et al. 2005, 2007).

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; Parpura et al. 2010; Parpura and Zorec 2010; Vardjan et al. 2012; Martineau et al. 2013; Potokar et al. 2013a). Their efficient delivery to the target destination in the cell is governed by vesicle mobility. It has been established recently that cytoplasmic vesicle mobility over distances of several micrometers is a tightly regulated process, involving cell signaling 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).

Single-vesicle trafficking studies in astrocytes have provided new insights into the roles of astrocytes in brain functioning. This review focuses on the mobility properties of exocytotic vesicles that transport gliotransmitters (glutamate, adenosine 5'-triphosphate [ATP], atrial natriuretic peptide [ANP], and brain-derived neurotrophic factor [BDNF]), membrane transporters (excitatory amino acid transporter 2 [EAAT2]; aquaporin 4 [AQP4]) and antigen-presenting receptors (major histocompatibility complex class II [MHC-II] receptors), and their role in health and disease. Although electron microscopy studies have revealed that astrocytes contain clear-core and, to a lesser extent, dense-core vesicles (Parpura and Zorec 2010), it is currently unknown whether the trafficking of these is distinct, therefore the aim here is to highlight studies in which vesicle mobility was monitored in real time in astrocytes.

3.2 Key Pathophysiologic Considerations of Vesicle Dynamics: from Signaling to Morphology of Astrocytes

Neurologic diseases are considered to emerge when disruption of the homeostatic balance between neural cell damage, neuroprotection, and regeneration occurs. A key function of astrocytes is to provide homeostatic support in the brain, hence these cells are involved in every kind of neuropathology (Seifert et al. 2006; Giaume et al. 2007). Moreover, these cells provide the first line of brain defense through regulation of the volume and composition of extracellular space (Kofuji and Newman 2004), such as extracellular levels of glutamate (Kirischuk et al. 2007). This function limits the excitotoxic burden of glutamate and is linked to the antioxidant system of astrocytes (Dringen 2000). However, astroglia may also contribute to neuronal damage through failure or reversal of various homeostatic cascades (Nedergaard and Dirnagl 2005; Giaume et al. 2007). Pathologic insults to the CNS trigger a specific astrocytic reaction known as reactive astrogliosis, which aims to protect the brain parenchyma, isolate the damaged area, reconstruct the BBB, and promote remodeling of the neural circuitry through hypertrophic morphological changes in astrocytes (Wilhelmsson et al. 2006).

3.2.1 Altered Vesicle Dynamics

How can vesicles contribute to the pathologic potential of astrocytes? Although vesicles are involved in relatively rapid processes, such as in neuronal synaptic transmission, the response of regulated exocytosis in astrocytes is at least one or two orders of magnitude slower (Guček et al. 2012). Furthermore, there are even slower vesicle-based processes than astrocytic-regulated exocytosis. It has been estimated that the cell surface area is turned over by constitutive vesicle traffic in 5

to 6 h (Kreft and Zorec 1997). Hence, if such slow constitutive plasma membrane turnover occurs in astrocytes in vivo, only very slight imbalances in the rate of vesicle delivery to the plasma membrane relative to retrieval from the plasma membrane may, over relatively long periods of time, affect the properties of astrocytes and their responsiveness to physiologic and pathologic stimuli. For example, tissue remodeling in Alzheimer disease (AD), where Aβ protein can be internalized into the astrocytes (Nagele et al. 2003, 2004; Olabarria et al. 2010), likely involves altered vesicle dynamics, which may contribute to the progression of AD. Another example of impaired vesicle traffic may give rise to conditions such as intellectual deficiency (ID), formerly known as human mental retardation, a common disorder characterized by an IQ lower than 85. Poor cognitive ability can be the only visible sign in nonspecific ID, whereas behavioral deficits accompanied by other clinical signs may compose a syndrome (e.g., Down syndrome), or may be associated with metabolic, mitochondrial or developmental disorders (Luckasson and Reeve 2001). Symptoms appear early in life and affect between 2 and 3 % of the population. Family studies have demonstrated a relatively large number of X-linked forms of ID (XLID) with an incidence of about 0.9 to 1.4 in 1000 males (Turner 1996). One of the first genes discovered to be mutated in patients with XLID is *GDI1* (D'Adamo et al. 1998). *GDI1* encodes for αGDI, a protein physiologically involved in retrieving inactive GDP-bound Rab from the membrane. The identification of *GDI1* as one of the genes causing human ID suggested that vesicular traffic in neuronal cells is an important pathway for development of cognitive functions (D'Adamo et al. 2002; Bianchi et al. 2009). Although the importance of αGDI in neuronal function has been demonstrated, it is unclear whether its role in glia vesicle trafficking contributes to the etiology of disease. αGDI protein regulates the function of Rab GTPases, such as Rab 4 and Rab 5, which have recently been shown to regulate the traffic of endosomes in astrocytes (Potokar et al. 2012), but it is likely that impaired vesicle traffic in astrocytes contributes to the ID.

3.2.2 Vesicles and Astrocyte Morphology

There are many mechanisms by which synaptic transmission is modulated by astrocytes, a partner of the tripartite synapse (Haydon 2001). These include morphological alterations of astrocytes. Because astrocytes ensheath synapses, local retractions or expansions of astrocytic processes, as observed by Theodosis et al. (2008), modify the geometry of the extracellular space, affecting neuron–glia interactions. Morphological changes in astrocytes also play an important role in pathologic states, since they become hypertrophic (Wilhelmsson et al. 2006). Astrocytes may swell and contribute to the development of brain edema and here vesicles carrying AQP4 seem important (Potokar et al. 2013a) as described below in Sect. 4.1.

What is the significance of morphological changes in astrocytes? The apposition of astrocyte membrane around the synaptic cleft seems, for example, to be an important determinant for the efficient removal of glutamate from the synaptic cleft, which determines the properties of synaptic signals (Marcaggi et al. 2003). Removal

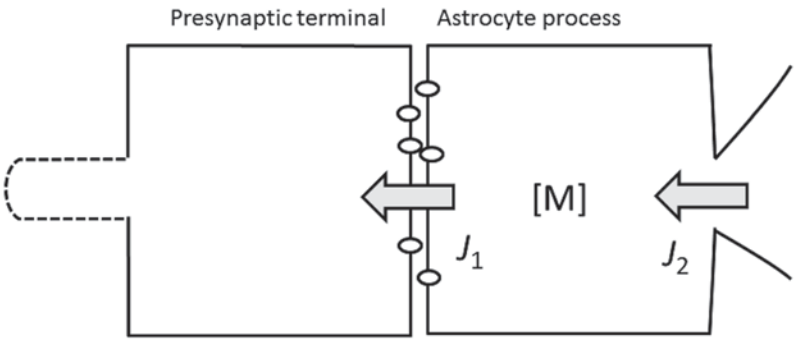

Fig. 3.1 Morphological metabolic coupling between the presynaptic terminal and the astrocytic process, i.e., glial microdomain (Grosche et al. 1999), but with a new metabolic course. These two compartments are considered to have equal volumes. Metabolite molecules M are transported into the astrocytic process (some may also be produced locally) through a conduit depicted by the narrow passage (J_2). The flux in the pathway to the presynaptic terminal (J_1) is mediated by transporters, depicted by spheroid symbols that permeate metabolite molecules M

of glutamate from the synaptic cleft consists of coupled diffusion of glutamate in the synaptic cleft and flux into the astrocyte via plasma membrane glutamate transporters; glutamate then diffuses in the cytoplasm to sites where it is metabolized. Another example of coupled transport between the astrocyte and the synapse is the transport of metabolites, such as glucose and lactate. The flux of metabolites increases during activation of the synapse. Given that the volume of an astrocyte is much larger than that of the presynaptic terminal, where energy is consumed during synaptic activity (Harris et al. 2012), one has to consider that the metabolite transport intensities between the two compartments differ due to the different surface-to-volume ratios in relatively small and large objects (McMahon 1973). However, the volume of astrocytes is fragmented into smaller compartments. For example, Bergmann glial cells send specialized appendages that cover several synapses and form relatively independent compartments enwrapping synapses, termed glial microdomains (Grosche et al. 1999), through which glial cells interact with neurons (Grosche et al. 2002). Such volume fragmentation likely improves the disparity of the surface-to-volume ratio between the neuronal and astrocytic compartments. In Fig. 3.1, the volumes of the presynaptic terminal and the astrocytic process are considered similar. However, the volume of an astrocyte process is open via a passage to the bulk volume of an astrocyte through which metabolites are delivered and may also escape (Fig. 3.1). The transport intensity of molecules M within the volume of the astrocytic process (J_2 in Fig. 3.1) is considered to be much higher than that through the membrane facing the presynaptic terminal (J_1 in Fig. 3.1). To maintain a stable and sufficiently high concentration of the metabolite (M) in the astrocytic process, the fluxes have to be balanced. During synaptic activation, the rate of metabolite consumption in the synaptic compartment increases and this may lead to a decline in the concentration of the metabolite, unless a compensatory increase in metabolic influx occurs or reserves (e.g., glycogen) are activated. Whether

such a process exists, however, remains to be investigated. Note that in Fig. 3.1, the transport capacity of molecule M between the compartments at rest likely favors that between the bulk volume of the astrocyte and the astrocytic process. However, the transport capacities may change if relative volumes of synaptic and astrocytic compartments are changed by putative regulatory processes. The narrower the passage connecting the astrocytic process to the bulk volume of the astrocyte, the smaller the potential loss of metabolites from the astrocytic process. An increase in the presynaptic terminal size was observed during functional inactivation of presynaptic terminals (Murthy et al. 2001). Changes in cell morphology have been observed during various active brain states (Shinohara et al. 2013). For example, in pathologic states such as trauma (Bardehle et al. 2013) and the late stages of AD (Verkhratsky et al. 2010), reactivated hypertrophic astrocytes (Wilhelmsson et al. 2006) are observed. Astrocyte atrophy was reported in some parts of the brain in the early stages of AD (Olabarria et al. 2010). These morphological cases likely mirror alterations in metabolic activity, whereby local fluxes of metabolites are corrected by morphological alterations due to altered neuron–glia interactions. However, the exact mechanisms of shape changes of astrocytes are unclear and are under active investigation by several laboratories.

One mechanism seems to involve G-protein coupled receptor (GPCR)-mediated processes. It has been known for some time that astroglial β-adrenergic receptors (β-ARs) functionally regulate astrocyte cellular morphology (Hatton et al. 1991). An increase in intracellular adenosine 3′,5′ cyclic monophosphate (cAMP) production on β-AR stimulation induces astrocyte stellation (Fig. 3.2), i.e., transformation from a flattened polygonal morphology to a stellate process-bearing morphology (Shain et al. 1987; Bicknell et al. 1989). How vesicle traffic contributes to these processes is largely unclear. β-ARs are abundant on astrocytes in both white and grey matter of the brain (Aoki 1992; Sutin and Shao 1992; Zeinstra et al. 2000; Catus et al. 2011) and are likely involved in a number of pathologic conditions. It is likely that during the early stages of AD, when a reduction of noradrenaline was reported (Hammerschmidt et al. 2013), this may result in reduced cAMP signaling and in astrocyte atrophy, the latter as observed in the triple transgenic animal model of AD (Olabarria et al. 2010). Such astrocytic atrophy may lead secondarily to synaptic loss due to insufficient metabolic support of synapses by astrocytes. In this particular case, astrocytes signal to neighboring cells likely using gliotransmitter vesicles.

3.3 Gliotransmitter Vesicles

Gliotransmitters are chemicals released from glial cells and are synthesized by and/or stored in glia (Parpura and Zorec 2010). Storage of gliotransmitters in membrane-bound vesicles as opposed to having them stored in the cytoplasm provides several advantages for cell-to-cell communication (Guček et al. 2012). In astrocytes, several types of gliotransmitter vesicles have been reported containing the amino acids glutamate and D-serine (Parpura et al. 1994; Bezzi et al. 2004; Kreft et al. 2004;

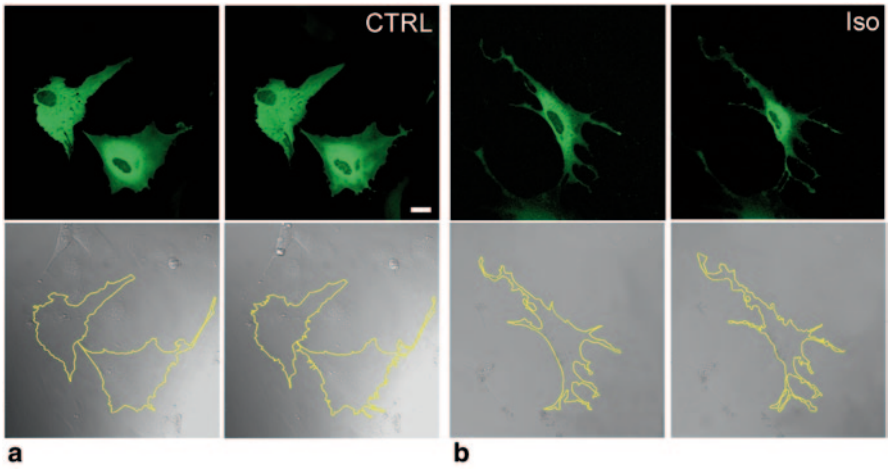

Fig. 3.2 Morphological changes in astrocytes (stellation) induced by β-AR activation, which increases cAMP. **a, b** Green fluorescing astrocytes transfected with the cAMP nanosensor Epac1-camps (Nikolaev et al. 2004) (*top*) and their corresponding differential interference contrast images (*bottom*) before (*left*) and within 30 min after (*right*) the addition of (**a**) extracellular solution as control (CTRL) and (**b**) 1 μM β-AR agonist isoprenaline (Iso), which increases intracellular cAMP levels through activation of β-ARs in astrocytes. **b** Rapid change in the cross-sectional area and perimeter (see the *yellow outline* in the bottom panels) of astrocytes, where thinning of processes indicates astrocyte stellation. The perimeter of individual cells expressing Epac1-camps was traced using LSM510 META software, which also outlines the cross-sectional area of the cell. Scale bar: 20 μm

Montana et al. 2004, 2006; Martineau et al. 2008, 2013), ATP (Coco et al. 2003; Pangrsic et al. 2007), and peptides such as ANP (Krzan et al. 2003; Kreft et al. 2004), BDNF (Bergami et al. 2008), and tissue plasminogen activator (tPA) (Cassé et al. 2012). Several vesicle mobility experiments have been performed on primary cultured astrocytes to explore how vesicle mobility is altered under different physiologic and pathologic conditions.It is not even a decade since the first spontaneous mobility of membrane-bound vesicles in astrocytes was described (Potokar et al. 2005), and subsequently confirmed (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, directional and nondirectional, of vesicle mobility have been described in astrocytes, and these modes of mobility were able to switch while a vesicle was being observed (Potokar et al. 2005) (Fig. 3.3a).

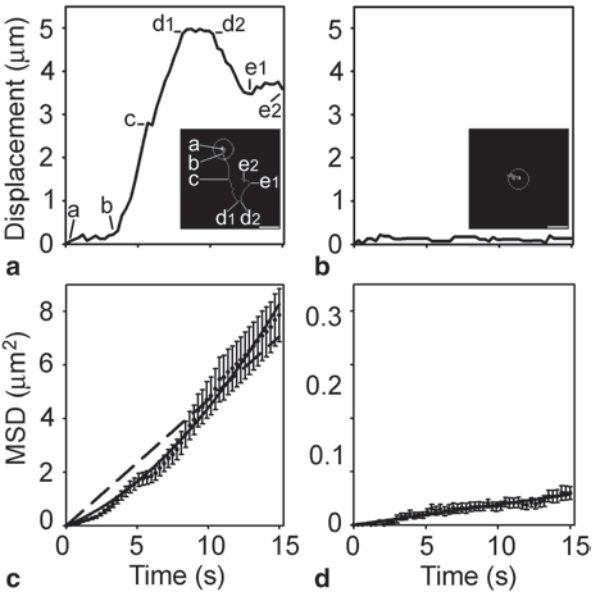

Fig. 3.3 Single-vesicle mobility tracks revealing directional and nondirectional vesicle mobility. a Displacement from the origin vs. time for a directional vesicle. The first quarter of the vesicle's mobility tracking time from the origin consists of almost constant displacements (a to b) and, during this time, the vesicle remains close to the origin of tracking (see inset (a)). In the next third of the tracking time (b–d1), vesicle displacements increase rapidly in a preferential direction with a brief pause (c). After a short period with equal displacements (d1–d2), the vesicle seems to move backwards, however, apparently not on the same track (d2–e1 and inset). b Displacement from the origin for a nondirectional vesicle. Minor mobility was observed and the vesicle did not translocate far from the origin of tracking (see inset). The mean square displacement (MSD) shown in (c, d) was calculated according to the equation MSD = [d(t) − d(t + Δt)]2. (c) The MSD of directional vesicles. The dashed line represents a linear function fitted to the data using an equation with the form MSD (µm2) = (0.4702 ± 0.0099) × time (s). The upwardly curving line represents a quadratic function fitted to the data following the equation MSD (µm2) (0.2189 ± 0.0148) × time (s) + (0.0221 ± 0.0013) × time2 (s2). d The MSD of nondirectional vesicles. The linear function was fitted to the data following the equation MSD (µm2) = (0.0038 ± 0.0001) × time (s). Bar: 2.5 µm. (Reproduced from Potokar et al. (2005); with permission)

3.3.1 Amino Acid-loaded Vesicles

In astrocytes, 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

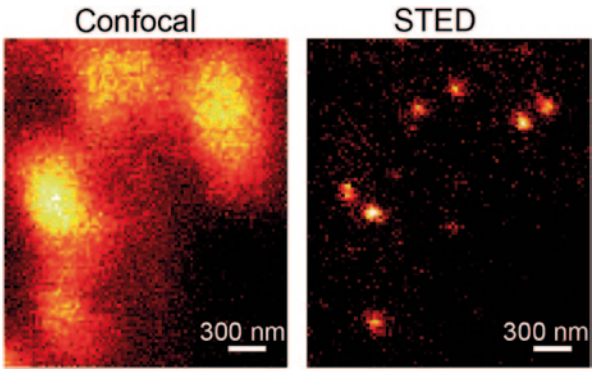

Fig. 3.4 Comparison of vesicles loaded with d-serine recorded by confocal microscopy (left) and by STED (right). Note that vesicles, which are positioned at a distance less than about half the emitting light wavelength (~300 nm), cannot be resolved with diffraction limited confocal microscopy. On the other hand, STED microscopy, which is the first far field microscopy to break the diffraction limit, successfully resolves individual vesicles

investigators. Figure 3.4 shows an example of vesicles fluorescently labeled by D-serine antibodies in fixed cultured astrocytes examined by confocal and stimulation emission depletion microscopy (STED, a superresolution 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.

In addition to studies of spontaneous vesicle mobility, experiments on astrocytes have revealed that vesicle mobility may be altered in stimulated cells. This was observed when glutamatergic vesicles were labeled 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 labeled 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 in native vesicles, because anti-VGLUT1 antibodies label the luminal part of vesicles (Almqvist et al. 2007; Stenovec et al. 2007). At higher intracellular concentrations of Ca^{2+} ($[Ca^{2+}]_i$) induced by4 μM ionomycin or 1 mM ATP, immunolabeling was more pronounced and the directional mobility of VGLUT1 vesicles was increased. Together with directionality, the fraction of fast-moving vesicles (>0.05 μm/s) 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). As discussed by Stenovec et al.

(2007), regulation of vesicle mobility after vesicle retrieval may be involved in various aspects of physiology, such as 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 contrasts the stimulation-induced attenuation of mobility of peptidergic vesicles and endosomal structures, which likely plays an important role under pathologic conditions (Potokar et al. 2008, 2010, 2011).

3.3.2 ATP-loaded Vesicles

ATP, an essential component of long-range Ca^{2+} signaling in the nervous system (Zimmermann 1994), is also an important astrocytic gliotransmitter (Parpura and Zorec 2010) and one of the major extracellular messengers for interastrocyte Ca^{2+}-mediated communication (Guthrie et al. 1999; Wang et al. 2000). In addition to nonvesicular 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), volume-sensitive organic osmolyte and anion channels (Blum et al. 2010), Ca^{2+}-dependent exocytotic ATP release from astrocytes has also been confirmed (Parpura and Zorec 2010). ATP-loaded astrocytic vesicles seem to be heterogeneous. So far in astrocytes, the vesicular distribution of ATP 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 in lysosomes (Jaiswal et al. 2007; Zhang et al., 2007; Li et al. 2008; Verderio et al. 2012). ATP seems to be co-stored together with classic neurotransmitters (acetylcholine in neurons and noradrenaline in neurons and chromaffin cells) (Zimmermann 1994) or with peptides (Calegari et al. 1999; Belai and Burnstock 2000; Bodin and Burnstock 2001; Coco et al. 2003; Pangrsic et al. 2007). In neonatal cortical rat astrocytes, ATP-containing vesicles seem to substantially co-store ANP (39 ± 7 %) (Pangrsic et al. 2007). Under spontaneous conditions, most of the ATP vesicles were located in close proximity to the plasma membrane (up to 150 nm) 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/quinacrine cargo by regulated exocytosis. This effect was obstructed by the presence of the dominant-negative soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) domain peptide, 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 the dominant-negative SNARE mouse model, it was shown that ATP release is reduced and this prevents tissue damage after a stroke (Hines and Haydon 2013).

3.3.3 Peptide-loaded Vesicles

ANP, which is stored in membrane-bound vesicles, was first shown to be released by Ca^{2+}-dependent exocytosis from astrocytes (Krzan et al. 2003). The cytoplasmic mobility in the secretory pathway was monitored using ANP.emd fluorescent recombinant protein (Han et al. 1999), whereas the mobility in the recycling pathway was monitored using an immunolabeling approach in living cells (Potokar et al. 2008).

The major difference between the mobility of ANP vesicles monitored in the different pathways in rat astrocytes was the traveling speed. In vesicles traveling from the cytoplasm to the plasma membrane, the speed was 0.4 ± 0.007 μm/s (nondirectional vesicles 0.3 ± 0.005 μm/s, directional vesicles 0.5 ± 0.01 μm/s), indicating that all vesicles were mobile but some displayed directional motion. Such rapid vesicle mobility is comparable with the directional movement of vesicles in some neurons (Potokar et al. 2005). However, their mobility was significantly attenuated after depolymerization of microtubules, actin filaments, and intermediate filaments (IFs) to 0.30 ± 0.0003 μm/s, 0.08 ± 0.01 μm/s and 0.21 ± 0.01 μm/s, respectively (Potokar et al. 2007). In mouse astrocytes, the measured parameters of mobility were lower compared with rat astrocytes (Potokar et al. 2013b). The slight difference in speed was also recorded between wild-type (WT) astrocytes and astrocytes without IFs, and the extent of directional mobility was also lower in astrocytes without IFs (Potokar et al. 2007). These data support the hypothesis that IFs are required for long-range directional vesicle mobility by acting as three-dimensional conduits. The importance of astrocytic IFs in vesicle mobility under pathologic conditions has also been confirmed by more recent studies (Vardjan et al. 2012).

The mobility of ANP vesicles in the recycling pathway has been monitored in rat astrocytes. Vesicle recycling has been considered for secretory granules, which are released by stimulated exocytosis (Taraska et al. 2003). During this process, 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 studying recycling ANP vesicles, they exhibited one order of magnitude slower mobility than secretory ANP vesicles (Potokar et al. 2013b). What is the physiologic 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. If sufficient time is allowed (during attenuated vesicle mobility), the vesicle cargo can be completely discharged from the vesicle lumen, especially if peptides in the

vesicle are aggregated into dense matrices (detected as electron-dense material on electron microscopy) and their discharge is inefficient unless the vesicles exhibit a fusion pore that permits prolonged discharge of the vesicle cargo. Furthermore, brain ANP content is significantly increased after experimental brain infarction, but not after brain hemorrhage, after contusion and in controls, indicating that ANP-positive astrocytes may increase in number and may be involved in the regulation of the cerebral blood flow in the infarcted brain area (Nogami et al. 2001). The altered cerebral blood flow thus also underlies enhanced delivery of ANP vesicles to the plasma membrane and/or release of ANP from astrocytic vesicles.

In addition, a specialized form of bidirectional communication involving signaling peptides exists between neurons and astroglia. BDNF secreted from neurons in its precursor form (pro-BDNF) is cleared from the extracellular space into nearby astrocytes, which internalize it via formation of a complex with the pan-neurotrophin receptor p75 and subsequent clathrin-dependent endocytosis. Endocytosed pro-BDNF is then routed into a fast recycling pathway for subsequent SNARE-dependent secretion triggered by glutamate (Bergami et al. 2008). Similarly, tPA appears as an element of the crosstalk between neurons and astrocytes. tPA released by neurons is constitutively endocytosed by astrocytes via the low-density lipoprotein-related protein receptor, and is then exocytosed in a regulated manner. Here, however, the exocytotic recycling of tPA by astrocytes is inhibited by extracellular glutamate. Kainate receptors of astrocytes act as sensors of extracellular glutamate and, via a signaling pathway involving protein kinase C, modulate the exocytosis of tPA (Cassé et al. 2012). Apart from this, BDNF is the most prevalent growth factor in the CNS and is widely implicated in psychiatric diseases, such as major depressive disorder (MDD), schizophrenia, addiction, and Rett syndrome (Autry et al. 2011). Notably, *N*-methyl-D-aspartate receptor (NMDAR) antagonists may produce fast-acting behavioral antidepressant effects in patients with depression and studies in mouse models indicate that these effects depend on the rapid synthesis of BDNF. At the cellular level, the blockade of NMDAR deactivates eukaryotic elongation factor 2 (eEF2) kinase/CaMKIII, resulting in reduced eEF2 phosphorylation and de-suppression of translation of BDNF (Autry et al. 2011). Although the regulation of protein synthesis has been identified as a valuable therapeutic target in the treatment of MDD, one cannot rule out the possibility that fast-acting antidepressants affect trafficking and/or release of presynthesized BDNF from brain cells; patients with depression report alleviation of MDD symptoms within 2 h of a single, low-dose, intravenous infusion of the antidepressant drug (Zarate et al. 2006).

Whether vesicles in intact tissue exhibit similar mobility to cultured cells was tested by labeling recycling vesicles in astrocytes in hippocampal tissue slices, because brain tissue slices represent a preparation that is physiologically closer to that occurring in vivo, i.e. cell-to-cell contacts and tissue architecture are preserved as present in the brain (Potokar et al. 2009). We incubated brain slices from mice with antibodies either against ANP or against VGLUT1. Vesicles in astrocytes from the CA1 region of the hippocampus were recorded. The recording of vesicle mobility was performed with two-photon microscopy. The fluorescent puncta exhibited two types of mobility: nondirectional and directional. The average velocity of ANP-

containing granules in slices from mice was approximately 0.04 μm/s, which is similar to that reported for recycling ANP granules in rat primary astrocyte cultures (0.06 μm/s) (Potokar et al. 2008), but one order of magnitude slower than the velocity of pro-ANP.emd-labeled granules (Potokar et al. 2005, 2007), where the average velocity was 0.4 μm/s. The mobility of VGLUT1 vesicles was analyzed similarly. The VGLUT1 vesicles in the slices were slightly slower than the ANP vesicles; their average velocity was approximately 0.03 μm/s. The velocity of recycling VGLUT1 vesicles in the slices was also slightly slower than recycling VGLUT1 vesicles from rat primary astrocyte cultures (0.05 μm/s) (Stenovec et al. 2007). Although vesicle mobility may differ in different brain regions, these results show that the experimental data obtained from cultured astrocytes closely resemble the properties of vesicle mobility observed in tissue slices.

3.4 Other Vesicles

The mobility properties of endosomes and lysosomes have been described in detail in mouse (Potokar et al. 2010) and rat astrocytes (Stenovec et al. 2011). These vesicles were labeled by LysoTracker dye (Ly) and exhibited slow mobility compared with other vesicle types (Potokar et al. 2013b). The direction and speed of Ly vesicles was shown to be influenced by the absence of astrocytic IFs. The trafficking of Ly-labeled vesicles seems to be regulated differently from glutamate-containing (VGLUT1-positive) and peptide-containing (ANP-positive) vesicles under different physiologic conditions. Cell stimulation to trigger an increase in $[Ca^{2+}]_i$ significantly reduced the mobility of Ly-labeled vesicles in WT astrocytes but not in astrocytes devoid of IFs (*GFAP*$^{-/-}$*Vim*$^{-/-}$ astrocytes) (Pekny et al. 1998; Eliasson et al. 1999; Wilhelmsson et al. 2004). Moreover, stimulation-dependent regulation of VGLUT1- and ANP-positive vesicles was attenuated by the absence of IFs. Because these filaments get overexpressed under pathologic conditions (Eliasson et al. 1999), it is likely that the traffic of distinct vesicle types is altered under these conditions (Potokar et al. 2010), likely leading to vesicle traffic jams (Potokar et al. 2011).

The regulation of endosome and lysosome mobility may exhibit completely different properties in pathophysiologic states. For example, if purified IgG antibodies harvested from patients with sporadic amyotrophic lateral sclerosis (ALS) are applied to astrocytes, the mobility of Ly-labeled compartment(s) is transiently increased, likely in a Ca^{2+}-dependent manner, indicating that acidic compartments may not represent a functionally homogeneous subcellular compartment, although endosomes and lysosomes were stained predominantly (Stenovec et al. 2011). The altered mobility is likely associated with altered Ca^{2+} homeostasis by ALS IgGs (Milošević et al. 2013). How do these results relate to the disease? ALS is a complex, incurable, and non-cell autonomous degenerative disease that affects upper and lower motor neurons located in a neighborhood enriched with nonneuronal cells; it occurs in adulthood (Haidet-Phillips et al. 2011) with a projected lifetime

risk of 1 in 2000 (Eisen 2009). The hallmark of ALS is selective death of motor neurons, although glial cells are also affected. In ALS, astrocytic function is compromised in several ways that impair neuronal survival and includes deficient release of (i) neurotrophic factors (Ekestern 2004); (ii) release of nerve growth factor or extracellular mutant superoxide dismutase 1 (SOD1) (Pehar et al. 2004; Urushitani et al. 2006); (iii) insufficient clearance of glutamate from the synaptic cleft, due to reduced density and loss of EAAT2 (Rothstein et al. 1995). Disturbance of the physiologic balance between neurons and astrocytes may therefore play a key role in motor neuron degeneration in ALS (Van Damme et al. 2005). In addition, activation of a systemic immune response in patients with ALS (Zhang et al. 2005) may play a role in the continuing pathology of ALS, once the BBB is compromised (Garbuzova-Davis et al. 2007). Correspondingly, motor neurons survived less when cocultured on astrocytes expressing the mutant form of Cu-Zn SOD1, as in the familial type of ALS, than on WT astrocytes (Di Giorgio et al. 2007). The application of conditioned medium from mutant SOD1-expressing astrocytes decreased the survival of motor neurons, suggesting the presence of astrocyte-secreting molecules that kill neurons (Nagai et al. 2007). Alterations in vesicle dynamics may thus reflect changes associated with the progression of the disease and may offer possibilities for the development of new diagnostic tests.

3.4.1 Aquaporin Transporting Vesicles

Recently, it was shown that the water channel AQP4 is also trafficking in vesicles that exhibit the properties of endosomes and lysosomes (Potokar et al. 2013a). This key molecule is involved in brain water homeostasis and is one of the three AQPs identified in brain cells in vitro and in vivo (Hasegawa et al. 1994; Jung et al. 1994; Nielsen et al. 1997; Badaut et al. 2002; Amiry-Moghaddam and Ottersen 2003). AQP4 isoforms in rodent and nonhuman primate brains are the most strongly expressed in astrocytic end feet surrounding the BBB (Nielsen et al. 1997; Nagelhus et al. 1998; Neely et al. 1999; Arciénega et al. 2010) and have also been identified in astrocytic processes in contact with synapses (Nielsen et al. 1997; Badaut et al. 2000a, b). Several studies have suggested an important role of AQP4 in water transport in several processes including astrocyte swelling and brain edema formation/resolution under various pathologic conditions, both in vitro (Yamamoto et al. 2001; Arima et al. 2003) and in vivo (Manley et al. 2000; Ke et al. 2001; Papadopoulos et al. 2004). Water transport through the cell membrane may be regulated by the permeability properties of AQP4 (Gunnarson et al. 2008; Nicchia et al. 2011), the heterogeneity of AQP4 crystalline-like orthogonal arrays of particles (Hirt et al. 2011), and, as recently suggested, by the mobility of the AQP4 vesicles that are delivered to and from the plasma membrane (Potokar et al. 2013a). The properties of AQP4 vesicle mobility are described in a study by Potokar et al. (2013a). AQP4 is one of the newly described basic AQP4 isoforms (Moe et al. 2008). In unstimulated conditions, the mobility of AQP4 vesicles resembled the mobility of slow recycling and endosomal vesicles (Stenovec et al. 2007; Potokar et al. 2008, 2010). After

dibutyryl-cAMP treatment, a model to induce reactive astrocytosis, an increased AQP4 signal was measured at the plasma membrane after 15 min (and remained increased even after 24 h) and the mobility of AQP4 vesicles was affected (Potokar et al. 2013a). These data indicate that the regulation of vesicle mobility in the relatively short time scale is an important regulatory mechanism to alter the delivery and removal ratio of AQP4 vesicles at the plasma membrane in reactive astrocytes. Decreased mobility with significantly lower directionality might contribute to restraining the AQP4 vesicles near the plasma membrane and may also be linked to dibutyryl-cAMP-induced rearrangements of the F-actin cytoskeleton mesh already considered to be one of the major factors responsible for increased AQP4 plasma membrane localization (Nicchia et al. 2008).

During the early stages of brain edema formation, astrocytes swell (Papadopoulos et al. 2004; Nase et al. 2008; Risher et al. 2009). A reduction in osmolarity triggers an increase in soma volume; this has been measured in tissue and in cultured rat astrocytes (Takano et al. 2005; Pangrsic et al. 2006; Thrane et al. 2011). The increase in cell volume may be accompanied by an increased rate of insertion of exocytotic vesicles in the membrane (Pasantes-Morales et al. 2002). Potokar et al. (2013a) reported that hypoosmotic conditions affected plasma membrane localization of AQP4 in rat astrocytes, in particular hypoosmotic stimulation triggered a transient increase in AQP4 plasma membrane localization. These changes were related to changes in AQP4 vesicle traffic; an increase in AQP4 plasma membrane localization overlapped with the observed decrease in mobility of AQP4 vesicles and the subsequent decrease in AQP4 plasma membrane localization overlapped with increased AQP4 vesicle mobility. The changes in mobility occurred predominantly in directional vesicles. These studies have shown that vesicle dynamics are playing a role in the cell swelling response at least by regulating the density of AQP4 channels in the plasma membrane. Whether cell swelling can be affected by manipulating vesicle traffic remains to be studied in the future.

3.4.2 Vesicles Delivering Plasma Membrane MHC Receptors and Transporters

Intracellular traffic of astrocytic vesicles, including endosomes and lysosomes, may also serve to deliver plasma membrane-associated MHC receptors (Soos et al. 1998; Vardjan et al. 2012) and transporters, such as glutamate transporter EAAT2 (Stenovec et al. 2008) where the nature of vesicles is not clear.

On exposure to the proinflammatory cytokine, interferon-γ (IFN-γ), otherwise immunologically silent astrocytes may begin to express MHC-II molecules and antigens on their surface and act as nonprofessional antigen-presenting cells (APCs). It has been suggested that IFN-γ-activated astrocytes participate in antigen presentation and activation of CD4 helper T cells in immune-mediated disorders of the CNS including multiple sclerosis (Fontana et al. 1984; Soos et al. 1998) and experimental autoimmune encephalomyelitis (Shrikant and Benveniste 1996).

In general, the delivery of MHC-II molecules from MHC-II compartments to the cell surface of APCs is mediated via a cytoskeletal network and is most likely completed with the fusion of MHC-II-carrying late endosomes/lysosomes with the plasma membrane. Actin microfilaments (Barois et al. 1998), microtubules (Wubbolts et al. 1999; Vyas et al. 2007), and their motor proteins (Wubbolts et al. 1999; Vascotto et al. 2007) have been shown to mediate trafficking of MHC-II compartments in APCs. Only recently, the role of IFs in MHC-II trafficking was investigated in IFN-γ-activated astrocytes (Vardjan et al. 2012), which as reactive astrocytes overexpress IFs (Junyent et al. 2011).

IFN-γ was shown to induce expression of MHC-II molecules on the astrocytic plasma membrane and late endosomes/lysosomes (Vardjan et al. 2012). The latter can be specifically labeled with Alexa Fluor® 546-conjugated dextran (Fig. 3.5a) (Jaiswal et al. 2002; Gabrijel et al. 2008; Vardjan et al. 2012). Time-lapse confocal imaging and fluorescent dextran labeling of late endosomes/lysosomes in WT astrocytes and in astrocytes devoid of IFs (*GFAP*$^{-/-}$*Vim*$^{-/-}$) revealed faster and more directional movement of late endosomes/lysosomes in IFN-γ-treated astrocytes than in untreated astrocytes (Potokar et al. 2013b). However, vesicle mobility was lower and less directional in IFN-γ-treated IF-deficient astrocytes than in WT astrocytes (Fig. 3.5b, c), indicating that the IFN-γ-induced increase in the mobility of MHC-II-carrying late endosomes/lysosomes is IF dependent. Application of ATP and the subsequent increase in $[Ca^{2+}]_i$ induced attenuation of the mobility of late endosomes/lysosomes was more apparent in the presence of IFs (Fig. 3.5b, c), implying a role for IFs in this process.

These data indicate that, in IFN-γ-activated astrocytes, upregulation of IFs allows faster and therefore more efficient delivery of MHC-II molecules to the cell surface. Reduced mobility of late endosomes/lysosomes due to increase in $[Ca^{2+}]_i$ may increase their probability of docking and fusion (Potokar et al. 2010), which, in astrocytes acting as APCs, may serve as an additional regulatory mechanism that controls the onset of late endosomal/lysosomal fusion and final delivery of MHC-II molecules to the cell surface (Vardjan et al. 2012). Besides IFN-γ, endogenous suppressors, including norepinephrine, have been shown to regulate the expression of MHC-II molecules in astrocytes (Frohman et al. 1988; De Keyser et al. 2004). The effects of norepinephrine are mediated through activation of G-protein-coupled β-ARs on astrocytes and subsequent activation of the cAMP signaling pathway. Our recent unpublished data suggest that the mobility and fusion of late endosomes/lysosomes involved in antigen presentation are also affected by activation of astrocytic β-ARs. Although these studies were carried out in vitro, all these regulatory mechanisms may enable antigen-presenting reactive astrocytes in vivo to respond rapidly and in a controlled manner during CNS inflammation.

Astrocytes play a key role in the uptake of glutamate, which is released into the extracellular space from glutamatergic neurons during synaptic transmission (Vesce et al. 1999; Amara and Fontana 2002) and from astrocytes themselves (Parpura et al. 1994). Physiologically, glutamate is cleared from the synaptic cleft via plasmalemma glial glutamate transporters GLAST (EAAT1) and GLT1 (EAAT2) (Danbolt 2001); its uptake is driven by the electrochemical gradient of sodium (O'Kane et al.

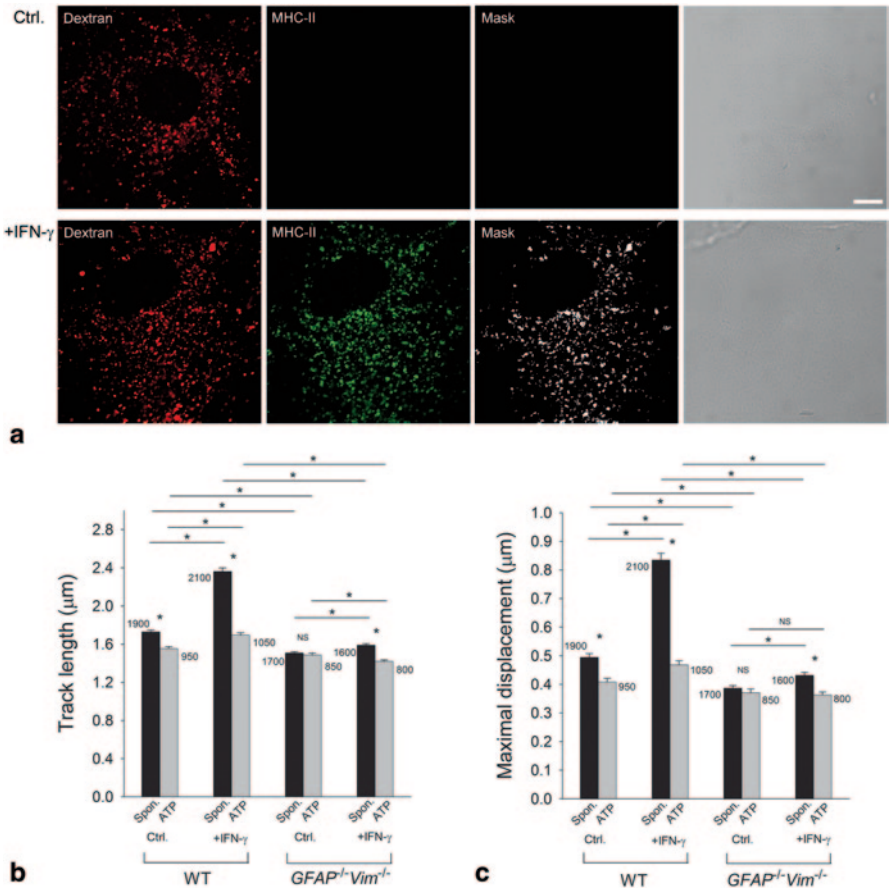

Fig. 3.5 The IFN-γ-induced increase in the mobility of MHC-II compartments in astrocytes is IF dependent. **a** Alexa Fluor® 546-dextran labels MHC-II-positive compartments in IFN-γ-treated WT and *GFAP*$^{-/-}$*Vim*$^{-/-}$(IF-deficient) primary mouse astrocytes. Fluorescence images of astrocytes labeled with dextran, fixed, and immunostained with antibodies against MHC-II molecules. *White pixels* (Mask) represent the colocalization mask of green (MHC-II) and *red fluorescence pixels* (Dextran). Scale bars: 10 μm. **b** Histogram of average vesicle track lengths in control (Ctrl.) and IFN-γ-treated (+IFN-γ) WT and *GFAP*$^{-/-}$*Vim*$^{-/-}$ cells. **c** Histogram of the mean maximal displacements of vesicles in control (Ctrl.) and IFN-γ-treated (+IFN-γ) WT and *GFAP*$^{-/-}$*Vim*$^{-/-}$ cells. The numbers on the bars are the numbers of vesicles analyzed. Values are mean ± SEM. *$P < 0.05$. (Adapted from Vardjan et al. (2012); with permission)

1999). The flux of transported molecules also depends on the density of transporters in the cell plasma membrane (Robinson 2002; Huang and Bergles 2004), which determines whether synaptic independence is compromised by the synaptic transmitter crosstalk. The density of EAAT2 in astrocyte plasma membrane is regulated by exo-/endocytosis in a Ca^{2+}-dependent manner (Stenovec et al. 2008). The altered trafficking of EAAT2 to/from the plasma membrane may result in diminished net uptake of extracellular glutamate, and an overabundance of glutamate accompanied

by failure of astrocytes to remove it may lead to neuronal excitotoxicity resulting in a selective loss of motor neurons as in ALS. Therefore, it may be useful to modify vesicle dynamics by drugs in order to minimize the adverse conditions associated with such a cell process.

3.5 Do Drugs Target Vesicle Dynamics in Astrocytes?

With advances in the understanding of the nature of vesicle mobility in astrocytes, a question has emerged whether there are any drugs that affect vesicle dynamics and could be used to treat neurologic diseases. Although the experimental evidence has revealed that different types of vesicles exhibit specific properties, it is possible that, under pathologic conditions, these may change. These include altered vesicle dynamics, changes in molecules that are released by vesicular mechanisms, alterations in the plasma membrane surface signaling landscape (altered densities of transporters, receptors, and associated signaling mechanisms) and changes in vesicle dynamics associated with morphology of astrocytes. All these factors may contribute to changes in communication between astrocytes and neighboring cells. However, one may consider that the least change may occur, if vesicle dynamics are attenuated, which may contribute to the minimization of vesicle-mediated changes in the cell. In line with this is the discovery that vesicle mobility was attenuated by fingolimod or FTY720 (Trkov et al. 2012). This drug was recently introduced as a therapeutic for the treatment of multiple sclerosis (Chun and Brinkmann 2011). It was shown that FTY720 accumulates in tissue hydrophobic pools (Foster et al. 2007), such as the white matter in the CNS, where it can reach concentrations that affect astrocytic vesicle mobility and consequently their ability to participate in regulated exocytosis (Trkov et al. 2012). This action may be part of its therapeutic effectiveness in patients with multiple sclerosis. The mechanism of reduction of vesicle mobility by fingolimod likely involves fingolimod-induced changes in $[Ca^{2+}]_i$ homeostasis. Figure 3.6 shows that the application of fingolimod augments $[Ca^{2+}]_i$ measured by the Fluo 4 fluorescent Ca^{2+} dye. In Fig. 3.6e, the time course of increased $[Ca^{2+}]_i$ is shown for 5 individual astrocytes. These increases in $[Ca^{2+}]_i$ are associated with the reduced mobility of peptidergic vesicles depicted in Fig. 3.6b–d. However, vesicle types other than peptidergic vesicles were also shown to be sensitive to fingolimod (Trkov et al. 2012). Moreover, astrocytes were considered to be the major source of a number of other molecules, including eicosanoids (prostaglandins, prostacyclins, thromboxanes, and leukotrienes) and these proinflammatory signaling molecules in the CNS are released via an ATP-dependent mechanism (Murphy et al. 1988). In astrocytes, ATP itself is released via regulated exocytosis, which participates in the neuroinflammatory and other pathologic states in the CNS. Thus, new therapeutics, such as FTY720 (Trkov et al. 2012), that affect vesicle mobility represent a novel possibility for the development of new therapeutics for neurologic diseases.

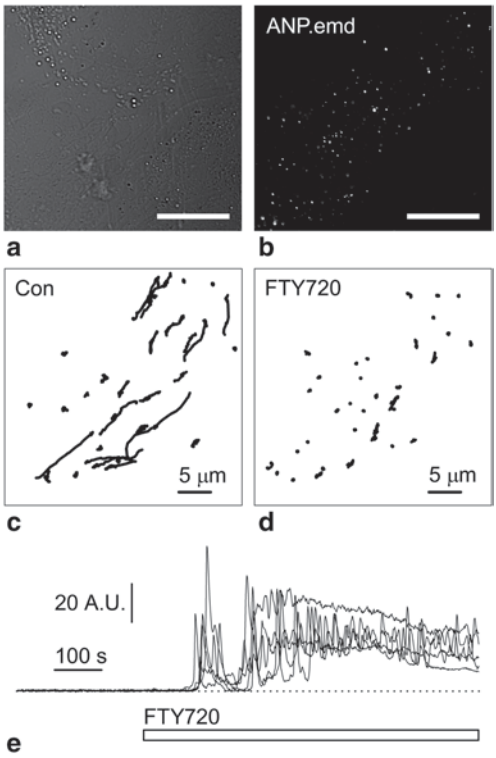

Fig. 3.6 The mobility of peptidergic vesicles is reduced by fingolimod (FTY720), which evokes prolonged increases in cytosolic calcium activity ($[Ca^{2+}]_i$) in cultured rat astrocytes. **a** Live astrocyte under differential interference contrast optics and **b** the confocal image of the same cell transfected to express ANP, which is fluorescently labeled by emd (ANP.emd) stored inside peptidergic vesicles (observed as fluorescent puncta); scale bars, 20 µm. **c** Vesicle tracks (n = 45) obtained in the same astrocyte before (Con) and **d** 10 min after treatment with 10 µM FTY720. Note many elongated vesicles tracks recorded during the 30 s epoch (**c**) indicating substantial vesicle mobility. After treatment with FTY720 (**d**), the mobility of vesicles is reduced as indicated by the absence of elongated tracks and the abundance of contorted tracks. **e** Superimposed time-resolved fluorescence intensity obtained in 5 cells treated with FTY720 (*white bar*). The *thin dotted line* indicates the zero fluorescence level (F_0)

3.6 Future Perspectives

Vesicle dynamics in astrocytes are altered in pathologic states. Although studied less intensely, vesicle dynamics may also contribute to shape changes in astrocytes. These occur under physiologic conditions and when astrocytes swell or become hypertrophic or atrophic, which occurs in several disease states. Second messenger cAMP, which is involved in shape changes in astrocytes, also plays an important role in regulating glucose metabolism (Prebil et al. 2011b). It will be important to

understand how energy support is regulated in view of vesicle dynamics and the morphological changes that take place in astrocytes in health and disease. Therefore, metabolic coupling measurements of D-glucose (Prebil et al. 2011a) and other metabolites, second messengers and measurements of shape changes will help us to understand the spatiotemporal coupling and interactions between neurons and glia.

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Conflict of Interest The authors declare no conflict of interest.

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Chapter 4
Glial Glutamate and Metabolic Transporters as a Target for Neurodegenerative Therapy and Biomarkers

Thomas Philips and Rita Sattler

Abstract Glial cells play a significant functional role in neuronal function and synaptic transmission and glial dysfunction contributes greatly to the development and progression of a large number of chronic and acute neurodegenerative disorders. Here, we report the importance of two glial plasma membrane transporter proteins, the astroglial glutamate/excitatory amino acid transporters (EAATs) and the oligodendroglial lactate/monocarboxylate transporters (MCTs), both of which have been shown to be dysregulated in disease. As action potentials invade presynaptic neuronal terminals, glutamate is released into the synaptic cleft, where it binds and opens postsynaptic ionotropic glutamate receptors. To limit excessive postsynaptic stimulation, glutamate is taken up by astrocytes through EAATs, a process coupled to Na^+ uptake and increased Na^+/K^+ pump activity leading to an increased metabolic demand, i.e. the production of ATP. To enhance ATP generation, glucose is taken up from blood vessels into the astrocytes through glucose transporters. The astrocytes convert glucose to pyruvate and lactate, generating ATP through glycolysis. Lactate, the end product of glycolysis could diffuse through gap junctions to oligodendrocytes. Alternatively, oligodendrocytes might generate lactate through glycolysis after glucose uptake from the blood stream. Oligodendrocytes export lactate through MCT1 transporters upon which neurons can import lactate through lactate transporters and use as a metabolic substrate. Any imbalance of the glutamate and/or lactate homeostasis can lead to neuronal degeneration, as shown in a number of neurodegenerative disorders.

Keywords Astrocyte-neuron · Astrocyte-oligodendrocyte · Biomarker · Gene expression · Glutamate · Glutamate transporter · Transporter · Lactate · Metabolism · Monocarboxylate transporter · Neuron-astrocyte · Neurotransmitter · Oligodendroglia · Oligodendrocyte · PET · Transcription · Translation

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4.1 Introduction

Glial cells are no longer considered the supporting cells in the central nervous system (CNS), but instead are recognized to play a significant functional role in neuronal function and synaptic transmission. With these important tasks comes great responsibility and recent studies confirmed that dysfunctional glial cells contribute greatly to the development and progression of a large number of chronic and acute neurodegenerative disorders. Here, we report the importance of the function and dysfunction of two glial plasma membrane transporter proteins (Fig. 4.1), the astroglial glutamate/excitatory amino acid transporters (EAATs) and the oligodendroglial lactate/monocarboxylate transporters (MCTs), both of which have been shown to be dysregulated in disease and therefore provide a potential target for therapeutic intervention.

Here, we briefly outline functions of these glial transporters (Fig. 4.1). As action potentials invade presynaptic neuronal terminals, glutamate is released into the synaptic cleft, where it binds and opens postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl D-asparate (NMDA) ionotropic glutamate receptors. To limit excessive postsynaptic stimulation, glutamate is taken up by astrocytes through plasma membrane glutamate transporters, a process coupled to Na^+ uptake and increased Na^+/K^+ pump activity leading to an increased metabolic demand, i.e. the production of ATP. To enhance ATP generation, glucose is taken up from blood vessels into the astrocytes through glucose transporters. The astrocytes convert glucose to pyruvate and lactate, generating ATP through glycolysis. Lactate, the end product of glycolysis could diffuse through gap junctions to oligodendrocytes. Alternatively, oligodendrocytes might generate lactate through glycolysis after glucose uptake from the blood stream. Oligodendrocytes export lactate through MCT1 transporters upon which neurons can import lactate through lactate transporters and use as a metabolic substrate.

4.2 Glutamate Transporters

The maintenance of low extracellular levels of the primary excitatory neurotransmitter glutamate at the neuronal synapse is crucial to ensure fast and efficient excitatory synaptic transmission in the CNS, particularly because there is no evidence for extracellular metabolism of glutamate. To be able to rapidly return to these homeostatic levels of glutamate after synaptic firing, plasma membrane spanning glial and neuronal high affinity glutamate plasma membrane transporters remove any excess glutamate via a sodium-potassium coupled uptake mechanism (Fig. 4.1a). Any unwanted rise in extracellular glutamate above normal leads to pathological activation of neuronal glutamate receptors, which in turn triggers intracellular events in the postsynaptic neuron leading to neuronal cell death. This phenomenon is known as glutamate excitotoxicity and is the underlying mechanisms of a number

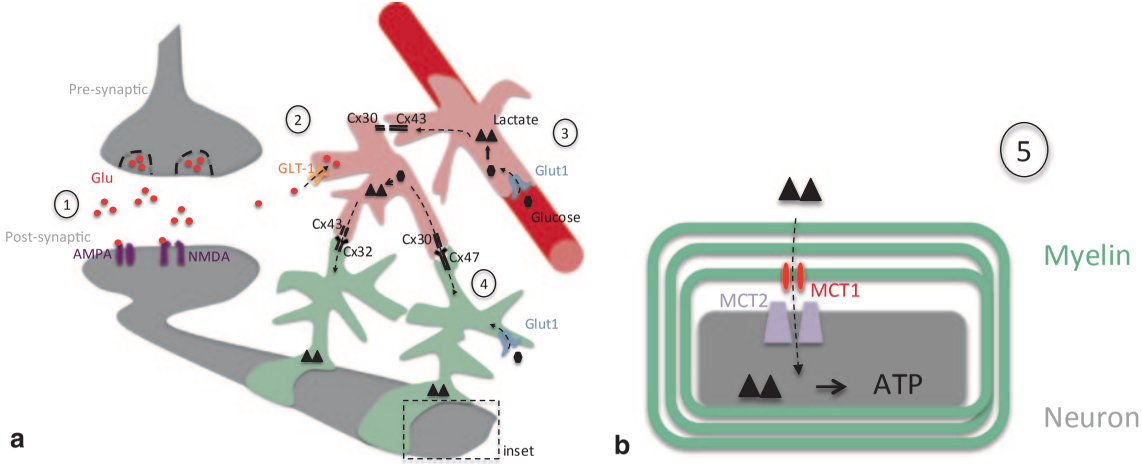

Fig. 4.1 Schematic depiction of glial glutamate and lactate transporter functions. **a** (*1*) Presynaptic neuronal impulses induce glutamate (Glu) release into the synaptic cleft. Glutamate binds and opens postsynaptic AMPA and NMDA receptors leading to enhanced Ca^{2+} influx and propagation of the presynaptic stimulus to the postsynaptic neuron. (*2*) To limit excessive postsynaptic stimulation, glutamate is taken up by astrocytes through GLT1 transporters. This process is coupled to Na^+ uptake and increased Na^+/K^+ pump activity leading to an increased metabolic demand, i.e. the production of ATP. (*3*) To enhance ATP generation, glucose is taken up from blood vessels into the astrocytes through glucose transporters (Glut1). The astrocytes convert glucose to pyruvate and lactate, generating ATP through glycolysis. (*4*) Lactate, the end product of glycolysis could diffuse through connexins (Cx) to oligodendrocytes. Alternatively oligodendrocytes might generate lactate through glycolysis after glucose uptake from the blood stream. **b** (*5*) Oligodendrocytes export lactate through MCT1 transporters upon which neurons can import lactate through MCT2 transporters and use as a metabolic substrate

of neurodegenerative as well as neuropsychiatric diseases. A loss of functional plasma membrane glutamate transport has been suggested to contribute significantly to excitotoxic neuronal cell death and regulation of glutamate transporters, and consequently the synaptic glutamate homeostasis, has therefore been an increasing interest in therapeutic development for neurological disorders.

4.2.1 Structure and Expression

Initial structure and function studies on EAATs have been based on molecular pharmacology approaches (Danbolt 2001). The recent crystallization of a prokaryotic orthologue Glt_{Ph}(Yernool et al. 2004) however provided an enhanced understanding into the structural details of EAATs as well as transport mechanisms (Boudker et al. 2007). The novel crystal structure predicted the presence of eight transmembrane domains and two re-entrant hairpin loops, with both, N- and C-terminal intracellular domains. While there is general agreement that the information obtained from the Glt_{Ph} will be informative for the structure and function of mammalian EAATs, future studies are needed to confirm commonality between these two transporter orthologues in regards to membrane topology, substrate preference, etc. (for a detailed review on glutamate transporter structure and function see (Jiang and Amara 2011)). Clearly, understanding the detailed mechanisms of neurotransmitter transport and potential conformational changes during this process will be important for the ongoing development of therapeutic compounds targeting these transporters.

Five plasma membrane glutamate transporter subtypes have been cloned and characterized thus far: EAAT1-EAAT5 (human nomenclature) or GLAST/GLT-1/EAAC1/EAAT4/EAAT5 (rodent nomenclature, respectively) (Danbolt 2001). The subtypes differ in their expression pattern with regard to cell type and CNS brain region. EAAT2/GLT-1 (Shashidharan et al. 1994) is primarily found on astrocytes and is the major glutamate transporter in the forebrain, estimated to be responsible for over 90 % of functional glutamate transport (Furuta et al. 1997b). EAAT1/GLAST (Shashidharan and Plaitakis 1993) is the major glutamate transporter present on astrocytes in the cerebellum (Furuta et al. 1997b), but also found in the inner ear (Furness and Lehre 1997), the circumventricular organs (Berger and Hediger 2000) and in the retina (Derouiche and Rauen 1995; Rauen 2000). Interestingly, EAAT1/GLAST is the highest expressed glutamate transporter during CNS development, but drops expression levels significantly when adulthood is reached (Furuta et al. 1997a). EAAT3/EAAC1 is a neuronal glutamate transporter with highest concentrations found in the hippocampus, cerebellum and basal ganglia (Furuta et al. 1997a, b). EAAT4 is also a neuronal transporter and for the most part expressed in the Purkinje cells of the cerebellar molecular layer (Barpeled et al. 1997; Furuta et al. 1997a) while neuronal transporter EAAT5 is mainly expressed in rod photoreceptors and bipolar cells of the retina (Arriza et al. 1997).

4.2.2 Physiology

Glutamate transport is facilitated by a co-transport of 2–3 molecules of Na+ and one molecule of H+. The binding of these molecules on the extracellular site of the transporter is thought to induce a conformational change of the protein, which then in turn allows for the counter-transport of one K+, before the transporter resumes its original confirmation (Zerangue and Kavanaugh 1996; Danbolt 2001). This transport cycle allows for the maintenance of a concentration gradient of extracellular versus intracellular glutamate of >10,000 fold. Most importantly, this transport is much slower than rapid excitatory synaptic signaling, allowing for maximum ligand-gated glutamate receptor response at the postsynaptic neuron (Wadiche et al. 1995; Bergles and Jahr 1997) and thereby reinforcing the impact that transporter function has on synaptic activity in the CNS (for reviews see Conti and Weinberg 1999; Huang and Bergles 2004). Experiments using antisense oligonucleotides or targeted gene disruption confirmed the critical role of efficient glutamate transporter activity in the maintenance of a healthy extracellular glutamate homeostasis (Rothstein et al. 1996; Tanaka et al. 1997). Mice lacking the prominent glutamate transporter EAAT2/GLT-1 showed significantly increased extracellular glutamate levels, excitotoxic neurodegeneration and progressive paralysis (Tanaka et al. 1997). While not fatal, knocking down EAAT3/EAAC1 *in vivo* led to epileptic seizures (Rothstein et al. 1996).

In addition to its role in excitatory neurotransmission, glutamate transport also contributes significantly to the metabolism of glutamate. Glutamate taken up by astrocytes can be recycled via the known glutamate-glutamine cycle: glutamate in astrocytes is converted to glutamine by the enzyme glutamine synthetase. Glutamine is then transported out of the astrocyte and back into neurons by cell type specific glutamine transporters. In neurons, glutamine is converted back to glutamate via glutaminase, where it is then re-packaged into synaptic vesicles and released by exocytosis back into the synaptic cleft (van den Berg and Garfinkel 1971) (for review see Danbolt 2001; Parpura and Verkhratsky 2012). While this metabolic pathway is widely accepted, there is sufficient evidence that glutamate in astrocytes can also be metabolized through different pathways, resulting not only in glutamine, but also lactate, thereby serving as a neuronal energy substrate (see discussion on astrocytic lactate below, also (Danbolt 2001)). In addition, neuronal glutamine is not only converted back to glutamate, but also used for the synthesis of γ-aminobutyric acid (GABA), which makes glutamate ultimately a precursor molecule for the formation of inhibitory GABA-ergic neurons.

4.2.3 Regulation

Given the crucial importance of efficient glutamate uptake activity for the maintenance of the extracellular glutamate homeostasis and prevention of glutamate excitotoxicity, significant efforts have been made to study the regulation of these transporters. Most importantly, any information gained from these studies should

facilitate the use of the glutamate transporter system as a pharmaceutical target for the treatment of neurodegenerative diseases that show impaired glutamate uptake activity.

Despite these efforts, the mechanisms of glutamate transporter regulation are still not well defined and only limited factors have been identified that regulate protein expression or transporter activity. The difficulty of identifying specific mechanisms arises from the diversity of glutamate transporter functions, reaching from very fast acute impact on signaling activity at the excitatory synapse to long term effects on excitotoxic neuronal cell death. Thus, regulation has been shown to occur on multiple levels, including DNA transcription and protein translation, as well as posttranslational modification, which, in turn, may affect glutamate transporter protein targeting, localization and transport activity. DNA transcription and protein expression are more likely to play a role in chronic glutamate transporter regulation, such as the one needed for the triggering of glutamate excitotoxicity, as these events require more time to induce changes in glutamate uptake activity (for detailed reviews see Sattler and Rothstein 2006; Kim et al. 2011). On the other hand, posttranslational modifications, transporter trafficking or direct transporter protein modulation can occur very acutely within minutes and therefore respond to fast synaptic event requirements (for detailed review see Robinson 2006; Sattler and Rothstein 2006).

Early in vitro studies showed that cultured astrocytes require the presence of neuronal soluble factors to express glutamate transporter EAAT2/GLT-1, and to a lesser degree EAAT1/GLAST (Drejer et al. 1983; Gegelashvili et al. 1997; Swanson et al. 1997; Schlag et al. 1998).The search for these soluble factors began, and the cloning of the glutamate transporter promoter regions allowed for the identification of more defined pathways regulating transporter expression while confirming earlier findings. For example dibutyryl- cAMP (db-cAMP), epidermal growth factor (EGF) as well as transforming growth factor-α (TGF-α), κB-motif binding phosphor protein or pituitary adenylate cyclase-activating polypeptide (PACAP) have all been shown to increase EAAT2/GLT-1 expression (Eng et al. 1997; Swanson et al. 1997; Schlag et al. 1998; Figiel and Engele 2000; Zelenaia and Robinson 2000; Yang et al. 2009). Inhibitors of phosphatidylinositol 3-kinase (PI3K) and nuclear transcription factor κB (NF-κB) blocked the effects of EGF and db-cAMP, and cloning of the promoter regions confirmed the presence of regulatory transcription factor binding sites including NF-κB, N-myc and nuclear factor of activated T cells (NFAT) for EAAT2/GLT1 and NF-κB, cAMP responsive element binding protein (CREB), activating protein 1 (AP1) and GC-box elements for EAAT1/GLAST (Kim et al. 2003; Su et al. 2003; Li et al. 2006). These new studies now confirmed a transcriptional activation by measuring increased mRNA levels of EAAT2/GLT-1 and EAAT1/GLAST (Kim et al. 2003; Su et al. 2003; Li et al. 2006) and supported the idea that glutamate transporter regulation differs between transporter subtypes but can also differ between activation and repression of one individual factor (Sitcheran et al. 2005), emphasizing the complexity of the glutamate uptake system.

In addition to transcriptional regulation, Lin and colleagues also found evidence for translational regulation of 5′-untranslated regions (UTRs) of EAAT2/GLT-1,

both in vitro and in vivo (Tian et al. 2007). These regions responded to exposure of corticosterone and retinol with increased glutamate uptake activity without increased mRNA levels of EAAT2/GLT-1, suggesting that aside from transcriptional activation, translational activation of glutamate transporters offers another avenue to regulate synaptic glutamate homeostasis. The idea of translational activation was further supported by a recent study implicating a role of neuronal micro RNA miR-124a in increasing EAAT2/GLT-1 protein expression levels, but not mRNA levels (Morel et al. 2013).

Other studies suggested a role of epigenetic regulation of glutamate transporters via DNA methylation (Zschocke et al. 2007; Yang et al. 2010). In human glioma cell lines, the EAAT2/GLT-1 promoter shows hypermethylation, which was suggested to be at least partially responsible for the decreased expression of EAAT2/GLT-1 in glioma cells (Zschocke et al. 2007). Similar results were found in ALS postmortem patient tissue samples, were low levels of methylation were found, concomitant with low levels of EAAT2/GLT-1 expression (Yang et al. 2010). On the other hand, neuron-dependent increase in EAAT2/GLT-1 expression in cultured primary mouse astrocytes was paralleled with decreased methylation of CpG sites of the EAAT2/GLT-1 promoter, suggesting that neuron-dependent epigenetic regulation of EAAT2/GLT-1 is different from disease-mediated dysregulation of transporter expression, which seems to be independent of EAAT2/GLT-1 promoter methylation (Yang et al. 2010).

4.2.4 Dysregulation

Given the importance of glutamate transporter function in the maintenance of extracellular glutamate homeostasis and consequently prevention of neuronal cell death via glutamate excitotoxicity, loss of glutamate transporter activity has been studied in a number of neurodegenerative diseases with the hope of the development of therapeutic interventions to prevent neurodegeneration by glutamate toxicity. Particular interest was given to astroglial transporter EAAT2/GLT-1 due to its high prevalence in the CNS and major contribution to overall glutamate removal.

Mechanisms leading to the loss of transporter protein are still unknown, but can be caused on both, the transcriptional level as well as the translational level of protein synthesis. Loss of EAAT2/GLT-1 has been reported in acute and chronic neurodegenerative disorders, as well as psychiatric diseases and addiction (For review see Kim et al. 2011; Lin et al. 2012): ischemia/hypoxia (Rothstein et al. 1996; Martin et al. 1997; Inage et al. 1998; Fukamachi et al. 2001; Rao et al. 2001; Chen et al. 2005; Yeh et al. 2005); Huntington's disease (HD) (Lievens et al. 2001; Behrens et al. 2002); Parkinson's disease (PD) (Ginsberg et al. 1995; Levy et al. 1995), epilepsy(Bjornsen et al. 2007; Sarac et al. 2009; Kong et al. 2012); Multiple Sclerosis(Mitosek-Szewczyk et al. 2008); Alzheimer's disease (AD) (Masliah et al. 1996, 2000; Li et al. 1997) and amyotrophic lateral sclerosis (ALS) (Rothstein et al. 1992; Bruijn et al. 1997; Howland et al. 2002); depression (Sanacora et al. 2004; Mineur et al. 2007; Sattler and Rothstein 2007; Gomez-Galan et al. 2013); alcohol

dependence (Rao and Sari 2012); and cocaine addiction (Fischer et al. 2013). Most of these diseases/conditions report a loss of glutamate transporter protein and mRNA, suggesting a dysregulation on a transcriptional level, possibly due to lack of specific transcription factor activation. No detailed mechanisms have been elucidated yet to explain this phenomenon. Some disorders show loss of glutamate transport function without a change in transporter mRNA, indicating dysfunctional translational regulation during disease progression. For example, animal models for AD had no changes in EAAT2/GLT-1 mRNA levels, while glutamate uptake and transporter protein levels were significantly reduced (Masliah et al. 2000). Similar observations were made in postmortem tissue of frontal cortex of AD patients (Li et al. 1997). The mechanisms underlying reduced translational activity are not yet fully understood either, but a possible explanation could be inhibition of translation via highly structured *cis* elements in the 5′UTR sequence (Tian et al. 2007).

Another possible cause for decreased glutamate uptake is abnormal splicing of EAAT2/GLT-1 mRNA during disease progression (Lin et al. 1996; Maragakis et al. 2004; Sullivan et al. 2004; Lee and Pow 2010). The altered splice product could lead to the production of truncated EAAT2/GLT-1 protein or lead to mistargeting of the transporter, which could then be responsible for reduced glutamate transport activity. The hypothesis of a role of alternate splicing in glutamate transporter dysfunction has been supported recently by the idea of a high prevalence of RNA toxicity and subsequent altered RNA metabolism due to sequestration of RNA binding proteins in ALS, but also other neurodegenerative disorders (Belzil et al. 2012).

4.2.5 Therapeutic Potential

Given the long list of neurologic disorders showing decreased glutamate transporter function, it becomes obvious that increasing glutamate transporter activity offers an interesting therapeutic target that could be applicable for multiple neurodegenerative disease. The hypothesis is that restoring functional glutamate uptake will prevent accumulation of toxic concentrations of extracellular glutamate and consequently protect against excitotoxic neuronal cell death. Two early studies support this hypothesis using transgenic mice overexpressing GLT-1 (1.5–5 fold over baseline levels) (Sutherland et al. 2001; Guo et al. 2003). Crossing these mice with mutant superoxide dismutase 1 (SOD1) mice, an animal model for ALS, resulted in delayed onset of motor neuron degeneration and increased survival, suggesting that preventing loss of glutamate uptake protects against neurodegeneration. Similar findings were made recently, when an EAAT2 overexpressing mouse was tested in pilocarpine-induced status epilepticus (SE), resulting in reduced epileptogenic symptoms, including chronic seizure frequency and post-SE mortality rate (Kong et al. 2012). These studies strongly suggest that enhancing EAAT2/GLT-1 protein expression and function is a promising therapeutic approach.

Searching for pharmacological drug-like compounds enhancing EAAT2/GLT-1 function, Rothstein and colleagues identified that a group of US *Food and Drug Administration* approved beta lactam antibiotics increased glutamate transporter

protein levels and function both in vitro and in vivo (Rothstein et al. 2005). Ceftriaxone, the beta lactam with the highest blood brain barrier penetration, was further characterized in the SOD1 mutant mouse model of ALS, resulting in increased protein levels of GLT-1, followed by increased survival, similar to what had been shown in the genetically altered mice (Rothstein et al. 2005). Since the initial discovery of ceftriaxone as a glutamate transporter enhancer and neuroprotectant, numerous animal models of neurodegenerative and psychiatric diseases, including Huntington's Disease, Parkinson's Disease, stroke, depression have been tested for ceftriaxone and reported neuroprotective activity concomitant with increased glutamate transporter expression and function (Lipski et al. 2007; Hota et al. 2008; Miller et al. 2008; Thone-Reineke et al. 2008; Ramos et al. 2010; Lai et al. 2011; Sondheimer and Knackstedt 2011). Furthermore, ceftriaxone has been tested in a National Institutes of Health-sponsored clinical trial resulting in a successful first and second phase of a multi-phase trial, but failing in the final 3rd phase due to lack of significant survival over the placebo group (Berry et al. 2013). In addition to ceftriaxone, we also identified harmine and thiamphenicol as EAAT2/GLT-1 transcriptional activator (Li et al. 2010; Sattler et al. 2011, 2013). From a drug discovery perspective, these compounds offer great opportunities for advancement towards lead compounds for future structure-activity relationship studies to improve potency as well as selectivity and drug delivery, as ceftriaxone e.g. cannot be administered orally.

While ceftriaxone was discovered as a transcriptional activator of EAAT2/GLT-1 (Lee et al. 2008), Lin and colleagues (Colton et al. 2010) were screening small molecule libraries for translational activators of EAAT2/GLT-1. Using a cell based enzyme-linked immunosorbent assay, the authors identified a novel lead series of active compounds, which are currently tested for in vivo efficacy in chronic and acute models of excitotoxicity (Lin et al. 2012).

Not many compounds have been identified that alter glutamate transporter function directly, by binding to the transporter protein. Parawixin 1, a spider toxin, was shown to increase EAAT2/GLT-1-mediated glutamate transport into the cells, not out of the cells, supposedly by speeding up the turnover of the glutamate transport cycle via conformational protein changes (Fontana et al. 2007; Torres-Salazar and Fahlke 2007). Drug discovery efforts on advancing this toxin towards the clinic are slowed down due to the lack of knowledge of the chemical structure of this protein.

4.2.6 Biomarker Potential

Reiterating the importance of a functional glutamate homeostasis it seems obvious, that opportunities to monitor functional glutamate uptake or the presence of glutamate transporters in the brain could present a valuable biomarker for both, disease detection as well as an evaluation of therapeutic efficacy during treatment in many neurological diseases, as suggested above. To our knowledge, our laboratory in collaboration with others has made the first progress on the development of two different biomarkers around the EAAT2/GLT-1 glutamate transporter protein.

The development of CNS biomarkers has been difficult partly due to the lack of availability of relevant tissue samples, i.e. live brain tissue. To overcome this challenge, we employed neural olfactory epithelial tissue to validate drug activity on astroglial proteins, including EAAT2/GLT-1. We validated this approach using the EAAT2/GLT-1 enhancing drug thiamphenicol, which was originally discovered together with ceftriaxone. We validated the effects of thiamphenicol on EAAT2/GLT-1 expression in olfactory epithelial tissue in a per-clinical rodent study as well as a phase 1 human trial, confirming biological activity of thiamphenicol at the drug target EAAT2/GLT-1 (Sattler et al. 2011).

In addition to the nasal biopsy approach, together with our collaborators from the University of Montana, we have been working on the development of an EAAT2/GLT-1 Positron Emission Tomography (PET) ligand biomarker. This PET ligand would allow us to visualize and quantify the levels of glutamate transporter protein in the CNS and spinal cord of patients. Ongoing studies have confirmed the specificity of this tracer in rodents and monkeys, moving this PET ligand towards first a first human safety trial by the beginning of 2015 (Gerdes and Sattler unpublished observation).

4.3 Monocarboxylate Transporters

The *SLC16* gene family of monocarboxylate transporters (MCTs) consists of 14 members implicated in the transmembrane transport of short-chain metabolites. Out of these 14, four of them are known to be involved in the proton-coupled transport of monocarboxylates like pyruvate, L-lactate and ketone bodies across plasma membranes. They are classified according to their functional characterization as MCT1, MCT2, MCT3 and MCT4. Alternatively, they have also been classified as SLCA1, SLCA7, SLCA8 and SLCA3, respecively. We will use the former nomenclature throughout the remainder of the text. Other MCTs like MCT8 are known to be involved in thyroid hormone signaling (Friesema et al. 2003) whereas MCT10 is involved in the transmembrane transport of aromatic amino acids (Halestrap 2013). Unlike the 'true' monocarboxylate transporters, the transport of these substrates is not proton linked. The 8 other members of the *SLC16* family have not been fully characterized so far and their implication under normal physiological situations or in disease still needs to be elucidated. Of all 14 monocarboxylates, only MCT1-MCT4 are known to be expressed in the CNS, with MCT1 and MCT4 mainly expressed in the glial cells, MCT2 in neurons and MCT3 specifically in the basolateral membrane of the retinal pigment epithelial cells (see below) .

4.3.1 Structure

MCTs have 12 transmembrane helices as based on hydropathy blots. Both the N-terminal as well as the C-terminal end is intracellularly located and there is a large

cytoplasmic loop between helices 6 and 7 (Halestrap 2013). It is suggested that MCTs are never glycolysated, unlike their ancillary protein (see below). Most of our knowledge of MCTs comes from studying the MCT1 isoform and it is thought that this structure is commonly shared by all MCTs. Using molecular modeling in combination with site directed mutagenesis, Halestrap and colleagues have suggested that MCT1 changes its conformation from an 'open' state, during which extracellularly one hydrogen ion binds to the transporter followed by the binding of the monocarboxylate L-lactate, to a 'closed' state during which MCT1 undergoes a reorganization of the N- and C-terminal 6-helix halves and lactate acid can diffuse intracellularly (Galic et al. 2003; Manoharan et al. 2006; Wilson et al. 2009). Mutations of specific residues within the MCT1 structure might lead to changes in substrate specificity. To function properly, MCTs need an ancillary protein as a chaperone, which enables MCTs to be expressed at the cell surface. Generally, MCT1, MCT2 and MCT3 use basigin as their ancillary protein, while MCT2 uses embigin (Wilson et al. 2005). Without these chaperones, MCT1 and MCT4 locate to the perinuclear area and are not expressed at the plasma membrane (Kirk et al. 2000; Wilson et al. 2005). Basigin/embigin have a single transmembrane domain and a very short intracellular C-terminal region (Iacono et al. 2007). They are highly glycosylated, unlike MCTs (Iacono et al. 2007). Through the glycosylated domains of their ancillary proteins, MCT expression might become more susceptible to expression regulation as would be predicted solely based on its own structure.

4.3.2 Expression

In mammals MCTs are widely expressed throughout different tissues. MCT1 is ubiquitously expressed with exception of the beta-cells of the endocrine pancreas (Halestrap 2013). MCT2 is widely expressed, highly in testis, and less so in spleen, heart, kidney, skeletal muscle and in the CNS (Garcia et al. 1995; Jackson et al. 1997; Halestrap 2013). MCT3 is exclusively expressed on the basolateral surface of the retinal pigment epithelial cells, regulating retinal osmotic pressure (Philp et al. 1995). MCT4 is mainly expressed in highly glycolytically active tissue, e.g., neonatal heart, chondrocytes and skeletal muscle (Hatta et al. 2001; Halestrap 2013). Other characterized monocarboxylate transporters like the thyroid hormone transporter MCT8 is also expressed ubiquitously (Visser et al. 2011), whereas the aromatic amino acid transporter MCT10 is mainly expressed in kidney, intestine and heart (Halestrap 2013). As mentioned earlier, In the CNS, four MCT isoforms have been identified; MCT1, MCT2, MCT3 and MCT4. MCT1, previously thought to be chiefly expressed in astrocytes and endothelial cells, is mainly expressed in oligodendroglial cells (Rinholm et al. 2011; Lee et al. 2012), the cells that myelinate axons to enable fast conduction of electric pulses along neurons. MCT1 is also expressed in some specific neuronal subpopulations, e.g. Purkinje neurons in the cerebellum (Lee et al. 2012). MCT4 is mainly expressed by astrocytes (Rafiki et al. 2003), the cells that are involved in a wide range of functions ranging from ion and energy homeostasis to glutamate uptake from the synaptic cleft (see below). MCT2

is mainly expressed by neurons (Pierre et al. 2000), especially in the mitochondrial enriched post-synaptic density, where it might be involved in trafficking of AMPA glutamate receptors (Bergersen et al. 2005; Maekawa et al. 2009), more specifically the sorting of their GluA2 subunits which absence determine AMPA channel Ca^{2+} permeability (Hollmann et al. 1991). It has to be mentioned here that for the localization of MCT2 and MCT4, the majority of the findings are based on antibody based immunodetection solely and have to be taken with caution (Pierre et al. 2000; Rafiki et al. 2003).

4.3.3 Physiology

In the CNS MCTs transport monocarboxylates bidirectionally, depending on the relative concentration of lactate and hydrogen ions across plasma membranes. MCTs can function as an exporter or importer. A net cellular flux is determined by the relative contribution of import versus export. Of the different MCTs expressed in the CNS, MCT2 has the highest affinity for its substrates (K_m for L-lactate 0.74 mM), followed by MCT1 (K_m for lactate 4.5 mM) and MCT4 (K_m for lactate 28 mM) (Halestrap and Price 1999; Halestrap 2012). Compared to the other MCTs, MCT4 has an extremely low affinity for substrates, making it more suitable for lactate export rather than import in highly glycolytic cells which produce large amounts of lactate. As this accumulation of lactate leads to a reduction of the intracellular pH, cells need to export lactate with hydrogen ions to protect them from acidosis. The MCT4 physiological characteristics make it ideal for this purpose. With its high K_m for lactate, it is only saturated at higher lactate concentrations. In addition, MCT4 has a high K_m for pyruvate, preventing pyruvate from being transported to the extracellular space. Pyruvate export would lead to a drop in intracellular NAD^+ levels and an impairment in maintaining glycolysis. In addition, intracellular pyruvate to lactate conversion restores the NAD^+ levels and enables glycolysis to continue. The low K_m values for lactate for MCT1 and MCT2 make them more suitable for lactate import rather than export and therefore allow the cells to use lactate for subsequent oxidation, lipogenesis or gluconeogenesis. Consequently, these transporters are found in cells whose metabolism relies on oxidative metabolism rather than glycolysis, e.g., in the adult heart and in neurons. MCT3's affinity for lactate is probably low, but is not well characterized. The specific physiological characteristics of the different MCT isoforms could allow for the discussion of subtype specific inhibitors which in turn could provide a great tool for a wide range of MCT research (see below).

Importantly, to date, most MCT inhibitors still lack specificity. An example is α-cyano-4-hydroxycinnamate (CHC), which blocks MCTs but is twice as potent in blocking the mitochondrial pyruvate transporter (Halestrap 2013). Similarly, the widely used MCT inhibitors 4,4'-di-isothiocyanostilbene-2,2'-disulphonate (DIDS) and 4,4'-dibenzamidostilbene-2,2'-disulphonate (DBDS) inhibit the chloride/bicarbonate transporter far more potently than MCTs (Poole and Halestrap 1991; Halestrap 2013; Kucherenko et al. 2013). Therefore their use has to be interpreted

with care. More recently, MCT inhibitor AR-C155858 was discovered with a very low K_i of around 2 nM, making it a very potent MCT blocker (Ovens et al. 2010). It inhibits both MCT1 and MCT2 but not MCT4 and has shown great therapeutic potential as an immunosuppressant (see below) (Ovens et al. 2010).

As mentioned earlier, different tissues express different MCT subtypes and cells express specific MCTs based on their physiological characteristics, i.e. whether they are more glycolytically active and produce lactate or whether they rather take up lactate from the extracellular space for oxidation, lipogenesis or gluconeogenesis.

In the CNS, some cell types are more glycolytically active while other cells are more oxidative. The most notable example is how glycolytically active, lactate producing astrocytes are metabolically coupled to neurons to sustain the latter's increased metabolic needs during neuronal activation, the so-called neuron-astrocyte lactate shuttle, as proposed by Magistretti and colleagues (Pellerin and Magistretti 1994). In fact not only are neurons unable to increase their glycolytic rate (Herrero-Mendez et al. 2009), they are also unable to store glucose as glycogen as their glycogen synthase enzyme is rendered inactive (Vilchez et al. 2007), and so might need other means to sustain their metabolism, especially under conditions of increased demand. Astrocytes are well known to upregulate glycolysis in situations of increased metabolic needs. As described above, astrocytes take up glutamate via glutamate transporters. This transport is coupled to three sodium ions, which activates the Na^+/K^+ pump in the astrocytic cell membrane in order to maintain its membrane potential. The Na^+/K^+ pump is ATP dependent, leading to increased glucose uptake through glucose transporters or increased glycogenolysis from their intracellular glycogen stores. Glucose is then converted to lactate to produce sufficient ATP levels. As astrocytes pile up lactate rapidly, they use the high lactate capacity MCT4 transporter to export lactate (Rafiki et al. 2003). Lactate could be taken up by neurons through MCT2 and converted back to pyruvate and used to sustain their own metabolism (Pierre et al. 2000). This enhancement of glucose uptake in response to glutamate uptake is now widely recognized (Takahashi et al. 1995; Keller et al. 1996; Bittner et al. 2010). The neuron-astrocyte lactate shuttle hypothesis does not rule out the role of glucose oxidation in maintaining neuronal metabolism (neurons express their own glucose transporter, Glut3 (Maher et al. 1991)), but lactate seems to be preferred over glucose, especially under conditions of increased demand, e.g., during neuronal activation, as has been shown both in vitro (Bouzier-Sore et al. 2003; Itoh et al. 2003; Bouzier-Sore et al. 2006; Ivanov et al. 2011) as well as in vivo (Smith et al. 2003; Serres et al. 2004; Wyss et al. 2011). Even more, lactate seems to have more neurotrophic potential than glucose in models of neuroinjury, as lactate, but not equicaloric glucose, is able to reduce stroke volume in an animal model of hypoxia (Schurr et al. 1997).

Recently Rothstein and colleagues have shaken up the astrocyte neuron lactate shuttle paradigm considerably as they have shown that another cell type in the CNS, the oligodendrocyte, is also involved in the metabolic coupling in the CNS. Unlike previous reports about MCT1 expression in the brain as mainly being detected in astrocytes and endothelial cells, they found using a *bacterial artificial chromosome* (BAC) transgenic mouse that it is the oligodendrocyte rather than any other cell

type who expresses MCT1 in vivo (Lee et al. 2012). In organotypic spinal cord slice cultures, lactate released through oligodendrocyte MCT1 sustains neuronal energy demands. Blocking oligodendrocyte MCT1 transporters leads to motor neuronal cell death, even while MCT4 expression in astrocytes was not affected. This neuronal cell death could be rescued by the addition of lactate to the culture medium. This data suggest that oligodendrocytes export lactate through MCT1 and provide this essential nutrient to neurons (Fig. 4.1b). Similar results were found in vivo (Lee et al. 2012). MCT1 heterozygous knockout mice, as well as mice injected with oligodendrocyte specific lentiviral knockdown of MCT1, showed signs of neuronal degeneration in the absence of overt oligodendrocyte pathology, indicating that a metabolic flux through MCT1 expressed by oligodendrocytes is an essential contributor to neuronal viability (Lee et al. 2012). One cannot rule out however that astrocytes themselves are also able to provide neurons to some extent with lactate directly through MCT4, as suggested by the astrocyte-neuron lactate shuttle hypothesis. On the other hand astrocytes, being able to enhance its own glycolytic rate, could provide oligodendrocytes with lactate through astrocyte-oligodendrocyte gap junctions (Fig. 4.1a). These gap junctions consist of various paired connexin (i.e., connexin hemichannel) isoforms formed by connexin-47 and connexin-32 expressed on oligodendrocytes and connexin-43 and connexin-30 expressed on astrocytes and enable exchange of metabolites between both cell types (Dermietzel et al. 1989; Nagy et al. 1999; Odermatt et al. 2003). After reaching the oligodendrocyte through these coupled hemichannels, lactate can be metabolized by oligodendrocytes to sustain their own metabolic needs or directly provided to neurons after export through MCT1. Another option is that oligodendrocytes produce lactate themselves through glycolysis after taking up glucose through gap junctions or from the extracellular space through their own glucose transporters. It has been shown that oligodendrocytes are able to maintain their myelin sheath and sustain axonal integrity in the absence of functional oxidative phosphorylation, when their function is dependent on glycolysis or connexin mediated import of astrocyte ATP (Funfschilling et al. 2012). It is not known at this point though, whether the lactate provided to neurons is being generated by astrocytes or oligodendrocytes. In fact it is not known whether oligodendrocytes are able to increase their glycolytic rate under conditions of increased energy demand just as astrocytes do. Therefore, it is suggested that astrocytes convert glucose to lactate first, after which lactate enters the oligodendrocytes through gap junctions and is then provided to neurons through MCT1 mediated export. This is further supported by the fact that astrocytes are able to enhance glycolysis and are efficiently coupled to blood vessels from where they take up glucose through glucose transporters (Nakazawa and Ishikawa 1998). Astrocytes also express the high capacity lactate transporter MCT4 to maintain its own viability when lactate levels rise high and express high levels of hexokinase, which converts glucose to glucose-6-phosphate in the first step of glycolysis (Rafiki et al. 2003). On the other hand, specific oligodendrocyte characteristics make these cells more suited to provide neurons with lactate. Unlike astrocytes, oligodendrocytes are very closely interacting with neurons all along their axons and can release metabolites to neurons through Schmidt-Lanterman incisures in their myelin

sheath. Astrocytes, with their end feet touching blood vessels are more ideal for glucose uptake from the blood, transporting glucose along their astrocytic syncytium to regions with increased energy demand, and providing oligodendrocytes with lactate for those regions not covered by astrocyte processes.

Although the validity of the glial-neuron lactate shuttle is accepted by many researchers in the field, it has also been challenged by many other investigators, and is still controversial to some extent. One can argue, e.g., that some of the findings are based on extensive in vitro studies using neuron-glia co-cultures and might not be relevant for in vivo neuronal metabolism. This critique can be challenged by several recent in vivo experiments. For example, in an anesthetized rat model, it has been shown that neurons prefer lactate over glucose as an energetic source, as artificial increased blood lactate levels reduces glucose consumption under both normal conditions as well as during neuronal stimulation (Wyss et al. 2011). Using two-photon in vivo imaging of 6-deoxy-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminoglucose in the somatosensory cortex of the rat, it has been shown that under resting conditions glucose is being taken up by both neurons and astrocytes, but in response to whisker stimulation, astrocytes enhance their glucose uptake while neurons do not, which is in agreement with the glial-neuron lactate shuttle (Chuquet et al. 2010). Also, in humans lactate is important for brain metabolism, as high as 10% under normal physiological conditions and increasingly so (up till 60%) in conditions of increased demand (Boumezbeur et al. 2010).

Secondly, some studies insist that glucose is the major energy source for neurons, rather than lactate, and might be used by neurons directly without astrocyte interplay through neuronal Glut3 transporters (Simpson et al. 2007; Mangia et al. 2009; DiNuzzo et al. 2010). Although it is not disputed that glucose itself is able to sustain neuronal energy demands, especially under normal physiological conditions, the contribution of lactate cannot be disregarded. Firstly, glucose transporter Glut3 knockout mice develop normally and do not show any obvious phenotype, which would be in discrepancy with glucose as a direct energy substrate for neurons (Schmidt et al. 2008; Stuart et al. 2011). On the other hand, knockout of the astrocyte glucose transporter Glut1 in mice, leads to a strong phenotype with motor impairment and seizures, in accordance that glucose has to be converted to lactate first by astrocytes, before lactate can be used as an energy substrate by neurons (Wang et al. 2006). In an elegant contextual learning study in mice, it was found that long-term memory was impaired when astrocytic MCT4 or MCT1 was knocked down in the hippocampus with antisense treatment (Suzuki et al. 2011). Interestingly, this impairment could be rescued by adding lactate but not equicaloric glucose to the mouse hippocampus. Even more, when the authors knocked down neuronal MCT2 expression, long term memory formation was also impaired which could not be rescued by adding lactate, suggesting that (1) astrocyte and their respective MCTs (MCT1 or MCT4) are essential in providing neurons with energy substrates and that (2) neurons use lactate, taken up by MCT2, rather than glucose, to maintain their long term memory formation. One cannot rule out however, that other metabolites like, e.g., ketone bodies might also be involved in maintaining the neuronal energy balance.

4.3.4 Regulation

Although there are some examples of short-term regulation of MCT expression, these are very rare. It has been suggested that MCT1 and MCT4 expression increases through the intracellular interaction with carbonic anhydrase 2 which increases transport activity, while MCT2 expression is enhanced through an extracellular interaction with carbonic anhydrase 4 (Klier et al. 2011). Most of what is known about MCT expression regulation are mechanisms involved in transcriptional and posttranscriptional regulation, which usually take long before exerting its effects. MCT1 expression in muscle is increased in response to enhanced physical activity and lactate transport is decreased in response to denervation (Pilegaard et al. 1998; Pilegaard et al. 1999). In the brain, MCT1 expression is upregulated in response to hyperglycemia and ketosis (Leino et al. 2001; Canis et al. 2009). Two mechanisms have been proposed that stimulate MCT1 expression, both through increased Ca^{2+} levels. One enhances calcineurin activity, which dephosphorylates and activates nuclear factor of activated T-cells (NFAT) which then regulates MCT1 expression levels by binding to NFAT consensus binding sites on the MCT1 promoter (Halestrap 2013). T-lymphocytes for example increase their MCT1 expression levels during proliferation to maintain their pH levels. Both inhibition of calcineurin activity with cyclosporin A as well as a MCT1 inhibitor are efficient in preventing T-lymphocyte proliferation (Murray et al. 2005). MCT1 expression is also regulated through AMP activated protein kinase (AMPK) (Takimoto et al. 2013), which is modulated by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) expression levels (Halestrap 2013). It is not clear whether AICAR increases or decreases MCT1 levels, as opposing results have been reported previously (Galardo et al. 2007; Halestrap 2013). It has further been reported that MCT1 promoter activity is also regulated by protein kinase C (PKC) levels, mediated by transcription factor activator protein 2 (AP2) (Saksena et al. 2009). Interestingly, in the beta cells of the pancreas, MCT1 is not expressed (Otonkoski et al. 2007). One possibility is that tissue specific methylation of CpG islands in the MCT1 promoter prevent MCT1 expression (Mueller and von Deimling 2009). For example, it has recently been reported that the extent of methylation of CpG islands in the MCT4 promoter is correlated with reduced MCT4 expression and prolonged survival in patients with cancer related death (Fisel et al. 2013). A recent study suggested that the MCT1 promoter activity is not regulated by CpG methylation, but rather by miRNA mediated gene suppression. The authors identified miRNA 29 isoforms which were instrumental in suppressing MCT1 expression in the beta cells of the pancreas (Pullen et al. 2011). Individuals with distinct mutations in these regions display exercise induced hyperglycemia due to increased insulin secretion from the beta cells in response to elevated blood lactate levels (Otonkoski et al. 2007) Lastly, intracellular pools of MCT1 might increase MCT1 expression at the plasma membrane irrespective of transcriptional regulation, as occurs in hypertrophied heart tissue (Johannsson et al. 2001).

Another notable example of transcriptional regulation exists for MCT4. The promoter for MCT4 comprises four hypoxia responsive elements which can bind to hypoxia-inducible factor-1α(HIF-1α) (Ullah et al. 2006). Under hypoxic conditions, MCT4 is highly upregulated, e.g. in sparsely vascularized brain tumors which are dependent on anaerobic glycolysis to sustain their metabolism (Ullah et al. 2006; Miranda-Goncalves et al. 2013). As lactate concentrations rise high in these cells, they need MCT4 to export lactate to avoid lactate acidosis, which would impair their cell viability. On the other hand, how MCT4 is regulated under normoxic conditions is unknown. Similarly it is not known why MCT1, which does not contain HRE elements in its promoter, is also upregulated during hypoxia (Cheng et al. 2012). Similar to MCT1 and MCT4, the MCT chaperone basigin is also highly upregulated under hypoxic conditions (Ke et al. 2012). Both HIF-1α binding and specificity protein 1 (SP1) binding to the basigin promotor is important for the upregulation of its activity (Ke et al. 2012). In a nude mice tumor xenograft model, basigin upregulation mediates tumor glycolysis in cooperation with MCT1 and MCT4, and promotes tumor growth, survival as well as tumor invasiveness (Ke et al. 2012).

MCTs might also be under control of post-transcriptional regulation. MCT1 has an unusually long 3′-UTR and several hexanucleotide elements which might interact with several unidentified proteins which regulate MCT1 expression (Pierre and Pellerin 2005). MCT2 expression is regulated by *brain-derived neurotrophic factor, Insulin-like growth factor 1* and insulin levels which involves the mammalian target of rapamycin (mTOR)/phosphoinositide 3-kinase pathway (Chenal et al. 2008; Robinet and Pellerin 2010). The identification of an expanding list of implicated pathways, preferentially the ones specific for a particular MCT might lead to a discovery of compounds with increasing therapeutical potential.

4.3.5 Dysregulation and Therapeutic Potential

To date, no known mutations in MCTs have been associated with disease. It has previously been suggested that a MCT1 variant is associated with a condition known as cryptic exercise intolerance, but the genetic evidence is not very strong (Fishbein 1986). A X-chromosome linked mutation in MCT8 is causative for psychomotor retardation (Friesema et al. 2004). MCT8 is responsible for transporting thyroid hormone across cell membranes and reduced uptake leads to abnormal brain development (Friesema et al. 2003, 2004). In the CNS, it is MCT1, expressed by oligodendroglia, that provides trophic support to neurons (Lee et al. 2012). Therefore, MCT1 is an attractive candidate for regeneration in neurodegenerative diseases like Parkinson's disease, AD and ALS. Both MCT1 and MCT4 levels have recently been evaluated in the CNS of patients with ALS, a motor neuron degenerative disease characterized by progressive muscular weakness, wasting and atrophy (Lee et al. 2012). Patients die between 3 to 5 years after disease onset due to respiratory insufficiency. In both brain and spinal cord of these ALS patients, there was a significant reduction in both MCT1 and MCT4 expression levels (Lee et al. 2012).

Studies from ALS animal models suggested that this reduction in MCT1 might occur early during disease progression (Lee et al. 2012). As oligodendrocytes are the main cell type expressing MCT1, it is hypothesized that lack of lactate mediated trophic support to the most vulnerable motor neurons early in disease might promote degeneration and cell death of these cells. The role of MCT1 and other MCTs in other neurodegenerative diseases has not been studied to date.

Most of what we know about MCT dysregulation in CNS diseases comes from studies in cancer research. Brain tumors range from the most common and most aggressive glioblastomas to gliomas like astrocytomas and oligodendrogliomas. Therapy for these diseases is still in its infancy, consisting of removal of tumor tissue combined with radiotherapy and chemotherapy. These tumors are characterized by excessive growth without a concomitant increase in functional vascularization to provide tumor cells with much essential nutrients. Sparsely perfused tumor cells therefore use anaerobic glycolysis to maintain their metabolism, growth rate and invasiveness. As lactate is piling up during this enhanced glycolysis, tumor cells increase expression of MCT1 and MCT4, to ensure that their pH levels do not rise too high and to ensure maintenance of glycolytic flux (Miranda-Goncalves et al. 2013). This essential upregulation of MCTs might be a direct target for pharmacological agents which are able to block MCT1/MCT4 and impair lactate efflux. This will lead to lowered pH levels in tumor cells and compromise their potential for further growth and metastasis. It is suggested that MCT1 is mainly upregulated in vascularized tumor tissue, at the border of the tumor, while cells deep within the tumor are sparsely vascularized and upregulate MCT4. In many biopsies of glioblastoma and astrocytoma brains, it has been shown that MCT1, basigin and MCT4 are upregulated, whereas MCT2 is downregulated (Cheng et al. 2012; Miranda-Goncalves et al. 2013). The reason for this MCT2 downregulation is still unsure, but the increase in MCT1 is essential for maintaining tumor cell metabolism, as in vitro treatment with a non-specific MCT1 inhibitor CHC reduces glycolytic metabolism, cell migration and invasion and induced cell death (Miranda-Goncalves et al. 2013). CHC treatment also reduced tumor size in vivo (Miranda-Goncalves et al. 2013). The effect of CHC was similar to that of MCT1 antisense treatment, suggesting that CHC, which is a non-specific MCT1 inhibitor, targets MCT1 (Miranda-Goncalves et al. 2013). It appears that MCT4 is the main driver for tumor growth, as tumor cells sensitive to MCT1/2 knockdown lose this sensitivity when MCT4 is overexpressed (Le Floch et al. 2011). Another study has recently shown that mild hypoxia induction in tumor cells protects them from acidosis by inducing pH regulatory mechanisms and enhancement of glycolysis, maintaining ATP levels (Parks et al. 2013). Therapeutics directed at increasing intracellular acidosis and metabolic disruption are therefore promising candidates to affect tumor cell survival. Another potential therapy might benefit from the MCT1 upregulation found in tumor cells. A toxic glycolysis inhibitor, 3-bromo-pyruvate has recently been shown to be internalized through MCT1 and exert its toxicity on tumor tissue, while cells that did not express MCT1 were insensitive to 3-bromo-pyruvate treatment (Nelson 2002; Queiros et al. 2012; Birsoy et al. 2013). The addition of butyrate, which activates MCT4, enhances the anti-tumor effects of 3-bromo-pyruvate (Queiros et al. 2012).

This indicates that selective MCT1 expression in cancer tissue can be exploited as a method to deliver toxic molecules. One should be careful though, as MCT1 expression is widespread in the brain, especially in oligodendrocytes and some neuronal subtypes, and therefore treatment with 3-bromo-pyruvate in vivo would probably cause widespread oligodendrocyte related side effects.

4.3.6 Potential as Biomarker

One of the metabolic enzymes classically used as a readout of cellular death is lactate dehydrogenase (LDH), which is involved in the reversible conversion of pyruvate into lactate. In several subtypes of cancer, increased LDH levels in patients serum has been shown to be indicative of poor prognosis, e.g. in renal cell carcinoma (Armstrong et al. 2012). Similarly, in patients with glioblastoma, increased LDH levels were found in 80 out of 234 patients analyzed, again showing a correlation between LDH levels and poor prognosis. Whether there is a similar correlation between LDH levels and prognosis in neurodegenerative diseases still needs to be elucidated.

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Chapter 5
Alexander Disease and Astrotherapeutics

Michael Brenner and Albee Messing

Abstract Alexander disease is a protein aggregation disorder resulting from mutations in the intermediate filament protein, GFAP. Progress in the past 15 years has defined numerous aspects of astrocyte function that are impacted by these mutations, and that might be amenable to correction. Since reversal of astrocyte dysfunction is likely to be valuable in a wide variety of conditions, Alexander disease offers unique opportunities for exploring the newly emerging field of astrotherapeutics.

Keywords Alexander disease · Neurodegenerative disorder · GFAP · GFAP mutations · Astrocytes · Astrotherapeutics · Protein aggregates

5.1 Alexander Disease: Clinical Manifestations and Etiology

Alexander disease (AxD) is a usually fatal neurodegenerative disorder resulting from astrocyte dysfunction (for reviews, see Brenner et al. 2009; Flint and Brenner 2011; Messing et al. 2012a). Its defining feature is the abundant presence in astrocytes of protein aggregates, called Rosenthal fibers, which are especially prevalent in subpial, periventricular and perivascular locations. Historically, AxD has been classified as a pediatric leukodystrophy because it was first and then most often diagnosed in young children, and because in this age group it is accompanied by massive myelination defects. Common clinical manifestations of childhood AxD include failure to meet mental and physical developmental milestones, megalencephaly, seizures, hyperreflexia and pseudobulbar signs, such as difficulty speaking

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and swallowing. Magnetic resonance imaging (MRI) criteria are highly diagnostic for childhood AxD, with a major feature being striking myelination defects bilaterally in the frontal lobes (van der Knaap et al. 2001). The classification of AxD was extended to adult cases based on similar findings of profuse astrocytic protein aggregates, despite these patients often having symptoms quite different from the childhood disorder. Adult AxD patients typically display normal development, normal head size, and rarely have seizures. They do share hyperreflexia and pseudobulbar signs with the infantile cases, but more uniquely often have ataxia, visual and autonomic abnormalities, sleep disturbances and palatal myoclonus. For both the childhood and adult forms, no single symptom is present in all cases, and both may also display multiple other symptoms with lesser frequencies (Prust et al. 2011; Yoshida et al. 2011). MRI diagnosis of adult cases is usually much more difficult than for children, since many of the criteria that apply for children do not hold for adults (van der Knaap et al. 2006). In particular, instead of the frontal predominance of pathology seen in the childhood cases, the pathology in adult AxD is typically more caudal, involving the brain stem, cerebellum and cervical spinal cord. Importantly, white matter lesions are not found in about half of the adult onset cases (Yoshida et al. 2011). Accordingly, AxD is now better considered as an astrogliopathy rather than a leukodystrophy.

Until recently, AxD was divided into three categories: infantile, with onset before 2 years of age; juvenile, with onset between 2 and 12 years; and adult, with onset at 13 years of age or later (Russo et al. 1976). The infantile and adult onset cases usually differed from each other as noted above, whereas the juvenile cases could be like either of these, or display selected symptoms of each. Statistical analysis of 215 cases by Prust et al. (2011) found the data are better fit by a two class model rather than by three classes, Type I having an early onset and largely mirroring the infantile form, and Type 2 usually having a later onset and largely mirroring the adult form. Average age at onset for Type I was 1.7 years, and average lifespan following onset was 14 years; for Type 2 average onset was at 22 years, and average lifespan thereafter was 25 years. Here we adopt this Type I and Type 2 classification, which are also referred to as early onset and late onset.

Due to the lack of specificity of its clinical presentations, the late onset form of AxD was diagnosed much less frequently than the early onset form until the discovery that approximately 95 % of both are due to heterozygous missense mutations in the gene encoding glial fibrillary acidic protein (GFAP), the primary and largely cell-specific intermediate filament protein in astrocytes (Brenner et al. 2001; Rodriguez et al. 2001; Gorospe et al. 2002; Li et al. 2005). With the advent of this simple genetic diagnostic, adult cases are now discovered at a frequency similar to or even greater than early onset cases (Pareyson et al. 2008; Yoshida et al. 2011), and include both de novo and familial occurrences. The cause of the remaining 5 % of cases for which a GFAP mutation has not been discovered is unknown. Since overexpression of wild type GFAP can produce AxD-like pathology in a mouse model (Messing et al. 1998), the possibility of GFAP gene duplication has been examined for several patients without a detectable mutation, but with negative results (Yoshida and Nakagawa 2012). The overall incidence of AxD has been estimated to be on the order of one in a million (Yoshida et al. 2011).

The disease-causing mutations have been found throughout the length of the protein, with the exception of the non-helical N-terminal domain, and nearly all are 100% penetrant. Genotype/phenotype correlations have been established for a few of the mutations and the clear absence of a correlation for several others, but the majority have been too rare for statistical analysis. As examples, mutations at R79 and R239 are associated with early onset and poor prognosis, whereas those at R88 and R416 have no correlation with age of onset or severity, suggesting other contributing factors to disease severity (Prust et al. 2011). The GFAP mutations appear to act by a dominant, gain of function mechanism, since mice lacking GFAP have only mild deficits which do not resemble AxD (Gomi et al. 1995; Pekny et al. 1995; McCall et al. 1996). This has implications for gene therapy, since provision of wild type GFAP to AxD patients is unlikely to be beneficial, and might instead exacerbate disease by increasing the GFAP load.

5.2 Disrupted Functions of Alexander Disease Astrocytes and Their Links to GFAP Mutations

What critical function (or functions) of astrocytes is disrupted in AxD, and how GFAP mutations cause that disruption, are active areas of research, which is being pursued by analysis of human tissues, by cell free assays, study of primary and stable cell lines, and use of *Drosophila* and mouse models. All these studies involve comparing the effects of expressing wild type and mutant GFAP. Functions found altered include decreased proteasomal activity, decreased plasma membrane glutamate transport [through GLT-1 (rodents)/EAAT2(human), commonly named (as per HUGO gene nomenclature committee) solute carrier family 1 (glial high affinity glutamate transporter), member 2 (SLC1A2)], increased autophagy, activation of the stress-activated protein kinase/c-Jun N-terminal kinase (JNK) pathway; presence of advanced glycation and lipid peroxidation end products, and activation of an oxidative stress response and iron accumulation (Hagemann et al. 2006; Tang et al. 2006; Castellani et al. 1997, 1998; Tang et al. 2008; Tian et al. 2010; Wang et al. 2011; Sosunov et al. 2013). In addition, AxD astrocytes are chronically reactive (Sosunov et al. 2013), and thus likely to be secreting cytokines such as tumor necrosis factor-α and interleukin-1 β, which can be toxic to other cells. Addressing any of these altered functions could potentially be therapeutic.

A mechanistic linking of GFAP mutations to proteasomal inhibition and stress pathway activation has been proposed by Tang et al. (2006) on the basis of cell culture studies. They found that expression of mutant GFAP inhibits proteasome function, that direct inhibition of proteasome function activates JNK, and that direct activation of JNK inhibits proteasome activity. This prompted the proposal of a positive feedback cycle in which mutant and/or increased GFAP inhibits proteasome activity, which in turn activates JNK, which further inhibits proteasome activity, resulting in yet higher GFAP levels. This is an attractive model because it links the primary defect in AxD, mutant GFAP, to physiological changes. It also could

explain why mutations present from conception might not have consequence until later in life—effects might remain subthreshold until some central nervous system (CNS) perturbation increases GFAP above a critical level that sets the positive feedback loop in motion. However, what players in the loop contribute to the clinical manifestations of the disease remains unclear.

Another proposed mechanism linking GFAP mutations to the disease process is inactivation of one or more critical proteins through sequestration in the Rosenthal fibers, a mechanism that has been proposed for other neurodegenerative disorders (Ali et al. 2010). Although GFAP is a major constituent of Rosenthal fibers, also associated with these aggregates are the small stress proteins HSP27 and αB-crystallin, the 20S proteasome subunit, plectin, p-JNK and p62 (Iwaki et al. 1993; Head et al. 2000; Zatloukal et al. 2002; Tang et al. 2006; Tian et al. 2006). Proteomic studies of Rosenthal fibers are currently underway to identify other candidate proteins (Heaven and Brenner, manuscript in preparation). Sequestration of the 20S proteasome has been suggested as a contributor to impaired proteasome activity in AxD, although as discussed below, it is also inhibited by a soluble GFAP oligomer (Tang et al. 2010).

Both sequestration of αB-crystallin in the Rosenthal fibers and its ability to debundle GFAP filaments (Koyama and Goldman 1999; Perng et al. 1999; Nicholl and Quinlan 1994) led to it being tested therapeutically in a mouse model of AxD. This model consists of mice that both overexpress human GFAP from a transgene and have a knocked-in GFAP R236H mutation, which is the mouse homologue of the particularly pernicious human R239H mutation. Separately, the overexpression and the knock-in each display some characteristics of AxD, including formation of Rosenthal fibers, activation of stress responses and seizure susceptibility, but have normal lifespans. When combined together, however, they produce lethality between about 25 and 35 days of age, presumably from seizures (Hagemann et al. 2006). In this lethal AxD mouse model, expression of αB-crystallin in astrocytes from a GFAP promoter-driven transgene to further increase the already elevated level present in the disease models resulted in complete rescue of viability, reduction of the stress response and partial restoration of GLT-1 levels (Hagemann et al. 2009). These marked and general therapeutic effects thus recommend elevation of αB-crystallin for further investigation as an AxD treatment. Indeed, systemic delivery or increased expression of αB-crystallin may have general applicability beyond AxD, as it appears protective in models of neuroinflammatory disease and stroke (Ousman et al. 2007; Arac et al. 2011; Shao et al. 2013). However, there are concerns about αB-crystallin as a therapy, primarily due to its identification as a negative risk factor in glioblastomas and several other types of cancer (Goplen et al. 2010). Pharmacological strategies for enhancing αB-crystallin expression would not be tissue specific, and therefore enhancing αB-crystallin expression may be challenging to achieve without unacceptable side effects unless an astrocyte-specific delivery method could be developed, such as with viral vectors (see below).

Presumably the causes of the pathological changes in AxD astrocytes have as their origin a misfolded state of GFAP, but the identity of the toxic species, and whether it is the same immediate cause for each of the pathological changes, is

not known. Correlations between the presence of Rosenthal fibers and disturbance of astrocyte functions have led to the tacit assumption amongst most investigators that Rosenthal fibers are the toxic species. However, by analogy with other protein aggregate neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease, the aggregate may be benign, and a smaller oligomer (which may be on the pathway to aggregate formation) may cause toxicity (Caughey and Lansbury 2003). Support for this has been obtained by Tang et al. (2010) with regard to proteasome inhibition. They found that 20S proteasome activity was inhibited more severely by in vitro polymerized R239C mutant GFAP than by wild type GFAP, and that an oligomeric fraction was much more potent than the filament fraction. Native gel separation of the wild type oligomer fraction indicated that it contained primarily monomers and tetramers, whereas the R239C oligomers were primarily tetramers and a more slowly migrating species that could be an octamer. Exposure to αB-crystallin converted the R239C size distribution to that of the wild type, and also reduced proteasome inhibition, suggesting that the larger oligomer is a primary inhibitory species, and that a therapeutic activity of αB-crystallin is to prevent its formation. If the toxic species is indeed an oligomer rather than the Rosenthal fiber aggregate, the possibility exists that treatments targeting dissolution of the Rosenthal fibers could contrarily exacerbate disease by increasing oligomer levels.

Studies in *Drosophila* have produced indications of what may be primary and what may be secondary pathological effects in AxD. Although *Drosophila* have neither astrocytes nor GFAP, targeting mutant GFAP expression to *Drosophila* glia produced many of the phenotypes of AxD, including formation of Rosenthal fiber-like protein aggregates, loss of glia and neurons, susceptibility to seizures, and activation of stress pathways (Wang et al. 2011). As in the mouse studies, over-expression of αB-crystallin decreased cell death, seizure susceptibility and aggregate formation, indicating that it acts at a fundamental level to reverse the AxD phenotype. On the other hand, although toxicity was decreased by elevating levels of catalase or superoxide dismutase to reduce oxidative stress, or by increasing plasma membrane glutamate transport, there was no effect on the burden of GFAP aggregates, suggesting that these treatments address downstream effects of the primary lesion. Also of therapeutic interest, the *Drosophila* studies found benefit from two compounds available for human use, vitamin E and 17-(allylamino)-17-demethoxygeldanamycin (17-AAG, an Hsp90 inhibitor that stimulates degradation of select proteins, and is undergoing clinical trials for cancer treatment).

5.3 Treatment Strategies

Thus for treatment strategy, reducing GFAP levels by inhibition of synthesis or increased degradation is likely to be most efficacious, followed by prevention of misfolding by agents such as αB-crystallin, followed by treatment of downstream consequences such as activation of stress pathways and reduced plasma membrane

glutamate transport. Both mouse and cell culture studies (Koyama and Goldman 1999; Mignot et al. 2007) (Messing unpublished observations) have found that Rosenthal fibers can disappear with time; thus to the extent that Rosenthal fibers serve as an indicator for the toxic GFAP species, limiting GFAP levels may not only prevent disease progression, but also reverse some symptoms. There is little concern about possible deleterious effects from extreme reduction in GFAP levels, since GFAP knockout mice have minimal neurological deficits. Tolerance of reduced GFAP expression in humans is suggested by the finding that 4 of 2026 healthy subjects were found heterozygous for deletion of the GFAP gene (Shaikh et al. 2009).

5.3.1 Reduction of GFAP Levels

In theory, GFAP synthesis could be curtailed by inhibiting participating transcriptional activators, but the transcriptional regulation of the gene is poorly understood. Many studies have been performed using cell culture and in the context of developmental activation of the gene. However, Yeo et al. (2013) have shown that cell culture studies of GFAP transcription are poor prognosticators of in vivo regulation; and factors necessary for activating GFAP transcription during development may act indirectly, and have no role in transcription in the adult (Herrera et al. 2012). Studies examining GFAP transcription in mice have identified AP-1, GATA, NF-1, NF-κB, SP-1 and STAT-3 as contributing to its activity, with others yet to be discovered (Steele-Perkins et al. 2005; Herrmann et al. 2008; Yeo et al. 2013). None of these is specific to GFAP transcription, making it likely that their inhibition would have unintended consequences. Indeed, general knockout of any of these six transcription factors is embryonic lethal or severely debilitating in mice (Johnson et al. 1993; Steele-Perkins et al. 2005; Fujiwara et al. 1996; Marin et al. 1997; Takeda et al. 1997; Weih et al. 1997). Perhaps partial inhibition of a combination of these transcription factors would be safe and efficacious, but we are unaware of such an approach having been used for reducing the activity of any gene.

An alternative, gene-specific method for reducing transcription is through use of inhibitory RNA (RNAi) or oligonucleotides. While this approach has been routinely used in cell culture, recently there has been exciting progress in the development of in vivo applications of this technique, and clinical trials for several diseases are now underway (Southwell et al. 2012). For CNS disorders, systemic delivery faces the usual hurdle of the blood-brain barrier, but recent work on models of Huntington's disease using intrathecal administration show remarkably long-lasting suppression by relatively short term treatments with antisense oligonucleotides (Kordasiewicz et al. 2012). In Huntington's disease there is concern that global suppression would itself cause problems, based on the embryo lethality associated with the null state of huntingtin in the mouse (Duyao et al. 1995; Nasir et al. 1995; Zeitlin et al. 1995). In the case of AxD, it may be possible to specifically reduce expression of the mutant allele while preserving expression from the wild type allele, although this would be technically challenging since it would require the development of appropriate

reagents for each of the more than 80 mutations now associated with disease. However, as noted above, general knock-down of GFAP expression is likely to be safe, recommending AxD as an excellent setting in which to explore the therapeutic value of antisense oligonucleotides.

Another approach already taken to reduce GFAP levels was a screen of small libraries of FDA-approved drugs. This was done using primary cultures of astrocytes prepared from a novel dual-luciferase reporter strain of mice (Cho et al. 2010). Several compounds were identified that met the criteria designated as hits for this study, but none of them has yet proved useful for reducing GFAP expression in the AxD mouse models (Messing unpublished observations). It should be noted that the astrocytes used in this screen expressed only wild type GFAP, and not the point mutants that are associated with disease, which will induce a reactive response. Although cultured neonatal astrocytes exhibit some properties of the reactive state (Nakagawa and Schwartz 2004; Cahoy et al. 2008; Zamanian et al. 2012), they do so only partially, and the transcriptional elements responsible for reactive upregulation of GFAP likely differ from those that control its basal level of expression (Yeo et al. 2013). In addition, subsequent comparisons of the dual-luciferase reporter with a single luciferase reporter demonstrate that expression from the latter correlates much more closely with GFAP mRNA (Jany et al. 2013). These two reporters differ in that a 2.2 kb human GFAP promoter fragment was used for the dual luciferase reporter, whereas a 12 kb mouse GFAP promoter was used for the single GFAP reporter. This finding and studies in progress (Brenner unpublished observations) indicate that important transcriptional elements are present in the 2.2–12 kb upstream region. Newer drug screens are now underway that utilize astrocytes derived from mice expressing the R236H point mutant of GFAP along with the more representative single luciferase reporter.

An alternative to inhibiting GFAP synthesis is to increase its degradation. Since inhibition of proteasome activity by mutant GFAP may be the initiating event in astrocyte dysfunction through activation of stress responses (Tang et al. 2010), stimulating proteasomal activity might not only reduce GFAP levels, but also attenuate the pathological response. On the other hand, the one mutant GFAP examined, R239C, was a poor substrate for proteasomal degradation (Tang et al. 2010), suggesting that increasing proteasomal activity may have little benefit. Also of concern is the finding that proteasomal inhibition unexpectedly decreases GFAP levels in cultured astrocytoma cells and rat brain, presumably due to preservation of a short-lived transcriptional repressor (Middeldorp et al. 2009). This raises the possibility that proteasome activation could actually increase GFAP levels and exacerbate AxD. Therapies that seek to inhibit proteasome activity are receiving major attention for other disorders, but those that seek to increase this activity are only in the early stages of development (Huang and Chen 2009).

GFAP degradation could also be augmented by stimulating autophagy. Increased numbers of autophagic vacuoles are already present in AxD patients, in the R236H mouse model and in transfected cells expressing R239C GFAP (Tang et al. 2008). However, no increase in protein degradation was observed in the cell culture systems, raising the possibility that the increase may be compensation for a partial

inhibition of autophagic flux or proteasomal activity, with the net result of reestablishing the normal steady state rate of degradation. Nevertheless, inhibiting autophagy pharmacologically with 3-methyadenosine or genetically with knockout of the *Atg5* gene led to accumulation of GFAP expressed from a CMV promoter, whereas activating autophagy by starvation, rapamycin, or decreasing mammalian target of rapamycin (mTOR) levels reduced GFAP levels, indicating that GFAP is an autophagy substrate and that increasing utilization of this pathway could be beneficial. Another player affecting autophagy is p38, whose activation was found to increase autophagy markers and reduce GFAP levels. Activation of p38 could occur through stimulation of the mitogen-activated protein kinase (MAPK) stress pathway already mentioned. As with proteasomal activation, a caveat for therapeutic efficacy of increased autophagy is that it might also increase GFAP levels through removal of a transcriptional repressor (Middeldorp et al. 2009). No attempt has been made to treat AxD by stimulating autophagy, although this is being investigated for other neurodegenerative disorders (Hochfeld et al. 2013).

Another possible route to reducing GFAP levels was discovered while pursuing the role in AxD of Nrf2, a transcriptional activator that regulates a large repertoire of genes associated with the response to toxic injury and oxidative stress. Activation of this pathway was first suggested by microarray analysis of olfactory bulb from the GFAP over-expressing mouse model of AxD, and subsequently confirmed by studies on tissues from human patients (Hagemann et al. 2005). Enhancing expression of Nrf2 showed clear evidence for protection in mouse models of amyotrophic lateral sclerosis (ALS) and α-synucleinopathy (Vargas et al. 2008; Gan et al. 2012). Most recently, beneficial effects of forcing over-expression of Nrf2 for the mouse models of AxD have been observed, with striking reductions in the expression of GFAP itself and other indicators of pathology in several areas of the CNS (LaPash Daniels et al. 2012). Surprisingly, however, placing the AxD models in the Nrf2-null background did not make the phenotype worse, and even reduced evidence of gliosis in the hippocampus (Hagemann et al. 2012). A similar pattern of protection from over-expression, but minimal effect from the null state, has also been observed with the ALS models (Guo et al. 2013; Vargas et al. 2013). The beneficial effects of increasing Nrf2 are of particular interest, since a drug that activates this pathway, BG-12, has recently been approved for use in multiple sclerosis (Scannevin et al. 2012; Linker et al. 2011; Stangel and Linker 2013). A clinical trial of BG-12 in AxD patients is therefore worth consideration.

5.3.2 Other Treatment Targets and Patient Testing

A decrease in GLT-1/EAA-2 as found in AxD also occurs in other neurodegenerative disorders, and may be associated with the astrocytic reactive response (Tian et al. 2010). GLT-1/EAAT-2 is the major glutamate transporter in astrocytes, and deficiency in glutamate transport causes seizures and excitotoxic death of both neurons and oligodendrocytes (Tanaka et al. 1997). Presumably with the rationale of restoring EAAT-2 levels, Sechi and colleagues treated a 39 year old late onset

AxD patient with ceftriaxone, an antibiotic identified as a potential therapeutic for ALS from a drug screen seeking enhancers of GLT-1 expression (Rothstein et al. 2005). The patient showed apparent improvement in some of her symptoms over a 20-month period (Sechi et al. 2010). More recently, this same group provided 4-year follow up data (~2 additional years of treatment) on the same patient, and claim continued improvement, particularly with respect to gait ataxia, dysarthria, and palatal myoclonus (for which blinded assessments were performed) (Sechi et al. 2013). However, these studies do not take into account the spontaneous remissions that have occasionally been reported in AxD patients, particularly those with bulbar signs (Messing et al. 2012b; Namekawa et al. 2012). Whether any other patients have successfully been treated with this drug is not known.

Despite this apparent success, there are several uncertainties concerning these findings with ceftriaxone. Although selected for its ability to elevate GLT-1 in test systems, the AxD patient treated had had no history of seizures, and in some models ceftriaxone has neuroprotective effects without any change in GLT-1 expression (Melzer et al. 2008). One hypothesis is that ceftriaxone might activate the Nrf2 pathway (Lewerenz et al. 2009; Nizzardo et al. 2011), one of the stress pathways shown to have beneficial effects in AxD as discussed above (LaPash Daniels et al. 2012). However, in our own studies with ceftriaxone administration to mouse models of AxD we have been unable to find effects on GLT-1 expression, protection from lethality, or activation of protective stress pathways (Messing unpublished observations). Seizures are a known side effect from ceftriaxone, raising the questions of whether it is suitable for use in patients who already have seizures as one of their symptoms.

Ishigaki and colleagues treated a 9 year old type II AxD patient with thyrotropin releasing hormone (TRH) based on its efficacy in treating cerebellar defects in mice (Ishigaki et al. 2006). Intravenous administration followed by oral dosing mitigated some of the symptoms in this child, although the effectiveness diminished over the 4 month period in which she was observed. More recently, in the context of a broad review on AxD, Yoshida and colleagues briefly mention that two other patients have been treated with TRH, without benefit (Yoshida et al. 2011). No other details were provided about these additional patients.

The spice curcumin has been tested in a cell culture system for its effects on aggregate formation. The rationale was that curcumin had been shown in other systems to increase levels of αB-crystallin and HSP27 in cells, to inhibit or activate proteasome activity depending on its concentration, to activate autophagy, and to reduce protein aggregate formation. In the AxD study, the effect of curcumin was tested in cells transfected with an R236C expression vector. Promising data were obtained indicating that curcumin could indeed increase both αB-crystallin and HSP27 levels in a glioma cell line and decrease GFAP expression. On the other hand, the absence of clear dose-response effects, a very narrow effective dose window, and variability of outcomes with time after treatment provide notes of caution. Additional mechanistic studies in cell culture and testing in animal models will be of interest. The safety of using curcumin has recently been questioned by Foxley et al. (2013), who found that CNS lupus erythematosus was aggravated by curcumin in a mouse model.

5.3.3 *Treatment Delivery Methods*

Whether treatment is by drugs or gene therapy, the therapeutic agent would either need to be delivered intracerebrally, or be able to cross the blood-barrier if given systemically. Systemic administration is certainly preferred for ease of delivery and safety concerns, especially since treatments may need to be chronic. Recent reports for recombinant adeno-associated virus (rAAV) suggest this may be feasible not only for drug treatments, but also for gene therapy. Foust et al. (2009) report that a single intravenous injection via the tail vein of an adult mouse resulted in transfection of about 64 % of astrocytes in the lumbar region of the spinal cord, while producing minimal neuronal labeling. They also observed, but did not quantify, extensive transgene activity in astrocytes throughout the brain. In contrast to the vascular route of delivery, this group found that direct injection of the rAAV9 into the striatum and dentate gyrus resulted primarily in neuronal labeling, suggesting that when delivered intravenously, astrocytes are labeled via their endfeet association with endothelial cells. This infection pathway might limit the percentage of astrocytes that can be genetically engineered as not all astrocytes have contact with blood vessels. On the other hand, those astrocytes which do associate with blood vessels (perivascular) are a prime site for Rosenthal fibers, and there is also a suggestion that reactive astrocytes are more prone to AAV transfection than resting astrocytes (Klein et al. 2008). Thus rAAV infection could have some specificity for those astrocytes affected by AxD. In contrast to the results of Foust et al. (2009), von Jonquieres et al. (2013) observed extensive astrocytic expression of rAAV1/2 in astrocytes delivered to P0 mice by striatal injection, with cell specificity being conferred by use of a GFAP promoter. In the immediate region of the injection site approximately half the astrocytes expressed the GFP transgene, and this number increased to approximately 65 % when P90 mice were injected. It is unclear whether this different result for intracerebral delivery is attributable to the different AAV serotype or some other variable.

These results are promising for the use of rAAV for AxD gene therapy; for example to deliver a mutant GFAP antisense RNA, a GFAP transcriptional inhibitor, or a constitutively active αB-crystallin gene. A critical unknown is the fraction of astrocytes that would need to be transfected to produce a clinical effect. In disorders due to loss of an enzymatic function, such as lysosomal storage diseases, restitution of activity in only a minority of cells may be sufficient to correct the metabolic defect. However, since AxD involves the gain of a toxic species, a high percentage of astrocytes may need to be successfully genetically engineered.

5.4 Evaluating Therapies

Whatever potential treatment strategy emerges as the best candidate for testing in human patients, a major question remains about how to determine efficacy. Indeed, simply measuring levels of GFAP may serve as a biomarker not only for treatments

directly targeting GFAP reduction, but also for other AxD therapies, since all should reduce the reactive state of astrocytes. A substantial literature exists on measurements of GFAP in both blood and cerebrospinal fluid (CSF) in the context of numerous disorders, including traumatic brain injury, stroke, and infectious/inflammatory diseases of the CNS (Liem and Messing 2009). One study of AxD patients demonstrated elevated levels of GFAP in the CSF of all three examined (Kyllerman et al. 2005). Other astrocytic biomarkers, such as S100β or aldehyde dehydrogenase 1, have not yet been reported, nor have any markers relevant to myelin or oligodendrocytes or neurons been studied in AxD. Biomarkers to monitor other targets such as the Nrf2 pathway do not yet exist, but would be very useful for that category of drugs.

A different problem exists with respect to the marked clinical heterogeneity in the patient population, reflecting diversity in age of onset and anatomic distribution of lesions. Indeed, only a few studies have reported on the natural progression of disease, either focusing on MRI findings in one (van der Voorn et al. 2009) or clinical symptoms in another (Zang et al. 2013). In addition, despite having been described from its inception as a "mental retardation" syndrome, only one study reports a full neuropsychological assessment, and this was for a young adult with a private mutation (Restrepo et al. 2011). Although it is widely assumed and often stated that the cognitive phenotype is more severe in young patients, and progressively milder and even absent in the older patients, the precise nature of the cognitive deficits in all groups deserves closer attention and better definition. One approach that may prove useful is to utilize more "patient-centered" measures to define severity and progression of disease, utilizing one or more well-validated self-report questionnaires that have been widely used for the study of other conditions, including intellectual disability syndromes such as autism, Fragile X and Down syndromes (Esbensen et al. 2013; Smith et al. 2012a, b). Such measures, although broadly worded, may actually serve to capture many of the neurological and other deficits that comprise the core phenotype in AxD patients of all ages and types, and could ultimately provide an important foundation for future natural history studies and therapeutic research.

Concluding Remarks

In just a little over a decade AxD has matured from a disorder without a cause to the poster child illustrating the critical role of astrocyte function. Accumulating knowledge of the mechanisms linking GFAP mutations to clinical presentations is providing multiple targets for intervention in this disease, offering hope that an effective treatment will soon be forthcoming. Although AxD is quite rare, the observation that many of its manifestations may be due to the reactive state of astrocytes, and thus have commonality with many neurodegenerative disorders (Sosunov et al. 2013), makes treatment of this disorder of paramount interest.

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Chapter 6
Role of Astrocytes in Central Nervous System Trauma

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Abstract In the blink of an eye, the life of a person who sustains a traumatic brain injury (TBI) or traumatic spinal cord injury (SCI) often changes forever. This chapter discusses the epidemiology of SCI and TBI followed by an overview of the key elements known to contribute to the pathophysiology. Next, astrocytic physiology thought to be involved in the acute post-injury phase is discussed, with particular focus on the mechanisms of protection.

Keywords Central · Nervous system · Astrocytes · TBI · Traumatic brain injury · SCI · Traumatic spinal cord injury · Glutamate · Kir4.1 · EAAT · Excitotoxicity · Inflammation · Plasma

6.1 Epidemiology of Traumatic Central Nervous System Injury

Traumatic spinal cord injury (SCI) is a major health concern in the United States (U.S.) and world not only due to the number of persons with SCI, but also due to the cost of long-term care of the SCI patient (Jackson et al. 2004). Estimates from 2013 of the incidence of SCI in the U.S. report that there were approximately 40 cases per million population, not including persons who die at the scene (National Spinal cord Injury Statistical Center 2013). This constitutes nearly 12,000 new SCIs occurring each year in the U.S. with most newly injured persons being in the 16 to 30 year old age group. Also, most persons (80.7 %) who sustain an SCI are males (National Spinal cord Injury Statistical Center 2013). The main etiology for SCI is motor vehicle crashes (36.5 %), followed by falls (28.5 %), and acts of violence (14.3 %), primarily gunshot wounds. Although often high-profile, SCIs caused by sports represent only 9.2 % of the total SCI annual incidence. A person who sustains an injury at one of the eight cervical segments of the spinal cord suffers from tetraplegia. An injury

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in the thoracic, lumbar, or sacral regions of the spinal cord results in paraplegia. The majority of persons with an SCI sustained tetraplegia (52.2%) (National Spinal cord Injury Statistical Center 2013; Jackson et al. 2004).

The cost of SCI is staggering on an individual, community, and national level. It is important to note that the annual average health care, living expenses, and estimated lifetime costs that are directly attributable to the injury vary greatly by severity of injury and do not include indirect costs such as lost wages and productivity, which average US$ 70,575 per year in 2013 (National Spinal Cord Injury Statistical Center 2013). The average yearly expenses for the first year post-SCI in the USA is US$ 1,044,197 for high tetraplegia, US$ 754,524 for low tetraplegia, and US$ 508,904 for paraplegia. The estimated life time cost (not including indirect costs) for a patient injured at age 25 is between US$ 3.3–4.6 million for tetraplegia and US$ 1.5–2.2 million for paraplegia (National Spinal cord Injury Statistical Center 2013). SCI significantly lowers the average life expectancy as well. For example, a person who sustains paraplegia at 20 years of age who survives at least 1 year post-SCI has an average reduction in life expectancy of up to 13.9 years as compared to a person without SCI. For a person with tetraplegia, the average reduction in life expectancy is 18.6 (National Spinal cord Injury Statistical Center 2013).

Traumatic brain injury (TBI) also constitutes a world-wide concern to human health. In the U.S., the annual incidence of TBI is estimated at 1.7 million, and TBI is a contributing factor to 30.5% of all injury-related deaths. Additionally, the yearly economic burden (includes direct and indirect costs) was estimated in 2000 as US$ 76.5 billion in the US, which does not include the social and emotional toll on patients, families, and the community (National Center for Injury Prevention and Control 2013). The age groups most likely to sustain a TBI are children aged 0 to 4, older adolescents aged 15 to 19, and adults aged 65 and older (National Center for Injury Prevention and Control 2013). When considering TBI in only persons 16 years or older, 48% of TBIs occur in persons age 16–35 and persons in the 36–56 year old categories constitute 40% of TBIs (National Data and Statistical Center 2013). As with SCI, injury occurs in males (74%) more than females (26%) at any age (National Data and Statistical Center 2013). Recent evaluation of the etiology of TBI in adults indicates that 53% of TBIs were the result of motor vehicle crashes, followed by 24% caused by falls, and 13% from violence. Evaluation of the blood alcohol (BAL) level at time of emergency department admission shows that 54% of persons who sustained a TBI were negative and 43% had a BAL of greater than 10 mg/dl (National Data and Statistical Center 2013) (note that 80 mg/dl is considered positive for driving under the influence in most states). Duration of loss of consciousness after TBI is highly correlated with injury severity and long-term outcome. The National Data and Statistical Center (NDSC) TBI model system data indicated that the average duration of loss of consciousness for that data set was 8.3 days. The average duration of post-traumatic amnesia is 24.3 days in the NDSC data. At 1 year post-TBI, 34% of persons who sustained a TBI exhibit moderate to severe disability and 36% exhibit mild or partial impairment. Data also show that a 1 year post-TBI, 30 % of persons who sustained a TBI show no disability. Also, 38% of persons who sustained a TBI require some level of supervision at

1 year post injury and 33 % at 2 years post-TBI (National Data and Statistical Center 2013).

With regard to injury severity, the NDSC TBI model system data indicates that 46 % of TBIs are severe, 16 % are moderate, and 39 % are mild. However, the U.S. Centers for Disease Control and Prevention (CDC) estimate that approximately 75 % of the 1.7 million brain injuries in the U.S. are mild TBI. The data source is one likely cause of this discrepancy as the NDSC TBI model system data is comprised from hospital emergency department records of persons 16 years of age or older at the time of injury. In contrast, the CDC data sources include all age groups with date obtained from the National Hospital Discharge Survey, the National Hospitals Ambulatory Medical care Survey and the National Vital Statistics Systems for the years of 2002 to 2006 (Faul et al. 2010).

In addition to TBI in the civilian populations, it is now being recognized that military TBI is an overarching concern. For example, evaluation of U.S. Army personnel deployed to Iraq and Afghanistan, including soldiers deployed in Operations Iraqi Freedom and Enduring Freedom (OIF/OEF), indicated that during the 10 year period from 1997–2006 over 110,000 military personnel experienced at least one TBI (Wojcik et al. 2010). Similarly, a RAND report suggested that 20 % of deployed service personnel suffered a TBI (Burnam et al. 2009). Terrio et al. (2009; Brenner et al. 2010) reported that 22.8 % of soldiers in a brigade combat team returning from Iraq had clinician-confirmed TBI and that the majority of these TBIs were defined as mild. They also reported that soldiers with confirmed TBI were significantly more likely to report post-deployment somatic and neuropsychiatric symptoms than those with no TBI, which suggest that the health consequences of even mild TBI (mTBI) can be enduring. Moreover, predominate cause of mTBI in military populations is exposure to blast (Hoge et al. 2008). Indeed from a broader perspective, over 63 % of casualties from October 1, 2001 through February 28, 2011 in OIF/OEF and Operation New Dawn were caused by detonation of an explosive device. Thus taken together, epidemiological data indicate that mTBI induced either by blast or direct impact is a significant occurrence affecting both military personnel and civilians, and that mTBI can have lasting effects on well-being.

Although an array of symptoms has been documented as occurring after blast-induced or impact-induced mTBI, evaluating and characterizing these neurological sequelae remains an area of active clinical research. Commonly described symptoms include retrograde and/or anterograde amnesia, learning impairments, reduction in executive function, reduced concentration, mood disturbances, disrupted sleep patterns, aggression, and anxiety (Hoge et al. 2008; Sayer et al. 2008). However, it remains an active debate as to the extent to which anatomical and biochemical alterations in the brain from blast exposure cause these impairments. Indeed, the question as to the mechanism(s) by which blast exposure induces lasting cognitive deficits (i.e. anatomical vs. psychological) has been actively debated clinically since the term “shell shock” was first coined during World War I and then raised again with the term “postconcussion syndrome” used in World War II (Jones et al. 2007). The clinical assessment of blast-induced mTBI is particularly complicated by the absence of an overt (detectable) focal lesion(s) which is combined with the high

comorbidity of psychiatric disorders such as post-traumatic stress disorder (Bryant 2001). Adding to this confusion is the idea that personnel in modern conflicts are exposed to blast at close proximity and with improved body armored protection which impacts the survivability as well as the physical properties of the blast wave (Cernak and Noble-Haeusslein 2010; Risling et al. 2011). Add the related problem of the impossibility of documenting blast characteristics such as blast wave propagation, secondary acceleration/deceleration, heating, and emitted gases on an individual basis and this makes the understanding of the relationship between biomechanical loading and the sequelae of mTBI extremely difficult to acutely assess in the clinical arena (Risling et al. 2011). Thus, several research groups (including ours) have developed animal models of blast-induced mTBI (Cernak and Noble-Haeusslein 2010) in order to systematically evaluate many of these complicated issues.

6.2 Overview of the Pathophysiology of Traumatic CNS Injury

The initiating event in a traumatic central nervous system (CNS) injury (TBI or SCI) is a mechanical insult and the resulting mechanical response at the cellular level, termed *primary injury*. Following this initial primary injury is a set of complex and interrelated biochemical *secondary injury* mechanisms which means that the array of deficits following a traumatic event is dependent on the mechanical response to the insult and complex secondary injury mechanisms. This concept has been extensively detailed and reviewed (see Bramlett et al. 1997; Bramlett and Dietrich 2002; Adelson et al. 2000; Hall and Wolf 1986; Hall 2003; Levin 1990; Popovich and Jones 2003; Povlishock and Katz 2005; Raghupathi 2004; Smith et al. 1994, 2003; Whalen et al. 1999). However, it is important to emphasize that the primary injury is the trigger for the secondary injury cascade and that differing biomechanical loading scenarios induce a different injury response (Schmitt et al. 2010).

For example, in TBI, the dynamic loading that is associated with contact and non-contact load differ and result in characteristically different brain pathologies. Specifically, contact loading caused by direct contact of the head to/from an object typically induces focal tissue damage oriented in the direction of the force vector. Moreover, rapid contact loading produces stress waves that propagate within the skull/brain that can lead to a pressure gradient which results in a positive pressure at the site of impact (coup) and negative pressure on the side opposite the impact (contrecoup). This pressure gradient gives rise to shear strain throughout the brain tissue. In non-contact scenarios, the head and brain are loaded by inertial forces, also termed acceleration/deceleration forces. Inertial loading can either be translational or rotational, with focal brain injury more associated with translational loading and diffuse brain injury with rotational loading. In reality, acceleration of the head also occurs in contact loading so the separation of these biomechanical forces is

somewhat academic as the mechanisms described for contact loading also occur in acceleration/deceleration brain injury. Thus, it is well-established that TBI caused by acceleration or a blow to the head (impact-induced TBI), results in isochoric deformation of the brain tissue and a significant biomechanics literature concludes that shear leading to stretch is the primary mechanical mechanism of damage in TBI (Ommaya et al. 2002; Zhang et al. 2010).

Although the primary injury is the triggering event in traumatic injury to the brain or spinal cord, the secondary injury mechanisms represent the area with the most complex pathobiology where in understanding the interactions between astrocytes and other brain cells could be critical to the development of therapeutic approaches. The initiating event in CNS injury is a physical insult and the resulting mechanical response at the cellular level (primary injury). Acute plasma membrane damage resulting from the primary injury can have detrimental effects on neuronal and astrocytic function, including disruption of ion homeostasis, synaptic transmission, and axonal transport (Pettus et al. 1994; LaPlaca et al. 1997; Stone et al. 2004; Shi and Whitebone 2006). Membrane damage may initially manifest as non-specific pores (Geddes et al. 2003; Farkas et al. 2006) and initiate secondary damage such as receptor dysfunction (Hardingham 2009) and phospholipid breakdown through free-radical production and inflammatory processes (Farooqui et al. 2007), leading to cell death (Whalen et al. 2008). Excitotoxicity is an acute secondary event stemming from depolarization and excess excitatory amino acid release, leading to further disruption of ion gradients, glutamate release, and intracellular calcium rise (Agrawal and Fehlings 1996; Arundine and Tymianski 2003; Sahuquillo et al. 2001). Energy deficits occur due to increased demand (Ahmed et al. 2000), causing mitochondrial dysfunction and a drop in ATP (Sullivan et al. 1998, 2004), hindering energy dependent ion pumps (Faden et al. 1987). Free radicals, including nitric oxide (Cherian et al. 2004) and reactive oxygen species (Hall et al. 2004) are liberated, damaging DNA, cytoskeleton, membranes, other proteins, and normal gene expression. Cell death can be either necrotic and/or apoptotic (Kovesdi et al. 2007; Raghupathi 2004) via caspase and calpain activation (Liu et al. 2006; Ringger et al. 2004). Inflammatory factors are also released from cells (Lenzlinger et al. 2001), including astrocytes (Israelsson et al. 2008; Laird et al. 2008; Suma et al. 2008) and resident microglia (Koshinaga et al. 2007), as well as infiltrating macrophages (Utagawa et al. 2008), resulting in exacerbation of pro-inflammatory cytokine production and more cell death.

There are several mechanisms in this pathological secondary injury cascade wherein astrocytes may be key therapeutic targets. As such, Table 6.1 summarizes several injury mechanisms that could potentially serve as therapeutic targets to break the progression from mechanical insult to cellular dysfunction and death. These target pathways are arranged into broad categories: (1) acute damage and excitotoxicity; (2) free radical damage and compromised energetics; and (3) inflammation which, although not exhaustive, cover a substantial portion of the injury cascade. In this chapter, we focus on the role of astrocytes in the excitotoxic phase of the secondary injury cascade (Table 6.1, Column 1).

Table 6.1 Categories of secondary injury mechanisms for selection of potential therapeutic targets

Excitotoxic	Energetic/free radical	Inflammatory
Mechanical damage	Decreased cell respiration/ induced ischemia	Activation of microglia → Production of pro-inflammatory cytokines
Membrane disruption	Decreased ATP production/ levels	Recruitment of macrophages → Production of pro-inflammatory cytokines
Increased $[Na^+]_i$, $[Ca^{2+}]_i$	Lipid peroxidation/formation of free radicals/nitrosylation	Activation of astrocytes → Production of pro-inflammatory cytokines
Disregulation of astrocytic K^+ and membrane potential	Mitochondria damage and opening of mitochondrial permeability transition pore	Subsequent cell death and production of pro-inflammatory cytokines
Excessive glutamate release	Cytochrome C release	
Ionotropic and metabotropic glutamatereceptors activation	Increased $[Na^+]_i$, $[Ca^{2+}]_i$	
Ionic disregulation activates ATP-dependent Na^+, Ca^{2+} pumps	Altered gene expression	
Alteration of glutamate uptake	Activation of caspases, calpains and apoptotic mechanisms	
Sequestration of Ca^{2+} into mitochondria		

6.3 Role of Astrocytic Glutamate Transporters in Excitotoxicity

The term "excitotoxicity" is credited to Olney in 1969 (Olney and Sharpe 1969; Olney 1969) with the seminal demonstration that injection of the primary excitatory neurotransmitter in the CNS, L-glutamate, into mouse or primate brain caused destruction of tissue. Since then, it has become well-established that primary injury in the brain or spinal cord rapidly leads to high extracellular glutamate (Faden et al. 1989a; Bullock et al. 1995; Choi 1992). In other words, the metabolic and cellular derangements in the secondary injury phase are largely initiated by massive and indiscriminant release of glutamate into the extracellular space (Katayama et al. 1990). Mechanisms for this glutamate release include mechanical disruption of the cell's plasma membrane, the subsequent disregulation of ionic gradients (i.e. sodium and potassium), and resulting indiscriminate electrical discharge (Katayama et al. 1990).Furthermore, the death of astrocytes, caspase mediated degradation of glutamate transporters, and reversal of sodium dependent transport have all been implicated in the pathology of glutamate release following TBI (Zhao et al. 2003; Floyd et al. 2005; Floyd and Lyeth 2007).

Glutamate extracellular concentration must be maintained at low levels by a family of plasma membrane sodium dependent excitatory amino acid transporters (EAATs), which are selectively expressed throughout the mammalian brain on both neurons and glia. EAATs 1 and 2 (in human, while GLAST and GLT-1 in rodents, respectively; here, used interchangeably) are expressed primarily in astrocytes while the remaining transporters reside on neurons (Shigeri et al. 2004). The astrocytic glutamate transporters are crucial for the proper maintenance of extracellular glutamate levels; antagonists for them can lead to toxic levels of glutamate and subsequent cell death similar to that exhibited in TBI (Shigeri et al. 2004). Of the astrocyte transporters, EAAT2 is responsible for approximately 90 % of the

clearance of glutamate from the extracellular space (Kanai and Hediger 2003). The function of this glutamate transporter is significantly reduced following TBI in rat cortical tissue, as illustrated by decreases in both mRNA levels and protein expression of EAAT2 following TBI (Rao et al. 2001). Other studies using TBI rodent models have reported comparable decreases in protein expression levels including decreases in EAAT2 expression by 29 % in the ipsilateral cortex 7 days post-injury (Goodrich et al. 2013). Additionally the use of antisense oligonucleotides to knockout expression of various subtypes of EAATs has indicated the transporters are crucial to maintaining concentrations below toxic levels. Knockout of either EAAT1 or EAAT2 in mice can produce excitotoxic levels of glutamate similar to that experienced following TBI and induce neuronal degeneration and loss of motor neuron viability (Rothstein et al. 1996). Similarly the introduction of knockdown antisense oligonucleotides for EAAT2 significantly increased hippocampal cell death compared to sense nucleotides and sham operated controls in a TBI model (Rao et al. 2001). Other studies have indicated comparable decreases in both subtypes of astrocytic glutamate transporters as well as their de novo expression in ramified microglia, possibly as a compensatory reaction to the increased extracellular glutamate levels and loss of transporter function (van Landeghem et al. 2001). Collectively, these studies demonstrate the crucial role of astrocytic EAATs, especially EAAT2, in the maintenance of extracellular glutamate within physiological concentrations and illustrate how the pathology of CNS injury can be exacerbated when function or expression of these transporters is compromised.

Although astrocytic EAATs are responsible for the majority of the removal of glutamate from the extracellular space, the neuronal EAATs are also affected by CNS injury. Increases in expression of EAAT4 have been reported in hippocampal astrocytes 3 to 7 days following TBI which may also be compensatory (Yi et al. 2007). This result is novel in that EAAT4 is typically expressed in neurons; particularly Purkinje cells, has a much higher affinity for glutamate, and has a unique chloride conductance that is not coupled to its glutamate transport function. The chloride ion conductance is believed to play a role in decreasing cellular excitability via influx of the ion resulting and cell hyperpolarization (Fairman et al. 1995). Thus, like the de novo expression of astrocytic glutamate transporters, the increased appearance of the typically neuronal EAAT4 in hippocampal astrocytes may represent an endogenous neuroprotective attempt to mitigate high extracellular glutamate levels following the loss of the primary removal mechanism (Yi et al. 2007). Other attempts to offset the loss of EAAT function following TBI have involved the use of therapeutic drugs that upregulate expression of the transporters. Goodrich and colleagues have indicated that intraperitoneal injections of the β-lactam antibiotic ceftriaxone reversed the loss of EAAT2 expression in the ipsilateral cortex of fluid percussion injured mice by 7 days post-injury and furthermore decreased astrocytic degeneration (as measured by GFAP expression) by 43 % when compared to ipsilateral cortex of non-treatment control mice (Goodrich et al. 2013).

Importantly in the acute response to CNS injury, the expression of EAATs appears to be modified on almost every level possible, from DNA transcriptional regulation, to mRNA splicing, protein synthesis, and post-translational modification

(Danbolt 2001). A primary means of regulating astrocytic expression of EAAT2 involves molecules secreted by neurons. As evidence of this concept, astrocytes cultured in the absence of neurons preferentially express EAAT1 with very little expression of EAAT2, while neuronal co-culturing with astrocytes increases expression of EAAT1 and induces the expression of EAAT2 (Danbolt 2001). This induction of EAAT2 in the presence of neurons appears to be the result of soluble factors given off by the neurons and not the result of direct cell-to-cell adhesion between astrocytes and neurons. Evidence supporting the idea that soluble factors represent a necessary and sufficient mediator of EAAT2 expression includes data demonstrating that the introduction of neuron-conditioned media to pure astrocyte cultures can induce EAAT2 expression (Gegelashvili et al. 1997). Additionally immunofluorescence staining of embryonic mouse cortical neurons demonstrates that the majority of EAAT2 containing glial cells are not in direct contact with neurons (Gegelashvili et al. 2000). The neuronal conditioned media induced-increases in EAAT2 expression can be mimicked by the addition of dibutyryl-cyclic AMP, epidermal growth factor (EGF), or pituitary adenylate cyclase-activating polypeptide (PACAP) (Swanson et al. 1997).These effects seem to be dependent on the activation of phosphatidylinositol 3-kinase (PI-3K) and nuclear transcription factor-κB (NF-κB), the latter of which has been demonstrated to directly regulate the glutamate transporter gene (Sitcheran et al. 2005). A key mediator between these two signaling molecules is the protein kinase Akt. Akt is activated by a number of growth factors and frequently functions downstream of PI-3K where it has the ability to increase NF-κB activity by decreasing the activity of the protein responsible for NF-κB's sequestration in the cytoplasm (Kane et al. 1999). Li and colleagues have demonstrated the importance of the Akt pathway in EAAT2 induction by using lentiviral vectors to create astrocytic cultures that express dominant-negative or constitutively active variants of Akt. The dominant-negative strain decreased the effects of EGF on EAAT2 expression while the constitutively active stain demonstrated a dose and time dependent increase in EAAT2 protein expression, mRNA levels, and transport activity thereby demonstrating that Akt can regulate the expression of EAAT2 by increasing its rate of transcription (Li et al. 2006).

Another method for EAAT induction by growth/neurotrophic factors involves preferentially targeting the receptor tyrosine kinase (RTK) pathway. Gegelashivili and colleagues have shown that inhibition of RTK by the cell permeable Typhostin A23 blocks the induction of EAAT2 in the presence of neuron-conditioned medium (NCM) (Gegelashvili et al. 2000). The RTK pathway appears to represent a primary starting point for a second messenger cascade, which converges on the mitogen-activated protein kinases (MAPK) p42 and p44, and ultimately results in increased expression of EAAT2. Binding of RTK by growth factors results in recruitment of the GTP-binding protein Ras and the stimulation of MAPK kinase kinases like Raf, which activates MAPK kinases MEK1 and 2, that in turn activate p42/p44 MAPK (Abe and Saito 2001). A crucial prerequisite of EAAT2 induction involves this phosphorylation of p42/p44 MAPKs. The double phosphorylation of p42/p44 MAPKs at threonine-202 and tyrosine-204 has been shown to correlate with expression of EAAT2 in the presence of NCM (Gegelashvili et al. 2000).

Phosphorylated p42/44 MAPKs can translocate to the nucleus and regulate gene transcription through activation of transcription factors like cyclic AMP (cAMP) responsive element binding protein (CREB) (Abe and Saito 2001). Growth factors can bypass the RTK-p42/44 MAPK pathway and still influence the expression of EAAT2 by directly activating transcription factors such as cAMP responsive element modulator, CREB, and activating transcription factor 1, although the induction of EAAT2 via these pathways is weaker than through the RTK controlled pathways. Additionally, the growth factor activation of PI-3K previously mentioned can lead to the activation not only of Akt, but also result in increased phosphorylation of p42/44 MAPKs demonstrating a convergence point in these two EAAT2 regulating pathways (Abe and Saito 2001). Further evidence supporting the importance of the p42/44 MAPK signaling pathway in the expression and regulation of EAAT2 comes from studies examining pure cultured cortical astrocytes exposed to varying concentrations of glutamate. Western blot analysis indicated that at increased concentrations of extracellular glutamate there were increased levels of phosphorylated p42/44 (Abe and Saito 2001). The changes in phosphorylated proteins occurred in a time and concentration dependent manner and did not induce changes in the total amount of cellular p42/44 (Abe and Saito 2001). Glutamate receptor agonists did not mimic the effects of glutamate-induced increases in phospho-p42/44, nor did glutamate receptor antagonists block them. However the effects of glutamate could be reproduced by molecules that could be transported into the cell by glutamate transporters; thus introducing aspartate or the transportable uptake inhibitors DL-*threo*-β-hyroxyaspartate (THA) and L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC) into the extracellular environment lead to increases in phosphorylated p42/44 almost as strongly as glutamate itself. Together, these results indicate that EAATs are capable of modifying their own expression in response to extracellular glutamate independently of the activity of glutamate receptors. Thus, the reuptake transporters are able to relay signals about the extracellular concentration of glutamate and activate second messenger systems leading to the recruitment of the p42/44 MAPK pathway and ultimately resulting in increases in the number of the transporters on the cell membrane (Abe and Saito 2001).

While RTK, Akt, and MAPK intracellular signaling molecules are predominantly involved in increases in EAAT expression and presence in the plasma membrane, glutamate transporters expression can also be regulated either through sequestration in intracellular storage sites or ubiquitin-mediated degradation. Of primary importance in selective EAAT downregulation is the activity of the signaling molecule protein kinase C (PKC). PKC has differential effects on the EAAT subtypes; in mixed neuronal and astrocyte cell cultures, the activation of PKC with phorbol ester caused a rapid (within minutes) decrease in cell-surface expression of EAAT2 (Kalandadze et al. 2002) and in increase surface expression in the neuronal glutamate transporter EAAT3 (Gonzalez and Robinson 2004). These differential effects are thought to represent a switching mechanism from astrocytic to neuronal glutamate uptake. However, as astrocyte transport represent the primary reuptake mechanism, the effect in a mixed cell culture would be overall reduced reuptake of glutamate and elevated extracellular levels of the neurotransmitter (Gonzalez and Robinson

2004). The deletion of amino acids 475–517 on EAAT2 abolishes the effects of phorbol ester-induced internalization, demonstrating a possible site of PKC phosphorylation on the transporter (Kalandadze et al. 2002). The decrease in cell-surface expression did not correspond with a reduction in total cellular levels of EAAT2, suggesting the immediate effect of PCK phosphorylation of EAAT2 involves internalization of the transporter to an intracellular sequestration site (Gonzalez and Robinson 2004). Internalization can be blocked in astrocyte cultures expressing a dominant-negative variant of clathrin (Susarla and Robinson 2008; Sheldon et al. 2008) or by inhibition of the ubiquitin enzyme E1 (Martinez-Villarreal et al. 2012), suggesting an ubiquitin-dependent, clathrin-mediated endocytic mechanism of sequestration. In contrast to short-term activation of PKC, long-term exposure to phorbol ester was accompanied by an overall decrease in total cellular EAAT2 expression (Susarla and Robinson 2008). This decrease was attenuated by lysosomal inhibitors, suggesting a cellular mechanism by which PKC can reduce EAAT2 levels under physiological or pathological conditions (Susarla and Robinson 2008). Indeed the PKC induced lysosomal degradation of EAAT2 could partially account for the rapid reduction in protein expression levels following TBI. Studies in astrocyte cultures have indicated that the half-life of EAAT2 is longer than 24 h (Zelenaia and Robinson 2000); therefore the observed decreases in EAAT2 expression in the hours immediately following TBI (Rao et al. 1998) cannot be accounted for solely by halting transcription of the transporter. Along with the aforementioned degradation of EAAT2 by caspase-3, the activity of PKC could possibly represent a mechanism by which the secondary injury phase of TBI induces dysfunction in glutamate reuptake transporters and exacerbate the deleterious effects of elevated extracellular glutamate.

6.4 Glutamate Receptors and Calcium Homeostasis

Under both physiological and pathophysiological conditions, glutamate acts on both ionotropic and metabotropic receptors. Glutamate binding to ionotropic receptors, particularly the N-methyl-D-aspartate (NMDA) receptor alters membrane permeability to a number of ions including sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) (Arundine and Tymianski 2003). The neurotoxic effects of TBI appears to be largely the result of NMDA receptor activation as blockage of the NMDA receptor attenuates some of the neurotoxic effects of TBI, especially by influencing calcium homeostasis (Demediuk et al. 1989; Faden et al. 1989b). In a seminal set of experiments, Choi and colleagues discriminated the neurotoxic effects of the calcium and sodium ions on neuronal survival by examining cell cultures exposed to toxic levels of glutamate in isolated extracellular environments (Choi 1987). In an extracellular environment that mimicked baseline physiological concentrations of both sodium and calcium, cells exhibited immediate morphological changes followed later by significant cell degradation and death. Removal of both ions from the extracellular environment showed large protective effects on the cells, even at prolonged

glutamate exposure. However, separation of the two ions indicated their unique roles in the excitotoxic cascade. Their results indicate that the acute event precipitated by sodium appears to be transient and largely non-toxic to cells. Cultures in a calcium free extracellular environment exhibited morphological changes including swelling and granulation to a greater degree than those in the baseline experiment, but were largely spared later cell death and returned to previous size within an hour (Choi 1987). Cultures in an environment where choline was substituted for sodium and calcium ions remained at physiological concentrations showed markedly less swelling and other morphological changes than those at baseline levels, but exhibited roughly the same amount of cell death as cultures with both ions at physiological levels (Choi 1987).

Metabotropic glutamate receptors (mGluRs) can also mediate changes in intracellular ion concentrations. These receptors are G- protein-couple receptors that signal via second messenger system including phosphoinositide-dependent processes, cyclic AMP, or PKC. Three groups of mGluRs have been characterized with group I mGluRs (mGluR1 and mGluR5) being positively coupled to phospholipase C (PLC) to induce phosphoinosidide hydrolysis and mobilization of intracellular calcium. Groups II and III are negatively coupled to adenylyl cyclase. Thus the group I mGluRs are thought to have the most direct role in modulating intracellular calcium after injury (Lea and Faden 2001). With regard increases in astrocytic intracellular calcium after injury, in vitro mechanical strain injury was shown to increase inositol trisphosphate (IP_3) up to 10-fold over uninjured controls acutely post injury. Importantly, pharmacological inhibition of PLC was shown to inhibit injury-induced increases in IP3 and the resultant increase in intracellular calcium (Floyd et al. 2001). It was subsequently shown that pharmacological inhibition of the group I mGluRs inhibited injury-induced increases in PLC and increases in astrocytic intracellular calcium (Floyd et al. 2004). Similarly, administration of a group I mGluR antagonist was shown to confer cellular protection and reduction in injury-induced learning and memory deficits in a rat model of TBI (Gong et al. 1995). This finding is mirrored in spinal cord injury as antagonism of the group I mGluRs was shown to increase tissue sparing and improve locomotor scores (Mills et al. 2002).

Regardless of the glutamate receptor type that induces pathological elevations in intracellular calcium, there are a number of immediate consequences of excessive influx of calcium into the intracellular space. Calcium entry to the cell directly activates a family of nonlysosomal, cysteine proteases, calpains, which bind calcium and begin to enzymatically cleave the αII-spectrin protein, a key component of the cytoskeletal architecture resulting in cellular degeneration (Saatman et al. 2010). Prolonged calpain levels have been indicated in necrotic degeneration associated with CNS injury (Wang 2000). Calpains are one of the earliest mediators of cellular death in the injury pathology with elevated levels of the proteins demonstrated as early as 15 min after injury, indicating that calpains may underpin a an early response to injury (Zhao et al. 1998). Other proteolytic enzymes activated by TBI include the caspases, a family of 14 cysteine proteases discovered to be related to gene products required for cellular apoptosis in the nematode (Thornberry 1997). Caspases function as either initiators, which respond to apoptotic cellular signaling, or effectors, which

directly participate in cellular degradation largely through the cleavage of multiple proteins responsible for proper cellular functioning (Thornberry 1997). Caspase levels increase following TBI while caspase inhibitors have been demonstrated to reduce apoptosis and neurological deficits following TBI (Raghupathi 2004). Additional reports have indicated that a specific effector caspase, caspase-3, is responsible for degradation of the principle glutamate transporter, EAAT2, by cleavage near the C-terminal domain at aspartate-505 resulting in significant loss of function (Boston-Howes et al. 2006). This loss of transporter function can further exacerbate the excitotoxic effects of elevated glutamate levels in the extracellular space and serve to prolong and exacerbate glutamate-mediated pathology.

While activation of calpains and caspases represent immediate reactions to apoptotic and necrotic signaling, delayed responses to the CNS injury include changes in genes involved in cell death and survival, including the *blc-2* gene family which codes for both pro-apoptotic proteins Bax and Bad and the pro-survival protein Blc-2 (Strauss et al. 2004). Following injury, increased mRNA levels of the anti-apoptotic gene *blc-2* are present in surviving neurons as early as 6 h after injury (Clark et al. 1997), while transgenic mice that overexpress the *blc-2* gene exhibit less neuronal degeneration after the injury (Raghupathi et al. 1998). Additionally in regions exhibiting increased loss of neurons, mRNA levels of *blc-2* were markedly decreased, while those of the pro-apoptotic *bax* gene were significantly increased (Strauss et al. 2004), demonstrating the role of the *blc-2* family and the ratio of the proteins that they code for are key in determining the fate of neurons following injury. Part of the determination of this ratio involves post-translational modification of several downstream proteins by Akt to promote cell survival (Zhang et al. 2006). Akt has the ability to phosphorylate Bad at serine-136 both in vitro and in vivo, inhibiting the ability to participate in the apoptotic pathway (Datta et al. 1997). Akt is itself regulated by phosphorylation at serine-473 and threonine-308. Decreased levels of phospho-Akt have been shown 1 h after TBI in the injured cortex, correlating with an increase in cell death, however in surviving neurons, phosphorylation of Akt was rapidly accelerated after 4 h post injury to encourage cell survival (Noshita et al. 2002). Phospho-Akt has a number of downstream effectors, including forkhead family transcription factors, andCREB, activation of these and other proteins by Akt shifts the balance of pro- and anti-apoptotic enzymes in favor of cell survival (Zhang et al. 2006).

Pro-apoptotic signaling pathways are also activated in response to the myriad of toxic stimuli activated during the secondary injury phase of CNS injury. One signaling molecule appearing to have primary importance for apoptosis in neuronal tissues is the c-Jun N-terminal Kinase (JNK). JNKs are phosphorylated and activated in response to DNA damage, axonal injury, and the formation of reactive oxygen species, all of which are present in the secondary injury phase. JNKs appear to represent a convergence point for a number of signaling molecules including MAPKs and mixed-lineage kinases (Xu et al. 2001). Additionally the activation of Akt appears to inhibit the activity of JNK, reducing its apoptotic influence while inhibition of JNK using geldanamycin reduces its activity and provides neuroprotection both

in vitro and in vivo (Kovesdi et al. 2007). Another pro-apoptotic protein, p53 also appears to increase its activity following DNA damage in TBI models. Elevated levels of p53 mRNA and associated protein levels have been reported in experimental models of TBI (Napieralski et al. 1999). These studies demonstrate that the secondary injury phase induces important changes in signaling molecules responsible for cell death and survival. As these changes in molecular signaling take place over an extended period following the injury, they represent potent targets for potential therapeutic intervention.

6.5 Role of Astrocytic Sodium and Potassium Regulation in Pathology

Clearly, intracellular calcium is a key component in the glutamate pathology in the astrocyte after CNs injury. However, other work has established that in the astrocyte, Na^+ and K^+ are also key modulators of glutamate-induced damage after injury. For example, using an in vitro model of strain-induced cellular injury, Floyd and colleagues (Floyd et al. 2005) demonstrated that either injury or exogenous glutamate application induced elevations of astrocytic intracellular Na^+ that were depended on injury severity and that these increases in intracellular Na^+ were significantly reduced, but not completely eliminated, by inhibition of glutamate uptake. Moreover, pharmacological blockade of the reversed mode of the sodium-calcium exchanger reduced intracellular calcium concentrations and astrocyte death after injury (Floyd et al. 2005). Similarly, in an in vivo model of TBI, pharmacological inhibition of the sodium-calcium exchanger or the type 1 sodium-proton antiporter were found to reduce astrocyte and neuronal death acutely after injury (Zhao et al. 2008). Taken together, these data indicate that astrocytic increases in intracellular sodium, particularly those associated with glutamate uptake, also contribute to injury-induced pathology.

Additionally, a growing body of evidence suggests that alteration in K^+ influx in astrocytes is critical to CNS injury pathology. It is well established that the highly negative resting membrane potential and high K^+permeability of astrocytes is maintained by the inwardly rectifying K^+ channel Kir4.1. Moreover, Kir4.1 is the channel that supports potassium spatial buffering and homeostasis, both vital astrocyte functions that maintain normal neuronal firing (Olsen et al. 2006, 2007. Kir4.1 also affects astrocyte clearance of glutamate released by neurons. Actually, nearly all glutamate uptake is achieved by sodium-dependent glutamate transporters on astrocytes that are electrogenic and more effective at negative membrane potentials. Thus, alteration in Kir4.1 signaling could have large implications for astrocyte-neuronal interactions in the normal or injured spinal cord (Olsen et al. 2010). Additional clues to the role of Kir4.1 in pathology come from the relationship between channel function and cell proliferation/differentiation. Previous work has shown that dividing and immature cells have a relatively positive resting membrane potential

that becomes more negative as cells mature and terminally differentiate and this transition is associated with the increased expression of Kir4.1 channels (Olsen et al. 2006, 2007). Several lines of evidence substantiate this relationship between Kir4.1 expression and cell division. For example, blockade of the Kir4.1 channel delays differentiation and exit from the cell cycle (MacFarlane and Sontheimer 2000), and glia in Kir4.1 knock-out mice display depolarized resting membrane potentials and immature morphologies (Djukic et al. 2007; Neusch et al. 2001). Also, malignant human glioma cells maintain a relatively depolarized membrane potential and have mislocalized/non-functional Kir4.1 channels, but transfection with functional Kir4.1 channels hyperpolarizes the cell membrane and arrests cell division (Olsen et al. 2006, 2007). With regard to injury, *in vitro* injury to spinal cord astrocytes decreased Kir4.1 activity and induced proliferation (MacFarlane and Sontheimer 1997). TBI was shown to induce abnormal K^+ accumulation that was similar to that achieved by blockade of Kir4.1 channels (D'Ambrosio et al. 2002). Similarly, it was shown in an SCI model that Kir4.1 expression and function was reduced by nearly 80 % after injury and that this down-regulation extended to spinal segments several millimeters from the lesion epicenter. Moreover, the loss of Kir4.1 was accompanied by functional loss of astrocytic glutamate transport (Olsen et al. 2010).

6.6 Summary

Traumatic brain and spinal cord injury are life-changing events that exact an enormous financial and societal toll. Currently, there are no widely accepted and clinically-approved treatments to reduce damage after traumatic CNS injury, making the development of novel treatments an important health care priority. Astrocytes play a critical role in the pathophysiology of traumatic CNS injury and therefore also represent potential therapeutic targets. Key components in the astrocytic response include astrocytic glutamate transporters, glutamate receptors, and modulators of calcium, sodium, and potassium homeostasis in the astrocyte. Indeed, a combination approach that targets several astrocytic mechanisms may also provide great therapeutic potential.

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Chapter 7
Astroglial and Neuronal Integrity During Cortical Spreading Depolarization

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Abstract In the minutes, hours and even days following stroke or traumatic brain injury (TBI), cerebral gray matter undergoes recurring waves of depolarization that spread across neurons and astrocytes. Originating near the site of injury, these waves of spreading depolarization migrate through regions of compromised blood flow, consuming precious energy and expanding the initial site of infarct or TBI. The collapse of ion gradients is the hallmark of spreading depolarization. This causes rapid astroglial and neuronal swelling (i.e. cytotoxic edema) and dendritic beading with spine loss which represents acute damage to synaptic circuitry. Spreading depolarization has long been associated with migraine aura and it was recently recognized as a novel mechanism of injury in stroke and TBI patients. Despite testing of numerous neuroprotective agents in clinical trials no neuroprotective drugs have proven helpful. This can be partially attributed to the inadequate understanding of the dynamic cellular processes that promote brain damage during recurring waves of spreading depolarization. A surprisingly large therapeutic window exists to diminish the depolarizing and hemodynamic activity of spreading depolarization waves and thus reduce subsequent secondary brain injury. In this chapter, the impact of spreading depolarization waves on neuronal and astroglial cellular integrity is described.

Keywords Stroke · Traumatic brain injury · Depolarization · Astrocyte · Neuron · Dendrites · Cytotoxic edema · Astroglial swelling · Neuronal swelling · Excitotoxicity · Polyanions · Repolarization · Ischemia

7.1 Introduction

Ischemic stroke injury evolves from a complex sequence of pathogenic events including a breakdown of transmembrane ion gradients, spreading depolarization, excitotoxicity, inflammation and apoptosis (Dirnagl et al. 1999). There is now unequivocal electrophysiological evidence for spreading depolarizations in patients

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with aneurysmal subarachnoid hemorrhage, delayed ischemic stroke after aneurysmal subarachnoid hemorrhage, malignant hemispheric stroke after middle cerebral artery occlusion, spontaneous intracerebral hemorrhage and traumatic brain injury (TBI) (Dreier et al. 2006, 2009; Fabricius et al. 2006; Dohmen et al. 2008; Hartings et al. 2011a, b). The full spectrum from short- to very long-lasting spreading depolarization waves has been recorded in the evolution of stroke and TBI not only in animals but also in the human brain (Dreier et al. 2009; Oliveira-Ferreira et al. 2010; Hartings et al. 2011a; Drenckhahn et al. 2012). Moreover, patients with multiple or prolonged spreading depolarizations have very poor prognoses for recovery (Dreier et al. 2006; Hartings et al. 2011a, b) pointing to spreading depolarizations as the important mechanism in acute human brain injury.

The first hours following stroke are crucial for reducing damage in the metabolically compromised penumbra, but despite hundreds of drugs having been clinically tested as anti-stroke agents, tissue plasminogen activator (tPA) remains the only Food and Drug Administration-approved treatment for stroke. The narrow window for tPA effectiveness (<4.5 h after stroke onset) has limited administration of this drug to less than 3% of patients, with those patients at an increased risk for intracerebral hemorrhage. A better understanding of spreading depolarization initiated events leading to neuronal, astroglial and vascular disruption will help to unravel mechanistic endpoints, assign treatments appropriately and detect significant treatment effects when they exist, especially in the salvageable penumbra.

7.2 Biophysical Basis of Spreading Depolarization

The cellular membrane is impermeable for polyanions inside the cell, such as proteins and peptides, but permeable for small ions such as sodium, potassium and chloride. The presence of impermeant polyanions in the cytosol attracts the small cations which will lead to the intracellular accumulation of the small cations while small permeant anions will be less concentrated inside the cell. A consequence of this ionic behavior is the development of Gibbs-Donnan equilibrium with unequal amounts of small cations and anions inside but equal outside the cell. In other words, the cell will acquire excess osmolytes as the sum of small cations and anions inside must be larger than that outside. What follows is a water accumulation and the cell swelling, i.e. the cytotoxic edema (Somjen 2004; Dreier et al. 2013). Instead, in healthy cells under physiological conditions the influx of ions driven by Gibbs-Donnan forces is opposed by active membrane transport, mainly by plasma membrane sodium-potassium pump. By extruding three sodium ions and bringing in two potassium ions with each cycle, the sodium-potassium pump maintains a balance between concentrations of small ions inside and outside the cell. This creates equal osmotic pressure inside and outside the cell, but at the expense of steady work requiring chemical energy in the form of ATP. Indeed, the human brain accounts for 20% of the body's resting metabolism (Rolfe and Brown 1997) with a majority of the energy (~50%) utilized by the sodium-potassium pump to create the steady

state of the physiological ion distribution across plasma membrane far away from Gibbs-Donnan equilibrium (Ames 2000). Hence, the energy released through ATP hydrolysis by the sodium-potassium pump is stored as electrochemical energy by the physiological ion gradients crucial for neuronal excitability and osmotic balance. Such sophisticated organization to maintain a non-equilibrium steady state creates the pathological side effect termed spreading depolarization, the phenomenon that describes the near-complete breakdown of these physiological ion gradients (Dreier 2011).

The energy requirements to maintain a steady state far away from thermodynamic equilibrium make the brain extremely vulnerable to oxygen and glucose deprivation caused by cessation of blood flow during ischemia. Changes in neuronal membrane potential during cerebral ischemia constitute a brief small hyperpolarization followed by a slow small depolarization and then by the rapid onset of spreading depolarization. There is general agreement that the initial transient hyperpolarization is generated by an increased potassium conductance (Hansen et al. 1982) involving activation of ATP-sensitive potassium channels (Ben Ari 1989) or a G-protein-dependent calcium-sensitive potassium channels independent of ATP (Erdemli et al. 1998). A slow depolarization is due to the inhibition of electrogenic sodium-potassium pump and an accumulation of extracellular potassium and glutamate (Somjen 2001). The mechanism of the generation of spreading depolarization and whether glutamate ignites it is still the matter of current debates. Yet, factors that restrain the accumulation of extracellular potassium and glutamate, such as astroglial uptake are critical for spreading depolarization initiation and effective uptake of these substances by astrocytes could prevent it (Kager et al. 2002). Indeed, metabolic poisoning of astrocytes increases susceptibility to spreading depolarization (Largo et al. 1996, 1997a). The failure of sodium-potassium and calcium pumps is the core process in generation of spreading depolarization which is triggered when the net dendritic current persistently turns inward as the cations outflux mediated by ATP-dependent pumps fails to compensate for the net cations influx (Kager et al. 2002; Dreier 2011). As simulated in a single neuron model, this persistent dendritic inward current initiates a positive feedback cycle producing abrupt sustained depolarization (Kager et al. 2002). Spreading depolarization is an all-or-none process and once it is triggered, its course is dictated by the parameters of underlying ion currents without regard of the noxious stimulus that had set the process in motion. The ion fluxes during spreading depolarization lead to an abrupt shift of transmembrane ion concentrations to a new pathological state closer to Gibbs-Donnan equilibrium. This new pathological state is characterized by a near-complete loss of the ion gradients with the extracellular $[K^+]_o$ raising from ~3 to ~60 mM whereas extracellular $[Cl^-]_o$ declines from ~130 to 80 mM, $[Na^+]_o$ from ~150 to ~50 mM and $[Ca^{2+}]_o$ from ~1.3 to ~0.08 mM (Kraig and Nicholson 1978; Hansen and Zeuthen 1981; Windmuller et al. 2005). Therefore, spreading depolarization signifies an energy state of the brain which may entail either recovery, dependent on sufficient sodium-potassium pump activity, or death (Dreier et al. 2013).

There is brief extracellular alkaline transient at the onset of spreading depolarization followed by pronged acidification (Mutch and Hansen 1984; Kraig and

Cooper 1987; Menna et al. 2000). The tissue acidosis is generated by metabolic processes, especially by activation of glycolytic pathways in astrocytes as evidenced by a raise in extracellular lactate (Dienel and Hertz 2005). Indeed, astrocytes are able to maintain ATP level and ion gradients during ischemic conditions longer than neurons (Rose et al. 1998; Xie et al. 2008). Neuronal intracellular $[Ca^{2+}]_i$ simultaneously increases a thousand fold from ~25 nM to ~25 μM and this $[Ca^{2+}]_i$ raise outlasts the other ionic changes (Dietz et al. 2008). Among these ionic changes, the net influx of Ca^{2+} is most important since it is thought to induce the cascades leading to neuronal death (Somjen 2004). Excessive Zn^{2+} release, damage to mitochondria and oxygen free radical production are also among the deleterious effects of spreading depolarization (Dreier et al. 1998; Dietz et al. 2008; Liu and Murphy 2009; Medvedeva et al. 2009; Dreier 2011). Spreading depolarization-induced breakdown of ion gradients causes astroglial glutamate transporters reversal, resulting in a massive release of glutamate(Rossi et al. 2000), triggering excitotoxicity (Choi and Rothman 1990). There is also spreading depolarization-induced release of other neurotransmitters such as γ-aminobutyric acid (GABA) and acetylcholine. During spreading depolarization the interstitial space shrinks dramatically (Perez-Pinzon et al. 1995; Mazel et al. 2002) reflecting the occurrence of cytotoxic edema (Klatzo 1987; Somjen 2004). Hence, spreading depolarization is a mechanism leading to the osmotic water imbalance during stroke which is widely accepted as an essential contributor to acute, irreversible brain injury (Kimelberg 1995; Somjen 2004; Mongin 2007).

Spreading depolarization is observed as near-complete sustained depolarization of neurons and astrocytes that can propagate a breakdown of ion gradients across the brain's gray matter at a rate of 2–6 mm/min (Leão 1944; Lauritzen et al. 2011). The extracellular negative slow potential change between −5 and −30 mV serves as a robust measure of spreading depolarization since it directly reflects the pronounced depolarization of a large population of cortical neurons (Canals et al. 2005). Unless spreading depolarization happens after arrest of spontaneous brain activity that occurs, for example during severe hypoxia or ischemia (Leão 1947; Fleidervish et al. 2001), it initiates a depolarization block of action potentials because transient conductances responsible for the generation of action potential are inactivated by the profound sustained depolarization (Kager et al. 2002). Hence, as a functional consequence, spreading depolarization causes spreading depression of the electrocorticographic brain activity. Indeed, the phenomenon of spreading depression of brain activity as a product of spreading depolarization was discovered by the Brazilian neurophysiologist Aristides Leão, working at the time at the Harvard Medical School, seven decades ago (Leão 1944). There is a large and continuous spectrum of spreading depolarization waves characterized by common biophysical features and ranging from long-lasting detrimental events under severe ischemia to short-lasting harmless events in the healthy brain (Oliveira-Ferreira et al. 2010; Dreier 2011; Dreier et al. 2013). Today, a term "spreading depolarization" is used as a generic term for the full spectrum of such brain waves describing the abrupt sustained depolarization of neurons and astrocytes (Dreier 2011, 2013; Hartings et al. 2011a).

Attempts to define a specific membrane channel solely responsible for spreading depolarization have failed because several ion channels cooperate in initiation, propagation and its maintenance (Somjen 2004). Indeed, depolarization approaches to near 0 mV pointing to mixed conductance (Na^+, $Ca^{2+}$and Cl^- influx, and K^+ efflux). The principal channels governing neuronal influx of sodium and calcium are persistent (slowly inactivating) sodium channels, glutamate-gated N-methyl D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-isoxazole propionate (AMPA) ionotropic receptors and the calcium-sensing nonspecific cation channels (Xiong and MacDonald 1999; Kager et al. 2002; Noh et al. 2005). Other key candidates facilitating influx of cations and ionic dysregulation during ischemia are large-pore pannexin 1 channels (Thompson et al. 2006), acid sensing ion channels (ASICs) (Xiong et al. 2004; Yermolaieva et al. 2004) and transient receptor potential (TRP) channels from Melastatin super family such as TRPM2 and TRPM7 (MacDonald et al. 2006). Delayed rectifier persistent potassium channels and small conductance calcium-activated SK-type potassium channels contribute to outward potassium flux opposing depolarization (Kager et al. 2002). Astrocytes are not active contributors to the triggering mechanism of spreading depolarization as they depolarize as a result of the rise of extracellular $[K^+]_o$ (Walz 1997; Somjen 2001; Zhou et al. 2010). Neurons depolarize first and astrocytes follow (Somjen 2004; Zhou et al. 2010).

The mechanism of spread has not been fully elucidated, but neither synaptic transmission nor neuronal firing is required for depolarization spread. The classic hypotheses interpreted a feed-forward cycle of depolarization as a consequence of an extracellular diffusion-reaction process involving either potassium (Grafstein 1956) and/or glutamate (Van Harreveld 1959, 1978), which when released into the extracellular space, first excite and then depress neurons. The rate of chemical diffusion of these substances to reach and act on the membrane of adjusted cells would account for the slow spread of depolarization. An alternative hypotheses proposed is that the propagation of spreading depolarization can be mediated by exchange of chemical signals not through the extracellular space but through intracellular gap junctions (Somjen 2004). One theory posited that the propagation of spreading depolarization is mediated by a calcium wave through the gap junction-coupled network of astrocytes (Nedergaard et al. 1995; Martins-Ferreira et al. 2000). However, abolishing calcium waves in brain slices by the removal of extracellular calcium (Basarsky et al. 1998) or *in vivo* by pharmacological treatment (Chuquet et al. 2007) did not block spreading depolarization. These studies suggest that an astroglial intercellular calcium wave is not essential for spreading depolarization initiation or propagation. Furthermore, interruption of gap junctions between astrocytes by genetic ablation of connexin 43 accelerated spreading depolarization propagation instead of preventing it (Theis et al. 2003). Moreover, astrocytes apparently delay spreading depolarization onset and propagation as spreading depolarization was enhanced in cortical tissue with selectively disrupted astroglial aerobic metabolism (Largo et al. 1997a, b). A contribution of neuronal gap junctions to spreading depolarization propagation is also possible as neuronal gap junctional communication provides an intracellular pathway for the long-distance synchronization of neuronal firing in the front of an approaching spreading depolarization wave (Herreras et al. 1994; Shapiro 2001). All these theories may actually complement each other as they are not mutually exclusive.

7.3 Neurovascular Coupling During Spreading Depolarization

Even in the healthy brain under physiological conditions, the energy demand of the sodium-potassium pump during spreading depolarization increases so markedly (LaManna and Rosenthal 1975) that the tissue ATP concentration falls to about 50 % of the normal level (Mies and Paschen 1984; Selman et al. 2004). What follows is vasodilation and an increase in regional cerebral blood flow by more than 100 % in order to match the raised neuronal energy demand (Lauritzen 1994; Piilgaard and Lauritzen 2009). This functional increase in blood flow in regions invaded by depolarization is called spreading hyperemia and it constitutes the normal hemodynamic response to spreading depolarization (Dreier 2011). The brief, about 2 min hyperemia is followed by a mild but more prolonged hypoperfusion (spreading oligemia) lasting between 1 to 2 h (Busija et al. 2008). The spreading hyperemia response has been suggested to have several similarities with physiological neuronal and astroglial signaling to blood vessels (Dreier 2011). Indeed, the depolarization-induced glutamate release and the raise in intracellular calcium in neurons and astrocytes evoke the release of vasoactive messengers but the amplitude of changes is larger and the relative contribution of any of these vasoactive stimuli might be modified during normoxic spreading depolarization (Busija et al. 2008). In neurons, normal glutamate-mediating signaling triggers nitric oxide synthase to release nitric oxide which dilates vessels (Busija et al. 2008). Increased intracellular neuronal calcium may generate arachidonic acid which is metabolized to prostaglandins that also dilate vessels (Attwell et al. 2010). In astrocytes, glutamate causes a rise in intracellular calcium through activation of metabotropic glutamate receptors (Porter and McCarthy 1996) evoking the production of arachidonic acid. Arachidonic acid derivatives (prostaglandins and epoxyeicosatrienoic acids) released from astrocytes dilate blood vessels, but 20-hydroxyeicosatetraenoic acid (20-HETE) formed in vascular smooth muscle cells from astrocytic arachidonic acid constricts blood vessels (Koehler et al. 2009).

A spreading depolarization-induced sharp rise in the extracellular $[K^+]_o$ to ~60 mM has a strong vasoconstrictor effect but under normoxic conditions it is counterbalanced by vasodilator effect of nitric oxide combined with vasodilator effect of extracellular acidification (Dreier 2011). Modulatory effect of nitric oxide to inhibit the synthesis of the vasoconstrictor 20-HETE should also boost the effect of the vasodilatory prostaglandin and epoxyeicosatrienoic acid metabolites of the arachidonic acid released from astrocytes (Attwell et al. 2010). In addition, local release of calcitonin gene related peptide from trigeminal afferents in the dura mater contributes to vasodilation during normoxic spreading depolarization (Busija et al. 2008). Generally cells are not short of energy as neurovascular response to normoxic spreading depolarization is regulated normally and the supply with oxidative substrates is sufficient (Dreier 2011). Yet, pockets of tissue hypoxia lasting for several minutes can develop during normoxic spreading depolarization in the most distant supply territories of cortical capillaries (Takano et al. 2007) where a rise in the cerebral metabolic rate of oxygen exceeds oxygen supply (Piilgaard and Lauritzen 2009).

The normal neurovascular response to spreading depolarization fails in pathology. In ischemic conditions inverse neurovascular coupling to spreading depolarization wave results in severe arteriolar constriction and a perfusion deficit called spreading ischemia which propagates together with spreading depolarization delaying recovery of transmembrane ion gradients and thus neuronal and astroglial repolarization (Dreier et al. 1998, 2000). A drop in oxygen availability during ischemia decreases nitric oxide formation that could impair the balance between direct vasodilator effect of nitric oxide and the vasoconstrictor effect of elevated extracellular potassium. Low nitric oxide availability is also expected to weaken the suppression of the 20-HETE formation further enhancing the vasoconstriction (Chuquet et al. 2007; Fordsmann et al. 2013) during ischemic spreading depolarization.

7.4 Spreading Depolarization in the Ischemic Penumbra

Within minutes of focal ischemia, a spreading depolarization erupts in the area of severely decreased blood flow in the stroke focus (Leão 1947). Without immediate reperfusion, the inadequate energy supply causes spreading depolarization to become long-lasting *terminal* depolarization (also called *anoxic* depolarization) throughout the ischemic core, where neurons do not repolarize (Dirnagl et al. 1999; Dreier 2011). Essentially, the spread of the pharmacoresistant terminal depolarization wave establishes the boundaries of the ischemic core (Kaminogo et al. 1998), the primary region of acute neuronal death. The duration of the spreading depolarization wave becomes shorter as it propagates away from the ischemic core throughout the metabolically compromised penumbra along gradients of perfusion, oxygen and glucose and into naïve cortex (Nedergaard and Hansen 1993; Hossmann 1994; Obrenovitch 1995; Back et al. 1996; Aitken et al. 1998). Here, in healthy tissue spreading depolarization is short-lasting (~1 min) and can be blocked by NMDA receptor antagonists (Hernandez-Caceres et al. 1987; Lauritzen and Hansen 1992). It results in a depolarization block of action potentials and therefore initiates the classic spreading depression (silencing) of electrocorticographic activity (Leão 1944). From this point, spreading depression propagates together with spreading depolarization, but the latter does no harm (Nedergaard and Hansen 1988) because energy resources are not severely compromised. Hence, there is a depolarization continuum between terminal and short-lasting spreading depolarization with biophysical features preserved as the depolarization wave spreads in tissue along the gradient of metabolic stress (Somjen 2004; Dreier 2011; Dreier et al. 2013). Recurring spontaneous spreading depolarizations (also called *peri-infarct* depolarizations) initiate at the perimeter of the ischemic core by elevated extracellular K^+ and glutamate, a product of maintained depolarization and necrosis within the core (Nedergaard 1996; Dreier 2011). These spreading depolarizations propagate throughout the penumbra for hours to days after the initial injury in animal models and patients (Hartings et al. 2003; Dohmen et al. 2008; Risher et al. 2010) and may even cycle repetitively around the ischemic core (Nakamura et al. 2010). The prolonged duration of recurring spreading depolarizations further elevates metabolic stress in the moderately ischemic penumbra due to the mismatch between energy supply and

increased energy needs for the recovery of ion gradients by ion pumps. Accompanying arteriole activity also contributes to the metabolic stress, because spreading depolarization in the penumbra results in severe arteriolar constriction and spreading ischemia (as described above) leading to the stepwise expansion of severely hypoperfused cortex (Shin et al. 2006; Strong et al. 2007). Over time, energy demands required for repolarization will not be matched and penumbral neurons and astrocytes remain depolarized and overloaded with Ca^{2+}, resulting in cellular death and penumbral recruitment into the infarct core (Mies et al. 1993; Ohta et al. 2001; Hartings et al. 2003; Dietz et al. 2009; Risher et al. 2010, 2012).

7.5 Water Movement Through the Osmotically Tight Membrane of Strongly Depolarized Neurons

Ion and water imbalance during stroke has long been thought as chief contributors to acute, irreversible brain injury (Kimelberg 1995; Somjen 2004; Mongin 2007). Spreading depolarization is a mechanism leading to abrupt cytotoxic edema but studies directly deciphering the neuronal and astroglial components of acute brain swelling during spreading depolarization are limited as they require viewing intact neurons and astrocytes and measuring their changing volume in real time. Strongly depolarized neurons swell during seizure (Somjen 2004), spreading depolarization (Obeidat et al. 2000; Andrew et al. 2007; Takano et al. 2007) or intense cooling (Volgushev et al. 2000; Kirov et al. 2004), but details explaining neuronal swelling are lacking. Astrocytes express abundant water-selective aquaporins (Nielsen et al. 1997; Amiry-Moghaddam and Ottersen 2003) whereas pyramidal neurons have not been reported to express functional aquaporins in their plasma membrane. Aquaporin 11 is present in several neuronal cell types but shows no water permeability when incorporated into the neuronal membrane (Gorelick et al. 2006). In line with these data it has been shown that pyramidal neuronal cell bodies, dendrites and axons resist volume change in the face of acute osmotic stress (Andrew et al. 2007), a benefit of the low water permeability of their plasma membrane. As a result, structure (Andrew et al. 2007) and the intrinsic electrophysiological properties (Rosen and Andrew 1990) of pyramidal neurons are stable during acute osmotic stress, even as the surrounding astrocytes swell (Risher et al. 2009) (Fig. 7.1). However, under ischemic conditions evoking spreading depolarization the same neurons rapidly swell, dendrites became varicose and many spines disappeared (Andrew et al. 2007; Risher et al. 2009) with associated synaptic failure and altered signal conduction along distorted neuronal processes (Douglas et al. 2011). It is these changes that are responsible for the acute dysfunction at stroke onset and contribute to the progressive deterioration generated by recurrent spreading depolarizations during several hours to days. Without functional aquaporins the molecular pathways by which bulk water molecules rapidly cross the osmotically tight neuronal membrane during spreading depolarization are not well understood (Andrew et al. 2007). The water cannot enter neurons as hydration shells of influxing $Na^{+}$and Cl^{-} ions be-

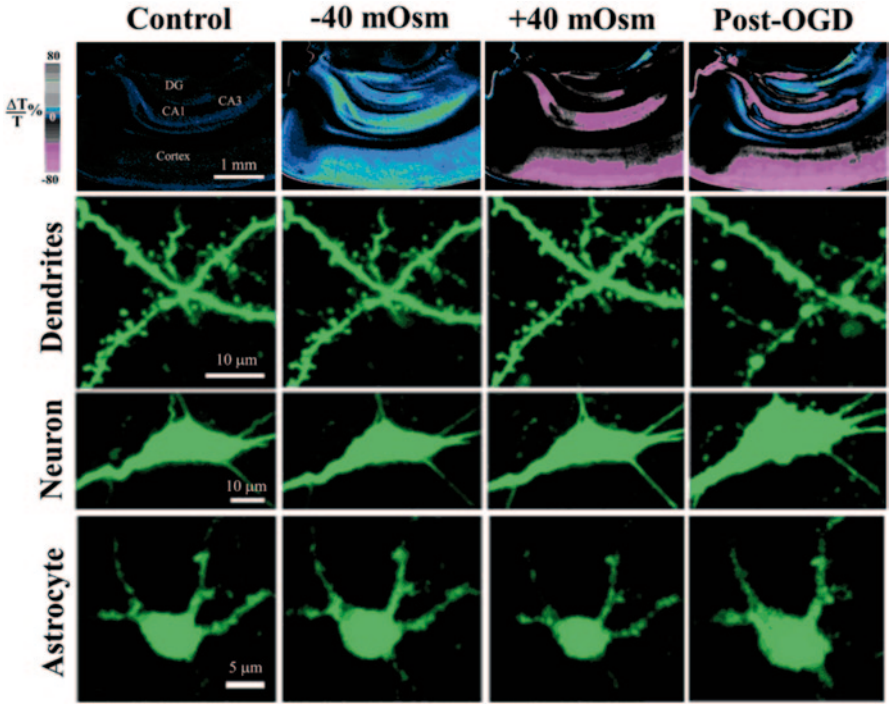

Fig. 7.1 Pyramidal neurons that do not express any functional membrane-bound aquaporins are osmoresistant. In contrast, aquaporin rich astrocytes are clearly osmoresponsive. Two photon laser scanning microscopy (2PLSM) imaging of dendrites, pyramidal neuronal cell bodies and astrocytes (*bottom three rows*; *green*, enhanced green fluorescent protein) in cortical brain slices showed that changes in intrinsic optical signals (*top row*) caused by osmotic challenge were associated with changes in astrocytic volume while neurons were not affected. Normosmotic measurements (column 1, control) were taken prior to 15 min of hypo-osmolar treatment (column 2, −40 mOsm) that increased light transmittance (LT, *ΔT/T%*) by neocortical gray matter, denoting tissue swelling. Dendrites and neuronal cell bodies did not change volume, whereas astrocytes swelled. Superfusion of slice with hyperosmotic artificial cerebrospinal fluid (ACSF) (column 3, +40 mOsm, 15 min) exposed the slice to an 80 mOsm step increase, that greatly reduced LT in the gray matter, signifying tissue shrinkage, but again dendrites and neuronal soma did not shrink, as astrocytes volume clearly decreased. Back in control ACSF (images are not shown), LT levels returned to near baseline as did the astrocytes. Following 10 min of oxygen-glucose deprivation (OGD), the gray matter displayed a propagating wave of elevated LT (not shown) caused by spreading depolarization-induced cell swelling. Pyramidal cell bodies and astrocytes swelled (column, 4). Dendrites also swelled but quickly form beads that reduced LT (Obeidat et al. 2000). Beading is considered an extreme version of focal dendritic swelling that paradoxically lowers LT in swollen tissue because the beads readily scatter light. This overcomes the elevated LT signal caused by neuronal and astroglial swelling. (Modified with permission from Andrew et al. (2007))

cause both ions are almost completely stripped of their hydration shells as they transit their channels (Hille 2001; MacAulay et al. 2004). The lipid bilayer can be considered water-impermeable over many minutes compared to the membrane containing functional water channels. Some small amounts of water will partition into

phospholipid bilayers in response to osmotic pressure and diffuse across with time through tiny transient pores probably arising from density fluctuations in the bilayers (Jansen and Blume 1995). Yet, in functional terms, plasma membranes lacking aquaporins such as those of oocytes (Preston et al. 1992; Agre et al. 2002), distal renal tubule cells (Somjen 2004; Tian et al. 2004)and epithelial lining of stomach and bladder (Krylov et al. 2001) do not pass osmotic water. It should be noted, however, that even without spreading depolarization metabolically compromised neurons eventually will swell due to the cation influx driven by Gibbs-Donnan forces (see above) and insufficient cation outflux caused by impaired ATP-dependent sodium-potassium and calcium pumps, but swelling will occur at a slower rate over many minutes as compared to rapid (<6 s) spreading depolarization-induced dendritic focal swelling (beading) (Zhang et al. 2005; Risher et al. 2010; Sword et al. 2013).

The reasons why neurons lacking aquaporins suddenly swell when strongly depolarized as during spreading depolarization represent an interesting question. One possibility is that bulk water influx might occur through pannexin 1 ion channels or other large-pore channels opened by ischemia(Anderson et al. 2005; Thompson et al. 2006). Pannexin 1 (Px1) is expressed predominantly in neurons where it forms large-pore channels that can pass substances up to 1000 Da (MacVicar and Thompson 2010). Px1 can be open by depolarization (Pelegrin and Surprenant 2006; Thompson et al. 2008), high extracellular $[K^+]_o$ (Silverman et al. 2009), strong elevation of intracellular $[Ca^{2+}]_i$ (Locovei et al. 2006) and by mechanical stretch (Bao et al. 2004). All of these conditions are present during spreading depolarization. It should be noted, that Px1 activation is not directly responsible for spreading depolarization generation (Madry et al. 2010; Bargiotas et al. 2011), but rather Px1 functions to maintain depolarization (MacVicar and Thompson 2010; Weilinger et al. 2012). Indeed, Px1 channels could be directly activated by ischemia in acutely isolated hippocampal neurons (Thompson et al. 2006), by ischemia-induced spreading depolarization in brain slices (Weilinger et al. 2012) and *in vivo* by spreading depolarization (Karatas et al. 2013). Px1 activation mediates neuronal death *in vitro* (Orellana et al. 2011), but most importantly *in vivo* (Bargiotas et al. 2011) during focal stroke. Hence, it might be anticipated that Px1 opening should make the neuronal membrane which poorly conducts water at its resting potential instantly permeable to water molecules during spreading depolarization leading to neuronal soma swelling accompanied by focal dendritic swelling (beading) and spine loss.

The synergistic contribution of Px1 channels and cotransporters to the neuronal and dendritic swelling with spine loss is another possibility. Several of the cotransporters that flux significant amounts of water are expressed in the neuronal membrane so cotransporters may also be responsible for water accumulation as well as recovery when water might be quickly translocated from neurons by cotransport proteins (MacAulay and Zeuthen 2010; Zeuthen 2010; Jourdain et al. 2011). The K^+/Cl^- contransporter KCC2, the $Na^+/K^+/2Cl^-$ cotransporter NKCC1 and the monocarboxylate transporter MCT2 are the dominant forms localized in neurons (Payne et al. 2003; Pierre and Pellerin 2005; Blaesse et al. 2009). The amount of water molecules cotransported per the protein turnover is about 500 and notably water can also be transported against an osmotic gradient (MacAulay and Zeuthen 2010).

These cotransporters are bi-directional and can carry a net ion and water influx or efflux, dictated by the free energy transmembrane gradients for the transported ions (Russell 2000; Rocha-Gonzalez et al. 2008; Blaesse et al. 2009) or H^+/lactate (Pierre and Pellerin 2005).

7.6 Neuronal Response to Spreading Depolarization

Since most excitatory synapses in adult brain occur on the dendritic arbor (Harris and Kater 1994), dendrites were predicted to be the initial site of acute excitotoxic injury leading to neuronal damage and death (Bindokas and Miller 1995). Indeed, sustained high calcium levels that were shown to develop in distal dendrites upon excitotoxic insult are capable of slowly spreading to the soma, ultimately resulting in acute neuronal injury (Shuttleworth and Connor 2001; Vander Jagt et al. 2008). Likewise, in the course of spreading depolarization dendritic calcium raises into the micromolar range (Dietz et al. 2008) and prolonged NMDA receptors activation during repolarization appears to underlie these calcium elevations (Aiba and Shuttleworth 2012). The formation of focal swelling or beading along the dendritic shaft separated from each other by thin dendritic segments ("beads-on-a-string" appearance) is an early morphological sign of acute neuronal injury typically associated with a variety of pathological conditions. Previously, dendritic beading has been reported in the ischemic condition (Hsu and Buzsaki 1993; Hori and Carpenter 1994; Zhang et al. 2005) and without recovery of sufficient blood flow it is a reliable indicator of an irreversible acute terminal injury to fine synaptic circuitry (Zhang and Murphy 2007; Li and Murphy 2008; Risher et al. 2010), and it is an early sign of cell death pathway activation (Enright et al. 2007).

Spreading depolarization-induced dendritic morphological alterations in the normoxic cortex are equivocal. Indeed, recent *in vivo* two photon laser scanning microscopy (2PLSM) studies have directly shown that in normal neocortex under physiological conditions, spreading depolarization can be accompanied by a complex pattern of dendritic beading or no beading (Takano et al. 2007; Sword et al. 2013). Importantly, several rounds of spreading depolarization caused no accumulating dendritic injury in normal neocortex as dendrites fully recovered from beading during repolarization (Sword et al. 2013). In normal cortex spreading depolarization is the pathophysiological correlate of the migraine aura (Lauritzen 1994), but even here it can cause a shortage of energy supply (Hashemi et al. 2009) and can be accompanied by a short period of tissue hypoxia with a complex pattern of distribution across the capillary bed (Takano et al. 2007; Yuzawa et al. 2012). This could possibly translate into a complex pattern of spreading depolarization-induced dendritic beading (Takano et al. 2007; Sword et al. 2013) thus reflecting patterns of tissue hypoxia across the capillary bed with perhaps no beading occurring in areas of luxury oxygen supply in the vicinity of penetrating arterioles (Kasischke et al. 2011).

Using a photothrombotic model of focal stroke other *in vivo* 2PLSM studies have shown that without recurrent spreading depolarizations injury to dendritic structure is gated by the degree of ischemia after local blood flow loss (Zhang et al. 2005; Risher et al. 2010). During severe ischemia (~90% reduction of blood flow) dendritic structure was lost within 10–40 min, but during moderate ischemia (~50% reduction of blood flow) dendritic beading developed within several hours (Zhang et al. 2005). Dendritic structure could be maintained in the ischemic tissue in the presence of a flowing capillary ~80 μm away (Zhang and Murphy 2007), and arterioles can supply oxygen even over longer distances, well above 100 μm (Kasischke et al. 2011). Spreading depolarization recorded *in vivo* under conditions of severe metabolic compromise, such as during global ischemia, rapidly damages fine synaptic circuitry (Murphy et al. 2008) and in the absence of reperfusion, as seen in the ischemic core, dendrites remain terminally beaded and spines are lost (Zhang and Murphy 2007; Risher et al. 2010).

Recurrent spontaneous spreading depolarizations greatly accelerated acute terminal dendritic injury in the moderately ischemic penumbra (Risher et al. 2010) (Fig. 7.2). Dendrites rapidly (<6 s) beaded and some spines were lost in concert with recurring spreading depolarizations. Dendrites quickly (<3 min) recovered between spreading depolarizations to near-control morphology, but it is conceivable that signals leading to neuronal death were triggered during this time. Hence, uncoupling of dendritic beading from spreading depolarizations could protect dendrites by delaying terminal injury (Risher et al. 2011). Preservation of dendrites and integrity of synaptic circuitry is the best indicator of the efficacy of neuroprotection strategy because preservation of neuronal soma alone but not dendrites does not result in proper function (Iyirhiaro et al. 2008).The likelihood of rapid dendritic recovery between spreading depolarizations was correlated with the presence of nearby flowing blood vessels. However, in about 1/3 of cases, dendrites did not recover even near a flowing capillary, suggesting that the energy demand of widespread repolarization following each spreading depolarization exceeded the energy supply of compromised blood flow. The accumulating stress of repeated spreading depolarizations eventually resulted in terminal dendritic injury (and thus permanent neuronal damage) dramatically accelerating recruitment of the penumbra into the infarct even if residual blood flow was present.

As it has been discussed above the most likely mechanism underlying spreading depolarization-induced dendritic beading results from a calcium-independent event such as the excessive influx of ions and obligatory osmotic uptake of water. Certainly, maintained depolarization during intense cooling results in dendritic beading with distinctive ultrastructural characteristics of focally swollen dendrites such as dramatically swollen organelles and electron lucent watery cytoplasm with holes indicating water entry (Kirov et al. 2004). Currently, there are no quantitative serial section electron microscopy (EM) studies directly examining dendritic disruption arising from spreading depolarization. Only a few single section EM studies have investigated *in vivo* changes in dendrites immediately after global cerebral ischemia without reperfusion (Yamamoto et al. 1986, 1990; Tomimoto and Yanagihara 1994). It is possible that ischemia-induced spreading depolarization has invaded

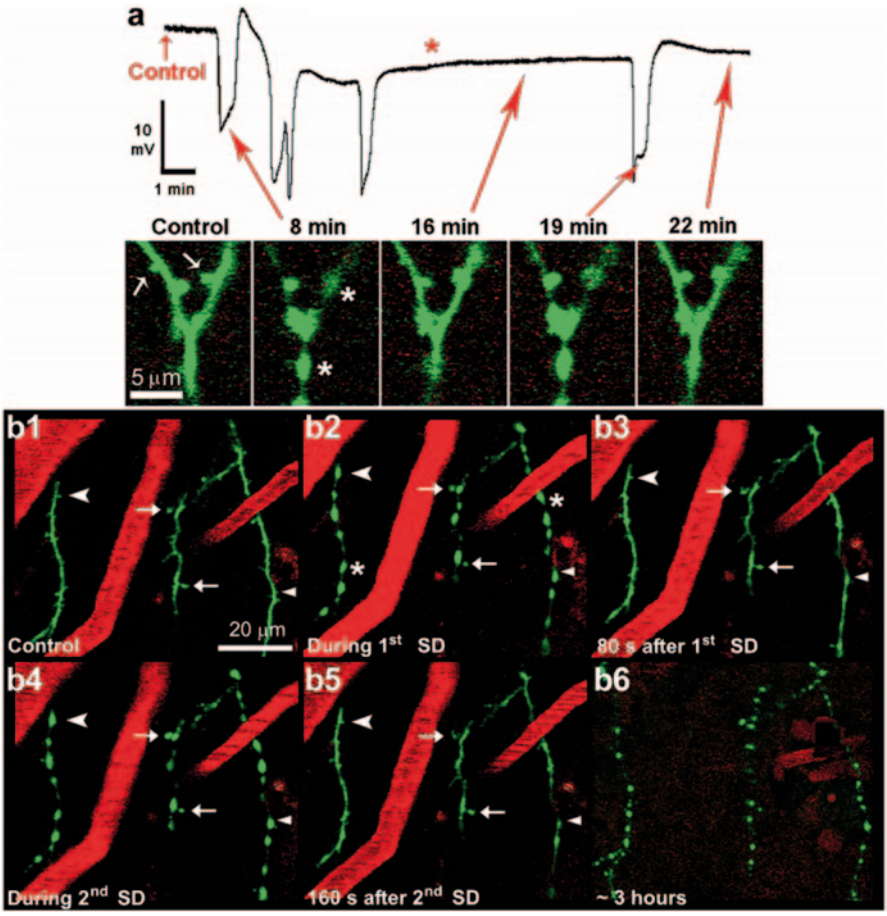

Fig. 7.2 Dendrites undergo a rapid cycle of beading and recovery coinciding with the passage of spreading depolarizations in the ischemic penumbra after focal photothrombosis in mice. **a** A high-magnification *in vivo* 2PLSM image sequence of a branched dendrite with *red arrows* corresponding to various time points on the above recording of spontaneous spreading depolarizations from microelectrode placed next to imaged dendrites. Dendritic spines are indicated by *white arrows* and *asterisk* indicates beading (8 min). The dendrite recovers at 16 min, but it is beaded again with spines lost by a subsequent spontaneous spreading depolarization at 19 min. Spontaneous spreading depolarizations are superimposed on a shallow ultraslow negativity (*red asterisk*) assumed to be an electrophysiological index of the cell death (Dreier et al. 2013). **b** Low-magnification image sequence showing dendrites (*green*, EGFP) and blood vessels (*red*, labeled with Texas Red dextran) from layer I of the somatosensory cortex. Blood flow within vessels is indicated by streaking caused by scanning of moving non-fluorescent red blood cells. The dendrites appear normal during control (b1) and undergo rapid beading (asterisks) and recovery coinciding with recurrent spreading depolarizations (b2, b4) similarly to the example in (**a**). Some dendritic spines are permanently lost (*arrowhead*), transiently lost (*chevrons*), or persistent (*arrows*) during spreading depolarization-induced dendritic beading. Appearance of dendritic beads is robust during passage of spreading depolarizations. Finally, nearly 3 h after photothrombosis, the dendrites undergo terminal beading coinciding with the passage of spreading depolarization and are no longer able to recover (b6) (vessels are no longer clearly seen at this time point due to Texas Red leaking out, necessitating a reduction in the red channel gain). (Modified with permission from Risher et al. (2010))

neuropil containing examined by EM dendrites, but this is unknown. Regardless, these EM analyses have only provided incomplete information about the extent of acute damage, limited mostly to mitochondrial swelling and disintegration of microtubules indicating calcium entry. Apparently, dendritic microtubules disassemble when intracellular calcium increases to the micromolar range, but although calpain can cleave microtubules, this calcium-activated protease was not involved in initial excitotoxin-induced dendritic beading as shown in brain slices (Hoskison and Shuttleworth 2006). Yet, the microtubule-stabilizing compound taxol prevented microtubule fragmentation and excitotoxin-induced dendritic beading (Hoskison and Shuttleworth 2006), suggesting that both the initial disruption of microtubules and beading may directly result from excessive water influx. It should be noted, however, that the shortage of metabolic energy associated with spreading depolarization may also result in unregulated polymerization of monomeric G-actin to polymeric F-actin (Atkinson et al. 2004). Such net conversion of G-actin to F-actin could contribute to dendritic structural rearrangements as beads are known to contain polymerized actin that redisperses during recovery from energy deprivation (Gisselsson et al. 2005).

7.7 Astroglial Response to Spreading Depolarization

Dendritic spines are present postsynaptically at the majority of excitatory synapses in the brain, where they are often partially ensheathed by astroglial processes to form the tripartite synapse (Harris and Kater 1994; Araque et al. 1999; Ventura and Harris 1999; Witcher et al. 2007, 2010). Through this close contact, astrocytes provide support for neurons via trophic factors (Yamagata et al. 2002) and maintain healthy synapses by clearing extracellular glutamate, thereby preventing neurotransmitter excitotoxicity (Anderson and Swanson 2000). Astroglial endfeet wrap around and completely ensheathe blood microvessels (Mathiisen et al. 2010), providing a critical link between neurons and the blood supply as part of the neurovascular unit (Haydon and Carmignoto 2006). Therefore, in recent real-time *in vivo* 2PLSM imaging study we have examined spreading depolarization-induced astroglial structural changes concurrently with signs of neuronal injury in the early periods of focal and global ischemia (Risher et al. 2012). In the penumbra, astrocytes underwent irreversible swelling in conjunction with spreading depolarization despite rapid dendritic recovery (Fig. 7.3). Astroglial swelling was often exacerbated in a stepwise manner by recurrent spontaneous spreading depolarizations and the magnitude of swelling strongly correlated with the total duration of depolarization. Such spreading depolarization-induced persistent astroglial swelling would greatly lessen their neuroprotective and supportive capabilities (Kimelberg 2005), making neurons more susceptible to damage from the propagating depolarization. Indeed, selective impairment of oxidative metabolism in astrocytes by inhibitors of the mitochondrial enzyme aconitase induces rounds of spreading depolarization sufficient to cause neuronal death (Largo et al. 1996). Astrocytes are relatively resistant to

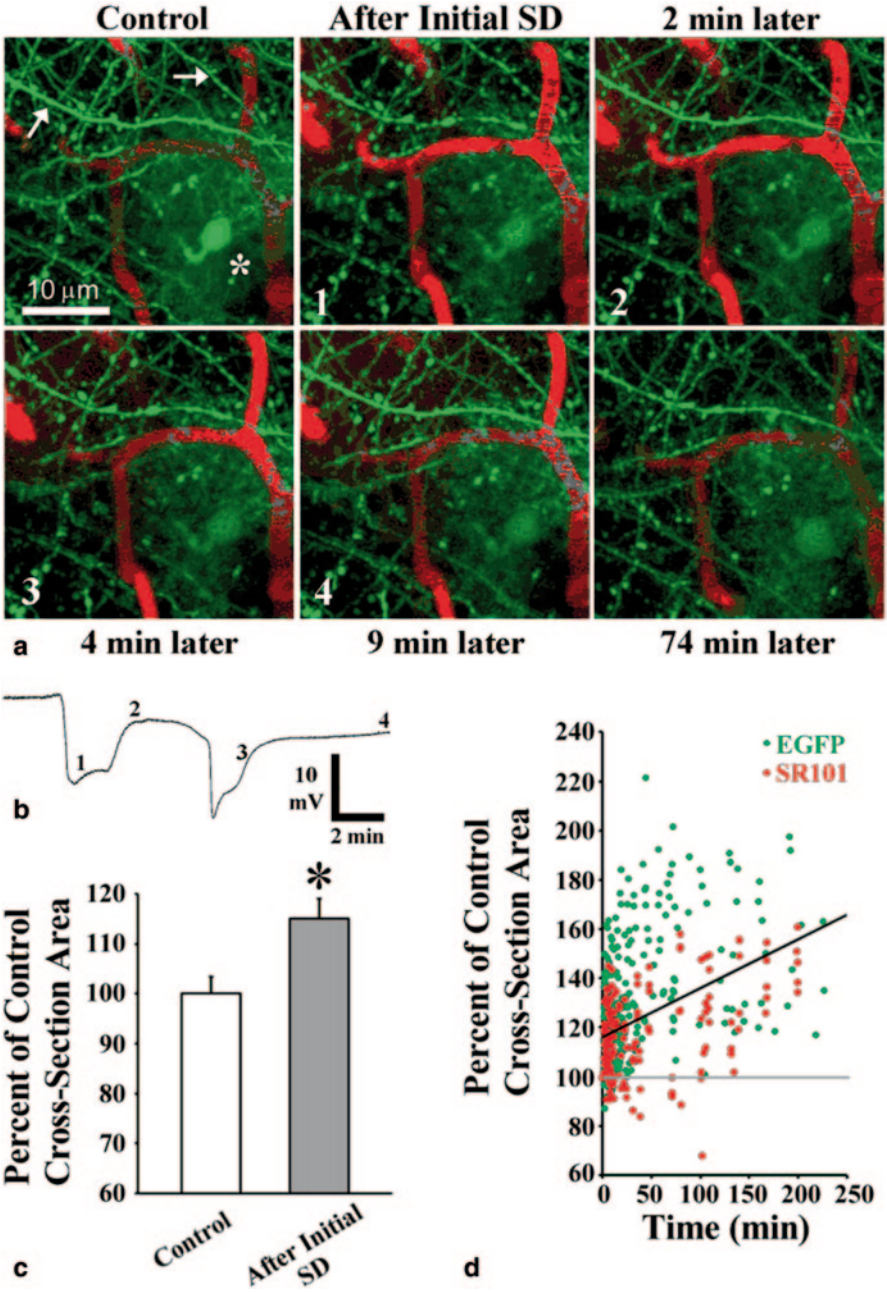

Fig. 7.3 Long lasting spreading depolarization-induced astroglial swelling in the ischemic penumbra after focal photothrombotic stroke. **a** 2PLSM *in vivo* images of an astrocyte (*asterisk*) along with nearby dendrites (*arrows*) and blood vessels (*red*, labeled with Texas Red Dextran) in layer I of mouse somatosensory cortex. Each numbered image corresponds with a time point indicated on the respective potential recording at the imaging site shown in (**b**). Negative deflections in the

hypoxia but become quickly injured if hypoxia is combined with acidosis and increased extracellular $[K^+]_o$ (Chesler 2005). It is conceivable that spreading depolarization-induced acidosis and elevated extracellular $[K^+]_o$ combine with hypoxia to compromise astroglial viability, promoting damage to neighboring neurons and thus contributing to a stepwise expansion of the infarct (Nedergaard and Dirnagl 2005).

In brain slices under normoxic conditions astrocytes undergo a passive transient swelling (Risher et al. 2009) in response to the spreading depolarization-elicited rise of extracellular $[K^+]_o$ (Zhou et al. 2010). Similarly, spreading depolarization results in transient astroglial swelling *in vivo* in healthy metabolically uncompromised neocortex (Risher et al. 2012). This transient astroglial swelling in normal healthy cortex is likely reflecting the protective work of these cells during the removal of extracellular $[K^+]_o$ and glutamate (Mongin and Kimelberg 2005). Yet here in healthy neocortex, astrocytes could be a critical link in the complex cascade of events linking spreading depolarization to activation of the trigeminal pain fibers that triggers migraine headache. This was recently found when spreading depolarization-induced transient opening of neuronal pannexin 1 channels resulted in the release of pro-inflammatory mediators with subsequent activation of nuclear factor κB (NF-κB) in astrocytes (Karatas et al. 2013). Activation of NF-κB could cause the formation and continuous release of cytokines, prostanoids, and NO to the subarachnoid space by astrocytes forming glia limitans and promote sustained stimulation of trigeminal nerves around pial vessels.

A transient global ischemia-elicited spreading depolarization induced either persistent or transient astroglial swelling alongside dendritic beading that was reversible with immediate reperfusion (Risher et al. 2012). Astroglial swelling was persistent during severe ischemia characterized by a long-lasting depolarization. Interestingly, when astroglial depolarization during oxygen/glucose deprivation in slices was combined with pharmacological blockade of ATP synthesis, astrocytes were not able to recover their membrane potential during re-oxygenation/normoglycemia, indicating irreversible damage (Xie et al. 2008). Perhaps these slice experiments closely mimic global ischemia when the sudden reduction in ATP and failure of the sodium-potassium pump results in long-lasting spreading depolarization, leading to persistent swelling of severely metabolically compromised astrocytes. In this respect, such an outcome is analogous to previously reported persistent swelling

potential represent spreading depolarizations. The dendrites bead during initial photothrombotically-induced spreading depolarization and rapidly recover (<2 min). By contrast, the astrocyte swells with no recovery seen. A spontaneous spreading depolarization occurring 4 min later is accompanied by another round of dendritic beading and recovery (shown at 9 min), while the astrocyte continues to swell. **c** Summary from 64 astrocytes in 20 animals showing the increase in astroglial soma size immediately after initial photothrombotically-induced spreading depolarization (posts-spreading depolarization images were taken 2.3 ± 0.2 min after initial depolarization onset). *P < 0.001, paired t-test. **d** Somata of EGFP-expressing astrocytes (*green dots*) and astrocytes labeled with sulforhodamine 101 (SR101) (*red dots*) continued to increase in size after initial spreading depolarization onset. Values are shown as percent of control cross-section soma area for each astrocyte. Regression line is shown (*black*) (r = 0.43, P < 0.001; 66 astrocytes from 21 animals). (Modified with permission from Risher et al. (2012))

of astrocytes during cardiac arrest and subsequent terminal spreading depolarization (Risher et al. 2009). Similarly to normoxic tissue, astroglial swelling was transient during short global ischemic periods distinguished by a short-lasting spreading depolarization. Therefore, it appears that the spreading depolarization-induced transient or persistent nature of astroglial swelling likely depends on the metabolic status of the surrounding tissue.

Astrocytes play a critical role in the clearance of potassium from extracellular space (Kofuji and Newman 2004) by several molecular mechanisms including inward rectifying K^+ channels, the $Na^+/K^+/2Cl^-$ cotransporter NKCC1 and the sodium-potassium pump (Mazel et al. 2002; Macaulay and Zeuthen 2012). Spreading depolarization-elicited astroglial swelling reflects the clearance of the accumulated extracellular potassium (Zhou et al. 2010) with associated water influx through aquaporins, mainly aquaporin 4 (Amiry-Moghaddam and Ottersen 2003; Kimelberg 2005; Leis et al. 2005). Accordingly, transgenic aquaporin 4-deficient mice display reduced edema and improved neurological outcome after experimental stroke (Manley et al. 2000). Activation of astroglial metabotropic glutamate receptors increases the water permeability of aquaporin 4 (Gunnarson et al. 2008). Water influx through aquaporin 4 triggers Ca^{2+} signaling in astrocytes and results in the activation of signaling cascades that could compromise astrocyte viability and exacerbate the pathological outcome (Thrane et al. 2011). Thus, astroglial swelling can be harmful and decrease the ability of astrocytes to maintain normal homeostatic function (Kimelberg 2005; Rossi et al. 2007).

Multiple mechanisms may participate in spreading depolarization-induced astroglial swelling. In normoxic brain tissue with a functional sodium potassium pump, intracellular water translocation by glutamate transporters during glutamate reabsorption and by NKCC1 cotransporter during the clearance of extracellular potassium should contribute to astroglial swelling during spreading depolarization (MacAulay and Zeuthen 2010). An increase in extracellular potassium during onset of spreading depolarization should provide a favorable driving force for the reversed direction of the transport by K^+/Cl^- contransporters with associated water influx and thus also contribute to the astroglial swelling (Macaulay and Zeuthen 2012). Then during repolarization heavy $[Cl^-]_i$ load should provide a favorable driving force for net extrusion of K^+, Cl^- and water by K^+/Cl^- contransporters aiding in the fast volume recovery.

Under ischemic conditions when the Na^+ and K^+ gradients run down due to inhibition of the sodium-potassium pump, voltage-sensitive Na^+-coupled transporters such as glutamate transporters will slow down, diminishing their contribution to the astroglial swelling during spreading depolarization (MacAulay and Zeuthen 2010). However, there is a strong indication that activity of the NKCC1 cotransporter is maintained even under disrupted ion gradients (Chen et al. 2005). Spreading depolarization-elicited extracellular acidification and increased lactate should also add to astroglial water accumulation due to the cotransport of water by the astroglial monocarboxylate transporter during lactate/H^+ clearance from the extracellular space (MacAulay and Zeuthen 2010). Intracellular acidification will similarly facilitate astroglial swelling mainly through activation of Na^+/H^+ and HCO_3^-/Cl^-

exchangers, leading to accumulation of intracellular osmolytes (Mongin and Kimelberg 2005). Importantly, Gibbs-Donnan forces should play an important role in the redistribution of ions across membranes of metabolically compromised astrocytes, leading to an uptake of the high extracellular K^+ followed by osmotically obligated water through aquaporins and thus persistent astroglial swelling (Kimelberg 2005).

Astroglial swelling may be a precursor to several detrimental consequences of ischemia, since swelling activates one or more membrane permeability pathways and leads to the release of neuroactive and neurotoxic molecules, including the excitatory amino acids glutamate and aspartate (Kimelberg et al. 1990; Abdullaev et al. 2006; Hines and Haydon 2013), ATP (Mongin and Kimelberg 2002; Liu et al. 2008; Orellana et al. 2011) and D-serine (Schell et al. 1995; Katsuki et al. 2004). Such release of ATP and glutamate through astroglial connexin 43 hemichannels could mediate neuronal death through activation of neuronal pannexin 1 channels (Orellana et al. 2011). Furthermore, spreading depolarization-induced astroglial swelling shown at such early time points after stroke may also be a prelude to devastating events occurring later on, as swelling eventually results in the rupture of the plasmalemma and organelles during delayed astroglial necrosis (Gurer et al. 2009). Loss of astrocytes would then be a crippling blow to the continued survival of neurons, since it has been well-established that neurons are dependent on the support provided by their glial neighbors both during development and following brain injury (Barreto et al. 2011).

Alternatively, astroglial swelling may be reflective of the protective work of these cells, including the removal of extracellular K^+ and glutamate (Mongin and Kimelberg 2005), increased glycogen metabolism (Hertz et al. 2007; Seidel and Shuttleworth 2011) and the release of glutathione to defend neurons from oxidative injury (Dienel and Hertz 2005). Equally, the release of ATP (Kimelberg et al. 2006) and subsequent extracellular accumulation of adenosine (Pascual et al. 2005) could provide neuroprotection by delaying the onset of spreading depolarization (Canals et al. 2008). This dual role of astroglial swelling may underlie the transition between injury and repair in tissue at risk and the ability of astrocytes to protect surrounding neurons may likely depend on the level of metabolic compromise.

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Chapter 8
Crucial Role for Astrocytes in Epilepsy

Peter Bedner and Christian Steinhäuser

Abstract Epilepsy is characterized by the periodic occurrence of seizures. Currently available anticonvulsant drugs and therapies are insufficient to controlling seizures in about one third of patients. Thus, there is an urgent need for new therapies that prevent generation of the disorder and improve seizure control in individuals already afflicted. The vast majority of epileptic cases are of idiopathic origin with their underlying mechanisms being unclear. Neurosurgical specimens from patients presenting with mesial temporal lobe epilepsy (MTLE) demonstrate marked reactive gliosis. Since recent studies have implicated astrocytes in important physiological roles in the central nervous system, such as synchronization of neuronal firing, it is plausible they may also have a role in seizure generation and/or seizure spread. In support of this view, various membrane channels, receptors and transporters in astrocytic membranes are altered in the epileptic brain. Excitingly, recent evidence suggests that in the course of the pathogenesis of MTLE, these glial changes alter homeostatic network functions and temporally precede the alterations in neurons. These findings might eventually classify MTLE as a glial rather than a neuronal disorder, and identify astrocytes as promising new targets for the development of more specific antiepileptic therapeutic strategies.

This chapter summarizes current evidence of astrocyte dysfunction in epilepsy and discusses presumed underlying mechanisms. Although research on astrocytes in epilepsy is still in its infancy, the review clearly demonstrate a critical role of astrocytes in the disturbance of K^+ and transmitter homeostasis and its impact on seizure generation.

Keywords Epilepsy · Astrocyte · Gap junction · Connexin · Pannexin · K^+ clearance · Kir channel · Gliotransmitter release · Adenosine kinase · Glutamine synthetase · Inflammation · AQP4 channel

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8.1 Introduction

Astrocytes are active partners in neural information processing. Advanced electrophysiological and Ca^{2+} imaging techniques unraveled that these cells express a similar spectrum of ion channels and transmitter receptors as neurons, which allows them to sense and respond to neuronal activity. Despite the fact that the pathways enabling activation of astrocytes under physiological conditions are still ill-determined, evidence is emerging suggesting a critical role of astrocyte dysfunction in the pathogenesis of neurological disorders (Seifert et al. 2006). In this review we will discuss recent work on specimens from patients with pharmacoresistant mesial temporal lobe epilepsy (MTLE) and corresponding animal models of epilepsy, which revealed alterations in expression, subcellular localization and function of astroglial K^+ and water channels, resulting in impaired K^+ buffering. Dysfunction of glutamate transporters and the astrocytic enzymes, glutamine synthetase (GS) and adenosine kinase (ADK), as observed in epileptic tissue suggested that impaired astrocyte function is causative of hyperexcitation, seizure spread and neurotoxicity. Increasing evidence suggests that proinflammatory mediators cause dysfunctions in astrocytes, which individually or in concert provoke neuronal hyperexcitability. Accordingly, astrocytes should be considered as promising targets for new therapeutic strategies. We will summarize current knowledge of astrocyte dysfunction in MTLE and discuss putative mechanisms underlying these alterations.

8.2 Impaired K^+ Buffering in Epilepsy

8.2.1 K^+ Uptake and K^+ Spatial Buffering

Intense neuronal activity elicits transient increases in the extracellular potassium concentration ($[K^+]_o$) which under pathological conditions like epilepsy can reach values of up to 10–12 mM (Heinemann and Lux 1977). Even moderate rises in $[K^+]_o$ have been shown to significantly increase neuronal excitability and synaptic transmission (Balestrino and Somjen 1986; Walz 2000), underscoring the necessity of tight control of K^+ homeostasis for normal brain function. This task is mainly accomplished by astrocytes which are characterized by a very negative resting potential and a high resting permeability for K^+. Responsible for these glial membrane properties are essentially inwardly rectifying K^+ channels of the Kir4.1 subtype (Seifert et al., 2009). Astrocytes control $[K^+]_o$ by two mechanisms: K^+ uptake and spatial buffering (for review see Kofuji and Newman 2004). Net uptake of K^+ is mainly mediated by Na^+/K^+ pumps and $Na^+/K^+/Cl^-$ cotransporters and to a minor extent by Kir4.1 channels (D'Ambrosio et al. 2002; Kofuji and Newman 2004; Ransom et al. 2000). It is rather unlikely that this mechanism alone is sufficient for efficient clearance of excess $[K^+]_o$ since intracellular K^+ accumulation results in water influx and cell swelling. The spatial buffering model (Fig. 8.1) (Orkand

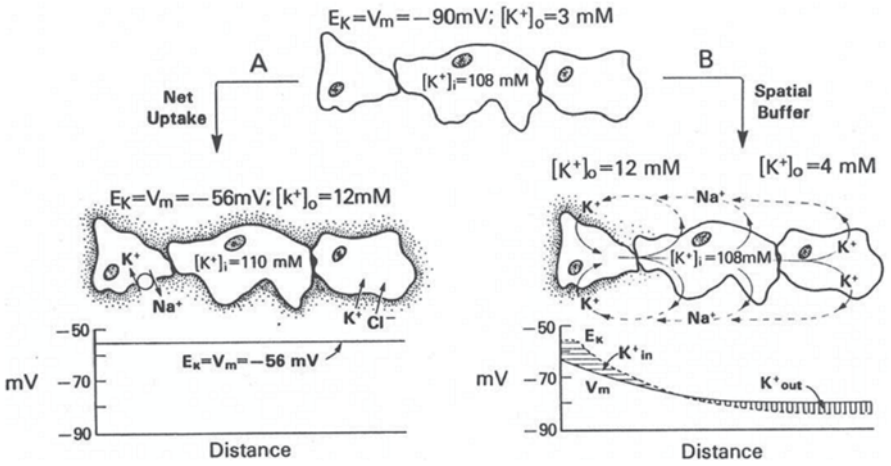

Fig. 8.1 Net uptake (*left*) and space-dependent spatial K^+ buffering (*right*). In case of spatial buffering (*right panel*), Activity in a group of neurons has produced local increase in $[K^+]_o$ to 12 mM (*shaded area, left*). This provokes a more positive membrane potential (V_m) that passively spreads through the coupled astrocytes. The positive shift of the K^+ equilibrium potential (E_K) is stronger than that of V_m of the cell exposed to high $[K^+]_o$ because the latter is "clamped" by the neighboring, more negative astrocytes exposed to lower (normal) $[K^+]_o$ (4 mM). The difference between E_K and V_m drives K^+ inward at the region where it is raised and outward at distant regions. The result is a net flux of K^+ away from the region where it has accumulated extracellularly. Average $[K^+]_i$ is not affected. The graph shows the distribution of E_K and V_m as a function of distance along the astroglial syncytium. (Modified from Orkand (1986), reproduced with permission)

et al. 1966) describes another, more effective mechanism for $[K^+]_o$ clearance. It is based on the fact that astrocytes are electrically connected to each other via gap junction (GJ) channels to form a functional syncytium. According to the model, excessive extracellular K^+ is taken up by astrocytes at sides of high neuronal activity redistributed through the astrocytic network to be released at regions of lower $[K^+]_o$. Here, uptake and release of K^+ occur passively, via passive diffusion through weakly-rectifying Kir4.1 channels. These channels are particularly well suited for this task because they possess a high open probability at resting potential and their conductance increases at high $[K^+]_o$ (Ransom and Sontheimer 1995). Intercellular K^+diffusion is also energy-independent, driven by the electrical gradient between the depolarized potential of glial cells at sides of K^+ entry and the more negative membrane potential of the glial syncytium (Orkand et al. 1966; Walz 2000; Kofuji and Newman 2004).

8.2.2 Kir4.1 Channels and K^+ Buffering in Epilepsy

Increased $[K^+]_o$ has been associated with the pathophysiology of epilepsy (Moody et al. 1974; Fisher et al. 1976; Lothman and Somjen 1976), and it is known that

high $[K^+]_o$ is sufficient to trigger epileptiform activity *in vitro* (Traynelis and Dingledine 1988). To assess the impact of Kir4.1 channels in K^+ buffering, the effect of Ba^{2+}-induced Kir channel block on stimulus-triggered rises in $[K^+]_o$ or iontophoretically applied K^+ was analyzed in sclerotic and non-sclerotic hippocampal slices from rat and man. It could be shown that Ba^{2+} significantly enhanced $[K^+]_o$ accumulation under control conditions, but had no effect in sclerotic hippocampi. These findings provided evidence for the disturbance of Ba^{2+}-sensitive K^+-uptake in sclerosis (Heinemann et al. 2000; Jauch et al. 2002; Kivi et al. 2000). Confirmation for this hypothesis came from patch clamp analysis, demonstrating significantly reduced Kir currents in the sclerotic CA1 region of neurosurgical specimens from patients presenting with MTLE (Hinterkeuser et al. 2000; Schröder et al. 2000; Bordey and Sontheimer 1998). Moreover, Western blot revealed a 50 % down-regulation of Kir4.1 protein in human hippocampal sclerosis (HS) compared to post-mortem controls (Das et al. 2012). In a recent study, Heuser et al. (2012) used immunohistochemistry to examine the distribution of Kir4.1 in hippocampi from MTLE patients. They found significantly reduced astrocytic Kir4.1 immunoreactivity in patients with HS compared to non-sclerotic and autopsy controls. Interestingly, the reduction was most pronounced around vessels and presumably caused by disruption of the dystrophin-associated protein complex in astrocytic endfeet (Heuser et al. 2012). Together, these studies imply that impaired K^+ clearance and increased seizure susceptibility in MTLE-HS result from reduced expression of Kir4.1 channels. However, it remains an open question whether this reduction represents cause, effect or adaptive response in TLE. In favor of a causative role for altered Kir channel expression in epilepsy, David et al. (2009) showed in an albumin model of epilepsy that Kir4.1 down-regulation occurs before the onset of epileptic activity. Another study, however, reported no changes in astrocytic Kir currents 7–16 days following systemic injection of kainate, implying that Kir down-regulation represents a consequential event in epilepsy (Takahashi et al. 2010).

Further support for the crucial role of Kir4.1 in glial K^+ buffering emerged from the phenotype of Kir4.1 knockout mice (Djukic et al. 2007; Kofuji et al. 2000). Global deletion of the Kir4.1 encoding gene, *KCNJ10*, resulted in marked motor impairments and premature death (postnatal day 8 (P8) to P24) (Neusch et al. 2001). Mice with glia-specific deletion of Kir4.1 (cKir4.1$^{-/-}$ mice) displayed a similarly severe phenotype, including ataxia, seizures and early lethality before P30. At the cellular level, these mice showed substantial depolarization of gray matter astrocytes and consequently severely impaired astrocytic K^+ and glutamate uptake (Djukic et al. 2007). Similar results were obtained after down-regulation of Kir4.1 by RNAi in cultured astrocytes (Kucheryavykh et al. 2007). Follow-up studies performed on cKir4.1$^{-/-}$ animals substantiated the crucial role of Kir4.1 channels in K^+ buffering and demonstrated that loss of Kir4.1 expression causes epilepsy (Chever et al. 2010; Haj-Yasein et al. 2011).

Missense variations in the *KCNJ10* gene have been linked to seizure susceptibility in man (Buono et al. 2004). Loss-of-function mutations in *KCNJ10* underlie an

autosomal recessive disorder characterized by seizures, ataxia, sensorineural deafness, mental retardation and tubulopathy (EAST/SeSAME syndrome) (Bockenhauer et al. 2009; Scholl et al. 2009; Reichold et al. 2010; Williams et al. 2010). Patients suffering from this disorder display focal and generalized tonic-clonic seizures since childhood. Another study reported that autism with seizures and intellectual disability is tentatively linked to gain-of-function mutations in *KCNJ10* (Sicca et al. 2011). Heuser et al. showed that a combination of three single nucleotide polymorphisms (SNPs) in the aquaporin 4 (AQP4) gene (encoding a water channel) together with two SNPs in the *KCNJ10* gene was associated with MTLE (Heuser et al. 2010). Association analysis in MTLE patients with a history of febrile seizures (FS) versus such without FS revealed that a combination of SNPs in *KCNJ10, AQP4*, and the area between *KCNJ10* and *KCNJ9* was significantly associated with MTLE-FS (Heuser et al. 2010).

8.3 GJ Communication in Epilepsy

8.3.1 Potential Roles of GJs in Epilepsy

Astrocytes in the adult brain are connected to each other via GJ channels composed of connexin43 (Cx43) and Cx30 (Nagy and Rash 2000), allowing intercellular exchange of ions, second messengers, nutritional metabolites and amino acids. The astroglial syncytium has important functions, including spatial buffering of K^+ ions (see above), trafficking and delivery of energetic metabolites to neurons (Giaume et al. 1997), intercellular propagation of Ca^{2+} waves (Scemes and Giaume 2006), volume regulation (Scemes and Spray 1998), and adult neurogenesis (Kunze et al. 2009; reviewed by Pannasch and Rouach 2013).

The role of interastrocytic gap junctional coupling in the development and progression of epilepsy is still controversial (for review see Nemani and Binder 2005; Carlen 2012; Steinhäuser et al. 2012). According to the spatial buffering concept (see above) the astroglial network is expected to possess antiepileptic function, since reduction of astrocytic coupling would result in accumulation of extracellular K^+ and, consequently, to neuronal depolarization and a lowered threshold for seizure generation. In line with this hypothesis are results from transgenic mice with coupling-deficient astrocytes ($Cx30^{-/-}$ $Cx43^{flox/flox}$ hGFAP-Cre mice; dko mice). In these mice, clearance of K^+ but also glutamate was disturbed. Consistently, these mice displayed spontaneous epileptiform events, a reduced threshold for the generating epileptic activity (Fig. 8.2), increased synaptic transmission and enhanced activity-induced astrocytic swelling (Wallraff et al. 2006; Pannasch et al. 2011). Although these findings strongly support an anticonvulsive role of glial GJ networks, a potential seizure-promoting role emerged from the results by Rouach et al. (2008). They elegantly demonstrated that astroglial GJs mediate activity-dependent intercellular trafficking of metabolites from blood vessels to sites of high energy

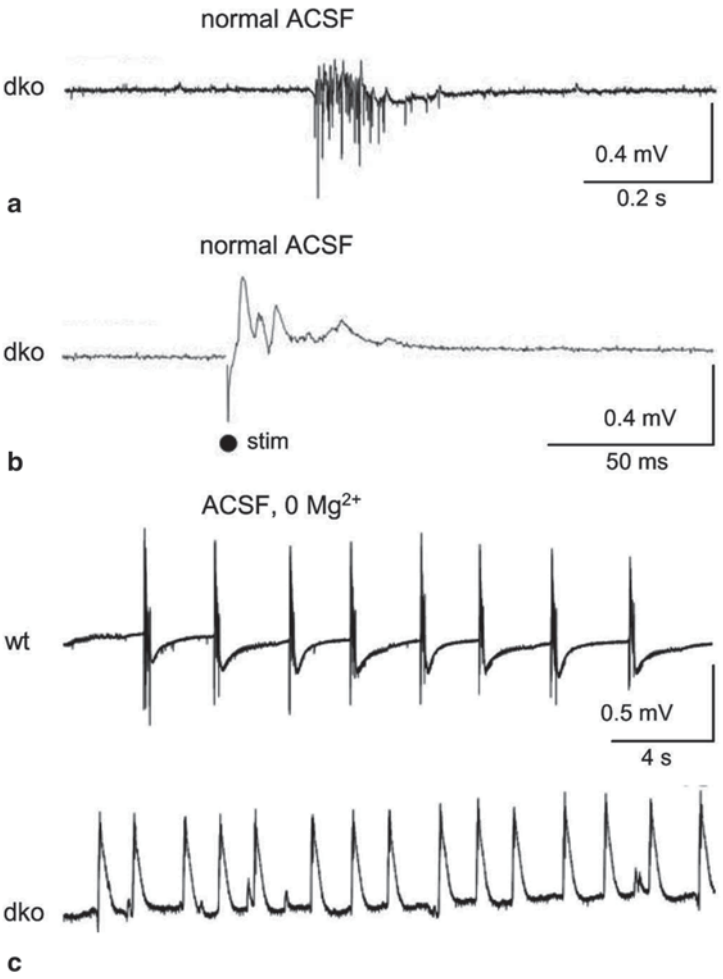

Fig. 8.2 Epileptiform field potentials (EFPs) in CA1 region of dko mice. **a** Spontaneous EFPs (CA1 region) occurred only in slices, bathed in artificial cerebrospinal fluid (*ACSF*), from dko mice but not in wild type (*wt*) mice (not shown). **b** Similarly, low-intensity Schaffer-collateral stimulation gave rise to EFPs only in dko slices. **c** EFPs induced by washout of Mg^{2+} (0 Mg^{2+}) were more frequent in dko slices and occurred with shorter latency. Original traces from wt (*top*) and dko (*bottom*) mice. (From Wallraff et al. (2006), reproduced with permission)

demand, suggesting that this process is essential for the maintenance of synaptic activity under pathological conditions such as epilepsy. Additionally, involvement of GJ channels in the intercellular spread of Ca^{2+} waves favors a proconvulsive role of the astroglial syncytium, since alterations in the astrocytic coupling would influence the propagation of Ca^{2+} waves and, therefore, neuronal synchronization and spread of ictal activity (Gomez-Gonzalo et al. 2010). Taken together, astroglial GJ networks might play a dual role in epilepsy, combining pro- and antiepileptic

properties. Further work is needed to elucidate which of the mechanisms prevails under various circumstances.

8.3.2 Connexin Expression and Coupling in Epileptic Tissue

Seizure-induced changes in Cx expression have been investigated in several studies using a variety of animal models and human tissue (for reviews see Nemani and Binder 2005; Giaume et al. 2010; Steinhäuser et al. 2012). The results are conflicting and do not allow drawing definitive conclusions. In animals, increased (Gajda et al. 2003; Samoilova et al. 2003; Szente et al. 2002; Condorelli et al. 2002; Takahashi et al. 2010; Mylvaganam et al. 2010), unchanged (Khurgel and Ivy 1996; Li et al. 2001; Söhl et al. 2000; Xu et al. 2009) and decreased (Elisevich et al. 1997a, 1998; Xu et al. 2009; David et al. 2009) Cx43 and/or Cx30 transcript and/or protein have been reported. This inconsistency might be explained by differences between animal models, seizure duration and investigated brain area. In human specimens, mainly up-regulation of Cx43 transcript and/or protein has been described (Aronica et al. 2001; Collignon et al. 2006; Fonseca et al. 2002; Naus et al. 1991), although unchanged levels have also been reported in one study (Elisevich et al. 1997b). However, Cx expression does not necessarily reflect the extent of functional coupling, since post-translational modifications, such as phosphorylation, might alter GJ channel unitary conductance, open probability, trafficking or internalization. Hence, functional coupling analyses are indispensable to receive reliable results. Increased astrocytic coupling has been reported in a post-*status epilepticus* (SE) rat model of epilepsy (Takahashi et al. 2010), and in hippocampal slice cultures chronically exposed to bicuculline (Samoilova et al. 2003). In contrast, Xu et al. observed significantly reduced coupling in the hippocampal CA1 region in a genetic mouse model of tuberous sclerosis complex (Xu et al. 2009). Human coupling studies have so far only been performed on primary astrocyte cultures derived from epileptic specimens (Lee et al. 1995). Using fluorescence recovery after photobleaching (FRAP), these authors found enhanced astrocyte coupling in cells from epileptic specimens.

Another approach to assess the role of GJ channels in epilepsy is pharmacological disruption of inter-astrocytic communication through GJ blockers, substances producing intracellular acidification or Cx mimetic peptides. Such experiments have been performed in a variety of *in vivo* and *in vitro* animal models of epilepsy (Bostanci and Bagirici 2006, 2007; Gajda et al. 2003; Gigout et al. 2006; Jahromi et al. 2002; Kohling et al. 2001; Medina-Ceja et al. 2008; Perez-Velazquez et al. 1994; Ross et al. 2000; Samoilova et al. 2003, 2008; Szente et al. 2002; Voss et al. 2009). Most of these studies reported anticonvulsive effects of GJ inhibition although opposite effects were observed in the study by Voss et al. (2009). In neocortical slices from patients with MTLE or focal cortical dysplasia, GJ blockers attenuated spontaneous and evoked epileptiform activity (Gigout et al. 2006). Major problems with using GJ blockers are their significant side effects and poor Cx isoform-specificity.

In conclusion, Cx expression studies, functional coupling analyses and uncoupling experiments yield an inconsistent picture on the role of the astroglial network in the pathophysiology of epilepsy. Further work is needed to clarify this issue.

8.3.3 Role of Cx Hemichannels and Pannexin Channels in Epilepsy

In addition to inter-cellular communication, functional membrane-spanning Cx hemichannels (HCs) have been demonstrated in astrocytes. These channels are non-selective and permeable for large molecules, such as ATP, glutamate, glucose and glutathione. Under normal conditions these channels are closed, but the open probability increases upon depolarization, altered intra- and extracellular Ca^{2+} concentration, metabolic inhibition or proinflammatory cytokines (reviewed by Theis and Giaume 2012; Orellana et al. 2009, 2013). It has been suggested that activated HCs promote neuronal hyperactivity and synchronization through excessive release of ATP and glutamate, which in turn increases excitability and Ca^{2+} wave propagation (Bedner and Steinhäuser 2013). GJ channels and HCs are oppositely regulated by proinflammatory cytokines (Meme et al. 2006; Retamal et al. 2007) and may play differential roles in epilepsy. Indeed, Yoon et al. (2010) showed in hippocampal slice cultures exposed to bicuculline that selective inhibition of HCs by low concentrations of mimetic peptides had protective effects on seizure spread while blockade of both HCs and GJs by high doses of the peptide exacerbated the lesion.

In addition to the Cx HCs, another family of proteins, termed pannexins (Panx1–3), can form functional transmembrane channels (but not inter-cellular channels) in different cell types, including astrocytes and neurons. Like Cx HCs, pannexins possess a very low open probability at rest, which increases upon elevated $[K^+]_o$, depolarization, increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), mechanical stress and $P2X_7$ receptor activation (Scemes and Spray 2012; Suadicani et al. 2012). It has been hypothesized that Panxs contribute to seizures by releasing ATP (Santiago et al. 2011). Indeed, increased Panx transcript levels have been found in an *in vitro* seizure model (Mylvaganam et al. 2010). Moreover, pharmacological inhibition or genetic deletion of Panx1 resulted in reduced seizure activity during kainate-induced SE (Santiago et al. 2011).

8.4 Aquaporin-4 Dysfunction and Epilepsy

8.4.1 Role of Aquaporin-4 in Epilepsy

The aquaporins (AQPs) are a family of small (24–30 kDa) integral proteins that mediate transmembrane water movement in response to osmotic gradients. So far, fourteen AQPs have been identified in mammals. AQP4 is the predominant water

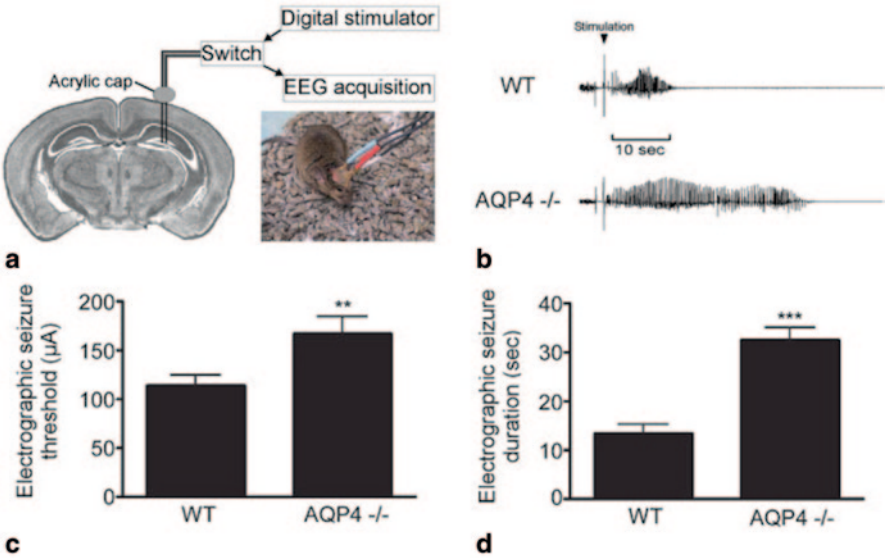

Fig. 8.3 Electrographic seizure threshold and duration in wt vs. AQP4$^{-/-}$ mice. **a** Bipolar electrodes implanted in the right hippocampus, and sealed to the skull by an acrylic cap, were connected to a stimulator and an EEG acquisition system. Mice were awake and behaved normally at the onset of stimulation (*inset*). **b** EEG from wt and *Aqp4*$^{-/-}$ mice. Baseline EEG prior to stimulation is similar (*left*). Hippocampal stimulation-induced electrographic seizures are shown for a wt (*top*) and an *Aqp4*$^{-/-}$ mouse (*bottom*). Note the prolonged seizure of the *Aqp4*$^{-/-}$ mouse. Behavioral arrest was observed in both animals during the seizure. Postictal depression is evident on the EEG in both mice. **c** *Aqp4*$^{-/-}$ mice had a higher electrographic seizure threshold than wt controls. **d** *Aqp4*$^{-/-}$ mice had remarkably longer stimulation-evoked seizures compared to wt controls. (From Binder et al. (2006), reproduced with permission)

channel in the brain, where it is mainly localized to astrocyte perivascular endfeet as well as perisynaptic processes (for review see Binder et al. 2012; Papadopoulos and Verkman 2013). AQP4 has been implicated in the pathogenesis of epilepsy mainly due to their role in regulating extracellular fluid osmolarity and extracellular space (ECS) volume (Schwartzkroin et al. 1998). Indeed, several studies have demonstrated that osmolarity-induced reduction in the ECS volume causes neuronal hyperexcitability (Dudek et al. 1990; Roper et al. 1992; Chebabo et al. 1995; Pan and Stringer 1996), while increasing the ECS volume attenuates epileptiform activity (Traynelis and Dingledine 1989; Dudek et al. 1990; Pan and Stringer 1996; Haglund and Hochman 2005). In addition to ECS volume regulation, the spatial overlap of AQP4 with Kir4.1 in glial endfeet gave rise to the hypothesis that AQP4 may be involved in K^+ homeostasis (Binder et al. 2012).

Mechanistic insight into the role of AQP4 in ECS volume regulation, K^+ clearance and neuronal excitability came from transgenic mice. AQP4-deficient (AQP4$^{-/-}$) mice display mild ECS volume expansion as assessed by FRAP and the tetramethylammonium method (Binder et al. 2004b; Yao et al. 2008). In acute seizure models, AQP4$^{-/-}$ mice exhibited elevated seizure threshold but prolonged seizure duration (Fig. 8.3) and enhanced frequency of spontaneous seizure during

the early phase (Binder et al. 2004a; Binder and Steinhäuser 2006; Lee et al. 2012). In addition, *in vivo* and *in situ* studies using K^+ sensitive electrodes or a fluorescent K^+ sensor revealed impaired stimulus-induced $[K^+]_o$ clearance in AQP4-deficient mice (Binder and Steinhäuser 2006; Padmawar et al. 2005; Strohschein et al. 2011). Interestingly, K^+ spatial buffering was enhanced in AQP4$^{-/-}$ mice, probably due to improved GJ coupling (Strohschein et al. 2011). The expanded ECS volume found in AQP4-deficient mice offers an explanation for the elevated seizure threshold of these mice, while the impaired K^+ uptake might account for the prolonged duration and increased frequency of seizures. As described in Sect. 8.2.1 above, insufficient $[K^+]_o$ clearance would result in neuronal hyperexcitability. However, the mechanistic link between AQP4 expression and K^+ homeostasis has not been resolved so far. The view of a functional interaction between the AQP4 and Kir4.1 (Nagelhus et al. 1999) is not supported by follow-up studies showing AQP4-independent Kir4.1 function (Ruiz-Ederra et al. 2007; Zhang and Verkman 2008; Strohschein et al. 2011). A more reasonable hypothesis implies that astrocytic K^+ uptake during neuronal activity triggers AQP4-dependent osmotic water uptake and, therefore, reduction of the ECS. ECS shrinkage, in turn, causes an increase of $[K^+]_o$ and consequently further K^+ uptake by astrocytes (Papadopoulos and Verkman 2013). Recently, this hypothesis was supported by mathematical modeling (Jin et al. 2013).

8.4.2 AQP4 Expression and Regulation in Epileptic Tissue

AQP4 expression was investigated in hippocampi from MTLE patients using rtPCR, immunohistochemistry and gene chip analysis (Lee et al. 2004). The authors found enhanced AQP4 levels in HS, but reduced expression of the dystrophin gene, which encodes the protein that is involved in anchoring AQP4 in perivascular endfeet, and speculated that polarity in astrocytic AQP4 distribution got lost. This finding was subsequently confirmed with immunogold electron microscopy and Western blot analysis (Eid et al. 2005). This locally restricted reduction of AQP4 was accompanied by a loss of perivascular dystrophin, indicating that AQP4 mislocalization was caused by a disrupted dystrophin complex. Similar loss of perivascular AQP4 and dystrophin were found in tissue from patients with focal cortical dysplasia (Medici et al. 2011). Further indication for the involvement of AQP4 in epilepsy and for the proposed interplay between AQP4 and Kir4.1 comes from genetic studies showing that several SNPs in the *KCNJ10* and *AQP4* genes are associated with MTLE (Heuser et al. 2010; see also paragraph 2.2).

In a recent study, Alvestad et al. (2013) explored, in the kainate model, whether the loss of perivascular AQP4 found in human MTLE is involved in epileptogenesis or merely represents a consequence of the condition. They could demonstrate that AQP4 mislocalization precedes the chronic phase of epilepsy, suggesting that astrocytic dysfunction is of pathophysiological relevance.

8.5 Altered Glutamate Homeostasis in Epilepsy

8.5.1 Extracellular Glutamate Levels in MTLE

Astrocytes modulate synaptic transmission and neuronal excitability by controlling extracellular neurotransmitter concentrations in the central nervous system (CNS). Rapid clearance of excessive glutamate from the ECS as well as its recycling is essential for survival and normal brain function. To prevent excitotoxic accumulation of glutamate in the ECS, glutamate is taken up by astrocytes via specialized transporters, converted to glutamine by GS and shuttled back to neurons for re-synthesis of glutamate. Dysfunction of the glutamate metabolism has been proposed to be critically involved in the pathophysiology of epilepsy (Eid et al. 2008b; Coulter and Eid 2012). This assumption is supported by the fact that glutamate and glutamate analogs cause seizures and neuronal loss in experimental epilepsy (Olney et al. 1972; Nadler and Cuthbertson 1980; Ben-Ari 1985; Fremeau et al. 2002). Moreover, increased interictal glutamate levels and stronger seizure-induced glutamate transients were found in the hippocampi of MTLE patients (Cavus et al. 2005; During and Spencer 1993). Among MTLE patients, those with HS displayed higher interictal extracellular glutamate levels (Petroff et al. 2003; Coulter and Eid 2012). However, as discussed by Coulter and Eid (2012), the source of glutamate in HS is obscure, since one of the hallmarks of this pathology is loss of glutamatergic neurons in the hippocampal CA1 region.

8.5.2 Astrocytic Glutamate Uptake in Epilepsy

Astrocytes take up glutamate from the ECS via high-affinity glutamate transporters (excitatory amino acid transporters, EAATs), which utilize the electrochemical gradient of Na^+ and K^+ as a driving force. Five transporter isoforms have been identified and two of them, GLAST (in rodents; in human EAAT1) and GLT1 (in rodents; in human EAAT2), are preferentially expressed in astrocytes. The impact of astrocytic glutamate uptake became obvious from the phenotype of genetically engineered mice devoid of transporter proteins. GLT1 knockout mice displayed lethal spontaneous seizures, increased susceptibility to acute cortical injury and seizures after administration of subconvulsive doses of pentylenetetrazole (PTZ) (Tanaka et al. 1997). Consistently, pharmacological inhibition of GLT1 in rat neocortex reduced the threshold for evoking epileptiform activity (Demarque et al. 2004; Campbell and Hablitz 2004). In contrast, antisense knockdown of GLT1 in adult rats caused increased extracellular glutamate, but not seizures (Rothstein et al. 1996). Mice deficient in GLAST showed no spontaneous seizures or electroencephalography (EEG) paroxysmal discharges, but amygdala kindling or PTZ-induced seizures were of longer duration, more severe and occurred after a shorter latency in mutant mice (Watanabe et al. 1999).

In MTLE patients, inconsistent data on the regulation of EAAT1 and 2 have been published; no changes in transporter expression (Tessler et al. 1999; Eid et al. 2004) or decreased levels of EAAT 1/2 were found in epileptic hippocampi (Mathern et al. 1999; Proper et al. 2002). In kindled rats, unchanged GLT1 and GLAST levels were described (Akbar et al. 1997; Miller et al. 1997; Simantov et al. 1999) while decreased levels were found in the pilocarpine (Lopes et al. 2013) and albumin models (David et al. 2009) as well as in a tuberous sclerosis epilepsy model (Wong et al. 2003). Finally, Guo et al. (2010) observed decreased GLAST but unaffected GLT1 expression in the hippocampus of spontaneously epileptic rats.

Taken together, the data described so far indicate that glutamate uptake by astrocytes plays a crucial role in protecting neurons from hyperexcitability and excitotoxicity. However, whether this mechanism is disturbed in epilepsy is still under investigation.

8.5.3 Regulation of GS in Epilepsy

In the CNS, GS is predominantly expressed by astrocytes where it converts glutamate and ammonia to glutamine. Impaired GS activity has been hypothesized to play a crucial role in the pathogenesis of MTLE (Eid et al. 2013b). This hypothesis is supported by the following observations: (i) unilateral intrahippocampal infusion with the GS inhibitor methionine sulfoximine causes recurrent seizures and neuropathological changes similar to human MTLE (Eid et al. 2008a; Wang et al. 2009; Perez et al. 2012); (ii) reduced GS protein and enzyme activity have been found in MTLE-HS patients (Fig. 8.4) (Eid et al. 2004; van der Hel et al. 2005); interestingly, in a recent study no reduction of GS mRNA could be found in HS in the CA1 region, suggesting posttranscriptional modification of GS in epilepsy (Eid et al. 2013a); and (iii) mutations in the GS encoding gene, *GLUL*, are associated with reduced GS activity and epileptic seizures (Haberle et al. 2005, 2006, 2011). One argument against a causative role of GS dysfunction in epileptogenesis, however, arises from a study performed in a kainate model (Hammer et al. 2008). The authors demonstrated that although GS protein level was reduced during the chronic phase (confirming the findings in human tissue), increased GS levels were found in the latent period (prior to seizure onset). The mechanistic link between GS downregulation and seizure development is provided by the assumption that GS deficiency and the resulting decrease in glutamate to glutamine conversion causes intracellular glutamate accumulation. This accumulation, in turn, slows down glutamate uptake, leading to increased glutamate levels in the ECS (Coulter and Eid 2012; Eid et al. 2013b). Support for this view emerges from recent work showing that GS inhibition causes indeed glutamate accumulation in hippocampal astrocytes (Perez et al. 2012).

The glutamate-glutamine cycle is essential for replenishing the neurotransmitter pool and maintenance of synaptic activity. Interruption of the cycle through GS

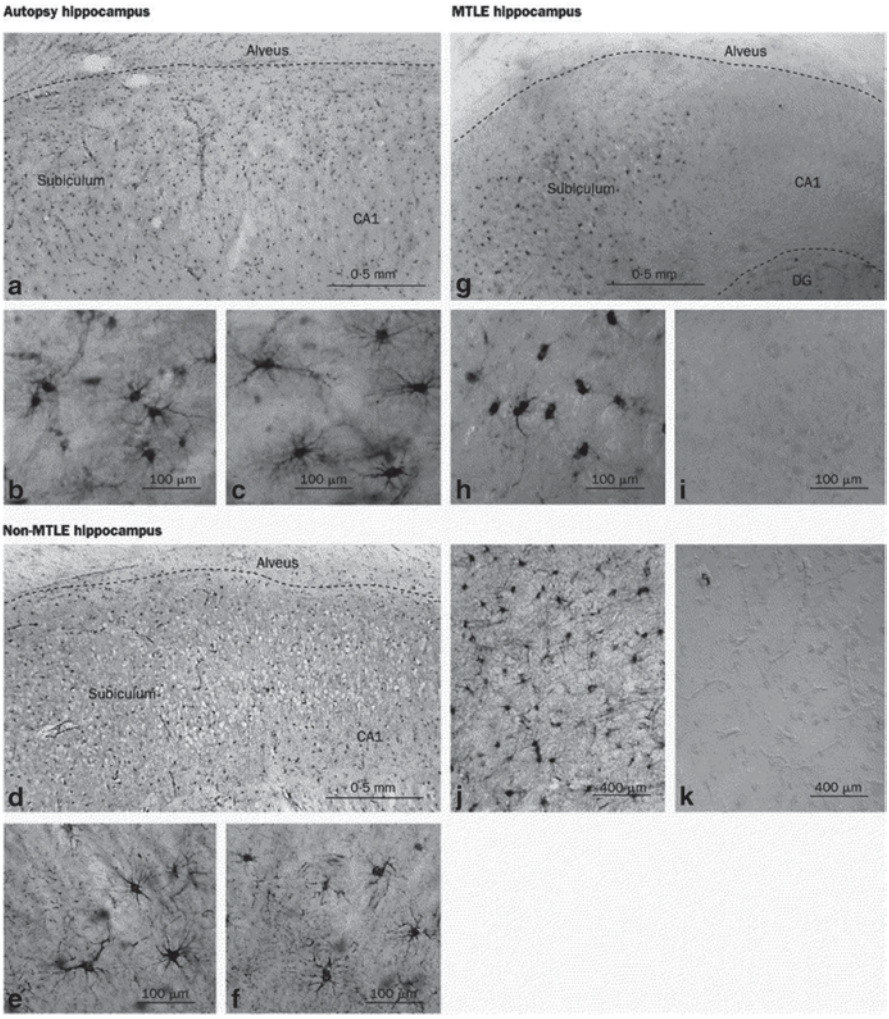

Fig. 8.4 Decreased GS immunoreactivity in the CA1 region of MTLE patients. There is dense and even distribution of GS-positive cells in the subiculum and CA1 region of autopsy (**a**) and non-MTLE hippocampi (**d**). High-power fields of subiculum in autopsy (**b**) and non-MTLE (**e**) hippocampi show that staining is confined to astrocytes. High-power fields of area CA1 in autopsy (**c**) and non-MTLE (**f**) hippocampi also show many GS-positive astrocytes. In MTLE hippocampus (**g**), there are many GS-positive cells in the subiculum but area CA1 is severely deficient in GS staining. High-power view of the subiculum in G (**h**) confirms presence of staining in astrocytes, which have fewer processes than GS-positive astrocytes in the corresponding areas of autopsy (**b**) and non-MTLE hippocampi (**e**). High-power view of area CA1 in G (**i**) confirms lack of GS staining in this region. Specificity controls with GS antiserum (**j**) and preimmune serum (**k**) on adjacent sections of the non-MTLE hippocampus shown in **d**–**f** reveal no staining in **k**. *DG* dentate gyrus. (Modified from Eid et al. (2004), reproduced with permission)

inhibition impairs inhibitory, i.e.γ-aminobutyric acid (GABA)-ergic transmission (Liang et al. 2006), but had little effect on excitatory (glutamatergic) synaptic function (Kam and Nicoll 2007). This phenomenon as well as its consequences on network excitability has been studied in detail in an *in vitro* model of astrocytic gliosis (Ortinski et al. 2010). In this study, virus-induced gliosis in the hippocampus caused downregulation of GS expression and deficit in inhibitory, but not excitatory synaptic transmission. Employing voltage-sensitive dye imaging the authors showed that these inhibitory deficits entail network hyperexcitability, which could partially be reversed by exogenously supplied glutamine. These data emphasize the importance of proper GS function for inhibitory neurotransmission and prevention of seizure generation (Ortinski et al. 2010).

8.6 Astrocyte Ca^{2+} Signaling and Gliotransmission in Epilepsy

8.6.1 Ca^{2+} Signaling

Already in the nineties of the past century it has been shown that astrocytes in culture (Cornell-Bell et al. 1990; Charles et al. 1991) and acute brain slices (Porter and McCarthy 1996) respond to glutamate with elevations in $[Ca^{2+}]_i$ which propagate through the astroglial network. Increased astrocytic $[Ca^{2+}]_i$, in turn, induced release of gliotransmitter, including glutamate, ATP, D-serine and GABA (Halassa et al. 2007; Crunelli and Carmignoto 2013). Hence, astrocytes not only sense, but also regulate synaptic transmission, neuronal excitability and plasticity. In human brain, bidirectional signaling between neurons and astrocytes has been demonstrated only recently (Navarrete et al. 2013). However, the mechanism by which astrocytes release gliotransmitter is still controversially discussed.

Evidence for the involvement of astrocytic Ca^{2+} waves and concomitant release of transmitters in epilepsy emerge from studies in brain slices and *in vivo,* demonstrating increased Ca^{2+} oscillations in astrocytes during epileptiform activity, which could be suppressed by anti-epileptic drugs (Tian et al. 2005; Fellin et al. 2006). In addition, metabotropic glutamate receptors, which mediate astrocytic Ca^{2+} signals, are up-regulated in experimental (Aronica et al. 2000; Ulas et al. 2000) and human (Tang and Lee 2001; Kandratavicius et al. 2013) epilepsy. Pilocarpine-induced SE caused long-lasting elevation in astrocytic $[Ca^{2+}]_i$ and the resulting glutamate release contributed to neuronal excitotoxicity (Ding et al. 2007).

8.6.2 Glutamate Release

Glutamate release from astrocytes synchronizes neuronal firing through activation of extrasynaptic NMDA receptors (Fellin et al. 2004; Angulo et al. 2004). Epileptic

discharges are characterized by excessive, hypersynchronous neuronal activity, leading to the suggestion that astrocytic glutamate release underlies the simultaneous activation of multiple neurons during such an event. Support for this view came from Tian et al. (2005) who studied paroxysmal depolarization shifts (PDSs) after inhibition of synaptic activity by tetrodotoxin (TTX) and Ca^{2+} channel blockers. PDSs, which underlie interictal activity, were largely insensitive to TTX but sensitive to α-amino-3-hydroxy-5-methyl-isoxazole propionate (AMPA) and N-methyl D-aspartate (NMDA) glutamate receptor antagonists, indicating that they were triggered by release of glutamate from extrasynaptic sources. Since photolysis of caged Ca^{2+} in individual astrocytes evoked local PDSs, the authors concluded that astrocytes are the primary source of glutamate in experimental seizure models (Tian et al. 2005). This conclusion was challenged by a subsequent study showing that astrocytic glutamate is not required for the initiation of epileptiform activity, but might have a modulatory role (Fellin et al. 2006). This view is supported by an elegant study of Gomez-Gonzalo et al. (2010), who combined patch clamp recording and Ca^{2+} imaging during *in vitro* seizures to assess the role of astrocytes in the generation of epileptiform activity. Their data revealed that ictal, but not interictal, discharges generate astrocytic $[Ca^{2+}]_i$ elevations (Fig. 8.5). In a new *in vitro* model of focal seizures induced by local application of NMDA in the presence of 4-aminopyridine and low Mg^{2+}, astrocytic Ca^{2+} signals preceded ictal discharges (Losi et al. 2010). These early Ca^{2+} signals appeared to have a causative role in the generation of focal ictal discharges, since their inhibition prevented NMDA-stimulated ictal discharges, while their stimulation enhanced discharges. In contrast to the findings by Tian et al. (2005), no association between $[Ca^{2+}]_i$ changes in astrocytes and interictal events could be found in this study. The authors concluded that bidirectional signaling between neurons and astrocytes in hyperexcitable networks generate a recurrent excitatory loop that promotes focal seizures (Gomez-Gonzalo et al. 2010).

8.6.3 Astrocyte Release of ATP and Epilepsy

In addition to its role in propagating inter-astrocytic Ca^{2+} waves, ATP release from astrocytes directly modulates synaptic transmission (Kumaria et al. 2008). Extracellular ATP is rapidly hydrolyzed to adenosine (Zimmermann and Braun 1996). Adenosine, in turn, potently suppresses excitatory synaptic transmission through activation of presynaptic A1 receptors (A1Rs) (Pascual et al. 2005a; Fredholm et al. 2005), indicating an anticonvulsive role of astrocytic ATP release (see Sect. 8.7).

In addition, extracellular ATP may activate purinergic receptors, which can have both pro- and anticonvulsant consequences. For instance, Torres et al. (2012) reported that the decrease in $[Ca^{2+}]_o$ accompanying excitatory synaptic transmission triggers ATP release from astrocytes through Cx43 HCs that, in turn, enhances inhibitory transmission by activating P2Y1 receptors on interneurons. In contrast, ATP released through Panx1 channels aggravates seizures and prolongs SE (Santiago et al. 2011). Astrocytic ATP has been proposed to possess seizure-promoting properties through activation of postsynaptic P2X receptors. The consequential

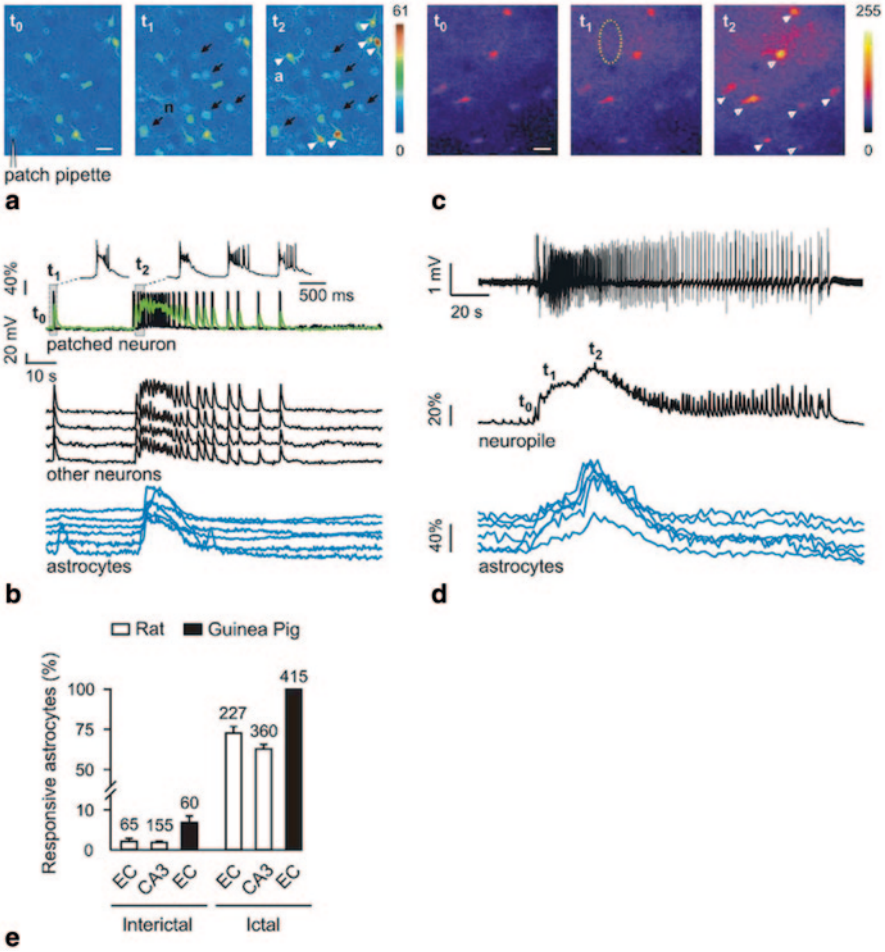

Fig. 8.5 Astrocytes respond with Ca^{2+} elevations to ictal, but not interictal, discharges. **a** Ca^{2+} changes at basal activity (t_0) in neurons (n, *black arrows*) and astrocytes (a, *white arrowheads*), during an interictal (t_1) or an ictal (t_2) event after perfusion with picrotoxin/zero Mg^{2+}. **b** Action potential bursts and Ca^{2+} change (*green trace*) of the patched neuron, and Ca^{2+} changes from other neurons and from astrocytes indicated in (**a**). Note the large Ca^{2+} rise in astrocytes upon ictal (t_2) but not interictal (t_1) events. **c** Ca^{2+} imaging in guinea pig entorhinal cortex before (t_0) and during (t_1 and t_2) ictal discharges induced by arterial perfusion with bicuculline. **d** Field potentials and Ca^{2+} changes from neuropil (*dashed oval* in **c**) and astrocytes (*arrowheads* in **c**) during ictal discharges. **e** Proportion of astrocytes activated during interictal and ictal events. Cell numbers are indicated above bars. (From Gomez-Gonzalo et al. (2010), reproduced with permission)

elevation in neuronal Ca^{2+} levels promotes the insertion of AMPA receptors and synaptic strength (Gordon et al. 2005).

Taken together, the role of gliotransmission in the pathophysiology of epilepsy is still unresolved. Clarification of the functional significance of the different

astrocyte-to-neuron signaling pathways represents an intriguing challenge and attracts increasing interest for developing new, anti-epileptogenic therapies.

8.7 Adenosine Dysfunction in Epilepsy

8.7.1 Role of Astrocyte-derived Adenosine in Epilepsy

The anticonvulsive action of adenosine has been demonstrated already three decades ago (Dunwiddie 1980; Lee et al. 1984; Dragunow et al. 1985). Since, various studies on experimental models and human epileptic tissue have confirmed and further elucidated these initial findings (Boison 2010, 2012). Extracellular adenosine levels rapidly rise during seizures, a process that probably mediates seizure termination and postictal suppression (During and Spencer 1992). Mice intracranially implanted with adenosine-releasing polymers, encapsulated cells or embryonic stem cells showed profound reduction of seizure activity in the kindling model (Boison et al. 1999, 2002; Huber et al. 2001; Li et al. 2007b). The anticonvulsive action of adenosine is mainly mediated via activation of G protein-coupled A_1Rs (Boison 2010, 2012). Consistently, A_1R agonists possess anticonvulsant and neuroprotective effects (Dunwiddie and Worth 1982; Barraco et al. 1984; Gouder et al. 2003; Li et al. 2013), while antagonists promote seizures and aggravate neuronal damage (Dunwiddie 1990; Ault et al. 1987; Avsar and Empson 2004; Vianna et al. 2005). Homozygous and heterozygous A_1R knockout mice exhibit spontaneous seizures in the CA3 area of the hippocampus (Li et al. 2007a). Furthermore, in the kainate model these animals displayed more severe SE and neuronal loss (Fedele et al. 2006). In human MTLE-HS (Glass et al. 1996) and rodent HS (Cremer et al. 2009; Aden et al. 2004) A_1Rs are downregulated.

Several studies have indicated that extracellular adenosine originates from hydrolyzed ATP released from astrocytes through Cx HCs, Panx channels (Kang et al. 2008; Santiago et al. 2011) or exocytosis (Pascual et al. 2005a). The latter study investigated the role of astrocytes in regulating synaptic transmission using transgenic mice, which overexpressed a dominant-negative soluble N-ethylmaleimide-sensitive factor attachment protein receptor (dn-SNARE mice) specifically in astrocytes. In these mice adenosine-mediated heterosynaptic depression was absent, confirming the astrocytic origin of extracellular adenosine (Pascual et al. 2005b).

8.7.2 Role of Glial ADK in Epilepsy

The ambient adenosine level is controlled by the activity of the enzyme ADK, which is critically involved in adenosine metabolism by phosphorylating adenosine to 5′-AMP, and which in the adult brain is mainly expressed in astrocytes. Since cytoplasmic and extracellular adenosine levels are tightly balanced by nucleoside

transporters, even small changes in ADK activity can influence extracellular adenosine concentrations and consequently neuronal excitability (Boison 2010; Aronica et al. 2013). Overexpression of ADK was found in human MTLE-HS (Aronica et al. 2011) and in experimental epilepsy (Gouder et al. 2004; Aronica et al. 2011; Li et al. 2008, 2012). Interestingly, in the intrahippocampal kainate model, Gouder et al. (2004) observed a transient reduction of ADK immunoreactivity during the first 24 h post SE, but pronounced overexpression during the later stages of epileptogenesis. The authors speculated that the initial reduction of ADK and the resulting increase in extracellular adenosine may contribute to termination of SE, while the enhanced ADK expression may contribute to chronic seizures by decreasing ambient adenosine. In experimental focal epilepsy, astrogliosis, ADK overexpression and spontaneous recurrent seizures are tightly associated (Li et al. 2008, 2007a, 2012).

In line with the expression studies, pharmacological inhibition of ADK potently inhibited seizures in experimental epilepsy (Kowaluk and Jarvis 2000; Gouder et al. 2004). Moreover, transgenic or viral overexpression of ADK in mice resulted in spontaneous recurrent hippocampal seizures, while viral knockdown of ADK prevented seizures. Since ADK overexpression was not accompanied by astrogliosis, these studies indicate that ADK overexpression is sufficient to trigger seizures and cause chronic seizure activity (Li et al. 2007a, 2008; Theofilas et al. 2011; Boison 2010, 2012; Aronica et al. 2013).

These above findings gave rise to the ADK hypothesis of epileptogenesis, which considers this enzyme both as a diagnostic marker as well as a potential therapeutic target to prevent epileptogenesis.

8.8 Astrocyte Immune Responses and Epilepsy

8.8.1 Astrocyte Changes Induced by Brain Inflammation

Brain inflammation has been implicated in the pathogenesis of epilepsy. Increased levels of inflammatory mediators, loss of blood-brain-barrier integrity and mono/lymphocyte infiltration were found in sclerotic tissue from MTLE patients as well as in animal models of TLE. There is growing evidence that inflammation is not only a consequence but also cause of epilepsy (Vezzani et al. 2011a). This assumption is supported by the following set of findings. (i) Cytokines like interleukin-1beta (IL-1β) and damage associated molecular pattern (DAMPs) like high mobility group box 1 (HMGB1) promote seizures in experimental epilepsy (Maroso et al. 2011). Preapplication of IL-1β or HMGB1 prolongs the duration of seizures induced by chemoconvulsant drugs (kainate, $GABA_A$ receptor antagonist bicuculline), while preapplication or overexpression of the corresponding antagonists or substances which inhibit IL-1β production attenuate seizure activity. Moreover, transgenic mice lacking functional receptors for the respective cytokines and

DAMPs displayed reduced susceptibility for seizures (Vezzani et al. 2000; Maroso et al. 2010). (ii) Seizures arising during fever (febrile seizures) are the most frequent type of seizures in children (Dube et al. 2012). (iii) Experimental induction of fever by lipopolysaccharide (LPS) injection or hyperthermia results in long-term enhancement of seizure susceptibility (Dube et al. 2012; Galic et al. 2008; Auvin et al. 2009, 2010). However, the mechanism linking inflammation and epilepsy is still unclear. Proinflammatory cytokines enhance neuronal excitability by directly modifying the function of neuronal voltage- and receptor-gated ion channels (Vezzani et al. 2012). However, IL-1β and other cytokines may also indirectly influence neuronal excitability by altering astrocyte function. For instance, in culture or acute rat slices, IL-1β and tumor necrosis factor α (TNFα) inhibit glutamate reuptake and increase glial glutamate release (Bezzi et al. 2001; Hu et al. 2000; Ye and Sontheimer 1996), which can be expected to produce hyperactivity. Furthermore, in cultured astrocytes cytokines inhibit Cx43 GJ channels, and open Cx43 HCs (Meme et al. 2006; Retamal et al. 2007). Intriguingly, cytokine-induced uncoupling in culture could be rescued by treatment with the anti-epileptic drug levetiracetam (Keppra®) (Haghikia et al. 2008). Whether astrocytic uncoupling and/or Cx HC activation is pro- or anticonvulsive needs to be clarified (see Sects. 8.3.2 and 3.3). Moreover, IL-1β and LPS increase ADK expression in human astrocyte cultures (Aronica et al. 2011). As described above (Sect. 8.7.2), ADK overexpression is sufficient to trigger seizures. Of note, glial Kir4.1 expression in culture is down-regulated after exposure to IL-1β (Zurolo et al. 2012). Given the essential role of Kir4.1 in K^+ buffering (Sect. 8.2), the consequence of this down-regulation on neuronal excitability seems to be evident.

Collectively, these findings demonstrate that proinflammatory mediators cause several dysfunctions in astrocytes, which individually or in concert provoke neuronal hyperexcitability.

8.8.2 Involvement of Astrocytes in Brain Inflammation

Astrocytes contribute to inflammation in the CNS by producing a variety of chemokines and cytokines (Aronica et al. 2012). Cytokines like IL-1β and TNFα are overexpressed by astrocytes in both human and experimental epilepsy (Vezzani et al. 2008). Importantly, during the latent period IL-1β was exclusively expressed by astrocytes in a rat model of epilepsy. In chronic experimental and human epilepsy about 10-fold more IL-1β-positive astrocytes than microglia were observed, indicating that astrocytes are the main source of IL-1β in the epileptic brain (Ravizza et al. 2008).

An important role in the astrocyte immune response has been suggested for the IL-1 receptor/toll-like receptor (IL-1R/TLR) superfamily (Maroso et al. 2010, 2011). In the human brain IL-1R and TLR4 are expressed at low levels in astrocytes under control conditions and are up-regulated in the sclerotic hippocampus from MTLE patients (Ravizza et al. 2008; Maroso et al. 2010). In the absence of

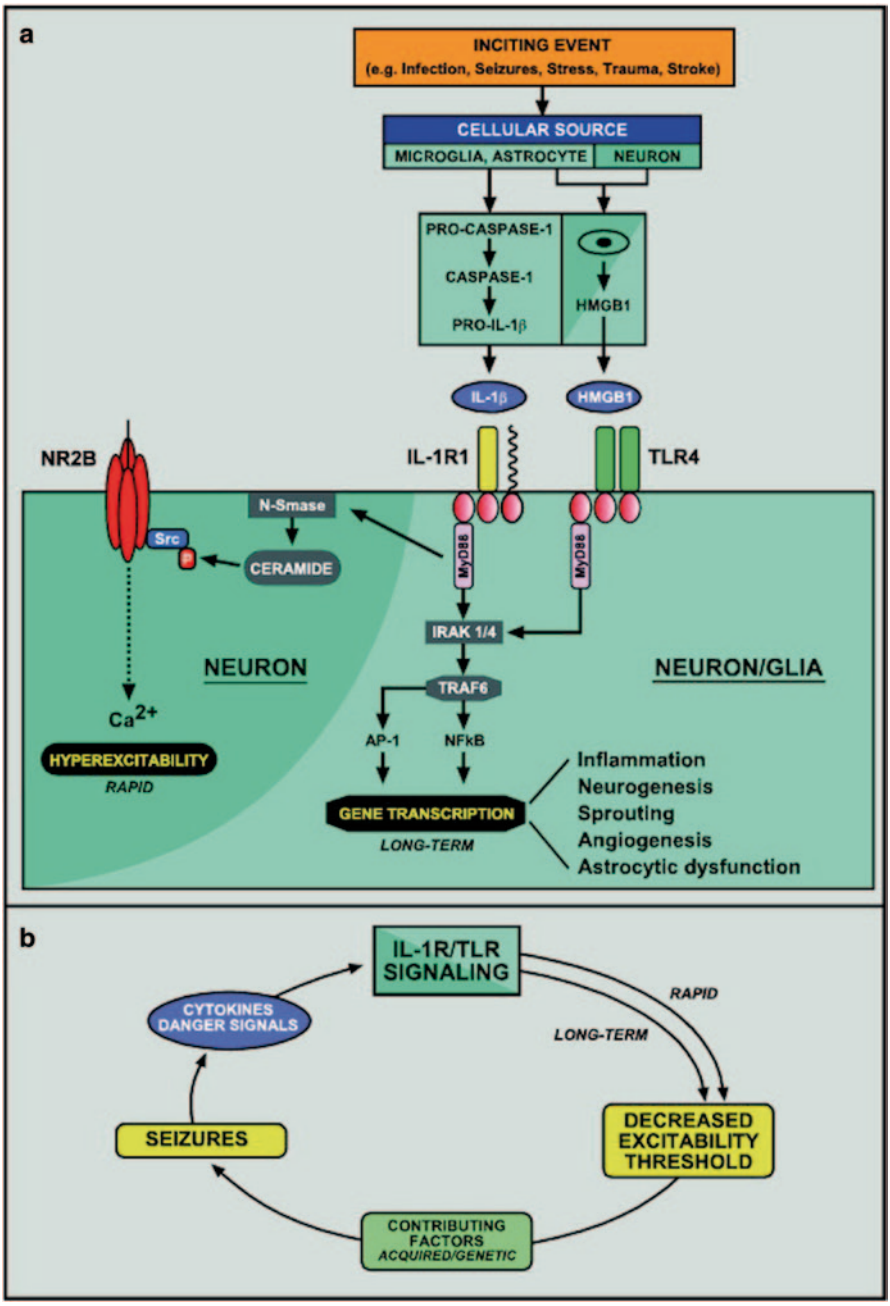

Fig. 8.6 Pathophysiological cascade mediated by IL-1R/TLR signaling in epilepsy. **a** Inciting events, initiated by local injuries or peripherally following infections, lead to activation of microglia, astrocytes and neurons. These cells release proinfammatory cytokines such as IL-1β, and danger signals such as HMGB1, thereby eliciting a cascade of inflammatory events in the target cells (i.e. neurons and glia) via activation of IL-1R1 and TLR4. This rapidly increases neuronal

pathogens, TLR signaling can be activated by DAMPs released by injured or dying cells. One member of the DAMP protein family, HMGB1, is released by cultured astrocytes upon IL-1β stimulation, and its nuclear to cytoplasmic translocation was observed in human and murine epileptic hippocampus (Maroso et al. 2010). Activation of IL-1R/TLR signaling induced Src kinase-dependent phosphorylation of the GluN2B subunit of the NMDA receptors, resulting in higher NMDA-dependent Ca^{2+} influx and neuronal hyperactivity (Vezzani et al. 2011b; Maroso et al. 2010). In addition, IL-1R/TLR signaling triggers, via nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) activation, transcription of several genes encoding downstream mediators of inflammation, like IL-6, TNFα or cyclooxygenase-2, which may further promote seizures (Vezzani et al. 2011b). Indeed, overexpression of NF-κB has been observed in the human sclerotic hippocampus (Crespel et al. 2002).

These findings led to a model for the involvement of the IL-1R/TLR axis in epileptogenesis (Fig. 8.6). An inciting event, like infection, seizures or stroke, leads to cellular stress and/or injury and release of IL-1β (from microglia and astrocytes) and HMGB1 (from astrocytes and neurons) from their constitutive endogenous pools. Activation of IL-1R/TLR signaling in neurons and astrocytes by the released molecules results in several cellular changes (such as GluN2B phosphorylation), which contribute to the generation of the first seizure. Seizures, in turn, induce *de novo* synthesis of IL-1β, HMGB1 and other proinflammatory mediators, which contribute to seizure recurrence (Vezzani et al. 2011b; Maroso et al. 2010). Thus, IL-1R/TLR signaling might represent promising targets for antiepileptic treatments.

Conclusions

The fact that astrocytes are now recognized as communication partners of neurons and do not merely represent "brain glue" has rekindled the question of the role of these cells in neurological disorders such as epilepsy. Compelling evidence is emerging demonstrating severe dysfunction of astrocytes in human and experimental epilepsy. However, several important questions still remain open. First, it is still unclear whether the reported alterations in astrocytes are causative of the condition or rather represent a compensatory phenomenon. Second, difficulties arise from the fact that the term "astrocyte" covers a heterogeneous group of cells, which complicates comparison of published work. Indeed, it is becoming increasingly clear that the molecular, functional and morphological properties of astrocytes do not

NMDA receptor Ca^{2+} conductance via ceramide/Src mediated phosphorylation of the GluN2B (NR2B) subunit, leading to hyperexcitability. Long-term decrease in seizure threshold results from activating transcription of genes, contributing to molecular and cellular changes involved in epileptogenesis and perpetuating inflammation. **b** Inflammation due to activation of IL1R/TLR signaling contributes to seizure generation by decreasing the threshold of neuronal excitability. Seizure recurrence, in turn, activates further inflammation, thereby establishing a vicious circle of events that contributes to the development of epilepsy. (From Vezzani et al. (2011b), reproduced with permission)

only vary across brain areas but even within a given subregion. Nevertheless, the combination of molecular genetics with functional approaches will help clarifying the specific roles of astrocytes in epilepsy and enable developing novel therapeutic approaches to better treat this disorder.

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Chapter 9
Microglial Biology in Neuroinflammatory Disease: Pharmaco-industrial Approach to Target Validation

Thomas Möller, Paul Dylan Wes and Dario Doller

Abstract Profound changes continue to shape scientific and business strategies in the pharmaceutical industry. Up until very recently, academic centers and corporations worked somewhat in isolation. However, over the last few years, two specific changes started to change this situation. On the one hand, academic organizations became more engaged in operations previously conducted mainly in the industry, such as actual drug design and high-throughput screening. Capabilities were enhanced, both human and technical, and consolidation of available "know-how" led to the establishment of several academic centers capable of influencing and making key contributions to early drug discovery research. Simultaneously, the pharmaceutical industry recognized the need to enhance their sources of innovation and engage in hitherto mainly unexplored areas of research, such as neuroinflammation. In the process, previously insular organizations became more open to collaborating, exchanging information and building knowledge with external partners. Challenges remain to maximize the productivity of these interactions, and to benefit the collaborating partners, and ultimately society, by boosting the success of drug discovery. Developing a common language to communicate respective views is a key step towards enabling the partners to learn from each other and work together. In recent years our company has established a variety of successful collaborations with external partners. This chapter summarizes at a high-level some of our current research processes, the learnings from our interactions with academic partners and our assessment of how to build strong academic-industry research partnerships.

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9.1 Introduction

Neuroinflammation has long been recognized as a pathological hallmark of a wide variety of neurological diseases. While activation of central inflammatory cells, particularly microglia, was initially interpreted as a reactive response to neuronal damage, growing evidence over the past decade suggests that neuroinflammation may contribute to disease progression (Weydt et al. 2002; Garden and Möller 2006; Hanisch and Kettenmann 2007; Möller 2010; Weinstein et al. 2010; Ellrichmann et al. 2013; Gandy and Heppner 2013; Nolan et al. 2013; Zhao et al. 2013). Consequently, efforts in the pharmaceutical industry have been mounting to target neuroinflammation as a therapeutic strategy to treat disorders of the central nervous system (CNS). This chapter aims to outline the high-level strategy by which companies approach drug discovery research, with a particular emphasis on novel microglial targets, and to address the critical criteria required to successfully bring new therapies to patients.

9.2 Target Identification and Validation

The successful development of a new drug is a herculean endeavor, with many potential pitfalls at each step of the way. On average it takes longer than a decade and more than a billion US$ to progress a drug discovery program from target identification to market (Bains 2004). Only a very small fraction of compounds make it through the whole drug discovery process and become *bona fide* drugs (Arrowsmith 2011). Therefore, it is critically important to minimize the risk of failure as early as possible, beginning with the selection of the best drug targets (Fig. 9.1).

One way to reduce this risk is to attempt to improve on existing drugs with well-established modes of action. However, to most significantly impact unmet medical need, new drug targets with novel mechanisms are required. New drug targets are discovered through a variety of means, from serendipitous discoveries in basic biology research to directed target identification screening efforts. Regardless of the source, it is critical to build a strong case that the target is relevant to the pathophysiology of the disease and that intervention would result in therapeutic benefit (Table 9.1). Furthermore, there must be a high likelihood that a successful drug can be developed with the appropriate pharmacological effect on the target. Often the first question is whether expression of the target is altered in the disease or associated with disease pathophysiology. Increased expression of a target in disease, for

Fig. 9.1 Stages in the drug discovery process. The drug discovery process often begins with the selection of a target using a variety of validation criteria. High-throughput screening (HTS) campaigns are then conducted as one of the ways to identify pharmacological modulators of the target. The output of high-throughput screens are relatively low potency, non-selective molecules called "hits". Hits are evaluated through a number of secondary assays and further optimized through chemical modifications to identify the most promising chemical matter, or "leads". Leads are further optimized to improve potency, increase specificity, and achieve the preferred physicochemical and metabolic properties

instance, not only increases confidence that the target contributes to the disease process, but also suggests that inhibition of the target may restore balance to a healthier state without resulting in significant adverse effects. Reduced expression of a target in the disease state, on the other hand, may point towards a need to augment functional activity. Unfortunately, it is particularly challenging to generate target expression data for CNS disorders compared to other disease areas, since human CNS tissue is obviously not as accessible as peripheral tissues where biopsies or blood draws are routine.

Alterations in expression are merely correlative and do not demonstrate any functional consequences. For this reason, demonstration that the target shows a reproducible genetic association with disease often provides more confidence of a mechanistic role. Mechanistic information can also be gleaned by pharmacologically or genetically assessing the function of the target in disease-relevant *in vitro* or *in vivo* disease models. As we discuss below, the latter criterion is fraught with many challenges, since multiple factors need to be met in order to ensure that the target function is indeed being accurately probed and that the models are indeed disease-relevant.

Table 9.1 Linking target to disease

Factors that support the role of a putative drug target in disease
Altered expression in disease
Component of disease pathophysiology
Mechanistic link to disease etiology
Genetic association with disease
Demonstration of beneficial effects in disease-relevant *in vitro* model
Knockout animals display beneficial phenotype
Pharmacological intervention using qualified probe compound(s) shows therapeutic benefit in animal model

Table 9.2 Criteria for selecting a new drug target

Target selection criteria	Examples
Link to disease	Genetic association, altered expression, preclinical or clinical validation
Chemically tractable	Precedents for target class; small molecule endogenous substrate/ligand
Likelihood for specificity	Some divergence from related family members
Safety	Restricted expression to target cell/organ; no overt knockout phenotype

There are other more pragmatic considerations for selecting a target, in addition to linking it to the disease (Table 9.2). Notably, the target must be considered chemically tractable, or "druggable". This is a somewhat amorphous term that is open to broad interpretation. Traditionally, a druggable target would be one in which a small molecule can bind with high affinity and specificity and mediate the desired biological effect. For instance, enzyme inhibition is considered a chemically tractable approach since a small molecule could be envisioned to occupy the active site and antagonize normal activity. Activation of a G-protein coupled receptor would also be considered feasible since a small molecule may be able to alter the conformation of the protein in the same way that the endogenous ligand would. By contrast, blocking a protein-protein interaction has traditionally been considered less druggable: since these are generally large surface interactions mediated by van der Waals forces as well as electrostatic interactions, they were thought to be difficult to disrupt with a small molecule. However, scientific advances are changing this view, and modulation of protein-protein interactions by small molecules is now an area of active growth (Higueruelo et al. 2013). Indeed, the scope of druggable targets continues to expand as new precedents are set, as drug screening technologies become more sophisticated, and as new drug modalities, such as monoclonal antibodies and antisense oligonucleotides, become available.

However, it is not merely necessary to be able to generate a potent pharmacological agent against the target; it is also important that the molecule can specifically modulate the target. Understanding the gene family to which the target belongs is useful in predicting the likelihood for specificity. For instance, if the target is highly related to another protein that it is not desirable to inhibit, then it may be difficult to identify specific inhibitors, and the value of the target will be diminished.

The final, and arguably most important, criterion for target selection is safety. Many drugs fail in the clinic, not because of lack of efficacy, but due to adverse safety events. Therefore, it is important to select targets with the greatest likelihood of being safe from the outset, and to address any potential safety concerns experimentally as early as possible. The first question to ask is how broadly the target is expressed. A target expressed specifically in the target cell and tissue presents some advantages, as there is less likelihood for adverse effects unrelated to the desired mechanism of action. Unfortunately, in the case of microglial targets, there are very few genes expressed in microglia that are not expressed in peripheral cells of monocytic origin, so immunosuppression is a persistent concern. This is in contrast to neuronal or astrocytic pathways, where it is often possible to identify targets with expression restricted to the CNS.

The potential safety of a target can also be assessed to some extent if a knockout animal for the target is available. If the knockout animal does not have any observable phenotypes, this is a good sign, with the major caveat that other genes may compensate for the complete loss of function of the target throughout development. A conditional knockout model where the target can be globally ablated only in the adult animal would be more informative than a constitutive knockout. If there are safety concerns, whether due to a knockout phenotype, suspected mechanistic roles, or some other information, it is important to address these directly in an experimental system. For instance, if immunosuppression is a concern, it might be important to challenge animals with an infection after drug administration to determine whether they are able to resolve it. Potential safety issues may not necessarily lead to immediate termination of a program, since there may be a large gap between doses that result in therapeutic efficacy and those that result in toxicity, thereby providing a therapeutic window. However, it is not sufficient to merely hope for the best and determine whether there is a viable therapeutic window in the clinic. The therapeutic window must be diligently determined in preclinical models using all tools available.

9.3 Assay Development

Once a target is identified, the next step is to screen for modulators of the target. A common approach is to employ high-throughput screening (HTS) of small molecule libraries to identify agonists, antagonists or allosteric modulators. In the pharmaceutical industry, an HTS campaign traditionally prosecutes libraries containing 10^5–10^6 compounds, and therefore must be run in a high-density plate format, usually 1,536-well plates. More recently, screening has become more sophisticated, employing smaller diverse libraries, fragment libraries, or other emerging technologies, rather than brute force approaches (Manly et al. 2008; Langer et al. 2009; Mayr and Bojanic 2009). The screens themselves are generally quite rapid. Most of the time and effort goes into developing the assay for the screen, and it is the quality of the assay that often determines the success of the screen.

Table 9.3 Assay development quality control

Factor	Measure
Precision of liquid handling	Coefficient of variation of volume transfer
Reagent stability	Assay performance remains the same over course of screen
Plate effects	Coefficient of variation of endpoint across plate; discernible patterns
Assay performance	Z′
Day-to-day consistency	Coefficient of variation of Z′ over different assay runs
Assay connectivity	EC_{50} or IC_{50} values comparable across multiple assays

EC_{50}, the concentration of a drug that gives half-maximal response; IC_{50}, the concentration of an inhibitor where the response (or binding) is reduced by half

High-throughput screening assays require more quality control (QC) than assays run in research labs (Table 9.3). This is in part because the assays must be robust enough to perform consistently from day-to-day executed by different scientists, and in part because each compound is run as a single replicate for practical reasons. A common metric used in research for assay performance is the coefficient of variation (CV), the ratio of the standard deviation (σ) to the mean (μ). However, this is not a sufficient metric for an HTS assay, since it does not incorporate the variability at the extremes of the assay and the size of the effect. A better metric to QC an HTS is the Z′ (Z-prime, also known as Z-factor) (Zhang et al. 1999). The Z′ is a measure of the magnitude of the window between the positive (p) and negative (n) controls as well as their variability:

$$Z' = 1 - 3\left(\sigma_p + \sigma_n\right) / \left|\mu_p - \mu_n\right| \tag{9.1}$$

The factor of 3 is chosen to multiply the sums of the standard deviations because in a normal distribution, 99% of values occur within 3 standard deviations of the mean. An assay with no variability ($\sigma = 0$) will have a Z′ of 1, and therefore 1 is the highest Z′ possible. However, due to the factor of 3 in the numerator, Z′s are very sensitive to assay variability. Indeed, in an assay with three replicates, a Z′ can be negative and still show highly significant differences between positive and negative controls, whereas a cutoff of 0.5 is generally required for HTS. This highlights the increased rigor required for screening compared to assays run in a more traditional basic research setting.

Developing *in vitro* assays in microglial cultures poses additional unique challenges. Microglia are specifically adapted to respond dramatically to the slightest signal of damage or infection. Therefore, even tiny amounts of contaminants, such as endotoxin present on most labware, can have major consequences on assay performance, even though they would not cause any noticeable effects on most other cell types. Standard protocols must be adapted so that special care is taken to minimize the effects of environment on microglia (Witting and Möller 2011).

There are additional considerations when establishing a screening assay. If automation is used, which is usually the case, the precision of liquid handling needs to be assessed. This can be achieved by transferring a dye, such as tartrazine (Petersen

and Nguyen 2005), at the desired volume to several plates and measuring the optical density against a standard curve. The assay endpoint should also be assessed across several plates to determine whether there are any artifacts amongst wells of a plate or from plate-to-plate. Since automation often adds time delays and assays are run over several hours, the stability of the assay components and of the assay itself needs to be determined. Finally, the assay must perform robustly from day-to-day over the course of the entire screen. Each run should include the appropriate controls, such as a whole column of the positive control and a whole column of the negative control on every plate, to ensure that the assay continues to perform with the appropriate metrics.

9.4 Hit-to-Lead and Lead Optimization

The output of screening campaigns using compound libraries are generally poor pharmacological agents, called "hits" or "actives", that serve as mere starting points in the drug discovery process. Hits must go through a number of quality assurance steps, secondary assays and some chemical optimization before promising lead compounds are identified (Fig. 9.2).

The first step is to retest a relatively large number of actives to confirm the biological activity found in the N = 1 screen. At this stage, some compounds will replicate the finding, and some will not. The former are considered "confirmed actives". Often a freshly-prepared sample is then tested to further confirm the *in vitro* activity. A number of counterscreens are performed to eliminate the possibility of false positives, such as tests on a parental cell line not expressing the receptor being studied, as well as a number of orthogonal tests which are usually run under more physiologically-relevant conditions than the screening assay.

This exercise often delivers up to a few hundred compounds ready to move forward. Sometimes an early readout of possible structure-activity relationships can be established. This is extremely valuable at this early stage, as it supports chemical tractability of the biological activity, which should not be taken for granted. Compounds are then typically analyzed based on a number of *in silico* physicochemical descriptors, which are used as surrogates of the drug-like quality of the compounds being studied. The most common descriptors used include molecular weight (MW), lipophilicity (cLogP), polar surface area (PSA), rotatable bonds (RB), and hydrogen bond donors and acceptors (HBD and HBA, respectively). For the specific case of CNS drugs a drug likeness central nervous system multiparameter optimization (CNS MPO) algorithm may be used (Wager et al. 2010).

After all these efforts, generally a number of distinct chemical series, called leads, are prioritized and the lead optimization work starts, aiming at delivering highly-optimized compounds known as drug candidates. The optimization process navigates through a number of hurdles to maximize confidence that the compound will be efficacious and safe when taken to the expensive clinical trials. For most projects this implies assuring oral bioavailability, good absorption and pharmacokinetics,

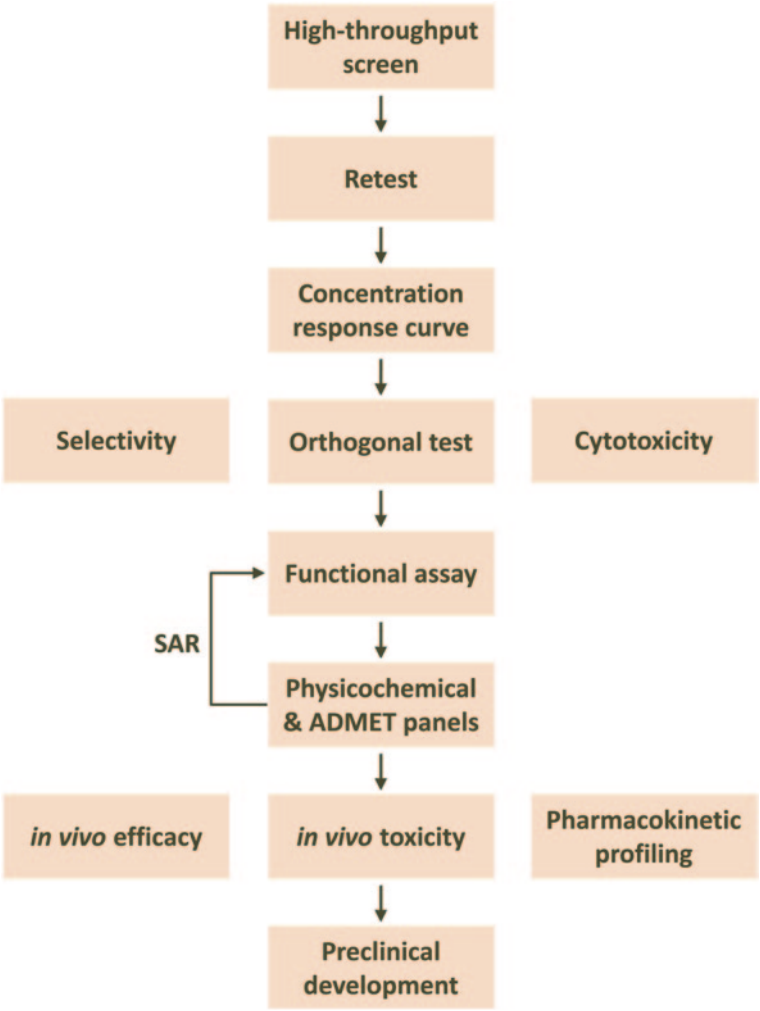

Fig. 9.2 Generic drug discovery screening cascade. After hits are identified from a high-throughput screen, they are first confirmed through retesting at the screening concentration, including parental cell line. Confirmed hits are then tested over a range of concentrations to generate concentration response curves and determine potency. Orthogonal tests confirm hits using technology completely independent of the screening assay to rule out potential screening artifacts. Orthogonal assays are usually more physiological than the HTS assay. At this point, compounds may also be evaluated for selectivity against proteins related to the target and, if they are going to be taken from a cell-free to cell-based assay, for general cytotoxicity. Secondary assays are then run to confirm the functional consequences of modulating the target. Compounds are then evaluated through a variety of *in vitro* physicochemical and ADMET (absorption, distribution, metabolism, excretion, toxicity) assay panels used to predict pharmacokinetic (PK) properties and potential safety liabilities. Structure-activity relationships (SAR) are determined via iterative medicinal chemistry to optimize PK, safety and activity, producing lead compounds. Lead compounds are tested *in vivo* to determine their PK, safety and efficacy profiles. The best compounds are progressed towards full preclinical development to identify candidates that will enter the clinic. It should be noted that this is a generic example. In keeping with the spirit and scope of this work, greater details are given for the early part of this process, and much less so for the later one. (For details on the later part of the preclinical drug development process, the reader is suggested to access a number of references (Wermuth 2008).)

efficacy at a reasonable daily dose, lack of drug-drug interactions derived from cytochrome P-450 (CYP) inhibition or induction, minimizing interactions with active transporters (*e.g.*, P-glycoprotein for CNS drugs), establishing an effect-exposure relationship *in vivo*, efficacy in phenotypic and mechanistic pre-clinical models (hopefully with good translational validity, *vide infra*), as well as a large battery of selectivity and safety tests *in vitro* and *in vivo*, to determine a therapeutic index and a maximum tolerable exposure during the clinical work (van de Waterbeemd 2009).

It is important to understand that, for the vast majority of new drug projects, the development candidate is not a compound that already exists as a member of a chemical library. On the contrary, medicinal chemistry efforts are often described as "threading the needle", to symbolize the highly sophisticated process that generally results in a very small number of molecular entities with acceptable attributes. Another point worth discussing is that *in vitro* potency should not be used in isolation as the key driver to rank-order compounds for further profiling. Indeed, it has been concluded that marketed oral drugs seldom possess single-digit nanomolar potency (50 nM is the average potency) (Gleeson et al. 2011). Therefore, a development candidate is often a compound with a number of balanced attributes rather than the molecule that performs significantly better in every possible test in the screening cascade.

9.5 Biological Validity Criteria: Predictive, Face and Construct Validities

A critical step in validating targets and evaluating lead molecules is to test them in preclinical animal models. A major pitfall has been the indiscriminate use of preclinical animal models without sufficient understanding of their validity. This paradigm worked for a while, when the exploited biological target space was a continuation of past successes. However, once these "low hanging fruit" of drug discovery were eventually depleted, and to continue to address unmet patient needs, industry had to venture into newer, more complex areas, and hence explore more sophisticated animal models (Meier et al. 2013).

The risk of failure is especially high for compounds reaching late clinical trials, where hundreds of millions of dollars may be spent for just one clinical study. In order to manage the risk involved in clinical translation of efficacy from preclinical models, a number of concepts were developed under the umbrella of the validity. While the concept is not new (Willner 1984), it is now a subject of increased focus (Fineberg et al. 2011).

There are three primary domains of validity that are sought after in animal models of disease: predictive, face and construct validity (Willner 1984; Markou et al. 2008; Becker and Greig 2010; Dzirasa and Covington 2012). Predictive validity refers of the ability of the model to predict pharmacological efficacy in the clinic. For instance, the Forced Swim Test (Porsolt et al. 1977) in which rodents are placed in a beaker of water has some predictive validity for depression because

monaminergic-based antipressants reduce animal immobility in this assay (although the observed rapid onset of efficacy in this model does not reflect the clinical experience). The major limitation for models with predictive validity is that they may not respond to drugs with novel, and maybe more efficacious, mechanisms of action. Furthermore, if there are no effective drugs in the clinic for a given disease, it would not be possible to know whether the model possesses any predictive validity for that disease.

Models with face validity overtly resemble the symptoms of the disease. For example, models in which dopamine neurons are ablated using exogenous toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (William Langston et al. 1984), exhibit some of the same motor symptoms as Parkinson's disease. The caveat for preclinical models with face validity is that they may not reflect the etiology of the disease in humans, and therefore may not respond to therapeutic interventions that target disease pathogenesis. They may however respond to treatments aimed at the symptoms of the disease.

The third type of biological validity is construct validity. Construct validity means that the model captures the underlying pathophysiology of the disease. This could be at the molecular level, such as when a transgenic animal model is generated by overexpressing a human mutant protein that causes an inherited monogenic disorder; or at the systems levels, such as by altering neuronal circuitry in a way that mimics alterations found in disease. The phenotypic manifestation of a model with construct validity may not resemble the disease at all. For instance, *Drosophila* mutants with loss of the *parkin* gene are arguably a model for early-onset Parkinson's disease with construct validity, yet the phenotype manifests as difficulty emerging from their pupal cases and defects in flight due to degeneration of muscle cells (Greene et al. 2003; Whitworth et al. 2005).

Ideally, a disease model will have all three types of biological validity. This is rarely, if ever, the case. Nevertheless, models with only one or two types of biological validity can be very useful in the drug discovery process, so long as their limitations are clearly understood and they are used to answer the right questions.

9.6 Additional Validity Criteria: Chemistry, Quantitative Pharmacology and Bias-free

In addition to the well-described types of biological validity, we propose three additional types of validity to consider when conducting preclinical studies using pharmacological probes in animal models. These are chemistry, quantitative pharmacology and bias-free validity (Fig. 9.3).

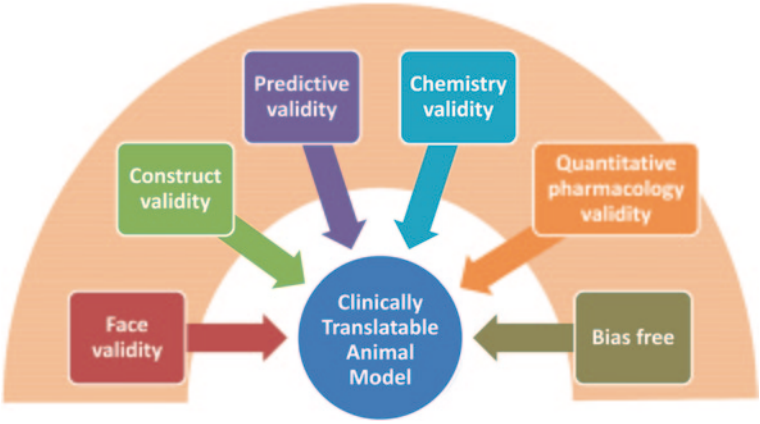

Fig. 9.3 Schematic proposal for enhancing biology-based validity criteria to include chemistry, quantitative pharmacology and decision-making principles useful in supporting scientific research aiming at validating biological targets for drug discovery projects

9.6.1 Chemistry Validity

The primary intent of a chemical probe is to establish the relationship between a molecular target and the broader biological consequences of modulating that target (Frye 2010). To this aim, a certain level of qualification of the compound being used across a number of areas is required. The goal of this exercise is to make sure the compound is actually doing what one thinks it must do, the way one expects it should do it, by ruling out other potential interferences of chemical origin.

Answering a number of key questions will go a long distance to support phenotypic observations and link these to a true pharmacological modulation of a biological target by a tool compound or drug candidate. For example,

- Is there a class effect? Do different compounds, with diverse chemical structures but similar target profiles, demonstrate similar pharmacology? Or is this a "one-off" effect, only seen with one compound and not seen with very close analogs?
- Does the tool compound have any druggability flaws derived from its chemical structure?

A number of properties related to the chemical structure of a certain tool compound may cloud the interpretation of phenotypic screens. Among these, the most common are shown in Table 9.4 (Davis and Erlanson 2013).

It is important to understand that these are not rigid rules, and are meant only to exemplify some of the weaknesses that may be encountered that bias data interpretation. For example, not all compounds containing a certain chemical moiety will be pan-assay interference compounds (PAINS) (Baell and Holloway 2010); it varies on a case-by-case basis and should be considered only as a risk management strategy.

Table 9.4 Some fundamental factors to be considered for available probe compounds depending on the nature of the study being planned

in silico	*in vitro*	*in vivo*, single-dose	*in vivo*, multiple-dose
Unequivocal structure	Functional activity and binding affinity at the target	Dosing routes: IV, IP, PO, SC, ICV	Dosing routes: IV, IP, PO, SC, minipump, ICV
Chemically synthesizable	Solubility in buffer used	Unbound drug concentration commensurate with *in vitro* activity	Understand exposure time profile and unbound drug concentration commensurate with *in vitro* activity at peak and trough
	Selectivity (binding and functional screens) against anti-targets and broad panel	Selectivity	Selectivity
		Possible active metabolites	Possible active metabolites CYP induction

IV intravenous, *IP* intraperitoneal, *PO* per os, i.e. oral administration, *SC* subcutaneous, *ICV* intracerebroventricular

9.6.2 What Type of Study Are You Planning?

Not all of a compound's properties are equivalent or critical in terms of qualifying it as an appropriate tool compound for the planned study. So, which molecular attributes are truly needed and how to define them? A useful way to address this issue is based on the type of pharmacological study to be conducted: *in silico*, *in vitro*, acute *in vivo* or multiple-dose (sub-chronic) *in vivo*.

For *in silico* work, one can of course design any virtual molecule "on paper" as long as its structure is unambiguously defined, but that does not mean the compound's structure can actually be put together, or that it will stay that way in the actual experiment. Care must be taken with chiral centers and other structural features that lead to an ambiguous definition of a molecule or an unstable arrangement of atoms (*e.g.*, tautomers, unstable atom arrangements, Fig. 9.4).

In vitro pharmacology is mostly conducted in buffered aqueous solutions, generally at pH values close to the physiologically germane value of 7.4 (unless a specific study is done to accommodate broader pH values that are found in certain tissues). If the test substance remains as a separate physical phase and cannot be brought in contact with the rest of the biological system under study (*i.e.*, it is insoluble) within the time frame of duration of the assay, the pharmacological experiment cannot be conducted. When this occurs, a number of solubilization techniques are routinely used to circumvent this major issue. However, one is frequently better off looking for a more water-soluble compound, as often times lack of solubility comes together

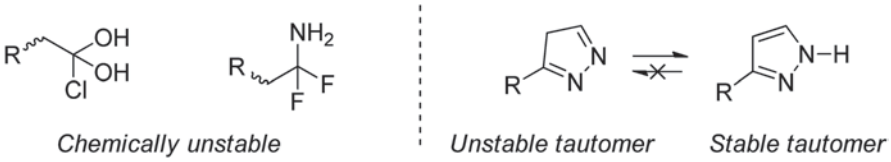

Fig. 9.4 Examples of chemical structures that may be drawn on "paper" for *in silico* studies but will not lead to synthesizable compounds due to chemical instability

with high non-specific binding (to plastic or glass surfaces as well as to the biological matrix, such as protein and lipids).

Importantly, selectivity of tool compounds against an anti-target (a biological receptor that must be avoided) or cell toxicity in toxicological screens is often assessed based on *in vitro* tests conducted at relatively high compound concentrations (in the 10–100 μM range). In this regards, solubility may hinder or confound the determination of these key enabling attributes.

If the compound can reach meaningful solubility in water and it interacts with the biological target being tested, the path forward is simpler. For overexpressed, recombinant systems used often for *in vitro* work, selectivity is not an issue. However, if then one turns to cell-based assays or *ex vivo* tissues for *in vitro* work, potential off-target interactions with other receptors that may now be expressed at similar levels as the target of interest must be kept in mind. Likewise, good cell membrane permeability is now an important requirement for the compound to reach certain types of targets (*e.g.*, intracellular enzymes).

For acute (single dose) *in vivo* studies, a different set of criteria comes to play to minimize the risk of misinterpreting phenotypic observations as related to the temporal concentration effects of the test compound. A number of routes of administration can be used, including intraperitoneal, intravenous, oral, subcutaneous, intracerebroventricular, etc. As long as the vehicle used is among those considered viable, any route may be fine. This is of course with the caveat that not all formulation vehicles are suitable to be administered in all routes. Capsules containing solid dosage forms for oral administration may be considered for some higher species like dog, with some distinct advantages.

The purpose of using an appropriate route of administration with relevant formulation is to deliver the drug to the desired site of action in large enough concentrations, as related to an *in vitro* measure of target affinity or functional potency, generally expressed as an IC_{50}, EC_{50}, etc. If the *free drug hypothesis* (*vide infra*) (Smith et al. 2010) is to hold within the target vicinity, then the unbound (free) drug concentration can be estimated. This estimation is calculated adjusting the total measured drug concentration by taking the unbound tissue fraction into consideration as shown in Eq. 9.2.

$$[Drug]_{unbound} = [Drug]_{total} \times [UB]_{tissue} \tag{9.2}$$

Unbound (UB) tissue fraction parameters (e.g., plasma protein or brain free fraction) are generally determined *in vitro* using relevant tissue homogenates. Often, for an antagonist (Kenakin 2009), receptor occupancy (RO) may be estimated based on Eq. 9.3.

$$\mathrm{RO\%} = 100/\left[1+\mathrm{IC}_{50}/[Drug]_{\mathrm{unbound}}\right] \tag{9.3}$$

So, when comparing similar compounds, a class effect may be established if a structurally diverse set of compounds provides a phenotypic effect at similar receptor occupancy values (Melhem 2013).

For multiple-dose *in vivo* work, in addition to the aforementioned criteria for single-dose studies, one has to consider the effects that prolonged drug exposure over time may have on the animal being studied. Thus, understanding the compound exposure-time profile is essential and achieving steady-state conditions may be of importance in these models. Potential changes range from those relatively simpler to explore, such as reduction of drug exposure due to CYP induction, or increases in drug exposure due to CYP inhibition or drug accumulation, to those more challenging to understand, such as changes in gene expression levels. In addition, the formation of circulating metabolites with their own pattern of biological activity, may contribute to the phenotypic readout otherwise attributed only to the parent drug. While this can certainly occur during single-dose studies, it becomes a much more significant risk during multiple-dose paradigm with *in vivo* work. If feasible, conducting a metabolite identification study with compounds of interest generally provides valuable information to mitigate this risk and design better multiple-dose studies.

Finally, we would like to express a word of caution about "repurposing" a compound used in human clinical work or even a marketed drug for use in preclinical studies. "Rats are not small humans." Their drug disposition mechanisms, as well as clearance pathways, may well not be the same, contributing to some of the caveats discussed earlier.

9.7 The Four Pillars of Target Validation

As discussed above, a full characterization of the chemical probes is essential to support the unbiased interpretation of biological experiments, which is a key element of rigorous preclinical target validation. It is to everyone's best interest that before making the commitments required to launch a drug discovery program, the biological target has been effectively validated using relevant assays. However, for a number of reasons this is not always the case, and several broadly used chemical probes exist which do not meet generally accepted potency and selection criteria, and therefore conclusions made from their use are suspect at best.

Pfizer scientists Mark E Bunnage, Eugene L Piatnitski Chekler & Lyn H Jones have put forward a framework for using chemical probes known as "the four pillars

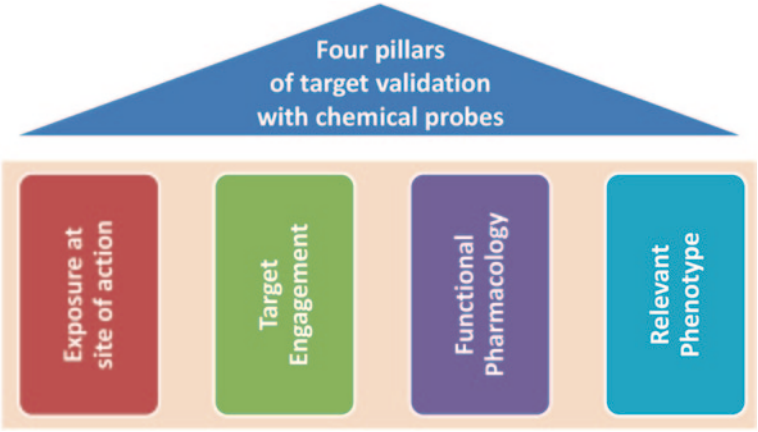

Fig. 9.5 The four pillars of target validation using chemical probes. Additional criteria may apply depending on the specifics of the target under investigation

of target validation" (Bunnage et al. 2013) (Fig. 9.5). While this is not meant to be a "one size fits all" solution to target validation, it is a good example of how strategic thinking can be applied during target validation using chemical probes. These key elements, which are not totally disconnected from those used in clinical drug studies (Morgan et al. 2012), are: establishing drug exposure at the relevant biophase, confirming target engagement, and confirming there is an actual functional pharmacology which is also relevant to the biological hypothesis under investigation.

Pillar 1: Exposure at the Site of Action This refers to the fact that regardless of the mechanism by which a tool compound exerts its effects, it must be able to reach the receptor localized at an appropriate bio-phase at pharmacologically relevant concentrations. For example, compounds exerting CNS effects *via* centrally-located receptors must be assessed for brain penetration. Importantly, the lack of exposure at the site of action does not rule out that the pharmacology observed may be real; it just negates the hypothetical mechanism of action. In particular, for intracellularly located biological targets, the ability of the chemical probe to penetrate the cell membrane (permeability) must be demonstrated. This is particularly important when assessing "false negatives" due to the probe's inability to enter a cell and reach its target rather than the lack of efficacy of a biological mechanism. Effective medicinal chemistry strategies exist to deal with this issue (Kerns and Di 2008).

Actual probe compound concentrations are often assumed to be "nominal concentrations" calculated based on those in a stock solution added to the test system. However, the presence of active uptake, transporter-mediated efflux or just slow permeability may hinder the probe compound from reaching the target. Hence, an experimental determination of the drug concentration *at the site of action* should be conducted. Exposures must also be commensurate with respect to the desired efficacious concentration of probe compound, as if these are in great excess over the on-target *in vitro* activity there may imply an erosion of the off-target selectivity, leading to misinterpretations.

Pillar 2: Target Engagement Receptor occupancy is a key element to link relevant drug concentrations to observed pharmacological effects. A number of different experimental techniques are routinely used to accomplish this task. Ideally, preclinical positron emission tomography studies provide valuable information in an *in vivo* setting (Linnman et al. 2013). A number of *ex vivo* techniques are often used. However, the interpretation of the data is not always straightforward (Grimwood and Hartig 2009). A possible approach to estimate preliminarily the levels of receptor engagement is to calculate the theoretical receptor occupancy (RO) using Eq. 9.4. This requires the determination of the free drug concentration in the appropriate biophase [A], and a measure of target affinity generally based in an *in vitro* assay (e.g., K_i).

$$\mathrm{RO\%}=100/\left[1+\mathrm{K_i}/[\mathrm{A}]\right] \tag{9.4}$$

During target validation, it is critical to minimize the risk that the activity measured in a given assay does not originate from an interfering biological target. In this regard, selectivity criteria for tool compounds are more stringent than for marketed drugs, where activity at a secondary target may be tolerable or even desirable.

Chemical probes acting *via* covalent binding to their biological targets deserve a special paragraph. While discouraged in the past due to potential for idiosyncratic toxicity, current views may be changing as can be inferred from an increasing number of drug candidates with a covalent mechanism of action progressing through clinical trials. It must be noted that technical challenges to fully characterize irreversible chemical probes are significant; a number of new strategies and tools have emerged and are expected to be expanded in the future (Mah et al. 2013)

Pillar 3: Expression of Functional Pharmacology Experimental means may be established to add support for the pharmacology derived from introducing a chemical probe into a biological system. Examples are the observation of changes of concentrations of an enzyme substrate or product (*e.g.*, the phosphorylated reaction product of a kinase) or measure changes in brain electrical circuitry using electrophysiology (*e.g.*, inhibition of post-synaptic excitatory currents).

Pillar 4: Proof of Phenotype Perturbation Preclinical-to-clinical translational challenges are well-known, especially for novel biological mechanisms of action. In part, this reflects a currentlyincomplete understanding of human and preclinical species disease and healthy-state physiology. Still, for a good number of indications, phenotypic tests exists that correlate with demonstrated efficacy in the clinic. These tests generally capture the most relevant biological changes in the context of the human disease. In such cases, probe compounds may be used to gain support for biological target validation.

Probe compounds may help achieve target validation even when providing negative results in a phenotypic screen. One such case, for example, is ruling out that a certain endpoint is derived from cell death instead of inhibition of a particular target (false positive). Another often used example is taking advantage of a chiral center in the chemical probe and when the biological activity resides mostly in one of the enantiomers. In such case, the biologically “inactive” enantiomer may be used

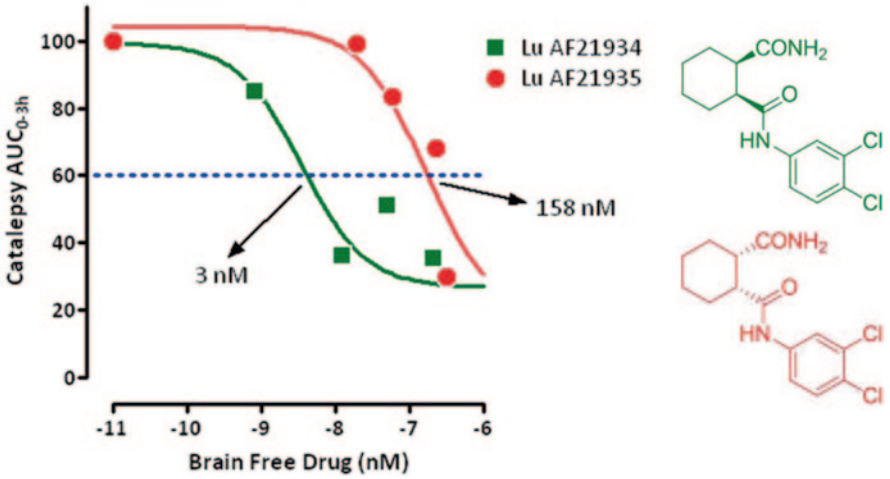

Fig. 9.6 Graph showing the a shift to the right for the relationship between the reversal of haloperidol-induced cataleptic response (phenotypic screen) and the unbound brain drug concentration of Lu AF21934 (in *green*) and Lu AF21935 (in *red*), the "active" and "inactive" enantiomers probe compounds, respectively, used in the validation of metabotropic glutamate receptor 4 as a Parkinson's disease target. (Bennouar et al. 2013)

as a negative control, as long as cross-reactivity and relevant tissue exposures are appropriately validated. These tests involve using probe compound doses beyond the minimum efficacy dose (MED), and often times a positive phenotypic response is obtained at a high dose or concentration, which originates as a result of a small percentage of the active enantiomer present as a contaminant, or due to potential cross-reactivity at probe high exposures. Practically, the extent of the "shift to the right" in the concentration- or dose-response graph must then be determined for the two enantiomers (Fig. 9.6). The observation of biological activity in a phenotypic screen *at high exposures* of the inactive enantiomer does not necessarily rule out a certain mechanism of action.

9.8 Quantitative Pharmacology

Not so long ago, *in vivo* pharmacological tests would be interpreted solely based on the phenotypic readouts. Due in part to advances in ADMET (absorption, distribution, metabolism, excretion, toxicity) science, and in part due to the extent of clinical failures, a view emerged postulating the need to take more integrative approaches, by linking the pharmacodynamic (PD) actions of a chemical probe to its pharmacokinetics (PK). This approach, known as 'quantitative pharmacology' or pharmacokinetics/pharmacodynamics (PK/PD), maximizes the information content and clarifies the temporal interdependence of the pharmacological properties and relevant tissue exposure characteristics (Gabrielsson et al. 2010).

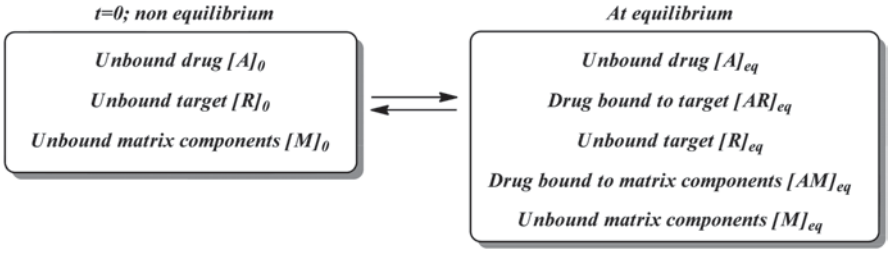

Fig. 9.7 Diagram showing changes in drug available to interact with the biological target. Non equilibrium concentrations correspond to nominal values. Equilibrium values take into account the non-specific binding that may occur to a variety of matrix components

An in-depth discussion of quantitative pharmacology is outside the scope of this work. Excellent discussions on practical PK/PD considerations for optimizing *in vivo* pharmacology studies have been published, and are highly recommended reading (Gabrielsson et al. 2010). However, two key points will be discussed in somewhat more detail here.

First, while human nature and probably our scientific training leads every one of us to simplify complex problems so that they become experimentally tractable and prone to be analyzed using mathematical models, we must not forget that Nature does not necessarily follow these principles. A large number of factors influence the way biological targets and their ligands interact in biological systems. Some of these are well-understood, but some are not. For example, for studies conducted *in vivo*, route of drug administration, vehicles used, rate and extent of drug absorption, dosing regimens, temporal differences and target turnover, receptor occupancy kinetics, differences in drug distribution, and non-specific tissue binding characteristics of drugs, all impact target engagement and therefore, are manifested in the observed pharmacology.

Second, in target validation studies, the *total* amount of drug at a given timepoint, in the relevant tissue is generally quantified using validated bioanalytical methods. However, as previously eluded to, the total concentration measured is often not what is available in the bio-phase to interact with the biological target. What is available is known as the *free (or unbound)* drug concentration. This difference is attributed to the vast majority of drug interacting with matrix components (*e.g.*, lipids and proteins *in vivo*, plastic or glass walls of labware used for *in vitro* tests) and as a consequence a new "equilibrium" state is generated (Fig. 9.7).

Thus, the free drug hypothesis is of particular importance when trying to establish the *relevant bio-phase* drug concentrations where the receptors are expressed (pillar 1, *vide supra*). Simply stated (Smith et al. 2010), the hypothesis is that:

a. at steady state, the free drug concentration is the same on both sides of any biomembrane
b. the free drug concentration at the site of action, the therapeutic target bio-phase, is the species that exerts pharmacological activity

The key implication is that measuring the total amount of drug in a certain tissue must be accompanied by a different measure of how strongly that drug interacts with matrix components. Exceptions are known to the free drug hypothesis, and include drugs with low passive permeability, or substrates of efflux or influx transporters present in the tissue expressing the therapeutic target.

9.9 Unpleasant Truths—Bias, Collaborations & (ir)Reproducibility

Sooner or later, data will be used to make decisions about a project. Given our imperfect understanding of disease biology, these decisions almost always imply incomplete knowledge. This is an area where better individual and team decision-making could enhance research performance. Indeed, it is well known that reproducible biases affecting human decision-making exist. Often known as cognitive biases, these jeopardize objectivity, and introduce a risk factor. These biases have been classified in overconfidence bias, calibration bias, availability bias, and excessive focus on certainty (Chadwick and Segall 2010). In a setting where projects compete against each other for (industry internal) resources, these biases pose a significant risk. Project leaders tend to start thinking of their projects as "their babies" and have a tendency to defend their projects beyond what would be considered reasonable. The *per se* laudable quality to "believe" in a project can turn into an obsession which keeps projects running against scientific evidence. In academia, where there is a constant need to publish, unsuccessful submission of manuscripts can have a limiting effect on work on "unpublishable" projects. In industry, where publications are usually a secondary aim and only happen with a delay during which intellectual property rights are secured, this external validation process is not in place. It is usually replaced by an internal review process which has to pay close attention not only to the scientific progress, but also needs to be aware of the "oh-so-human" biases. This internal review process is usually on an annual or biannual cycle. The outcome of such a project review determines the resources available to a project—they could be steady, increased or decreased. Due to the nature of pharmaceutical research, most projects will eventually close. The closure of projects leads to redistribution of resources and usually also affects academic collaborations.

Many investigators in academia have been in situations where they had industry contacts or even what they thought to be a successful collaboration with industry, and suddenly the industry partner walks away. Some academics may perceive this as "they were picking my brains and now they are secretly pursuing my idea". While we cannot speak for all in the pharmaceutical industry, we have not come across any case like this. Not only would we consider this unethical, this would deny our projects access to a valuable resource, the academic partner, who most likely is one of the leaders in their field. It is much more likely that a project which was working on a specific target got closed for the reasons discussed in earlier sections of this chapter.

The ultimate goal for the pharmaceutical industry is to develop safe and efficacious medication for patients with a high, unmet need. New projects are usually initiated with a survey of the scientific landscape around a disease or proposed disease biology mechanism. Many papers are read, discussed, prioritized, compared to our own expertise and in-house data and soon a picture emerges. Confidence builds around a target and its role in particular disease biology. However, one frequently lamented (Mullard 2011; Prinz et al. 2011; Begley and Ellis 2012; Nature-Editorial 2013b), but hitherto unresolved, issue might raise its ugly head: irreproducibility. This is certainly a challenge for industry where usually larger teams (and not only a sole graduate student) are used to pursue a project, and research costs quickly escalate. Yet, this issue affects the biomedical research community at large. The awareness around the topic has substantially increased in the recent years and the Nature publishing group has started a campaign in 2013 which is aptly called "Reducing our irreproducibility" and "Raising standards" (Nature-Editorial 2013a; Nature Neuroscience-Editorial 2013). Furthermore there is a web special on the issue (Nature-Editorial 2013b). In 2012 Nature had an editorial headlined "Must try harder" which bemoaned the perceived sloppiness of some research (Nature-Editorial 2012). Due to the touchiness of the topic, hard data is difficult to come by. However two recent non-peer reviewed correspondence pieces in Nature have tried to shed some light on the topic. In the first, titled "Believe it or not: how much can we rely on published data on potential drug targets?", a team from Target Discovery at Bayer Healthcare reports that out of 67 target validation studies, 43 (i.e. 64 %) were not reproducible (Prinz et al. 2011). In the second publication, results from a team at Amgen were reported. This group tried to validate 53 of what they considered landmark studies. Only six (i.e. 11 %) were successfully replicated (Begley and Ellis 2012). Based on these reports and the response they triggered from academia and industry alike, C. Glenn Begley wrote another comment which he termed the "Six red flags for suspect work" (Begley 2013). Based on his experience in trying to replicate published research, he raised the following six questions which might help identify "irreproducible" results. (1) Were experiments performed blinded? (2) Were basic experiments repeated? (3) Were all the results presented? (not just those that fit the story). (4) Were there positive and negative controls? (5) Were reagents validated? (6) Were statistical tests appropriate? These questions should resonate with any diligent reviewer for a scientific journal. However, it should come at nobody's surprise that many publications, including ones in high profile journals, do not hold up to these standards. Table 9.5 summarizes some of the key criteria we consider constitute a well-designed preclinical target validation study using pharmacological tools.

As detailed in the cited publications the reasons for irreproducibility can be multiple and our comments on this topic should not be perceived as finger-pointing. We simply want to raise awareness on the topic. In some instances, preclinical papers, which ultimately could not be reproduced, spawned an entire field. Sometimes, hundreds of follow-up publications expanded on elements of the original publication, without validating or falsifying its fundamental basis. More troubling though, some of the research led to the initiation of clinical studies implying that some

Table 9.5 Check-list for a well-designed preclinical pharmacological CNS study for target validation

Criteria	Importance
Drug exposure measured in brain at relevant time-points	Essential
Free fraction in target organ corresponds to compound potency	Essential
Pharmacodynamics endpoint	Essential
Researcher blinded to compound identity	Essential
Study size appropriately powered	Essential
Study repeated on independent cohorts of animals	Essential
Animals and groups randomized	Essential
Side-effect profile that may confound efficacy readout	Essential
Compounds tested in dose-response	Important if feasible
Evidence for target engagement (e.g., receptor occupancy)	Important if available
Multiple compounds of different chemotypes tested	Greatly improves confidence
Compounds tested in multiple disease models	Greatly improves confidence

patients might have been subjected to a trial of a drug or regimen that in all likelihood would not work. There is substantial difference if the end product of an effort is a publication or a drug which is given to people.

Conclusions

The beginnings of the twenty-first century are witnessing a strong wave of downsizing among corporations conducting new drug discovery. This is especially so in the area of central nervous system diseases. The lack of detailed knowledge about the biology of devastating diseases such as Alzheimer's disease, depression, or Parkinson's disease is a key determinant of extremely costly failures in clinical trals of drug candidates. Simultaneously, neuroinflammation biology is increasingly being recognized as playing a key role in these diseases, potentially enabling key progress in this area.

While several targets can be interrogated repositioning previously developed drugs for peripheral inflammation processes, a significant amount of new neuroinflammation targets require target validation and the discovery of new chemical probes to interrogate the relevant biologies. We hope the information provided in this chapter will aid organizations which have taken a leadership role and are making strides in the search for new drugs.

Scientists working in drug research projects end up making multitude of decisions—hopefully data-driven, good decisions. Given the time and efforts that are invested in the endeavor, it is not unexpected that personal biases will play a role and increase the chances of making a poor decision. Strategies to reduce sources of bias when assessing evidence exist (PLoS-Medicine-Editorial 2005). Understanding these "yellow flags" and becoming aware of them should also improve the probability of success. This may require special training and new approaches to analyzing data, data mining and visualization techniques. Drug discovery is a

task filled with uncertainty, and researchers must reach a level of comfort dealing with this issue. Differentiating preliminary from confirmatory studies or refutations, negative results, understanding biases and study design limitations, and potential confounding factors in the system under study should help managing the multiple inherent risks existing in target validation exercises.

Lastly, we would like to use our own experience pool (two of us are career pharmaceutical industry scientists, one is a recent transplant from academia) to suggest ways to optimize academia-industry interactions. Sometimes there seems to be too much focus on each other's weaknesses and very little is known about each other's strength. This can lead to misunderstandings, misconceptions and frustrations on either side. We believe there is tremendous potential for these collaborations to positively impact the future of human healthcare. To fulfill this potential, our interactions suggest that both parties should see each other as equal partners, and share as much knowledge and information as possible. To facilitate this aim, clear research collaboration agreements need to be in place, enabling the open exchange of data and ideas in both directions. Private parties, who must have a laser-focused objective to generate practical applications for human health, should clearly decide what deliverables they must own in order to justify their investment. Academic parties, typically more concerned about discovering new knowledge and making it available to the broader scientific community, need to clearly communicate their priorities and have a robust legal frame work to engage in frank discussions without the fear of being treated unfairly by the industrial partner. The key is to leverage each other's strength and compensate for potential weaknesses and generate mutually beneficial outcomes.

Nobody can discover drugs in isolation. It is our sincere hope that this book chapter highlights some of the needs and approaches of one of the partners and helps improve mutual understanding.

Acknowledgments We would like to sincerely thank Vlad and Alex to have given us the opportunity to write this chapter. We are grateful to all our colleagues in the Neuroinflammation Disease Biology Unit of Lundbeck Research USA for the enthusiasm with which they take every step in the exciting journey of uncovering the secrets of the intriguing and complex science required to improve our understanding of Neuroinflammation, and hopefully deliver new and effective treatments for underserved patients. In particular, we recognize Drs. Robb Brodbeck, Bob Nelson and Gamini Chandrasena for their encouragement. We acknowledge Drs. Stevin H. Zorn and Klaus Bæk Simonsen for their support.

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Chapter 10
The Role of Astrocytes in Huntington's Disease

Michelle Gray

Abstract Huntington's disease (HD) is a progressive autosomal dominant neurodegenerative disorder characterized by psychiatric disturbances, cognitive impairment and choreiform movements. It is caused by a repeat expansion in the gene encoding the widely expressed protein HTT. This protein is present throughout the nervous system in neuronal and non-neuronal cell types. The mutant HTT (mHTT) protein has been implicated in multiple cellular processes. To date, however, no single mechanism has been shown to be the primary mechanism that leads to neuronal dysfunction and death. Instead, it is believed that multiple mechanisms together may contribute to HD pathogenesis. At present, there is no effective neuroprotective treatment for HD. Although mHTT is found in astrocytes, most of the focus to date has been on understanding processes that may be dysfunctional in neurons. Nonetheless, there is substantial evidence for abnormal astrocytes in HD. In this chapter we present a review of the current understanding of astrocyte involvement in HD. We describe observations made in HD patients concerning morphological and molecular changes in astrocytes as disease progresses. Additionally, we describe the recapitulation of some of the phenotypes observed in HD patients in various mouse models expressing the mHTT protein. Together, the data from patients and the mouse models strongly implicate astrocyte-specific mechanisms as players in the pathogenesis of HD.

Keywords Astrocytes · Huntington's disease · Mutated huntingtin · Glutamate · Excitotoxicity

Abbreviations

CAG	Cytosine-adenosine-guanine
CPN	Cortical pyramidal neurons
CT	Computed tomography
EAAT2	Excitatory amino acid transporter 2
GFAP	Glial fibrillary associated protein
GLT-1	Glutamate transporter 1

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GS	Glutamine synthetase
HD	Huntington's disease
Hdh	Huntington disease homologue
HTT	Huntingtin
MAP2	Microtubule associated protein
MRI	Magnetic resonance imaging
MSN	Medium spiny neurons
PC	Pyruvate carboxylase
PET	Positron emission tomography
polyQ	Polyglutamine
TCA	Tricarboxylic acid cycle

10.1 Introduction

Huntington's Disease (HD) is a progressive and fatal neurodegenerative disorder characterized by motor dysfunction, psychiatric disturbances and cognitive impairment for which we have no neuroprotective therapies. The age of onset of HD is in the mid-forties, with death usually occurring 15–20 years after diagnosis (Gomez-Tortosa et al. 2001). The first published clinical description of HD was by George Huntington in 1872 in which he described sufferers of this disease quite accurately (Huntington 2003). He described a "*hereditary* chorea" where chorea is defined by "*dancing* propensities", and "with no loss of volition attending these contractions….the will is there, but its power to perform is deficient". He noted that the disease appeared hereditary in nature, with a tendency to insanity and suicide and that it manifested in adult life (Huntington 2003).

These descriptions of the disease, which now bears his name, have stood the test of time with more information having been added over time. To date, the clinical diagnosis of HD is based on the development of the classic movement deficit, chorea, which can present in combination with other movement deficits, including dystonia and bradykinesia. While the motor symptoms are the most visible symptoms of HD, the cognitive and behavioral problems are also very prominent in this disease. The cognitive changes can manifest early in the disease process and can include deficits in emotional recognition, time production, and speed of initiating thought processes (Hinton et al. 2007). HD patients also display changes in learning and working memory (Solomon et al. 2007). They have difficulties in learning new information and in memory recall (Montoya et al. 2006).

10.2 Huntington's Disease Genetics

HD is one of the most common single gene dominantly-inherited neurodegenerative disorders. While it is rare, meta-analysis of studies of prevalence worldwide shows it affects 2–3 persons per 100,000. In areas with populations largely of European

descent, the overall prevalence is 5–6 persons per 100,000 (Pringsheim et al. 2012). HD is caused by a repeat expansion in the highly conserved huntingtin (*HTT*) gene (Group 1993). The triplet repeat, cytosine-adenosine-guanine (CAG), is found in exon 1 of the gene and encodes glutamine. The disease allele results in the production of an expanded polyglutamine (polyQ) stretch in the N-terminal portion of the large 3144 amino acid protein (Group 1993; Zoghbi and Orr 2000). When someone carries a CAG stretch with less than 35, repeats there is no risk for getting HD. Alleles with CAG repeat lengths in the 36–40 range are incompletely penetrant. Those persons with CAG repeat lengths in this range will likely not develop HD symptoms, but if they do, it tends to be at a very advanced age (Snell et al. 1993; Rubinsztein et al. 1996; Quarrell et al. 2007). When the CAG repeat expansion is greater than 40, the carriers will develop HD (Andrew et al. 1993; Group 1993; Zoghbi and Orr 2000). The average age of onset in HD is in the mid- forties and is inversely correlated to the length of the CAG repeat expansion in HD (Duyao et al. 1993; Zoghbi and Orr 2000).

10.3 Huntingtin Expression and Huntington's Disease Neuropathology

The HTT protein is expressed throughout the nervous system (Landwehrmeyer et al. 1995; Schilling et al. 1995; Sharp et al. 1995). It can be found in neuronal as well as non-neuronal cell types (Singhrao et al. 1998). While the mutant protein is found in cells throughout the nervous system, the classic HD neuropathology is characterized by degeneration of the γ-aminobutyric acid (GABA)ergic medium spiny neurons (MSNs) in the striatum, with the vast majority of these cells degenerating at advanced stages of disease (Fig. 10.1; Vonsattel and DiFiglia 1998). HD disease grades are determined post-mortem based on the degree of striatal degeneration, with little degeneration assigned as Grade 0 and the most severe atrophy and degeneration assigned to Grade 4 with ~95% of neurons lost in the striatum (Vonsattel et al. 1985). Within the striatum itself, the earliest degeneration is observed in the tail of the caudate nucleus, and progresses in a caudal to rostral and dorsal to ventral gradient. There is also selectivity with MSNs based on neurochemical and anatomical features, with the MSNs expressing enkaphalin and dopamine receptor D2 (and projecting to the globus pallidus and substantia nigra pars reticulata) being lost earlier than MSNs expressing dopamine receptor D1 (Vonsattel et al. 1985; Richfield et al. 1995). Magnetic resonance imaging, positron emission tomography, and computed tomography have been used to image brains of presymptomatic *HTT* mutation carriers. Atrophy of the striatum is shown prior to overt motor symptoms and a clinical diagnosis of HD (Aylward et al. 2000). These studies also helped to confirm degeneration of extra-striatal regions, with the cortex being the next most affected brain region in HD. There is significant cortical atrophy that can be detected early in disease (Rosas et al. 2003, 2005). There is degeneration of cortical pyramidal neurons especially those in cortical layers III, V and VI, including those that project directly to the striatum (Hedreen et al. 1991; Halliday et al. 1998). The

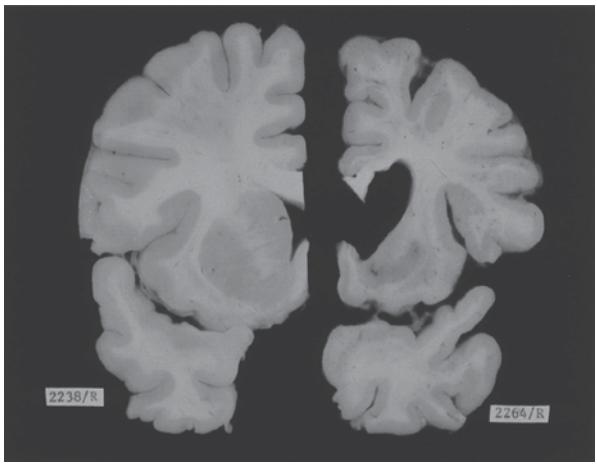

Fig. 10.1 Post mortem human brain at the level of the caudate-putamen. Coronal brain sections taken through the caudate-putamen of a normal (*left*) and a Huntington's disease patient (*right*). The Huntington's disease brain on the right shows degeneration of the caudate nucleus adjacent to the lateral ventricle, which has enlarged in response to the striatal atrophy. (Courtesy of J-P. Vonsattel. Reproduced from Alexi et al. (2000), with permission from Elsevier)

atrophy of these structures seems to appear long before the onset of overt motor dysfunction, as the imaging studies demonstrate atrophy prior to clinical diagnosis. Although HD affects most prominently the MSNs in the striatum, there is also significant atrophy of other brain regions as disease progresses, including the nucleus accumbens, globus pallidus, thalamus, and parts of the hypothalamus (Kremer et al. 1991; Tabrizi et al. 2009; van den Bogaard et al. 2011).

As with many other neurodegenerative diseases, another hallmark of HD is the progressive aggregation or inclusion body formation of mutant HTT (mHTT). These aggregates/inclusions were initially identified in a mouse model harboring an expanding CAG repeat stretch within exon1 of a human *HTT* transgene (Davies et al. 1997). Subsequently, these inclusions were identified in the neurons of HD patients (DiFiglia et al. 1997). The initial descriptions of these inclusions in HD tissue largely identified them as neuronal in nature, cytoplasmic/neuropil with a few intranuclear locations, and found primarily in gray matter (Gutekunst et al. 1999). The largest number of inclusions identified in patient tissue in those studies was observed in the deeper cortical layers, with many fewer and much smaller inclusions found in the striatum.

10.4 Cell Autonomous and Non-cell Autonomous Toxicity in Huntington's Disease

The most prominent area of neurodegeneration in HD is the striatum. The dysfunction of the MSNs in this region and their ultimate degeneration is at the core of the motor abnormalities that exist in this disease. The striatum is the central input area of the basal ganglia, receiving excitatory glutamatergic input from both the cortex and thalamus and dopaminergic input from the substantia nigra (Graybiel 1990; Wilson et al. 1990; Bolam et al. 2000). Although the mHTT protein is expressed

throughout the nervous system, much of the focus of HD research has been in the striatum. However, a series of studies in mice demonstrates the importance of other cell types in HD and their effect on neuropathological and behavioral manifestations of disease phenotypes in mice. There is clear evidence for non-cell autonomous mechanisms of toxicity in HD. In a mouse model with inducible expression of a mHTT-exon1 fragment (the Rosa/HD mouse), induction of expression throughout the brain (using Nestin-Cre) in neurons and glia results in neuropathological abnormalities, including gliosis and neurodegenerative changes in cortex and striatum. However, when the expression of mHTT expression was restricted to the cortex (using Emx1-Cre) or the striatum alone (using Dlx5/6-Cre), there were no significant behavioral or neuropathological changes at the ages examined (Gu et al. 2005, 2007). In another model, conditionally expressing a different mHTT fragment in MSNs under the control of the Darpp32 promoter (DE5 mice), there is late onset motor abnormalities but no evidence of neurodegenerative changes (Brown et al. 2008), whereas mice expressing this fragment throughout the brain showed extensive neuropathological changes (Yu et al. 2003).

10.5 Huntingtin Expression in Astrocytes

10.5.1 Expression in Human Astrocytes

The HTT protein is found throughout the nervous system. The majority of studies of pathogenesis in HD has centered on its dysfunction in neurons. However, RNA in situ hybridization identified positive signal in astrocytes from normal brain tissue (Landwehrmeyer et al. 1995). Furthermore, brain tissue from HD patients that was stained with antibodies to HTT and glial fibrillary acidic protein (GFAP) revealed the presence of HTT in astrocytes (Singhrao et al. 1998), although to a lesser degree than what is seen in neurons. Astrocytes in various brain regions, including the striatum (caudate nucleus and putamen), as well as white matter from HD patients, also contained mHTT positive aggregates (Singhrao et al. 1998; Shin et al. 2005; Bradford et al. 2009; Faideau et al. 2010).

The Htt protein is also found in astrocytes from mice (Bradford et al. 2009; Lee et al. 2013). The normal function of this protein in astrocytes remains to be completely elucidated. However, in mouse *Hdh* (encoding endogenous mouse huntingtin) knock-out neural stem cells treated using a neuronal differentiation protocol, there was a significant increase in the number of GFAP positive cells and a decrease in the number of microtubule associated protein 2 positive neurons when compared to control cultures. This finding suggests that wildtype Htt is involved in controlling the differentiation of neuronal and glial cells and that production of neurons from neural stem cells requires a normal level of wildtype Htt (Conforti et al. 2013). This data is in line with previous studies showing that wildtype Htt plays a role in central nervous system development and neuronal survival (Reiner et al. 2003; Lo Sardo et al. 2012). The wildtype Htt expressed in these stem cells could be acting in an

instructive or repressive role to promote neuronal fate and/or repress the glial fate. However, the exact role of wildtype Htt in astrocytes in the nervous system will need to be further assessed in conditional knock-in mouse models, where one can specifically reduce the expression of endogenous Htt only in astrocytes.

10.5.2 Expression in Mouse Astrocytes

Mutant HTT positive aggregates are found in astrocytes in the brains of HD mouse models. These mice contain aggregates in the white matter as well as the gray matter (Reddy et al. 1998; Yu et al. 2003; Shin et al. 2005). Like the aggregation found in neurons in these mice, aggregation in the astrocytes also appears to be progressive, with the number of mHTT aggregates increasing as the animal ages. The aggregates are found not only in the striatum and cortex of these mice, but also in the corpus callosum (Shin et al. 2005; Bradford et al. 2010). In addition, in a mouse model expressing a fragment of mHTT only in astrocytes, driven by the human GFAP promoter, there are aggregates in the astrocytes in the cortex, striatum, brainstem, and spinal cord (Bradford et al. 2009).

10.6 Astrogliosis in Huntington's Disease

10.6.1 Humans

One prominent feature found upon neuropathological examination of HD patient tissue is the presence of astrogliosis (Vonsattel et al. 1985; Faideau et al. 2010) (Fig. 10.2). In neurodegeneration, it is generally believed that astrogliosis is a response to dysfunction or death of neurons. In HD patient brains, there is a significant increase in the number of reactive astrocytes as disease grade (neuropathological severity) increases (0–4). Astrogliosis was assessed in striatal tissue from all disease grades using GFAP immunohistochemistry. These studies reveal that GFAP immunoreactivity is present throughout the striatum in all disease grades. Furthermore, the GFAP level seems to increase as a larger number of astrocytes are expressing GFAP as disease grade increases (Faideau et al. 2010). The pattern of astrogliosis in the striatal tissue from these patients seems to follow the pattern of neurodegeneration, where it is first seen in the dorsal striatum and then in the ventral striatum. Since the dorsal striatal astrogliosis is so early in the disease process (Grade 0), at least as far as obvious neuropathological changes are seen, this suggests that there is likely a cell-autonomous change in the astrocyte. The increase in GFAP staining intensity exists with characteristic reactive astrocytic phenotypes, including hypertrophic cell bodies. As disease progresses, the reactive phenotype becomes more severe with hypertrophic and overlapping protrusions from the astrocytes (Fig. 10.2; Faideau et al. 2010).

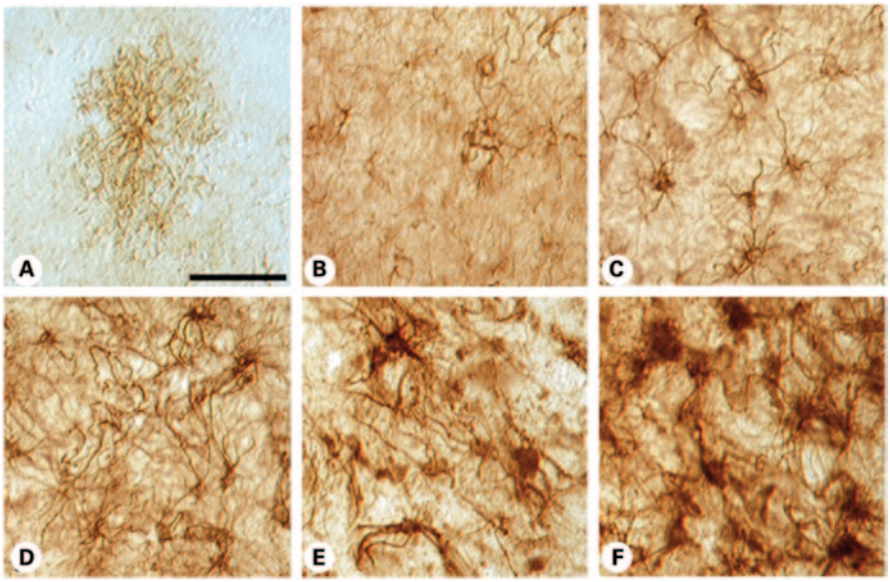

Fig. 10.2 GFAP immunohistochemistry in 50 μm tissue sections from the caudate nucleus in non-neurological control (**a**), and in increasingly severe HD specimens, Grades 0–4, Grade 0 (**b**), Grade 1 (**c**), Grade 2 (**d**), Grade 3 (**e**) and Grade 4 (**f**) HD subjects. Normal astrocytes present as faintly GFAP-stained cells with a short lace-like branching pattern distributed symmetrically around the cell soma. With increasing disease progression, there was greater GFAP immunoreactivity, twisting and thickened arbors, with larger somal size. The degree of astrogliosis became so great as to mask their individual appearance. The magnification bar in (**a**) represents 100 μm and is the same in all photomicrographs. (Reproduced with permission from Faideau et al. (2010); Oxford University Press)

10.6.2 Mouse Models

The reactive astrocyte phenotype has been observed in many of the mouse models expressing mHTT. Neuropathologically, these models display varying degrees of pathological changes, with region atrophy, cellular atrophy, dark neuron degenerative changes, and mHTT aggregation. There are multiple mouse models that express some form of the mutant protein, either a full-length or truncated protein by either a knock-in or transgenic approach. Many of these models also display some degree of astrogliosis. In the RosaHD/Nestin-Cre model, which expresses mHTT throughout the nervous system in both neurons and glia, there is significant astrogliosis in the cortex and striatum (Gu et al. 2005). Studies of astrogliosis in models where the mHTT protein is expressed in the cortex with RosaHD/Emx1-Cre and striatum with RosaHD/Dlx5/6-Cre revealed no significant astrogliosis when mHTT was restricted to neurons (Gu et al. 2005, 2007). The data from these models suggested that cell-cell interactions were important for the development of the reactive astrocyte phenotype.

In the mouse model HTT171-82Q, where mHTT expression was targeted to astrocytes in the striatum through lentiviral expression, there was an increase in GFAP staining and astrocytes exhibiting a reactive phenotype. This phenotype increased in severity as the animal aged, with an increased astrocyte soma size (Faideau et al. 2010). This indicates a cell-autonomous effect of the mHTT protein within astrocytes, as this reactive phenotype is not due to the expression of mHTT in neurons. This data reinforces the idea that reactive gliosis is not merely a consequence or response to sick or degenerating neurons in neurodegenerative diseases. In the knock-in mouse model, *Hdh* CAG 150, there is extensive astrogliosis in the striatum (Lin et al. 2001). Together, these mice demonstrate that the reactive astrocyte phenotype can be elicited from expression of mHTT specifically in the astrocytes or observed when mHTT expression is also found in neurons; therefore the mHTT protein is able to elicit both cell-autonomous and non-cell autonomous phenotypes.

10.7 Excitotoxicity in Huntington's Disease

There are many possible mechanisms that may contribute to toxicity in HD; one of these is excitotoxicity. Excitotoxicity leading to neuronal dysfunction and death is caused by excessive activation of glutamate-gated N-methyl D-aspartate receptors (NMDARs) due to increased exposure to glutamate. This leads to Ca^{2+} overload and mitochondria energy failure (Coyle and Puttfarcken 1993). This mechanism had been hypothesized for HD many decades ago. In HD, this mechanism has primarily focused on the cortico-striatal synapse, with the pre and post-synaptic neuron receiving the most attention. The MSNs in the striatum receive glutamatergic excitatory input from both the cortex and thalamus (Fonnum et al. 1981a, b). The excitotoxicity hypothesis of HD pathogenesis is supported by the existence of these extensive inputs and the presence of high densities of glutamatergic receptors in striatal neurons (Albin et al. 1990; Beal 1994; Landwehrmeyer et al. 1995). Many of the initial studies in HD used excitotoxins to mimic HD pathology. One of the first rodent models of HD used injections of the excitotoxin kainic acid into the striatum to selectively destroy MSNs (Coyle and Schwarcz 1976; McGeer and McGeer 1976). Quinolinic acid, a selective NMDAR agonist, was also used to replicate the features of HD including selective degeneration and morphological changes in MSNs, including loss of dendritic spines in rodents and non-human primates (Sanberg et al. 1989; Beal et al. 1991; Ferrante et al. 1993; Zeron et al. 2002). This selective NMDAR agonist produced specific toxicities for striatal MSNs, without causing degeneration of striatal interneurons, further reinforcing the idea that excitotoxicity caused by activation of NMDARs is an important mechanism in HD.

The excitotoxicity hypothesis is further supported by data from multiple HD mouse models. There is increased response of MSNs to NMDAR activation. When quinolinic acid was injected into the YAC72 and YAC128 mice, there was a significant difference in lesion size as compared to wildtype mice, although as disease progressed in these models, this phenotype did not persist in older mice (Zeron

et al. 2002; Graham et al. 2009). Furthermore, there is increased glutamate level in the striatum of YAC128 mice upon cortical stimulation (Joshi et al. 2009), although other studies suggest no such change in young YAC128 mice not yet displaying behavioral or neuropathological features of HD (Milnerwood and Raymond 2007; Cummings et al. 2010). These data support the hypothesis that altered NMDAR function early in the course of disease in these mouse models, and altered striatal NMDAR signaling likely contributes to the deficits seen in HD.

10.8 EAAT2 Expression and Glutamate Level

10.8.1 Humans

The glutamate transporter is critical for regulating glutamate levels at the synapse. The uptake of glutamate and its conversion into glutamine reduces the level of glutamate in the synaptic space. In support of the excitotoxicity hypothesis, HD brains show a decrease in the level of the excitatory amino acid transporter 2 (EAAT2; human)/Glutamate transporter 1 (GLT-1; rodent) (Cross et al. 1986; Arzberger et al. 1997). *In situ* hybridization studies on HD brain tissue revealed a decrease in EAAT2 mRNA labeling that correlated with disease severity (Arzberger et al. 1997). In the tissue, a decrease in the number of cells expressing EAAT2 mRNA was seen in the remaining tissue of both the caudate and putamen, although the putamen seemed to have the greater decrease. In addition, immunohistochemistry performed on grade 0 to grade 4 tissues with an EAAT2/GLT-1 antibody revealed a grade-dependent decrease in protein levels (Faideau et al. 2010). This data reveals that there is a loss of EAAT2 early in the disease process, which can implicate the transporter as a primary component in the initiation of disease. To properly maintain synaptic function and glutamate neurotransmission, there must be coordinated activity of the pre- and post-synaptic cells, but also the astrocyte. The role of this transporter in astrocytes is to remove glutamate from the synaptic cleft after it is released (Danbolt 2001; Maragakis and Rothstein 2001). With excitotoxicity as one of the proposed toxic mechanisms in HD, due to increased levels of glutamate in striatal tissue that is hypothesized to come from the cortico-striatal inputs, the ability to efficiently uptake glutamate from the synaptic space is of vital importance.

10.8.2 Mouse Models

Based on the decrease in the level of the astrocyte-specific glutamate transporter EAAT2/GLT-1, this has been a major focus of studies aimed at understanding astroyctic contribution to HD. Much of this work has been performed in multiple mouse models expressing various forms of the mHTT protein. There is a reduction in the level of *Glt-1* mRNA in the R6/2 mice, which express an exon1 fragment with

an expanded CAG repeat, when compared to littermates (Behrens et al. 2002; Shin et al. 2005). The decrease in *Glt-1* mRNA levels seem progressive as a decrease can be seen in R6/2 animals in 6 week old animals, in both cortex and striatum, and further declines at 12 weeks (Behrens et al. 2002). In addition to mRNA levels in the R6/2 mice, there is a significant reduction in the protein level of GLT-1 at 11–12 weeks of age (Behrens et al. 2002), and although there is a likely a decrease in protein levels early as well, it does not reach statistical significance (Shin et al. 2005). Glutamate uptake is decreased in the striatum of the R6/2 transgenic model (Lievens et al. 2001; Behrens et al. 2002; Shin et al. 2005; Estrada-Sanchez et al. 2009). Another mouse model, the *Hdh* CAG 150 knock-in mouse, did not show a significant difference in the *Glt-1* mRNA at 9 months of age, although there was a slight reduction in *Glt-1* mRNA levels (Shin et al. 2005). This result is likely true given the slower progression in this full-length mHTT mouse model that exhibits no obvious neuropathological changes at this age (Lin et al. 2001).

Mice were generated using the human GFAP promoter to specifically express a mHTT exon 1 fragment carrying 160Q repeat in astrocytes (GFAP-HD) (Bradford et al. 2009). There is a significant decrease in the level of GLT-1 protein in the brains and cultured astrocytes from these mice. As a consequence of the decrease in the levels of *Glt-1*, there is also decreased glutamate uptake in the GFAP-HD mice (Bradford et al. 2009). In mice that expressed a mHTT fragment containing 82 CAG repeats (viral HTT-82Q) specifically in astrocytes using lentiviral vectors, there was a decrease in the level of *Glt-1* mRNA in the striatum of these mice 12 weeks after injection (Faideau et al. 2010). The level of the Glt-1 protein was assessed by immunohistochemistry in the mHTT positive astrocytes and showed a significant and progressive decrease in the striatum. Interestingly, there was also a decrease in GLAST, but this was not significant until late in the disease process in this animal. Glutamate transport was also decreased in the astrocyte HTT171-82Q expressing mice, and no decrease in glutamate transport or Glt-1 levels was observed with neuronal expression of the HTT171-82Q (Faideau et al. 2010). Perhaps the most interesting observation from these mice was the decrease in the levels of two neuronal proteins DARPP-32 and GluN2B subunits of NMDARs, thus indicating that the presence of mHTT in astrocytes is likely sufficient to alter neuronal activity and function. The exact mechanism whereby the mHTT in the astrocytes caused the decrease in the levels of these proteins is unclear, but implicates the inability of GLT-1 to properly buffer extracellular glutamate as a possible mechanism for decreased expression of DARPP-32 and GluN2B in neurons.

The mechanism for decreases in GLT-1 levels in the mouse models include a change in Sp1-dependent transcription of *Glt-1* and palmitoylation of GLT-1. In mHTT expressing astrocytes from GFAP-HD transgenic mice, there is a reduction of the transcription factor Sp1 occupancy at the *Glt-1* promoter as compared to littermate controls (Bradford et al. 2009). This reduction in this model is likely due to increased association of mHTT with Sp1 as shown by more mHTT precipitating with Sp1 than with HTT with a polyQ repeat in the normal range (Bradford et al. 2009). The YAC128 model expresses full-length human mHTT and recapitulates aspects of HD neuropathology and behavior that becomes progressively worse as

the animal ages (Hodgson et al. 1999; Slow et al. 2003). There does not appear to be a decrease in the levels of GLT–1 protein in the brain of these mice, even as disease progresses. Interestingly, glutamate uptake was decreased in the striatum of these mice as early as 3 months of age, and at 12 months is also seen in the cortex (Huang et al. 2010). EAAT2 was identified in a proteomics study of palmitoylation (Kang et al. 2008), which involves the thioesterification of palmitic acid to cysteine residues and functions in tethering proteins to membranes or sorting them to lipid microdomains. Palmitoylation was reduced on GLT-1 in the brains of YAC128 mice (Huang et al. 2010). The decrease in palmitoylation of GLT-1 was found to impair glutamate uptake without affecting is localization to the membrane. Thus, exactly how palmitoylation affects the function of this receptor is still up for debate; nonetheless, these data provide two mechanisms for decreased GLT-1 levels and activity in HD mouse models.

With the alterations in GLT-1, studies have been performed to determine if increasing the expression of GLT-1 would alleviate phenotypes caused by the presence of mHTT. When *Glt-1* was overexpressed by using a lentiviral vector in astrocytes also expressing HTT-171-82Q, the level of GLT-1 increased and the reactive astrocyte previously seen in those mice significantly decreased (Faideau et al. 2010). Furthermore, the use of ceftriaxone, a β-lactam antibiotic in R6/2 mice raised Glt-1 levels and reversed the glutamate uptake deficit in these mice. Ceftriaxone has improved some of the motor deficits found in the R6/2 mice. However, use of ceftriaxone must be approached with caution as it has been found to affect long term-depression in the hippocampus (Omrani et al. 2009) and impairs prepulse inhibition (Bellesi et al. 2009). Nonetheless, while the appropriate approach to take has yet to be determined, increasing the levels of GLT-1 in HD may have beneficial effects on glutamate uptake deficit and motor impairment.

10.9 Glutamate Release from Mutant HTT Expressing Astrocytes

Much of the study in HD on astrocytes centers around the decrease in EAAT2/Glt-1 levels, and the ability of the astrocyte to take up excess glutamate from the synaptic cleft, and how that may contribute to excitotoxicity. However, there has been a lack of appreciation for the ability of the astrocyte to release glutamate and whether that ability is changed in HD. The astrocyte is the only cell in the brain that can synthesize glutamate *de novo* (Hertz et al. 1999). It is capable of releasing glutamate through various mechanisms, including but not limited to Ca^{2+} dependent vesicular exocytosis (Parpura and Zorec 2010). Glutamate released from astrocytes has been shown to act on extrasynaptic NMDARs to modulate neuronal excitability and synaptic transmission (Araque et al. 1999; Fellin et al. 2004). Based on this ability of astrocytes to modulate neuronal excitability, it is possible that a change in this function due to the presence of mHTT in astrocytes could contribute to changes in the activity at the most critical excitatory synapses in the brains of HD patients.

To date, only one study has been performed to determine whether mHTT expression in astrocytes had any affect on the levels of Ca^{2+}-dependent glutamate release (Lee et al. 2013). This study used cultured solitary astrocytes from the full-length human mHTT expressing BACHD mouse. This study employed a previously validated system where astrocytes were first grown in culture flasks and purified solitary astrocytes were then mechanically stimulated resulting in glutamate release (Hua et al. 2004). Mechanical stimulation of astrocytes allows one to assess the exocytotic release of glutamate (Montana et al. 2004). Mechanical stimulation of full-length mHTT-containing cortical astrocytes from BACHD mice resulted in an increase in the level of glutamate released into the extracellular space near the solitary astrocytes as compared to wildtype astrocytes (Lee et al. 2013). Astrocytes exhibiting Ca^{2+}-dependent exocytotic release of glutamate do so by increased cytosolic Ca^{2+} responses, which are usually in proportion to the level of glutamate released from these cells (Parpura and Haydon 2000). While these BACHD cells responded to mechanical stimulation with a rise in cytosolic Ca^{2+} levels, there was not a significant difference between wildtype and full-length mHTT expressing cortical astrocytes in cytosolic Ca^{2+} levels upon mechanical stimulation.

Multiple mechanisms could account for the change in levels of glutamate released from astrocytes. These include the trafficking of glutamate containing vesicles and glutamate synthesizing machinery within these cells. However, analysis of glutamate containing vesicles revealed no change in mHTT expressing astrocytes when compared to wildtype astrocyte, suggesting other mechanisms are involved to cause the increase in levels of glutamate released from these astrocytes.

Glutamate can be synthesized in astrocytes *de novo* as a by-product of the tricarboxylic acid cycle. In addition, a decrease in the enzyme glutamine synthetase could result in an increase in the levels of glutamate present intracellularly in these astrocytes, which ultimately could contribute to an increase in the amount released. Further examination of these mHTT expressing astrocytes revealed an increase in the level of the mitochondria resident enzyme pyruvate carboxylase and no change in the glutamine synthetase. The increase in pyruvate carboxylase provides a mechanism for the increase in glutamate released from these cortical astrocytes. The exact mechanism leading to the increase in pyruvate carboxylase remains to be elucidated and validated in patient samples.

Concluding Remarks

The importance of astrocytes in HD has yet to be fully uncovered. While the presence of reactive astrocytes has been found in post-mortem tissue and seems to progress with age, whether this phenotype is truly an intrinsic phenotype or a response to the neuronal changes in HD brains remains to be completely determined. The astrocyte functions in and impacts a variety of processes in the brain. The uptake of excess glutamate from the synaptic space by astrocytes is critical to proper neural circuit function, and its decreased uptake in HD could contribute to

disease pathogenesis. Both the levels of the receptor responsible for this uptake into astrocytes and post-translational modification of the receptor are likely important mechanisms that could contribute to excitotoxicity. The recent identification of an increase in the levels of glutamate released from mHTT expressing cortical astrocytes also provides another mechanism whereby mHTT in astrocytes could lead to excitotoxicity. Although neurotransmitter uptake/levels have been widely studied, many of the processes that astrocytes are involved in have not been fully explored in HD. These processes, including the release of glutamate (Lee et al. 2013), astrocyte roles in inflammatory response (Benveniste 1992; Soulet and Cicchetti 2011), and sleep (also abnormal in HD) (Fellin et al. 2012; Morton 2013), need further exploration. More study of these astrocytic processes in HD will require the manipulation of normal HTT and mHTT in both cell and animal models of HD. Many of these studies can likely be performed using the many genetic models of expressing mHTT that have been generated to date. However, proper performance and interpretation of these studies will require cooperation of expert glial biologists and those focused on the elucidation of pathogenic mechanisms in HD.

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Chapter 11
Amyotrophic Lateral Sclerosis: A Glial Perspective

Chiara F. Valori, Liliana Brambilla and Daniela Rossi

Abstract The lack of effective disease-modifying therapies for the treatment of Amyotrophic Lateral Sclerosis (ALS) demands for major research investments aimed at investigating novel mechanistic hypotheses as well as at validating unprecedented cellular and molecular targets for therapeutic intervention. Within this framework, glial cells have recently acquired great importance in view of the growing body of evidence indicating that motor neuron degeneration involves non-cell autonomous mechanisms in ALS, including the interaction with various glial cell populations. These observations not only have drawn attention to the physiopathological changes glial cells undergo during ALS progression, but they have moved the focus of the investigations beyond the neuronal compartment towards glia-neuron interactions. With this in mind, in this chapter, we dissect the specific contribution of the different glial subtypes to the dreadful chain of events leading to motor neuron sufferance and death in various forms of ALS. Furthermore, we discuss the possibility of targeting specific molecular defects in glial cell physiology and glia-neuron communication for the treatment of this disorder.

Keywords Amyotrophic lateral sclerosis · Glia · Astrocytes · Microglia · Oligodendrocytes · Schwann cells · $NG2^+$ cells · Neurodegeneration · Transgenic animals

11.1 Amyotrophic Lateral Sclerosis: A Brief Introduction

Amyotrophic Lateral Sclerosis (ALS) is a chronic and incurable disease characterized by the impairment of motor function due to weakness, atrophy and spasticity of voluntary muscles. Disease signs and symptoms stem from the combined

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degeneration of corticospinal and spinal motor neurons, leading to progressive muscle denervation.

ALS is the most common form of adult-onset motor neuron disease, with an incidence and prevalence of about 1–3 and 4–6 per 100,000 individuals each year, respectively. Generally, it manifests between 40 and 60 years of age, with symptoms reflecting the early involvement of spinal (fasciculation, tremors, muscle weakness) or bulbar (dysphagia and dysarthria) motor neurons.

The diagnosis of ALS is made upon meeting the El Escorial World Federation of Neurology Criteria, namely clinical signs of both upper and lower motor neuron degeneration, along with evidence of spreading of the motor syndrome within a region or to other regions. These criteria should be combined to the absence of electrophysiological and neuroimaging signs of other disease processes that might justify the observed symptoms (Brooks et al. 2000).

Although cognitive functions are not affected in most instances, about 50 % of patients show behavioural and linguistic abnormalities. The cognitive decline is generally mild, but, in about 15 % of patients, the impairment becomes so severe to call for an additional diagnosis of Frontotemporal Lobe Dementia (FTLD) (Lomen-Hoerth et al. 2002; Ringholz et al. 2005).

The progression of the disease is usually rapid, and it is monitored by the Revised ALS Functional Rating Scale (ALSFRS-R)(Hardiman et al. 2011), a validated rating tool that allows to assess the progression of the patient's disability. A decline in the ALSFRS-R score is basically considered as a predictor of reduced survival.

Symptomatic therapy can help to relief muscle spasticity, excessive drooling, body weight loss and depression, thus contributing, at least to some extent, to the preservation of quality of life. Yet, the sole drug approved by the US Food and Drug Administration (FDA) for the cure of ALS is currently riluzole, which can extend the patient survival only by 3–6 months (Hardiman et al. 2011). Thus, despite a certain variability, the patient death generally occurs within 3 to 5 years from the diagnosis, mostly by respiratory failure. As a consequence, the discovery of novel effective disease-modifying therapies for the treatment of this devastating condition has presently a high priority in the scientific community. In this context, the identification of novel cellular and molecular targets for therapeutic intervention is highly desirable, and may importantly contribute to design unprecedented strategies for the treatment of ALS patients.

In the vast majority of cases, ALS appears sporadically (sporadic ALS, sALS). Presumed risk factors include aging, environmental agents (e.g., exposure to heavy metals) and life habits (e.g., smoking). Yet, in about 5–10 % of instances, the disease is inherited, mostly as an autosomal dominant trait (familial ALS, fALS). Familial ALS has been linked to mutations in various genes, including *Superoxide dismutase 1* (*SOD1*) (Rosen et al. 1993), *TAR DNA binding protein 43* (*TARDBP*) (Kabashi et al. 2008; Sreedharan et al. 2008), *Fused in sarcoma* (*FUS*) (Kwiatkowski et al. 2009; Vance et al. 2009), *Alsin* (Yang et al. 2001), *Optineurin* (Maruyama et al. 2010), *C9orf72* (DeJesus-Hernandez et al. 2011; Renton et al. 2011), *Ubiquilin 2* (Deng et al. 2011) and *Profilin 1* (Wu et al. 2012).

During the last few years, several mechanistic hypotheses have been formulated to explain the origin of ALS. Among others, the involvement of oxidative stress, excitotoxicity, impaired axonal transport, mitochondrial dysfunction and/or alteration of RNA metabolism has been contemplated (reviewed in Ferraiuolo et al. 2011a). While most of these mechanisms appear to suggest that neurodegeneration arises from intrinsic defects in motor neurons, there is accumulating evidence supporting the concept that non-neuronal cells of the central nervous system (CNS) contribute to the dreadful chain of events ultimately causing motor neuron demise.

This observation has brought attention to the abnormalities affecting glial cells in ALS. At the histopathological level, the loss of upper and lower motor neurons, in both sporadic and familial cases, is accompanied by massive activation of glial cells in the affected areas, a phenomenon commonly described as "reactive gliosis". Furthermore, in ALS patients and transgenic animals, there is evidence of ubiquitinated protein inclusions in motor neurons as well as in glial cells (Bruijn et al. 1997; Pasinelli et al. 2000; Mendonca et al. 2006). Noteworthy, the ubiquitinated inclusions identified in various forms of fALS and sALS have a distinct pattern of protein composition, suggesting that different ALS-linked gene products may trigger distinctive neurodegenerative mechanisms.

To predict the deleterious potential of glial cells, it should be, however, noted that neuroglia include distinct cell populations characterized by an independent embryological origin and exerting different functions (reviewed in Allen and Barres 2009). These include astrocytes, which represent the main effectors of the brain homeostatic system; microglia, the immunocompetent and specialized brain macrophages; oligodendrocytes and Schwann cells, which form layers of myelin around neuronal axons in the central and peripheral nervous system, respectively; and $NG2^+$ cells, a peculiar type of glial cells that express the NG2 proteoglycan and receive direct synaptic input from neurons.

In this chapter, we present recent mechanistic studies, performed in cellular and animal models of the disease, emphasizing the contribution of distinct glial cell populations to the development and progression of ALS. Furthermore, we recapitulate the evidence for glial cell dysfunction in the human pathology. Lastly, we highlight the importance of testing glia-targeted agents for their potential to slow down or halt the progression of this disorder (reviewed in Valori et al. 2014).

11.2 ALS Genetics and Experimental Models

The landmark discovery, achieved in the early 1990s (Rosen et al. 1993), that *SOD1* presents various mutations in a certain percentage of fALS cases (ALS-SOD) raised the possibility to generate transgenic animal models of the disease, which express mutant forms of the human Copper, Zinc Superoxide Dismutase (hSOD1) enzyme. Several different hSOD1 mutations were introduced into these genetically modified animals, mostly causing the development of an ALS-like syndrome *in vivo* (reviewed in Turner and Talbot 2008). Since the disease arose even in the presence

of normal or increased dismutase activity, it was early proposed that mutant SOD1 toxicity is due to a gain-of-function rather than to the loss of the normal enzymatic activity of SOD1 mutants. The nature of such aberrant function of mutant SOD1s remains mostly elusive, though several mechanistic hypotheses have been proposed.

The first ALS mouse model to be produced was the one carrying a mutant form of hSOD1 harbouring a glycine with alanine substitution in position 93 of the amino acid sequence (Gly93→Ala; hSOD1^{G93A} mice; Gurney et al. 1994). Extensive characterization of these animals revealed that they exhibit a phenotype that recapitulates several aspects of the human condition, being characterized by tremor, progressing to muscular weakness, paralysis, and eventually premature death. On a neuropathological standpoint, the behavioural abnormalities of hSOD1^{G93A} mice are strictly linked to motor neuron degeneration in the ventral horns of the spinal cord (Turner and Talbot 2008), an event that is accompanied by reactive astrocytosis and microgliosis (Hall et al. 1998). Moreover, in this mouse model, there is evidence of the presence of Lewy body-like inclusions containing SOD1 and ubiquitin in motor neurons as well as in various glial subpopulations (Bruijn et al. 1997; Pasinelli et al. 2000; Stieber et al. 2000). Such glial inclusions were originally proposed to be the earliest indicator of the disease in the hSOD1^{G85R} mice (Gly85→Arg substitution), another transgenic line developing a late onset, ultrarapid disease progression (Bruijn et al. 1997). Similar inclusions, containing active caspase-3, were subsequently reported in the same hSOD1^{G85R} mice, as well as in hSOD1^{G93A} mice, which show earlier onset, but analogously fast disease progression (Pasinelli et al. 2000). Yet, the significance of these glial protein aggregates in the context of ALS pathology remained unclear until recently (Rossi et al. 2008). The theory that glial cells can contribute to ALS pathogenesis was corroborated only in 2003 owing to the revolutionary discovery, achieved in chimeric mice, that motor neuron degeneration involves non-cell-autonomous events, including the interaction with mutant SOD1-expressing glial cells (Clement et al. 2003). As we shall discuss in the next sections, this observation moved the focus of the investigations from intrinsic defects and weakening of motor neurons to glia-neuron interactions. This paved the way for a new series of experiments apt to clarify the specific contribution of the different glial cell types to both motor neuron cell death and ALS pathogenesis (reviewed in Ilieva et al. 2009).

While most of the currently available mechanistic information on ALS is based on observations made on mutant SOD1-expressing experimental models, the landscape of ALS genetics has greatly expanded over the last few years, thus prompting new investigations apt to clarify the functions of the newly identified disease proteins. Thus, much effort is currently being invested to study TDP-43, the protein encoded by the *TARDBP* gene. This protein was originally discovered as a major component of ubiquitinated protein inclusions in FTLD and sALS cases in 2006 (Neumann et al. 2006). Two years later, mutations in the *TARDBP* gene were identified in fALS patients (Kabashi et al. 2008; Sreedharan et al. 2008), thus strengthening the hypothesis that TDP-43 may be involved in the development of the disease (ALS-TDP). At the cellular level, TDP-43 is normally concentrated in the nucleus, but can also shuttle back and forth between the nucleus and the cytoplasm (Ayala

et al. 2008). It is a global regulator of gene expression and is involved in the control of transcription as well as in multiple aspects of RNA processing and functioning. Besides, this protein can redistribute to cytosolic granules in response to neuronal stress, although nuclear localization is restored after recovery (Dewey et al. 2011; McDonald et al. 2011). Concerning ALS, there is no clear consensus of how pathological TDP-43 operates within diseased cells (Halliday et al. 2012).

In 2009, mutations in the *FUS* gene were also identified in about 4% of fALS cases (ALS-FUS) (Kwiatkowski et al. 2009; Vance et al. 2009). This gene codes for a ubiquitously expressed, predominantly nuclear, protein that is involved in DNA repair and regulation of transcription, RNA splicing, and export to the cytoplasm. Analyses of ALS-FUS cases, expressing different forms of the mutant protein, allowed to identify two distinct clinical and neuropathological patterns (Mackenzie et al. 2011), which correlate with a different extent of cytoplasmic FUS mislocalization (Dormann et al. 2010). Because many of the mutations identified in fALS patients cluster in the C-terminal domain of the FUS protein, and this includes a non-classic nuclear localization signal, it was shown that *FUS* mutations impair the physiological nuclear import of FUS (Dormann et al. 2010). However, similar to TDP-43, it is presently highly debated whether FUS mutations induce gain of toxic functions or loss of protective activities (Halliday et al. 2012). The discovery of these novel ALS-related genes led to a new generation of transgenic animal models suitable to explore the function of TDP-43 and FUS *in vivo* as well as to perform mechanistic studies (Lanson and Pandey 2012; Tsao et al. 2012). As outlined below, some of these animals have been used to specifically investigate the impact of ALS glia on the disease outcome.

Because most of the currently available evidence on glial pathology relates to mutations/mislocalization of SOD1, TDP-43 or FUS, herein we specifically focus on the forms of ALS linked to these proteins, and we discuss the potential of glial cell dysfunction towards neurodegeneration and disease progression in these contexts.

11.3 Astrocytes

Considerable evidence indicates that astrocytes represent the main type of glia in the CNS and critically control the brain homeostasis, being responsible for all aspects of metabolic support, nutrition, control of ion and transmitter environment, regulation of brain-blood barrier (BBB), and defense of the CNS (reviewed in Rossi et al. 2011). Considering the diversification and complexity of such astrocytic activities, it is reasonable to postulate that any astroglial dysfunction may impact the maintenance and performance of the CNS. This hypothesis has been corroborated by experimental evidence suggesting that astrocytes are critically involved in the development and progression of several neurological disorders, including ALS (reviewed in Parpura et al. 2012). As we shall see in the next paragraphs, astroglial cells were shown to be implicated in various pathogenetic pathways in ALS, where

they appear to exert a dual role: on the one hand, they were shown to actively contribute to motor neuron damage by secreting neurotoxic factors; on the other hand, astrocytes were reported to loose some of their physiological functions and, ultimately, undergo themselves degeneration. Thus, it seems that the pathological process of ALS transforms astrocytes from supportive friends for neurons into noxious foes, and ultimately eliminates them. The most straightforward consequence of these events is that neurons, deprived of their interactive partners, start suffering and eventually undergo accelerated cell death.

11.3.1 Direct Contribution of the Astrocytes to the Development of Neuronal Cell Death and Disease Manifestations

Several approaches *in vivo* and *in vitro* have been implemented in the last few years that aim to clarify the role of astrocytes in ALS. The initial generation of transgenic mice with restricted expression of the murine G86R SOD1 mutation (G85R in humans) in astrocytes resulted in astrocytosis, but failed to reproduce the neurodegenerative phenotype. This led to the early conclusion that the limited expression of mutant SOD1 in astrocytes is not sufficient to trigger the disease (Gong et al. 2000). At variance with this, subsequent studies using chimeric mice revealed that wild-type non-neuronal cells, located in the microenvironment of mutant SOD1-expressing motor neurons, increase neuronal survival. In turn, the presence of mutant SOD1-expressing non-neuronal cells, in the motor neuronal neighbourhood, is sufficient to induce the formation of ubiquitinated protein inclusions and to elicit sufferance within wild-type motor neurons (Clement et al. 2003). These seminal findings introduced the original idea that it is actually the interaction between motor neurons and their non-neuronal neighbours that critically impact the neurodegenerative process in ALS (Clement et al. 2003). In this context, alterations to astrocytes became very important, considering that these cells cover a wide range of functions that are critically involved in the maintenance and activity of neuronal cells. Several studies *in vivo* and *in vitro* have been then undertaken to clarify the impact of astroglial cells towards ALS-linked neurodegeneration and pathology. In particular, two distinct experimental strategies have been exploited to address these issues *in vivo*. Firstly, a variety of animal models have been generated by ablating mutant SOD1 expression selectively in astrocytes by means of the *Cre*/loxP recombination system (Yamanaka et al. 2008). This genetic approach allowed to unveil that reducing the expression of the hSOD1^{G37R} protein (Gly37→Arg substitution) within the astrocytes positively modulates the phenotype of mutant SOD1 transgenic mice by slowing down ALS progression (Yamanaka et al. 2008). Such results were soon expanded by the observation that depletion of the hSOD1^{G85R} protein from astrocytes postponed the onset of the disease, in addition to extend the mouse lifespan (Wang et al. 2011). Secondly, the neurotoxic potential of mutant SOD1 astroglial cells has been directly investigated by means of cell transplantation strategies. The introduction of hSOD1^{G93A}-expressing astrocyte precursors

into the spinal cord of wild-type rodents revealed that astrocytes alone can trigger motor neuron degeneration and ALS symptoms *in vivo* (Papadeas et al. 2011). Complementary to this, the transplantation of wild-type astrocyte precursors into rodent models of ALS delayed disease progression and extended the animal life span, thus opening the perspective of astrocyte replacement-based therapeutic approaches (Lepore et al. 2008a).

Although the vast majority of the investigations addressing the contribution of astrocytes to ALS pathogenesis was performed on experimental models of ALS-SOD, the recent generation of new transgenic animals, which mimic other forms of fALS, allowed to gain further insights into the impact of these cells on the disease outcome. Some important clues came from the recent characterization of a transgenic rat line showing tetracycline-inducible expression of the mutant TDP-43^{M337V} (Met337→Val substitution) protein selectively in astrocytes (Tong et al. 2013). Upon doxycycline withdrawal, these animals exhibited selective expression of the transgene in astrocytes, and this led to the first signs of motor weakness in as little as 20 days. The phenotype worsened rapidly resulting in complete paralysis within additional 20 days. Histopathological analysis revealed denervation atrophy of skeletal muscles, progressive loss of spinal cord motor neurons, microglial activation and ubiquitin-positive inclusions within astrocytes (Tong et al. 2013). In keeping with this, another recent report provided complementary information by investigating the impact of the expression of four different variants of human TDP-43 (D169G, G298S, A315T and N345K) in glia *in vivo*, using *Drosophila* as a model. Remarkably, glial expression of mutant TDP-43 resulted in significantly smaller neuromuscular junctions (NMJs), with a decreased number of synaptic boutons. This correlated with a considerable impairment in the locomotor performance (Estes et al. 2013).

A distinct subpopulation of highly proliferating astrocytes, with an enhanced neurotoxic phenotype, has been then isolated from mutant $hSOD1^{G93A}$-expressing transgenic rats (Diaz-Amarilla et al. 2011). Increased proliferation of astroglial cells in the spinal cord of $hSOD1^{G93A}$ ALS mice was reported also in a different study, which suggested the implication of the Wnt signalling pathway in the development of the proliferating glial phenotype (Chen et al. 2012). Wnt is a family of highly conserved signalling molecules that, in the CNS, play complex and diversified roles during development, but it is also involved in the maintenance of synaptic integrity (reviewed in Rosso and Inestrosa 2013). Of note, alterations of this transduction pathway have been associated to several neurological disorders (reviewed in Al-Harthi 2012). In the $hSOD1^{G93A}$ ALS mouse model, the expression of several members of the Wnt family was shown to be de-regulated (Yu et al. 2013). In particular, some of these proteins resulted to be over-expressed in astrocytes, thus suggesting that the enhanced proliferative potential of astroglial cells might be a direct consequence of increased Wnt signalling (Li et al. 2013; Yu et al. 2013). Yet, the relevance of proliferating astroglial cells towards the ALS phenotype was not corroborated *in vivo*, as the selective ablation of dividing astrocytes by genetic approaches was shown not to affect any measures of the disease outcome in mice (Lepore et al. 2008b).

To gain new mechanistic insights into the contribution of astrocytes to motor neuron degeneration *in vitro*, several groups then established astrocyte-motor neuron co-culture systems, which allowed direct monitoring of the impact of ALS astroglia towards neuronal viability, with no interference by other neural cell types. By this means, they demonstrated that diseased astrocytes can impact motor neuron survival by releasing noxious factors (Di Giorgio et al. 2007; Nagai et al. 2007; Bilsland et al. 2008; Di Giorgio et al. 2008; Marchetto et al. 2008; Ferraiuolo et al. 2011b; Haidet-Phillips et al. 2011; Phatnani et al. 2013). Although the identity of these toxic agent(s) remains mostly elusive, different molecules have been proposed to play a role in this dreadful intercellular cross-talk (Table 11.1). The first candidate, in terms of deleterious molecules, was initially indicated in the excitatory amino acid glutamate, which is well known to trigger neurodegeneration by excitotoxic mechanisms. This hypothesis was initially fuelled by multiple evidence pointing out a defect in the astrocyte-specific plasma membrane glutamate transporter EAAT2 (also known as GLT-1 in rodents) in both ALS patients and mutant SOD1 transgenic animals (Rothstein et al. 1992, 1995; Bruijn et al. 1997; Howland et al. 2002; Guo et al. 2003; Pardo et al. 2006). EAAT2 is a high-affinity glutamate transporter that is critically involved in the clearance of glutamate from the synaptic cleft, and it is responsible for interrupting the activity of this excitatory neurotransmitter at the synapse (Danbolt 2001). However, genetic studies revealed that EAAT2 plays a major role also in maintaining extracellular glutamate below excitotoxic levels (Rothstein et al. 1996; Tanaka et al. 1997). Based on this, it was reasonably postulated that its impairment can cause abnormal increases in the extracellular concentration of glutamate, and this leads to neuronal cell death. Such hypothesis was boosted by the observation that abnormal levels of glutamate were detected in the cerebrospinal fluid (CSF) and spinal cord of sporadic ALS patients when compared to control individuals (Rothstein et al. 1990). Remarkably, defects in the glutamate uptake system were lately confirmed also in transgenic models of ALS, other than ALS-SOD. Thus, rats expressing the mutant M337V form of TDP-43 in astrocytes exhibited a progressive depletion of the astroglial glutamate transporters EAAT1 and EAAT2 in the spinal cord (Tong et al. 2013). Furthermore, a recent study in *Drosophila* revealed that both depletion or overexpression of TBPH, the *Drosophila* homologue of human TDP-43, in glial cells caused detrimental effects *in vivo*. While the selective depletion of TBPH from glia led to age-related motor abnormalities, its over-expression caused premature lethality during the larval status. Importantly, both loss and gain of *Drosophila* TDP-43 were reported to alter the expression levels of the glutamate transporters EAAT1 and EAAT2 (Diaper et al. 2013).

As yet, the mechanisms leading to EAAT2 depletion in ALS have not been fully elucidated, although several mechanistic hypotheses have been postulated. Initially, it was reported that the loss of this transporter was the consequence of aberrant splicing of its messenger RNA (mRNA) (Lin et al. 1998). Yet, this event was later proved unspecific for ALS cases, as it was observed also in control and Alzheimer's Disease patients (Honig et al. 2000). EAAT2 was subsequently proposed to be a substrate of caspase-3 (Boston-Howes et al. 2006), an enzyme executing the

Table 11.1 Factors released by astrocytes and potentially responsible for enhanced motor neuron degeneration. A list of factors released by astrocytes and presumably able to contribute to motor neuron degeneration in ALS is tabulated. These factors are subdivided by chemical class. Experimental evidence achieved in animal models and/or human ALS cases is indicated

Chemical class	Toxic molecule	Animal models	Supporting evidence from human ALS cases	References
Amino acids	Glutamate[a]	hSOD1^{G85R} mice hSOD1^{G93A} mice TDP-43^{M337V} rats TBPH depleted or over-expressing *Drosophila*	Defective uptake in synaptosomes prepared from ALS patients due to selective down-regulation of EAAT2 Increased levels in the CSF of ALS patients.	(Rothstein et al. 1992, 1995; Bruijn et al. 1997; Howland et al. 2002; Guo et al. 2003; Pardo et al. 2006; Diaper et al. 2013; Tong et al. 2013)
	D-serine	hSOD^{G93A} mice	Mutation in D-amino acid oxidase in a fALS pedigree.	(Sasabe et al. 2007, 2012; Mitchell et al. 2010)
Pro-inflammatory mediators	PGD2	hSOD^{G93A} mice: co-cultures of astrocytes and motor neurons from embryonic stem cells		(Di Giorgio et al. 2008)
	Interferon γ	hSOD^{G93A} mice		(Aebischer et al. 2011)
Growth factors	TGFβ	hSOD^{G93A} mice		(Phatnani et al. 2013)
	Pro-NGF	hSOD^{G93A} mice	Increased levels in the CSF of ALS patients.	(Ferraiuolo et al. 2011b)
Other	Lipocalin 2	Mutant SOD1, mutant TDP-43, mutant FUS rats		(Bi et al. 2013; Tong et al. 2013)

PGD_2 prostaglandin D_2, *TGFβ* transforming growth factor β, *Pro-NGF* pro-nerve growth factor, *EAAT2* excitatory amino acid transporter 2, *CSF* cerebrospinal fluid

[a] Glutamate accumulation does not depend on enhanced release from glial cells, rather to defective clearance

proteolytic phase of apoptotic programmed cell death. Cleavage generates a C-terminal fragment, and leads to inhibition of the transporter activity (Boston-Howes et al. 2006). The product of EAAT2 cleavage was also described to undergo additional post-translational modification, namely SUMOylation (Gibb et al. 2007), and to accumulate in spinal astrocytes from hSOD1^{G93A} mice at the symptomatic stage (Foran et al. 2011). More recently, an additional theory has been proposed, based on the occurrence of aberrant editing events in intron 7 of the EAAT2 pre-mRNA in the affected areas of ALS patients (Flomen and Makoff 2011). Such events appears to lead to alternative polyadenylation, ultimately causing premature transcriptional

termination and reduced levels of EAAT2 (Flomen and Makoff 2011). Down-regulation of EAAT2 was also suggested to be the result of defective signalling from neurons. For example, presynaptic terminals were shown to transcriptionally control the expression of EAAT2 by modulating the astrocytic levels of the nuclear factor Kappa-B Motif-Binding Phosphoprotein (KBBP). The loss of presynaptic signalling, following axonal degeneration, resulted in diminished EAAT2 expression (Yang et al. 2009). Finally, experiments in culture suggested that neuronal cells have the capacity to release exosomes containing the microRNA 124a (miR-124a) (Morel et al. 2013). Such organelles can be internalized by astrocytes, where they increase the astrocytic miR-124a. In turn, the latter up-regulates EAAT2 expression through a yet unidentified mechanism (Morel et al. 2013). Since miR-124a expression is down-regulated in the spinal cord of $hSOD1^{G93A}$ mice, it was speculated that its reduced expression may be responsible for the diminished levels of EAAT2 *in vivo* (Morel et al. 2013). While this amount of evidence globally failed to provide definitive conclusions about the mechanism driving EAAT2 loss in ALS, it prompted the idea that the relevance of the transporter in ALS may be determined by directly modulating its expression *in vivo*. This hypothesis was pursued by genetic and pharmacological approaches. Thus, transgene-driven expression of EAAT2 in the $hSOD1^{G93A}$ mouse line was shown to delay neurodegeneration and disease onset (Guo et al. 2003). Furthermore, double transgenic mice obtained by crossing $hSOD1^{G93A}$ ALS mice with animals showing ubiquitous over-expression of the Peroxisome Proliferator-Activated Receptor γ Coactivator 1α (PGC1α) exhibited enhanced EAAT2 expression, and this correlated with improved motor function (Liang et al. 2011). From a pharmacological standpoint, the screening of a panel of US FDA-approved drugs revealed that beta-lactam antibiotics, and ceftriaxone in particular, are capable to enhance EAAT2 expression (Rothstein et al. 2005). Chronic administration of ceftriaxone in $hSOD1^{G93A}$ transgenic mice extended the animal lifespan (Rothstein et al. 2005), thus raising great hope that this compound may successfully control disease progression in ALS patients. It is to mention that a clinical trial with ceftriaxone is currently ongoing, and results from Phase I and II indicate that this drug is generally well tolerated and can successfully reach the CNS at therapeutic doses (Berry et al. 2013). In an attempt to identify other compounds that enhance EAAT2 expression, a further screening was undertaken by the same group, which led to the identification of harmine (Li et al. 2011), a beta-carboline alkaloid, as a promising agent to be tested *in vivo*, in preclinical trials.

Further support to the excitotoxic hypothesis of ALS was provided by the landmark observation that mutant SOD1-expressing astrocytes can release high levels of D-serine, a co-activator of the N-methyl-D-aspartate (NMDA) receptors, and this exacerbates glutamate toxicity on motor neurons *in vivo* (Sasabe et al. 2007, 2012). The relevance of this amino acid was corroborated also by the identification, in a fALS pedigree, of a unique mutation (Arg199→Trp substitution) in the gene coding for D-amino acid oxidase (DAO^{R199W}), an enzyme that regulates the levels of D-serine (Mitchell et al. 2010). *In vitro* characterization revealed that this mutation not only causes the loss of DAO enzymatic activity, but also promotes both the formation of ubiquitin aggregates and apoptosis in neuronal cells. Remarkably, the detrimental effect of the mutant protein was observed even when motor

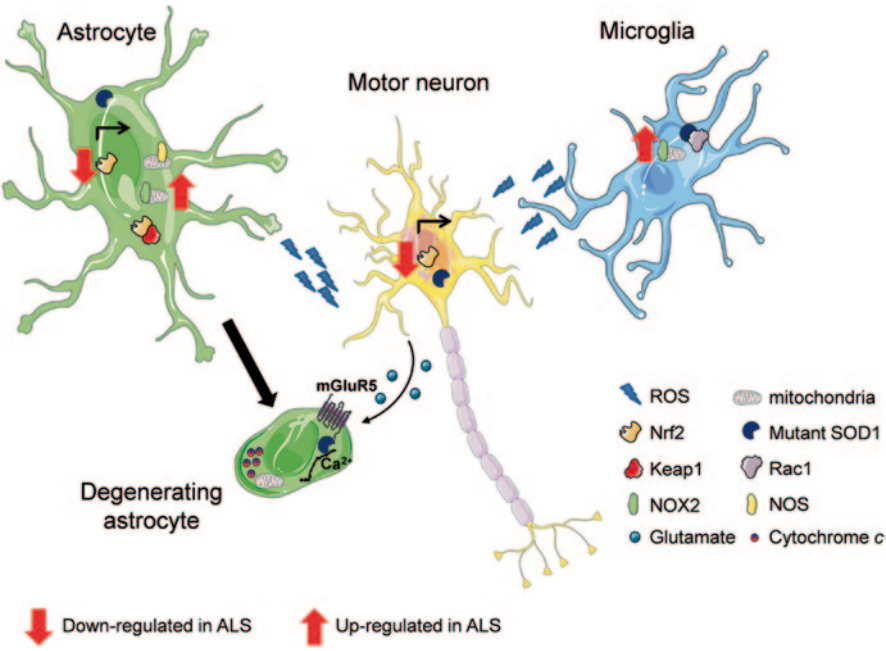

Fig. 11.1 Impact of glial cells on motor neuron sufferance in ALS-SOD. In normal conditions, healthy astrocytes support the welfare of motor neurons by supplying trophic and metabolic factors as well as by scavenging potentially harmful agents. The transcription factor Nrf2 mediates the expression of several antioxidant proteins. Normally, its activity is repressed by the Keap1 protein in cytoplasm. However, when cells are exposed to chemical or oxidative stress, Nrf2 escapes Keap1-mediated repression, translocates into the nucleus, and promotes the expression of enzymes involved in the detoxification response. In the presence of mutant SOD1, the Nrf2-Keap1 pathway is down-regulated, and both oxidant species and Nitric Oxide Synthase (NOS) products are release from astrocytes following mitochondrial dysfunction (*top*, in *green*). During ALS, mutant SOD1 expression greatly impacts on the astroglial function, and some astrocytes (*bottom*, in *green*) display an enhanced susceptibility to physiological concentrations of the transmitter glutamate. This latter triggers a gliodegenerative process via the activation of its metabotropic receptor type 5 (mGluR5). mGluR5 activation induces aberrant Ca^{2+} release from the intracellular stores, mitochondrial disarrangement with cytochrome *c* release, and apoptotic cell death. In microglial cells (in *blue*), the activation of NADPH oxidase (NOX2) participates to the establishment of a condition of oxidative stress. SOD1 normally contributes to the regulation of NOX2 activity by binding to its regulatory protein Rac1. In the presence of mutant SOD1, a stronger interaction with Rac1 is established, and this leads to aberrant NOX2 activation with the consequent over-production of reactive oxygen species

neurons were co-cultured on DAO^{R199W}-expressing astrocytes, thus suggesting that the neurodegenerative process can be mediated by both cell autonomous and non-cell autonomous events (Mitchell et al. 2010).

Increased production of reactive oxygen species (ROS), following mitochondrial dysfunction of mutant $hSOD1^{G93A}$ astrocytes, was also counted among the mechanisms contributing to motor neuron sufferance (Fig. 11.1). Oxidant species and Nitric Oxide Synthase (NOS) products were proposed to be involved in the neurotoxic process *in vitro* (Cassina et al. 2008). In keeping, the over-production of ROS by

human astrocytes expressing the hSOD1^{G37R} mutant protein was corroborated by the observation that activation of NADPH oxidase (NOX2) contributes to the establishment of a condition of oxidative stress (Marchetto et al. 2008). Interestingly, pharmacological inhibition of NOX2 activity by the drug apocynin reversed both ROS production and motor neuron cell death driven by mutant SOD1-expressing astrocytes (Marchetto et al. 2008). In addition, pharmacological (Cassina et al. 2008) or genetic (Vargas et al. 2008) manipulations apt to support the mitochondrial activity of astrocytes (Miquel et al. 2012), or to potentiate the antioxidant defenses of the cells, (Vargas et al. 2008) showed a neuroprotective potential, thus establishing a direct link between the astrocytic production of free radicals and the process of neuronal cell death.

The idea that targeting oxidative stress in astrocytes might be an efficient therapeutic option for ALS was further corroborated by a number of recent studies focused on the nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a transcription factor that mediates important cellular defense mechanisms against oxidative stress, through its capacity to regulate the expression of an array of antioxidant proteins. Under normal condition, Nrf2-dependent transcription is repressed by the inhibitor Kelch-like ECH-associated protein 1 (Keap1), which sequesters Nrf2 in the cytoplasm and promotes its degradation by the ubiquitin proteasome pathway. However, when cells are exposed to chemical or oxidative stress, Nrf2 escapes Keap1-mediated repression and translocates into the nucleus. This allows its interaction with the antioxidant response element (ARE), a *cis*-acting regulatory sequence located in the promoter region of a number of genes. By this means, Nrf2 promotes the expression of enzymes involved in the detoxification and antioxidant response (Bryan et al. 2013). The fact that the Nrf2-Keap1 signalling pathway may be involved in neuroprotective mechanisms in ALS was initially suggested by multiple investigations *in vitro*, in motor neuronal cell culture systems expressing either hSOD1^{G93A} or various TDP-43 mutants (Kirby et al. 2005; Pehar et al. 2007; Duan et al. 2010). Similar conclusions arose from additional studies investigating the expression of Nrf2 and Keap1 in post-mortem tissue samples from ALS patients (Sarlette et al. 2008) and hSOD1^{G93A} transgenic animals (Mimoto et al. 2012). Thus, the impact of different compounds acting on this signalling cascade was investigated in the hSOD1^{G93A} animal model of the disease. Among the activators of the Nrf2/ARE system taken into consideration, there are DL-3-n-butylphthalide (Feng et al. 2012), *S*[+]-Apomorphine (Mead et al. 2013), and triterpenoids (Neymotin et al. 2011). While all of these molecules resulted effective in improving the animal motor performance (Neymotin et al. 2011; Feng et al. 2012; Mead et al. 2013), the overall impact on survival was either modest (Neymotin et al. 2011; Feng et al. 2012) or even null (Mead et al. 2013). Consistent with this, genetic approaches apt to modulate neuronal Nrf2 expression *in vivo*, in the hSOD1^{G93A} and hSOD1^{G85R} mouse models, moderately delayed disease onset without affecting the animal lifespan (Vargas et al. 2013). Similarly, a gene therapy protocol apt to induce Nrf2 overexpression in neurons failed to mitigate the detrimental phenotype in hSOD1^{G93A} ALS mice (Nanou et al. 2013). At variance with these results, Nrf2 activation in astrocytes gave more promising results. Firstly, increasing Nrf2 activity in astrocytes,

by transfection or pharmacological methods, resulted neuroprotective in astrocyte-motor neuron co-culture experiments (Vargas et al. 2005, 2006). Secondly, transgene-driven expression of Nrf2 in astrocytes prevented motor neuron degeneration, delayed disease onset, and extended survival of hSOD1^{G93A} ALS mice (Vargas et al. 2008) (Fig. 11.1).

Although not all studies agree (Guo et al. 2013), these reports suggest that the Nrf2-Keap1 pathway may represent an attractive pharmacological target for therapeutic intervention in ALS. More importantly, they emphasize the concept that Nrf2 activation should be selectively achieved in the astrocytes.

Besides glutamate and oxidant species, additional neurotoxic candidate molecules of astrocytic origin were indicated in different inflammatory mediators. For example, abnormal signalling in astrocytes from different prostaglandins (PGs) was initially proposed as a likely contributor to motor neuron demise (Di Giorgio et al. 2008). Analysis of the expression of the PGD_2 and PGE_2 receptors in fact revealed that both proteins are up-regulated in hSOD1^{G93A} astrocytes (Di Giorgio et al. 2008; Liang et al. 2008). Besides, the relevance of the PGE_2 EP2 receptor was directly investigated *in vivo*. Importantly, its genetic ablation was reported to limit the pro-inflammatory response, to delay the onset of motor impairment, and to extend the lifespan of hSOD1^{G93A} ALS mice (Liang et al. 2008). An abnormal signal transduction pathway, leading to motor neuron cell death, was identified also upon the release of interferon γ (IFNγ) from hSOD1^{G93A} astrocytes in co-culture experiments (Aebischer et al. 2011). More recently, an investigation of the gene expression profile of mutant SOD1-expressing astrocytes identified striking changes even in a panel of growth factors. Thus, analysis of hSOD1^{G93A} astrocytes and motor neurons in co-culture revealed a complex and interconnected alteration in the transcriptome of these cells, suggesting a potential role for Transforming Growth Factor β (TGFβ) signalling in ALS (Phatnani et al. 2013). Furthermore, an up-regulation of the TGFβ type II receptor (TGFβ–RII) was described in motor neurons from hSOD^{G93A} mice, with a peak of expression occurring before the onset of the disease. Interestingly, such increase in TGFβ-RII immunoreactivity temporally paralleled the progression of reactive astrocytosis, as indicated by the presence of thicker and hypertrophic astrocytes around TGFβ-RII-immunopositive motor neurons (Phatnani et al. 2013). Transcription profile analysis of astrocytes isolated by laser-capture microdissection from the lumbar spinal cord of pre-symptomatic hSOD1^{G93A} mice further revealed an up-regulation of the gene encoding the Nerve Growth Factor (NGF) (Ferraiuolo et al. 2011b), a molecule that triggers apoptotic cell death in the neighbouring motor neurons through the activation of the p75 neurotrophin receptor (Pehar et al. 2007). An increased release of NGF from astrocytes expressing the mutant hSOD1^{G93A} protein was confirmed *in vitro*, and immunodepletion of this peptide was shown to rescue the neurotoxic effect of hSOD1^{G93A} astrocytes in co-cultures (Ferraiuolo et al. 2011b). It should be, however, mentioned that the contribution of glial cells to motor neuron degeneration *in vitro* was not only shown in those forms of the disease linked to *SOD1* mutations. In fact, TDP-43 was reported to interact with the Nuclear Factor-kB (NF-kB), a master regulator of several genes involved in the inflammatory response, in both neuronal and glial cells (Swarup et al. 2011). The

direct consequence of this event is that, under stress conditions, TDP-43-expressing microglia and astrocytes produce enhanced pro-inflammatory cytokines as well as neurotoxic mediators (Swarup et al. 2011). A role for the astroglial NF-kB signalling complex in mediating the inflammatory response was further supported by a recent gene expression study made on astrocytes from familial and sporadic ALS patients (Haidet-Phillips et al. 2011).

Among the reported neurotoxic factors secreted by ALS astrocytes, there is also lipocalin 2 (lcn2), a protein characterized by the ability to bind and transport lipids and other hydrophobic molecules. While lcn2 is involved in diverse cellular processes, there is considerable evidence suggesting that this protein is able to induce cell death by stimulating an apoptotic program (Devireddy et al. 2001, 2005). Remarkably, Bi and colleagues recently reported that lcn2 is released from reactive astrocytes in cultured organotypic brain slices from transgenic rats with neuron-specific expression of mutant human $TDP43^{M337V}$. Furthermore, in the brain of transgenic rats expressing mutant forms of TDP-43, FUS, or SOD1, this protein was strongly induced in reactive astrocytes (Bi et al. 2013; Tong et al. 2013). *In vitro*, lcn2 is cytotoxic to primary neurons, and toxicity is enhanced in cells that express disease genes, such as mutant FUS or TDP-43 (Bi et al. 2013).

Altogether, these observations suggest that the dynamic interactions between astrocytes and motor neurons become impaired at different levels during ALS progression.

11.3.2 Astrocyte Dysfunction and Sufferance

Several lines of evidence indicate that some of the neurosupportive functions of the astrocytes can be lost during the progression of the disease. As mentioned in the previous paragraph, astrocytes were consistently reported to exhibit a dysfunction of the glutamate uptake system in ALS patients and transgenic rodents, and this was proposed to contribute to excitotoxic motor neuron cell death (Rothstein et al. 1992, 1995; Bruijn et al. 1997; Howland et al. 2002; Guo et al. 2003; Pardo et al. 2006). In addition, astroglia were reported to modulate the intrinsic susceptibility of motor neurons to glutamate by fine-tuning the expression of the GluA2 subunit of the glutamatergic α-amino-3-hydroxy-5-methyl-isoxazole propionate receptors (AMPARs) (Van Damme et al. 2007). Remarkably, the presence of GluA2 in the AMPAR subunit composition critically impacts the biophysical properties of the receptor and determines its permeability to calcium ions (Ca^{2+}). In particular, the expression of GluA2 normally renders AMPARs impermeable to Ca^{2+}, because of an RNA editing event of the Q/R site (Q, unedited; R, edited) in the GluR2 mRNA (Sommer et al. 1991). Yet, astrocyte expressing the mutant $hSOD1^{G93A}$ protein appear to lose their capacity to modulate the expression of the GluA2 subunit of the AMPARs in the neighbouring motor neurons, thereby exposing them to enhanced Ca^{2+} influx and cell death (Van Damme et al. 2007). Besides their involvement in excitotoxic mechanisms, ALS astroglia were described to hold additional functional deficits, including mitochondrial defects as well as a reduced capacity to

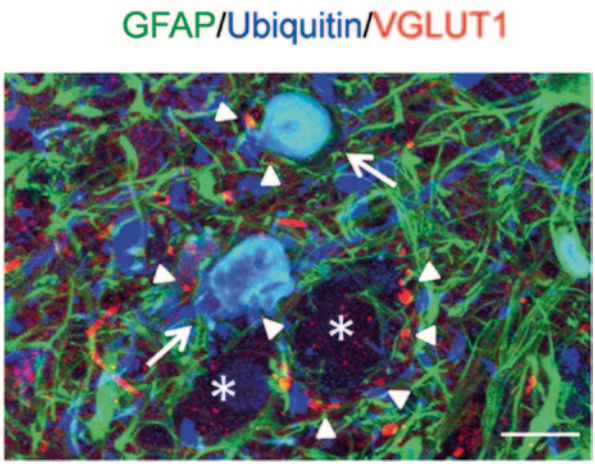

Fig. 11.2 Degenerating astrocytes appear in the hSOD1^{G93A} ALS mouse model at the pre-symptomatic stage of the disease. *Arrows* show ubiquitin-positive (in *blue*) astrocytes labelled with the astroglial marker Glial Fibrillary Acidic Protein (GFAP, in *green*). Degenerating astroglial cells are located in the motor neuronal microenvironment and became visible early during disease progression in the lumbar spinal cord of hSOD1^{G93A} mice. They are enclosed by glutamate-containing presynaptic terminals immunolabelled for the Vesicular Glutamate Transporter 1 (VGLUT1, in *red*). *Arrowheads* indicate VGLUT1-positive glutamatergic vesicles and *asterisks* show motor neuronal somas. Scale bar, 20 μm

release the metabolic substrate lactate (Cassina et al. 2008; Ferraiuolo et al. 2011b). Furthermore, the early observations of ubiquitinated SOD1- and active caspase-3-immunopositive inclusions in these cells, in the two hSOD1^{G93A} and hSOD1^{G85R} mouse models of the disease, raised the original idea that mutant SOD1 can be toxic to astrocytes themselves (Bruijn et al. 1997; Pasinelli et al. 2000). In subsequent experiments, our group investigated the significance of such astrocytic inclusions in hSOD1^{G93A}- expressing transgenic mice. We found that these unusual cells are specifically located in the motor neuronal microenvironment in the ventral horns of the spinal cord (Fig. 11.2). Furthermore, they display morphological and biochemical features reminiscent of degenerating cells, including an atypical spheroid morphology with an increased diameter and a reduced number or even the absence of cell processes immunopositive for the Glial Fibrillary Acid Protein (GFAP) (Rossi et al. 2008; Martorana et al. 2012). Dying astrocytes were first observed at the pre-symptomatic stage (Rossi et al. 2008), i.e. at a time when motor neurons exhibit axonal damage, but their somas are still vital (Pun et al. 2006). The number of spheroid astrocytes significantly increased concomitant with the onset of neuronal degeneration and the appearance of ALS symptoms (Rossi et al. 2008; Martorana et al. 2012). The relevance of this phenomenon was more recently reinforced in the context of the human disease by the identification of cells with an analogous aberrant morphology in the spinal cord of ALS patients (Martorana et al. 2012). Interestingly, we realized that degenerating astrocytes, in the spinal cord of hSOD1^{G93A}

mice, were surrounded by glutamatergic terminals (Fig. 11.2), as to indicate that these cells can sense the neurotransmitter glutamate spilled over during synaptic activity (Rossi et al. 2008; Martorana et al. 2012). Mechanistic studies *in vitro* revealed that astrocytes expressing different SOD1 mutations, either G93A or G85R, displayed an enhanced susceptibility to physiological concentrations of glutamate. The glutamatergic mechanism responsible for the deleterious event was found to involve metabotropic glutamate receptor 5 (mGluR5) signalling, which triggers an apoptotic gliodegenerative pathway in culture (Rossi et al. 2008). More recently, the signalling prompted by the activation of group I mGluRs, including mGluR5, was further characterized.

In normal astroglial cells, the activation of group I mGluRs is well known to cause the formation of inositol 1,4,5 trisphosphate (IP_3), followed by IP_3-mediated release of Ca^{2+} from the endoplasmic reticulum (ER) stores, which results in intracellular Ca^{2+} oscillations (Zur Nieden and Deitmer 2006; Gunnarson et al. 2009). Yet, mutant $hSOD1^{G93A}$-expressing astrocytes responded to the receptor stimulation with an aberrant and persistent Ca^{2+} release from the intracellular stores. This correlated with mitochondrial disarrangement, cytochrome *c* release from mitochondria, and astrocyte degeneration (Martorana et al. 2012). These results are in line with the mitochondrial dysfunction previously described by Cassina and colleagues in mutant SOD1-expressing astrocytes (Cassina et al. 2008).

The anti-apoptotic members of the Bcl-2 family, particularly Bcl-2 and Bcl-X_L, were largely reported to confer cell death resistance by fine-tuning intracellular Ca^{2+} signalling through direct interaction with the IP_3 receptor (IP_3R) channels (Chen et al. 2004; White et al. 2005; Zhong et al. 2006; Li et al. 2007; Rong et al. 2008, 2009). Interestingly, structure-function analysis of Bcl-2 homologues revealed that their N-terminal homology domain 4 (BH4) is essential for inhibition of apoptosis (Hunter et al. 1996; Lee et al. 1996; Huang et al. 1998). Our group thus investigated the impact of the BH4 domain of Bcl-X_L on astrocyte Ca^{2+} signalling by exploiting a biologically active BH4 peptide fused to the protein transduction domain of the HIV-1 TAT protein (TAT-BH4). We realized that TAT-BH4 modulates the IP_3R-dependent Ca^{2+} release from the ER, and restores spontaneous Ca^{2+} oscillations in mutant SOD1-expressing astrocytes. This tight control of IP_3Rs by the peptide prevents group I mGluR-driven aberrant release of Ca^{2+} from the intracellular stores, precludes the release of cytochrome *c* from mitochondria, and protects the cells from excitotoxic damage (Martorana et al. 2012). Furthermore, chronic treatment of $hSOD1^{G93A}$ transgenic mice with TAT-BH4 reduces degeneration of spinal cord astrocytes and shows a positive impact on the disease manifestations (Martorana et al. 2012).

Consistent with a specific vulnerability of ALS astrocytes, functional astroglia deriving from human induced pluripotent stem cells (iPSC) expressing the mutant $TDP\text{-}43^{M337V}$ protein were recently generated and shown to exhibit reduced cell survival (Serio et al. 2013). Yet, in co-culture experiments, such cells were not harmful to wild-type motor neurons, possibly because *in vitro* differentiation may not have captured some of the detrimental properties that diseased astroglia harbor *in vivo*

(Serio et al. 2013). In line with this conclusion, in transgenic rats expressing the same mutant form of TDP-43 in astrocytes, the loss of unhealthy astroglial cells paralleled progressive degeneration of motor neurons, denervation, atrophy of skeletal muscles, and progressive paralysis (Tong et al. 2013). Taken together, these findings support the view that glial cell sufferance can play a role in motor neuron degeneration also in ALS-TDP, though the evidence in this clinical subtype of the disease is still limited.

Another important function of the astrocytes that is worth mentioning concerns their association with the BBB, a complex structure tightly regulating the exchange of molecules between the brain parenchyma and the blood stream (reviewed in Daneman 2012). On a structural standpoint, the BBB is made of endothelial cells, astroglia, pericytes and neurons, which globally establish a "neurovascular unit". In this context, astrocytes project off their processes, and their endfeet ensheath the blood vessels. This strategic location suggests that they can importantly contribute to the maturation and maintenance of the BBB. Consistent with this vision, multiple studies *in vitro* have provided consistent evidence that astrocytes can influence the transendothelial resistance, a measure of BBB permeability; the organization of tight junctions between brain endothelial cells; and the luminal polarization of transporters, such the glucose transporter Glut-1 and the P-glycoprotein (Dehouck et al. 1990; Rubin et al. 1991; Hayashi et al. 1997; Sobue et al. 1999; Al Ahmad et al. 2011). Although the integrity of the BBB is of outstanding importance for the maintenance of the optimal neuronal microenvironment, alterations of this structure have been described in a number of neurological disorders, including ALS (Daneman 2012). Early studies in the hSOD1^{G93A} ALS mice identified ultrastructural damage to astrocytes and endothelial cells, leading to vascular leakage of intravenously injected Evans Blue (EB) dye already at the pre-symptomatic stage (Garbuzova-Davis et al. 2007). EB extravasation abnormalities were found in both the cervical and lumbar spinal cord (Garbuzova-Davis et al. 2007), and correlated with altered expression of critical proteins for the regulation of the efflux of catabolites from the brain parenchyma, such as Glut-1 and P-glycoprotein (Garbuzova-Davis et al. 2007). Evidence of BBB damage and microhaemorrhages was later extended to other transgenic mice, harboring the G37R and G85R SOD1 variants (Zhong et al. 2008), and to sporadic ALS cases (Garbuzova-Davis et al. 2012; Winkler et al. 2013). Given the importance of the BBB in tuning the access of drugs to the CNS, these findings should be taken in consideration when designing new therapies for ALS.

In conclusion, taken together, these studies confirm a multifaceted role for astrocytes in ALS, even though they fail to provide conclusive evidence as to whether the multiple and diverse contributions of astroglia are due to distinct astrocyte subpopulations or to the same population receiving different external inputs (Molofsky et al. 2012; Oberheim et al. 2012). We suggest that a better understanding of the impact of the distinct astrocytic mechanisms to ALS pathogenesis will be certainly beneficial to develop targeted therapeutics.

11.4 Microglia

Microglia are the glial cell population deputed to the immune surveillance of the CNS. They typically respond to injury or disease with a massive activation in areas affected by neuronal degeneration. This condition is commonly known as "reactive microgliosis". Reactive microglia have the capacity to release a wide variety of substances that can either limit or exacerbate neuronal sufferance (reviewed in Aguzzi et al. 2013). In both autoptic ALS cases and animal models, microgliosis was early recognized as a typical hallmark of the disease (Hall et al. 1998). Yet, it was only with the recent advent of modern imaging technologies, coupled to the development of radiolabelled ligands, that the extent of this phenomenon could be assessed *in vivo*, in ALS patients, at the time of disease diagnosis. A number of positron emission tomography (PET) studies has been performed by neuroimaging the 18 kDa translocator protein (also known as the "peripheral benzodiazepine receptor") (Turner et al. 2004; Corcia et al. 2012), which is highly expressed in phagocytic inflammatory cells, including activated microglia (Papadopoulos et al. 2006).

Several investigations, using mutant SOD1-expressing cellular and animal models of the disease, were then performed in order to elucidate the mechanism(s) that trigger the activation of such cells. These studies suggested that reactive microgliosis is prompted by the secretion of mutant SOD1 (Urushitani et al. 2006), and the aggregated protein was described to be more efficient than the monomeric form in this activity (Roberts et al. 2013). The relevance of microglia towards the disease manifestations was then tackled *in vivo* by genetic (Boillee et al. 2006; Gowing et al. 2008) and cell transplantation (Beers et al. 2006) approaches. Thus, reducing the expression of the mutant $hSOD1^{G37R}$ protein selectively in microglial cells was reported not to affect the disease onset, although it slowed down ALS progression in transgenic mice (Boillee et al. 2006). In agreement with these results, transplantation of donor-derived microglia from the spinal cord of $hSOD1^{G93A}$ mice into wild-type animals did not trigger ALS disease (Beers et al. 2006). Yet, transplantation of wild-type microglia induced neuroprotective effects on $hSOD1^{G93A}$-expressing motor neurons and prolonged the survival of transgenic mice (Beers et al. 2006). Altogether, these studies strengthen the idea that microglial cells modulate ALS progression, rather than influencing the development of the disease.

At the molecular level, the mechanism(s) by which microglia contribute to motor neuron sufferance still needs to be elucidated in details. Yet, a transcriptional profile analysis recently performed on acutely isolated spinal cord microglia from $hSOD1^{G93A}$ transgenic mice revealed that these cells exhibit an ALS-specific phenotype characterized by the concomitant up-regulation of both potentially neurotoxic and neuroprotective factors (Chiu et al. 2013). Among the molecules presumably mediating microglial toxicity, there are for example reactive oxidant species. In both human and mouse autoptic tissues, the ROS-generating NOX2 enzyme was in fact shown to be up-regulated specifically in microglial cells (Wu et al. 2006). This event correlated with the appearance of oxidation products and protein carbonylation adducts, including oxidative modifications of insulin-like growth factor 1

(IGF1) receptors, which are critically involved in neuronal survival. The relevance of NOX2 in disease progression was then confirmed by the evidence that genetic ablation of its catalytic subunit ameliorated the phenotype and extended the lifespan of hSOD1^{G93A} ALS mice (Wu et al. 2006). Further insights into the mechanism of NOX2- driven toxicity have been provided a few years later by Harraz and colleagues, who unveiled a new function for the SOD1 enzyme. Along with its dismutase activity, SOD1 was shown to participate to the regulation of NOX2 function by binding to its regulatory protein Rac1. Under reducing conditions, this interaction appears to stabilize Rac1 activation, thus leading to NOX2 activation and superoxide production. However, when the local concentration of oxidant species increases, SOD1 is displaced from Rac1, and NOX2 activity appears to be negatively regulated. Yet, certain mutant forms of SOD1 display a stronger interaction with Rac1, and this leads to both sustained NOX2 activation and excessive superoxide production. Because chronic treatment with the NOX2 inhibitor apocynin tremendously increased the lifespan of hSOD1^{G93A} ALS mice, this mechanism was proposed to contribute to disease progression (Harraz et al. 2008). The promising outcome of this study subsequently encouraged further investigations on NOX2 inhibitors in the context of ALS. Thus, the neuroprotective properties of diapocynin, a potent NOX2 inhibitor, were addressed *in vitro* and *in vivo* (Trumbull et al. 2012). Despite this drug proved to prevent motor neuron death at lower doses in culture experiments when compared to apocynin, the treatment of hSOD1^{G93A} transgenic mice failed to extend the animal lifespan. Moreover, the dramatic effect on mouse survival originally observed with apocynin could not be replicated in this study (Trumbull et al. 2012), thus raising serious concerns about the therapeutic potential of these agents. Nonetheless, the pathway(s) leading to NOX2 activation, and their contribution to ALS pathogenesis, remain of great interest for the scientific community. In fact, the enzyme protein disulfide isomerase, which assists the oxidative protein folding in the ER, was recently shown to be up-regulated in microglia from hSOD1^{G93A} mice. This suggests that the unfolded protein response (UPR) does not occur only in ALS motor neurons, but also in microglial cells (Jaronen et al. 2013). Importantly, UPR was reported to trigger the activation of microglial NOX2 and, thus, to increase the production of superoxide in culture experiments (Jaronen et al. 2013). These findings suggest that UPR, initiated by protein misfolding, may lead to NOX2 activation and ROS generation. However, recent studies propose alternative pathways leading to the activation of NADPH oxidase. For example, experiments *in vitro* showed that stimulation of the purinergic receptor P2X7 in mutant hSOD1^{G93A}-expressing microglia results in enhanced NOX2 activity and ROS production, thus suggesting a deleterious role for the P2X7 receptor in ALS (Apolloni et al. 2013b). At variance with these results, genetic ablation of the P2X7 receptor in hSOD1^{G93A} mice, however, exacerbated gliosis and motor neuron cell death; anticipated the clinical onset of the disease; and worsened ALS progression (Apolloni et al. 2013a). The protective effect of P2X7 suggested by these results *in vivo* was completely unexpected, given that stimulation of the P2X7 receptor had been linked also to the induction of a neurotoxic phenotype in hSOD1^{G93A} astrocytes (Gandelman et al. 2010) and to motor neuron apoptosis (Gandelman et al. 2013).

Altogether, these observations reveal that the P2X7 receptor exerts a complex dual role in ALS. Evidence in favor of a neuroprotective role for microglia in the context of ALS was provided by a recent study addressing the biological significance of the glycosaminoglycan keratan sulfate (KS) (Hirano et al. 2013). In both autoptic ALS tissues and hSOD1^{G93A} transgenic mice, KS was found to be highly expressed by a specific subpopulation of microglial cells exerting anti-inflammatory functions during the early phase of the disease. Noteworthy, genetic ablation of KS in the CNS of hSOD1^{G93A} mice resulted in marked reduction of anti-inflammatory microglia, and this correlated with acceleration of the clinical symptoms and shortening of the animal lifespan (Hirano et al. 2013).

More recently, microglia was also described to dialogue with the peripheral immunocompetent cells, an interaction that proved to be both beneficial and deleterious (reviewed in Appel et al. 2010). Several lines of evidence indicate that T cells infiltrate the spinal cord of ALS patients (Troost et al. 1989; Kawamata et al. 1992; Engelhardt et al. 1993) and mutant SOD1 transgenic mice (Beers et al. 2008; Chiu et al. 2008). Of note, infiltrating T lymphocytes were described to slow down disease progression in mice by modulating the microglial inflammatory response (Beers et al. 2008, 2011; Chiu et al. 2008; Henkel et al. 2013). Yet, spinal cord microglia were also reported to recruit peripheral monocytes to the CNS, a process that critically impairs neuronal viability and the mouse survival (Butovsky et al. 2012).

In conclusion, these findings globally suggest that the neuroinflammatory response driven by microglia includes both neuroprotective and neurotoxic aspects in ALS. Therefore, the possibility of establishing a successful therapeutic effect relies on the selective targeting of the toxic components of the immunoreaction, rather than on the non-specific suppression of the immunocompetent response.

11.5 Oligodendrocytes and Other Glial Cell Types

While astrocytes and microglia have been at the center of the investigations on ALS for almost a decade, other glial cell populations have attracted the attention in more recent times. Among others, these latter include oligodendrocytes and Schwann cells, the myelin-forming cells of the central and peripheral nervous system, respectively. Although early analyses of mutant SOD1 transgenic mice revealed SOD1-immunopositive inclusions in oligodendrocytes (Stieber et al. 2000), this observation was not pursued immediately thereafter, and these cells have been involved in ALS pathogenesis quite recently. In both ALS patients and mutant hSOD1^{G93A} transgenic mice, it has been consistently reported a loss of the monocarboxylate transporter 1 (MCT1), a protein that is highly expressed in oligodendrocytes, and which is deputed to provide motor neurons with the metabolic substrate lactate (Lee et al. 2012; Philips et al. 2013). Noteworthy, ubiquitous genetic ablation of MCT1 in mice was described to cause axonopathy (Lee et al. 2012). Furthermore, selective reduction of its expression in oligodendrocytes resulted in axonal sufferance

(Lee et al. 2012). This suggests that the shuttling of lactate from oligodendrocytes, through MCT1, is crucial for the energy supply to axons, and any disturbance of this transport can lead to axon dysfunction and, eventually, to neuronal cell death.

More recently, oligodendrocytes themselves were described to be a direct target of the disease. An apoptotic oligodendrocytic phenotype was, in fact, detected in the ventral grey matter of the spinal cord from mutant hSOD1^{G93A} transgenic mice before actual motor neuron loss became evident. Surprisingly, the overall number of oligodendrocytes was fully preserved (Philips et al. 2013). In demyelinating diseases, mature oligodendrocytes are typically replaced by the differentiation of NG2^{+} cells, which behave as precursors committed to the oligodendrocyte lineage (Chang et al. 2000). In keeping with this, investigations of the NG2^{+} cell fate in symptomatic mutant SOD1 mice revealed that they exhibit an increased proliferation rate (Magnus et al. 2008; Kang et al. 2010, 2013; Philips et al. 2013). The occurrence of this event is accompanied by a more frequent differentiation of NG2^{+} cells into oligodendrocytes (Magnus et al. 2008; Kang et al. 2010, 2013; Philips et al. 2013). Yet, at the time when mice show overt signs of the disease, the concomitant degeneration of early-born oligodendrocytes and, thus, the accelerated turnover of these cells, result in grey matter demyelination in ALS mice and human CNS (Kang et al. 2013). In addition, newly generated oligodendrocytes display reduced MCT1 expression, and thus fail to provide motor neurons with metabolic support (Kang et al. 2013; Philips et al. 2013). Since oligodendrocyte myelination is regulated by lactate (Rinholm et al. 2011), it was proposed that reduced levels of MCT1 in oligodendrocytes may affect the process of myelination, in addition to depriving motor neurons of critical metabolic substrates (Magnus et al. 2008; Kang et al. 2010, 2013; Philips et al. 2013). A role for NG2^{+} cells and their oligodendrocyte progeny in ALS development was corroborated also by gene targeting experiments. Thus, diminishing mutant SOD1 expression in these cells was shown to delay the onset of the disease and to extend the life span of mutant hSOD1^{G37R} transgenic mice (Kang et al. 2013).

In the peripheral nervous system (PNS), individual axons are myelinated by the Schwann cells, the major glial component of the PNS. An initial indication of the involvement of this glial cell population in the pathogenesis of ALS was provided by the early observation that femoral nerves from post-mortem cases displayed a certain degree of myelin disruption (Perrie et al. 1993). Later studies in mutant hSOD1^{G93A} mice provided further insights into the role of these cells in ALS by revealing that they exhibit signs of distress at the asymptomatic stage (Keller et al. 2009). Yet, investigations addressing the impact of Schwann cells on ALS pathogenesis *in vivo* by cell-specific expression or ablation of mutant SOD1 provided slightly divergent outcomes. Thus, transgene-driven expression of mutant hSOD-1^{G93A} protein in Schwann cells resulted neither detrimental for motor neurons nor deleterious to the disease manifestations (Turner et al. 2010). On the other hand, removal of mutant hSOD1^{G37R} from Schwann cells by gene excision experiments accelerated disease progression *in vivo* (Lobsiger et al. 2009). Considering the intimate relationship between motor neuronal axons and myelin, these results indicate that Schwann cells certainly deserve further attention in order to definitely elucidate their contribution to the disease.

11.6 Glial Abnormalities in Human Neuropathology

The growing awareness that non-cell autonomous mechanisms play a role in both the process of motor neuron degeneration and the manifestations of ALS disease prompted an in-depth assessment of glial abnormalities also in the human pathology. In addition to the specific alterations outlined in the previous paragraphs, there are a number of other neuropathological anomalies in glial cells related to the identification of misfolded protein inclusions in different molecular variants of the human disease. For example, misfolded SOD1 inclusions, the typical hallmark of ALS-SOD, have been recently described in various glial cell populations from both familial and sporadic ALS cases (Forsberg et al. 2011). In addition, ubiquinated protein inclusions immunopositive for TDP-43 were found in neurons, but also in the cytoplasm of glial cells (Nishihira et al. 2008; Zhang et al. 2008), particularly oligodendrocytes (Neumann et al. 2007; Seilhean et al. 2009), of classic ALS, FTLD and ALS linked to mutant optineurin (ALS-OPTN) (Ito et al. 2011). Similarly, abundant inclusions within oligodendrocytes have been identified in ALS-FUS, as revealed by double immunofluorescence labelling (Mackenzie et al. 2011). FUS-related glial pathology appears to be a distinctive feature of patients harboring mutations that mildly impair the nuclear localization of the FUS protein (Hewitt et al. 2010; Yamamoto-Watanabe et al. 2010; Mackenzie et al. 2011; Robertson et al. 2011). Remarkably, in the corticospinal tract of patients affected by ALS-OPTN, there are signs of demyelinization, further supporting the hypothesis of oligodendrocyte impairment in this form of fALS (Maruyama et al. 2010). Taken together, this amount of evidence suggests that oligodendrocyte pathology might be a common feature of those forms of ALS characterized by misfolded/mislocalized protein inclusions. This lead to the speculation that factors responsible for protein aggregation might be expressed by both oligodendrocytes and motor neurons, and their identification may open new perspectives for therapeutic intervention.

Conclusions

Several lines of evidence indicate that ALS is a disease with a very complex and multifactorial etiopathogenesis. An effective therapeutic intervention on patients is still prevented by the lack of early biomarkers of the disease as well as by the absence of an effective pharmacological strategy. Among others, this situation is conditioned by the incomplete knowledge of the mutual regulation and interactions between the cellular structures affected by the disease, particularly motor neurons and glial cells. Yet, recent breakthroughs in neuroscience have led to the increasing awareness that neuron-glial interactions are much more critical for the correct brain functioning than previously thought, and disruption of such intercellular cross-talk impairs the performance of the CNS. The perception that glial cells are actively involved in CNS function and dysfunction clearly offers a wide range of new

possibilities to unravel physiological and pathological mechanisms. In this chapter, we have summarized the currently available knowledge on the contribution of the different glial cell types to various forms of ALS. These observations convincingly demonstrate that the development of the disease involves the de-regulation of a finely tuned interplay between multiple neural cell populations. Thus, it seems that a substantial improvement of the outcome of ALS treatments may depend on a better understanding of the mechanisms governing the detrimental glial responses. The elucidation of such aspects may open new perspectives for innovative therapeutic strategies specifically targeting these cells.

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Chapter 12
Neurodegeneration and Neuroglia: Emphasis on Astroglia in Alzheimer's Disease

Alexei Verkhratsky, Vladimir Parpura and José J. Rodríguez

Abstract Neurodegenerative diseases, which affect almost exclusively humans, are chronic disorders that ultimately result in atrophy of the brain and profound cognitive deficit. Neurodegenerative process reflects a profound failure of brain homeostasis. Neuroglial cells, being primarily the cells responsible for brain homeostasis and defense, naturally contribute to an overall homeostatic failure underlying neurodegeneration. In this chapter we shall deliver a brief on astroglial contribution to common neurodegenerative disorders and then continue with a detailed account on the pathological potential of astroglia in Alzheimer's disease. Astrocytes undergo complex alterations in Alzheimer's disease, which are represented by region-specific atrophy and asthenia at the early stages and reactivity at the late stages of the disease. These complex changes can be considered as pathologically relevant because they may define the early cognitive deficits and the later neurotoxicity in Alzheimer's disease. Targeting astroglia in neurodegeneration may result in new therapeutic strategies aimed at preventing and delaying the progression of Alzheimer's disease.

Keywords Astrocytes · Astrogliosis · Alzheimer's disease · Homeostatic failure neurodegeneration

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12.1 Neurodegeneration and Neuroglia

Neurodegenerative diseases, which affect almost exclusively humans, are chronic disorders that result in a progressive loss of function, structure and number of neural cells, ultimately resulting in atrophy of the brain and profound cognitive deficit. Etiology of neurodegeneration is multifaceted including trauma (caused by physical, chemical or infectious attack), genetic predisposition, metabolic insufficiency or the combination of the above likely with some other, yet unidentified factors. Similarly, cellular and molecular mechanisms of neurodegeneration are many and because of their complexity it is often almost impossible to identify the single leading cause. At the cellular level neurodegenerative processes are often associated with aberrant handling of various proteins leading to the accumulation (both intra- and extracellular) of abnormal proteins such as β-amyloid, tau or α-synuclein (Jellinger 2008). All in all, however, neurodegenerative process reflects a profound failure of brain homeostasis, which results in a functional and structural decline in the connectivity of neural networks, which ultimately destroys information processing. Neurodegeneration begins with a functional weakening of synapses and a neurotransmission disbalance, the combination of which affects the flow of information through the neural networks. As a neurodegenerative disease progresses, the functional abnormalities worsen leading to the disappearance of synaptic contacts, alterations of cellular integrity and ultimately to death of subpopulation of the brain cells. These structural-functional changes are reflected by a generalized atrophy of the brain accompanied with profound cognitive deficiency (Terry 2000; Selkoe 2001; Knight and Verkhratsky 2010; Palop and Mucke 2010).

The common and prevailing point of view considers neurons as the main substrates of pathological progression of neurodegeneration, and it is generally assumed that failures in neuronal protein synthesis and/or direct neuronal damage caused by various factors assume the leading role in the pathogenesis of neurodegenerative disorders. These neuron-centric doctrine has been challenged only recently (Rodriguez et al. 2009; Rossi and Volterra 2009; Salmina 2009; Heneka et al. 2010; Verkhratsky et al. 2010; Rodriguez and Verkhratsky 2011; Verkhratsky et al. 2012, 2013; Brambilla et al. 2013;), when data begun to accumulate indicating a pathogenic potential of neuroglia. Neuroglial cells, being the primary cells responsible for brain homeostasis and defense, naturally contribute to an overall homeostatic failure underlying neurodegeneration.

We shall start this chapter with a brief narration on astroglial contribution to common neurodegenerative disorders and continue with a detailed account on the pathological potential of astroglia in Alzheimer's disease (AD)

12.2 Astroglial Atrophy and Astrogliosis in Neurodegenerative Diseases

Pathological changes in astroglia that occur in the course of neurodegeneration include astroglial atrophy, both morphological and functional, and astrogliosis. These pathological reactions are differentially observed at different stages

of neuropathological progression; often astroglial loss of function is observed at the early stages of the disease, whereas at the advanced stages a development of disease-specific lesions (such as, for example, senile plaques) and neuronal death trigger astrogliotic response. Pathological remodeling of astroglia is accompanied by changes in microglia similarly represented by either microglial loss of function with subsequent fading of neuroprotective capacity, or with the activation of microglia that contributes to neuroinflammation. Neurodegeneration also affects oligodendroglia and NG2 cells leading to a failure of myelination, which further exacerbates the alteration of connectivity in the central nervous system. These complex changes in neuroglia are documented for all major types of neurodegenerative disorders.

12.2.1 Neurodegeneration Associated with Toxic Encephalopathies

Astroglial dysfunction lies at the core of acute and chronic neurodegeneration associated with brain poisoning by various toxic agents. The primary mechanism of astroglial-dependent neurotoxicity that results in a profound neuronal death is associated with a failure of astroglial glutamate uptake. Astrocytes selectively express two types of glutamate transporters, the excitatory amino acid transporters 1 and 2 (EAAT1 and 2); with the aid of these transporters astroglial cells remove about 80% of glutamate released during synaptic transmission. The same mechanism is critical for astroglial protection against glutamate excitotoxicity; silencing of astroglial glutamate uptake greatly increases neuronal damage following exposure to glutamate (Danbolt 2001). Deficient astroglial glutamate transport is almost invariably present in neurodegeneration and can be considered as one of the common mechanism of this process (Kim et al. 2011).

Toxic poisoning of the brain with metals triggers neuronal death, which causes psychotic symptoms and deteriorates cognition. Astrocytes that express a complement of specific transporters are primary targets of heavy metals. Astrocytes, for example, accumulate methylmercury, which inhibits glutamate, glutamine and cystine transporters, thus severely compromising glutamate homoeostasis (Yin et al. 2007; Ni et al. 2012). These changes are primary pathogenetic elements of methylmercury-induced encephalopathy (or Minamata disease (McAlpine and Araki 1958)) manifested by cognitive decline, impaired vision and hearing, as well as motor symptoms. Astrocytes, which express high capacity manganese transport system, also appear as a main target for manganese toxicity; accumulation of manganese inhibits astroglial glutamate uptake with subsequent excitotoxic neuronal damage (Sidoryk-Wegrzynowicz and Aschner 2013). Astrocytes are primary targets for other heavy metals, such as arsenic, lead and cadmium, which all reduce the expression of glial fibrillary acidic protein (GFAP) and trigger astroglial apoptosis (Rai et al. 2013). Aluminum toxic encephalopathy (which proceeds with cognitive deficits and speech alterations) is mediated through astrocytes, which accumulate aluminum; this metal impairs glutamate transporters and gap junctions and causes astrocytic

death (Suarez-Fernandez et al. 1999). Astroglial demise through apoptosis plays a leading role in the encephalotoxic damage caused by cypermethrin, a class II pyrethroid insecticide (Maurya et al. 2012).

12.2.2 Wernicke Encephalopathy

Wernicke encephalopathy, which represents the pathological substrate for Korsakoff syndrome (ante- and retrograde amnesia, apathy and confabulation (Wernicke 1881–1883; Korsakoff 1889)) is a rapidly progressing thalamo-cortical neurodegeneration. Failure of astroglial glutamate uptake (resulting from ~60–70% decrease in the expression of EAAT1 and EAAT2) is the key pathogenetic element of Wernicke encephalopathy. A decrease in glutamate transporters expression was identified in postmortem samples, as well as in the rat thiamine deficiency model of the disease (Hazell 2009; Hazell et al., 2009); in this model astrocytes showed several atrophic signs including decrease in GFAP profiles, the expression of glutamine synthetase (GS) and GAT-3 γ-aminobutyric acid (GABA) transporter.

12.2.3 Post-infectious Neurodegeneration: Human Immunodeficiency Virus-1 (HIV-1) Associated Dementia

The HIV-associated dementia (HAD) is a primary neuroglial pathology; the HIV-1 infects microglial cells, which sustain virus propagation and through the release of neurotoxic factors precipitate neuronal death (Mattson et al. 2005; Kaul and Lipton 2006). In recent years, despite the overall success in containing HIV infection, the incidence of HAD is on the increase (Kaul 2009). In HAD, astrocytes show signs of both astrodegeneration and reactive astrogliosis. Substantial depletion of astroglial population has been recorded in the basal ganglia, with a correlation between the speed of cognitive impairments and the degree of astroglial death (Thompson et al. 2001). Astrogliotic reactions are most prominent in the entorhinal cortex and in the hippocampus (Vanzani et al. 2006).

12.2.4 Non-AD Dementiae

Astroglial pathology is documented for various forms of non-AD dementiae, such as fronto-temporal lobar degeneration and Pick's disease. These pathological remodeling include both astroglial atrophy with apoptotic death (Broe et al. 2004) and astrogliosis, the latter being, for example, prominent in the frontal and temporal cortices of patients with fronto-temporal dementia (Kersaitis et al. 2004). In thalamic dementia, profound astrogliosis was suggested to represent a key pathogenetic factor (Potts and Leech 2005).

12.2.5 Amyotrophic Lateral Sclerosis (ALS)

Astrodegeneration seems to be a key factor defining the early stages of experimental ALS; atrophic changes in astroglia and astroglial death in the human superoxide dismutase 1 (hSOD1) G93A mutation transgenic ALS model mice precede neuronal death and clinical symptoms (Rossi et al. 2008; Rossi and Volterra 2009). Furthermore, selective silencing of hSOD1 gene in astrocytes delays ALS progression (Yamanaka et al. 2008). Neuronal death, occurring at the later stages of ALS triggers astrogliotic response (Rossi and Volterra 2009).

12.2.6 Parkinson's Disease

Little is known about the contribution of neuroglia to the pathogenesis of Parkinson's disease (PD). In recent years, neuroinflammatory component begun to be considered in the context of PD and there are indications of specific role for activated microglia in causing the death of dopaminergic neurons (Depboylu et al. 2012), although there are also data showing that the activation of microglia follows the death of neurons, rather than causing it (Henry et al. 2009). Pharmacological inhibition of microglial activation was found to be neuroprotective against 6-hydroxydopamine (6-OHDA) neurotoxicity (Lazzarini et al. 2013), the treatment with 6-OHDA being employed to generate one of the most common animal model of PD. Astrocytes may also contribute to PD development, being generally protective of dopaminergic neurons at least in vitro (Mena et al. 2002; Mena and Garcia de Yebenes 2008). Similarly, astrocytes in neuronal-glial co-cultures convert L-DOPA, the immediate precursor of dopamine, from neurotoxic to neurotrophic substance, and hence astroglia can be important for L-DOPA substitute therapy (Mena et al. 1996).

12.3 Astrocytes in Alzheimer's Disease

Alzheimer's disease (Alzheimer 1907), characterized by specific histopathological lesions represented by senile plaques (extracellular depositions of β-amyloid) and interneuronal tangles resulted from abnormal phosphorylation of tau protein (Braak et al. 1998; Armstrong 2009), is a frequent cause of dementia in aging world population. The ultimate endpoint of the disease is an atrophic shrinkage of the brain accompanied with severe cognitive decline. The main current hypothesis of AD pathogenesis puts main emphasis on β-amyloid (Gerlai 2001; Hardy and Selkoe 2002; Korczyn 2008; Karran et al. 2011); criticism of which is, however, mounting (Hardy 2006, 2009; Biochemical Society 2011; Reitz 2012). The progression of the disease (according to the spread of β-amyloid load and damage to the grey matter)

begins in the transentorhinal cortex and then senile plaques spread to the entorhinal cortex, hippocampus and the temporal, frontal, and parietal lobes (Thompson et al. 2001, 2003).

12.3.1 Note on Astroglia in Aging

Aging is the major risk factor for AD; with the exception of family forms of the disease, which account for an exceedingly small number of cases (<1%), the incidence of sporadic AD correlates with age. Our knowledge of age-dependent changes in astroglia is rudimentary. There is a general consensus that aging is associated with profuse astrogliosis (Schipper 1996; Unger 1998; Lynch et al. 2010), although this notion is not based on systematic studies of either humans or animals. Majority of histological reports are based on counting GFAP positive astrocytes or on morphometry of GFAP positive profiles. Thus, the findings might be misleading given that in many brain regions healthy astrocytes do not show GFAP immunoreactivity. Consequently, an increase in the number of GFAP positive cells may not reflect actual changes in the quantity of astrocytes. Nonetheless, an increase in the number of astroglial cells was reported for the hippocampus of female C57BL mice (Mouton et al. 2002), for the CA1 area of the hippocampi of old Sprague-Dawley rats (Amenta et al. 1998), and for the parietal cortex and dentate gyrus of Wistar rats (Pilegaard and Ladefoged 1996; Peinado et al. 1998). At the same time age-dependent changes in the number of astrocytes were observed neither in the primary visual cortex of rhesus monkeys (Peters et al. 2008) nor the number of astrocytes changed in the aged human cortex (Pakkenberg et al. 2003). Increase in GFAP expression, however, was identified in the white matter of old monkeys which may signify axonal damage (Hinman and Abraham 2007). In male SV129/C57BL6 aged mice, systematic study with three astroglial markers GFAP, S100β and GS revealed rather heterogeneous changes in various brain regions (Figs. 12.1, 12.2, 12.3). In the hippocampus, for example, a prominent increase in the surface and volume of GFAP positive profiles in old (24 month) mice was not paralleled with substantial increases in the morphology of GS and or S100β positive profiles; in the entorhinal cortex, aging resulted in a significant reduction of the surface and volume of GFAP-positive profiles with an increase in the expression of S100β-positive astrocytes (Figs. 12.4, 12.5, 12.6) (Rodriguez et al. 2013b).

12.3.2 Astrocytes and β-Amyloid

There are several sporadic reports implicating astrocytes in the accumulation of β-amyloid through either compromised β-amyloid clearance or increased β-amyloid production. Reactive astrocytes, surrounding senile plaques, were suggested to accumulate and degrade β-amyloid (see (Guenette 2003; Nicoll and Weller 2003) for

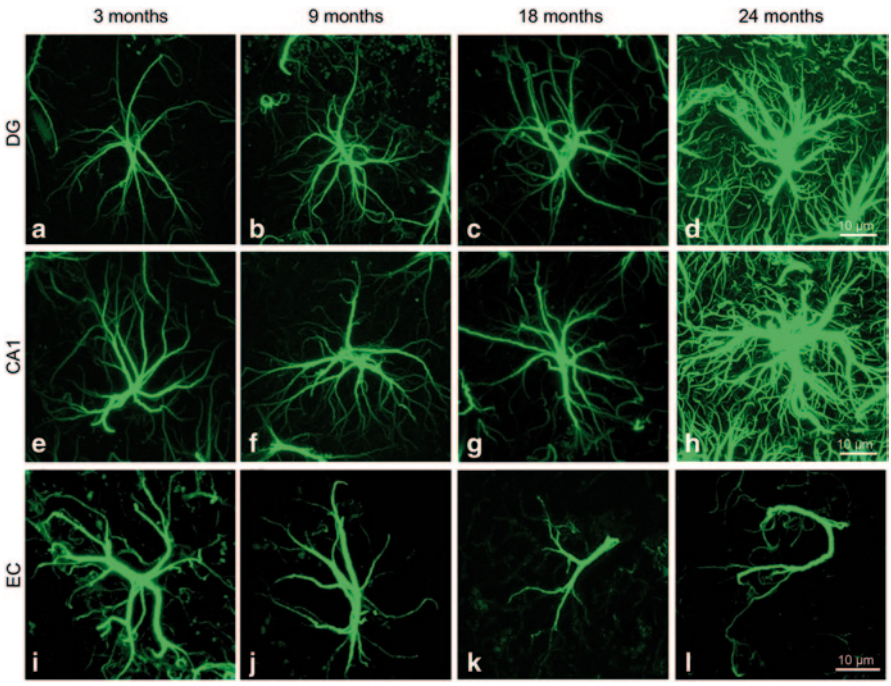

Fig. 12.1 Representative confocal 3-dimensional reconstructed images showing GFAP-immunoreactivity (IR) astrocytes in the dentate gyrus (DG), cornus ammonis 1 (CA1) and entorhinal cortex (EC) of animals at 3 months (**a**, **e** and **i**), 9 months (**b**, **f** and **j**), 18 months (**c**, **g** and **k**) and 24 months of age (**d**, **h** and **l**), respectively. (Reproduced from Rodriguez et al. (2013b) with permission.)

details and references). These reactive astrocytes, at least in the transgenic mice expressing mutant amyloid precursor protein (APP), were found to express the amyloid degrading enzyme neprilysin, a zinc metallopeptidase (Apelt et al. 2003). Cultured primary astrocytes, isolated from healthy mice brains, were able to actively accumulate β-amyloid; at the same time, astrocytes obtained from transgenic mice bearing a mutant APP were not capable of taking β-amyloid up (Wyss-Coray et al. 2003), this being another example of functional astroglial asthenia in the context of AD. Accumulation of β-amyloid was detected in astroglial cells from the entorhinal cortex of AD patients (Nagele et al. 2003) although β-amyloid was rarely found in astrocytes from the triple transgenic-AD (3xTg-AD) mice (Olabarria et al. 2010), harboring mutated presenilin 1 M146V, APP Swedish mutation (K670N/M671L) and mutated tau P301L transgenes (Oddo et al. 2003).

Healthy astrocytes do not express the main component of β-amyloidogenic pathway, the β-site APP-cleaving enzyme 1 (BACE 1; generally known as β-secretase), which seems to be exclusively expressed by neurons. Exposure of astrocytes to chronic stress, however, was reported to induce BACE1 expression, thus, potentially enabling astrocytes with β-amyloid producing capability; this was, for example, reported for astrocytes activated following immune lesion of cholinergic

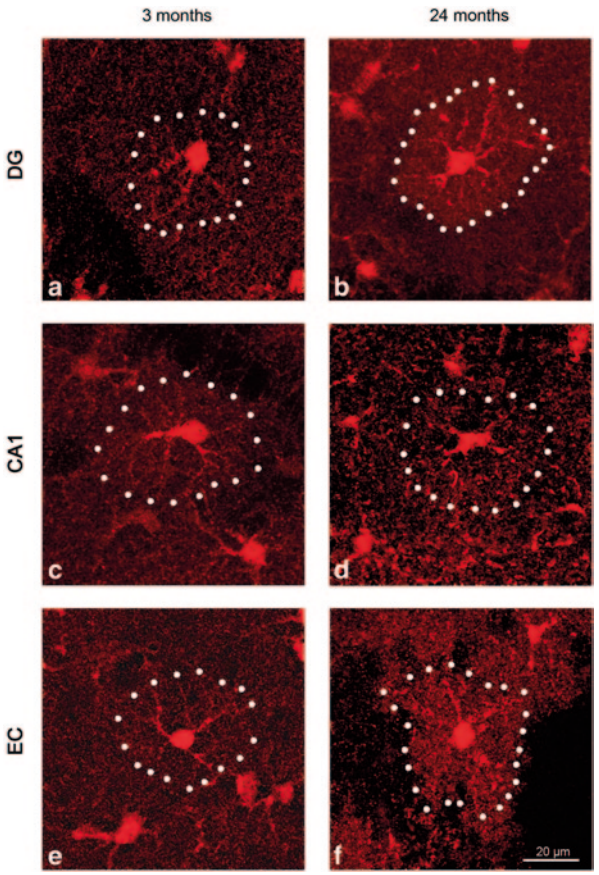

Fig. 12.2 Representative confocal 3-dimensional reconstructed images showing S100β-IR astrocytes in the DG, CA1 and EC of animals at 3 months (**a**, **c** and **e**) and 24 months of age (**b**, **d** and **f**), respectively. (Reproduced from Rodriguez et al. (2013b) with permission.)

septohippocampal afferents or occlusion of middle cerebral artery (Rossner et al. 2005). Expression of BACE1 was identified in reactive astrocytes in AD mice models (Tg2576, K670N/M671L APP or APP V717I) expressing mutated human amyloid precursor protein (Rossner et al. 2001; Hartlage-Rubsamen et al. 2003; Heneka et al. 2005). Incidentally, increase in APP production was described in a rat model of chronic neocortical astrogliosis, induced by grafting fetal cortical tissue in the midbrain of neonatal animals; these chronically activated astrocytes were immunopositive for APP, as well as for another AD-related marker apolipoprotein E (Martins et al. 2001). Nonetheless, the role of astroglia in β-amyloid turnover needs further confirmation and investigation.

12.3.3 Astrogliosis in AD

Reactive astrogliosis is generally considered to be a feature of the AD brains, and, indeed, Alois Alzheimer had found association of glia with damaged neurons; he

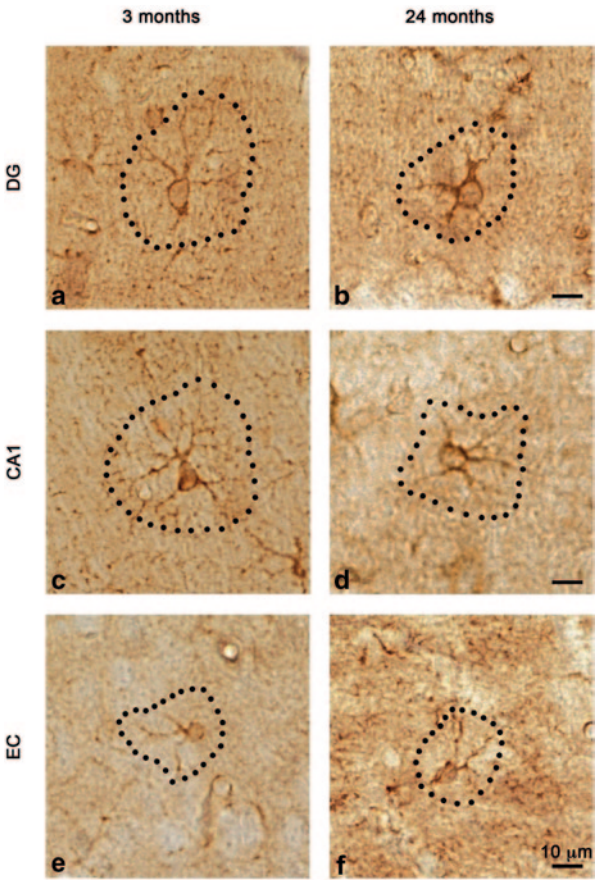

Fig. 12.3 Light micrographs showing the morphology and cell area of GS positive astrocytes in the DG, CA1 and EC of 3-month-old mice (**a**, **c** and **e**, respectively) and 24-month-old (**b**, **d** and **e**, respectively). (Reproduced from Rodriguez et al. (2013b) with permission.)

also observed glial cells abundantly populating senile plaques (Alzheimer 1910). Astrogliotic changes, mainly documented by an increase in the expression of GFAP and astroglial S100 β protein, have been observed in post-mortem tissues from AD patients (Beach and McGeer 1988; Griffin et al. 1989; Meda et al. 2001; Mrak and Griffin 2005; Rodriguez et al. 2009). Some reports claimed a degree of correlation between the astrogliosis (defined as increase in GFAP expression) and the Braak stage of AD, although there was no correlation between astrogliotic changes and β-amyloid load (Simpson et al. 2010). Reactive astrocytes were found to be associated with some senile plaques, but they were also identified in plaque free regions of the grey matter (Simpson et al. 2010). At the same time, no differences in GFAP expression was found between demented and non-demented brains (Wharton et al. 2009). Reactive, hypertrophic astrocytes, associated with senile plaques and perivascular β-amyloid deposits, were also observed in the brains of AD-models mice (Rodriguez et al. 2009; Olabarria et al. 2010; Verkhratsky et al. 2010) (Figs. 12.7 and 12.8).

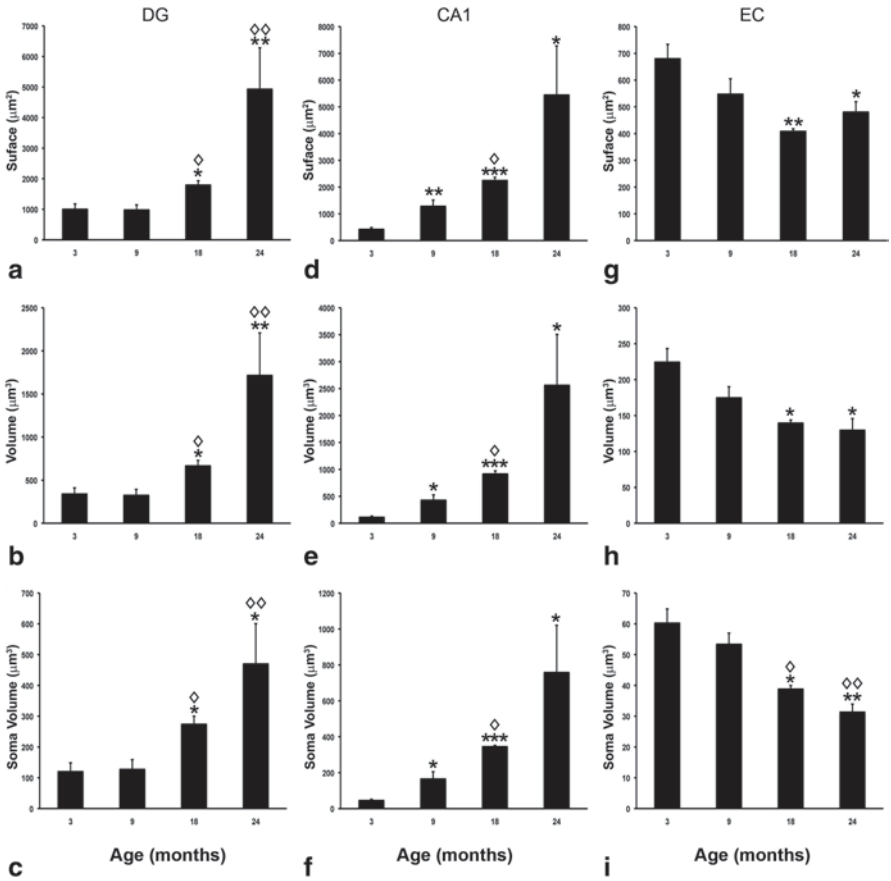

Fig. 12.4 Bar graphs showing the regional comparisons of GFAP surface, volume, and soma volume in the DG (**a**–**c**), CA1 (**d**–**f**) and EC (**g**–**i**) across ages. *Bars* represent mean± SEM (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.0001$ compared with 3 months of age; ◊ $p \leq 0.05$; ◊◊ $p \leq 0.01$ compared with 9 months of age; in DG, $n = 6$, 7, 3 and 4 for 3, 9, 12 and 18 months, respectively; in CA1, $n = 4$, 3, 3, 4 for 3, 9, 12 and 18 months, respectively; in EC, $n = 4{,}5$, 3, 3 for 3, 9, 12 and 18 months, respectively). (Reproduced from Rodriguez et al. (2013b) with permission.)

Changes in GFAP expression reported in the AD tissue may, however, not only reflect the disease-specific changes but also the age-dependent remodeling of astrocytes, which, as narrated above, remains incompletely characterized. Furthermore, it has to be emphasized that reactive astrogliosis in AD is of a rather mild variety; astrocytes in the grey matter preserve their domain organization and there is no indication of anisomorphic gliosis and the formation of glial scars. Reactive astrocytes in AD animal models show aberrant physiology. These astrocytes, associated with senile plaques, were reported to generate spontaneous Ca^{2+} oscillations and abnormal Ca^{2+} waves (Kuchibhotla et al. 2009).

Molecular cues initiating astroglial reactivity in AD are multiple and may include extracellular β-amyloid as well as factors released by damaged cells. Soluble

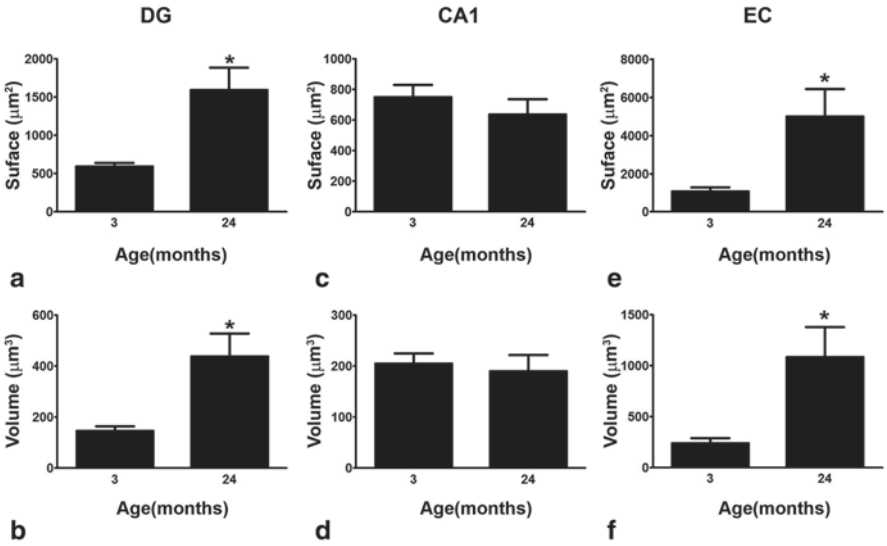

Fig. 12.5 Bar graphs showing the regional comparisons of S100β-IR surface area and volume in the DG (**a** and **b**), CA1 (**c** and **d**) and EC (**e** and **f**) at 2 and 24 months of age. *Bars* represent mean ± SEM (*$p \leq 0.05$ compared with 3 months of age; in DG, $n = 3$ and 4 for 3 and 24 months, respectively; in CA1, $n = 3$ for both 3 and 12 months; in EC, $n = 4$ and 3 for 3 and 12 months, respectively). (Reproduced from Rodriguez et al. (2013b) with permission.)

β-amyloid is reported to trigger reactive changes in astrocytes in vitro (DeWitt et al. 1998). Exposure of cultured astrocytes to β-amyloid also modifies signaling cascades. For example, extracellular β-amyloid triggers abnormal oscillatory Ca^{2+} fluctuations in cultured primary astrocytes (Abramov et al. 2003, 2004). Incubating primary astrocytes with pathologically relevant concentrations of soluble β-amyloid affects the expression of Ca^{2+} toolkit components; importantly, this remodeling differs for astrocytes derived from different brain regions (Grolla et al. 2013). Similarly, exposure to β-amyloid was claimed to down-regulate glutamate uptake in astroglial cells in vitro (Matos et al. 2008). All in all, however, the precise mechanisms of astroglial activation and remodeling of astroglial physiological signaling cascades in AD remains virtually unknown.

12.3.4 Astrodegeneration in AD: Reduction in Astroglial Profiles

Effects of AD pathology on astroglia, however, are not limited with astrogliotic response, to the contrary, astrogliosis most likely occurs in later stages of the disease, and reactive astrocytes are mainly associated with senile plaques (Olabarria et al. 2010). Recent studies of transgenic AD mice models revealed a profound astrodegeneration that occurs at the early stages of AD progression (Olabarria et al. 2010; Yeh et al. 2011; Kulijewicz-Nawrot et al. 2012; Beauquis et al. 2013).

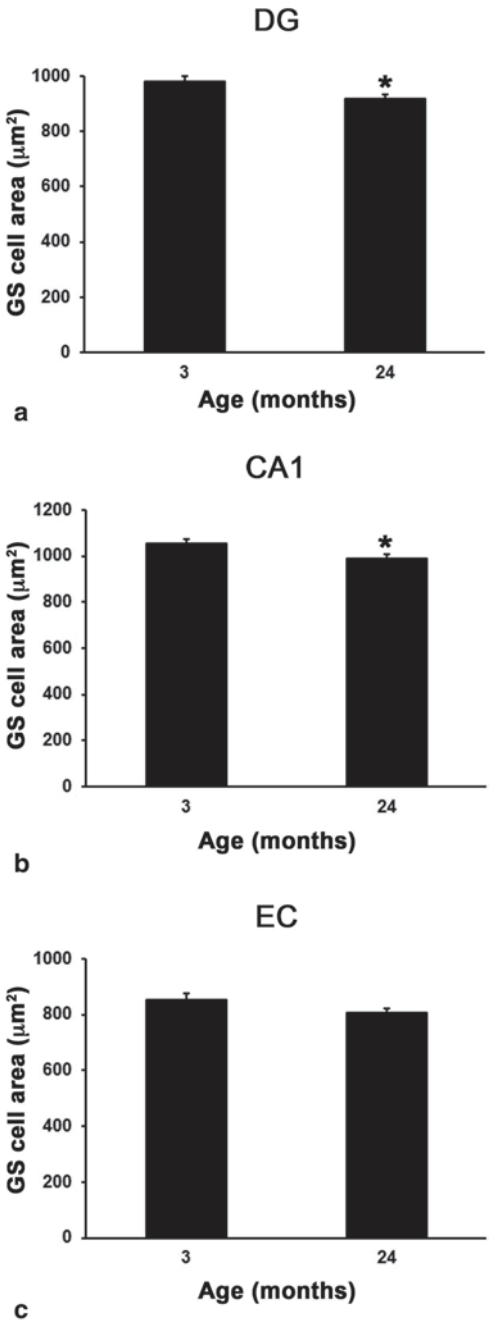

Fig. 12.6 Bar graph showing regional comparison of GS-positive cell area in the DG (**a**), CA1 (**b**) and EC (**c**) at 3 and 24 months of age (*$p \leq 0.05$ compared with 3 months of age; in DG, $n=4$ for both 3 and 24 months; in CA1, $n=4$ and 5 for 3 and 12 months, respectively; in EC, $n=4$ for both 3 and 12 months). (Reproduced from Rodriguez et al. (2013b) with permission.)

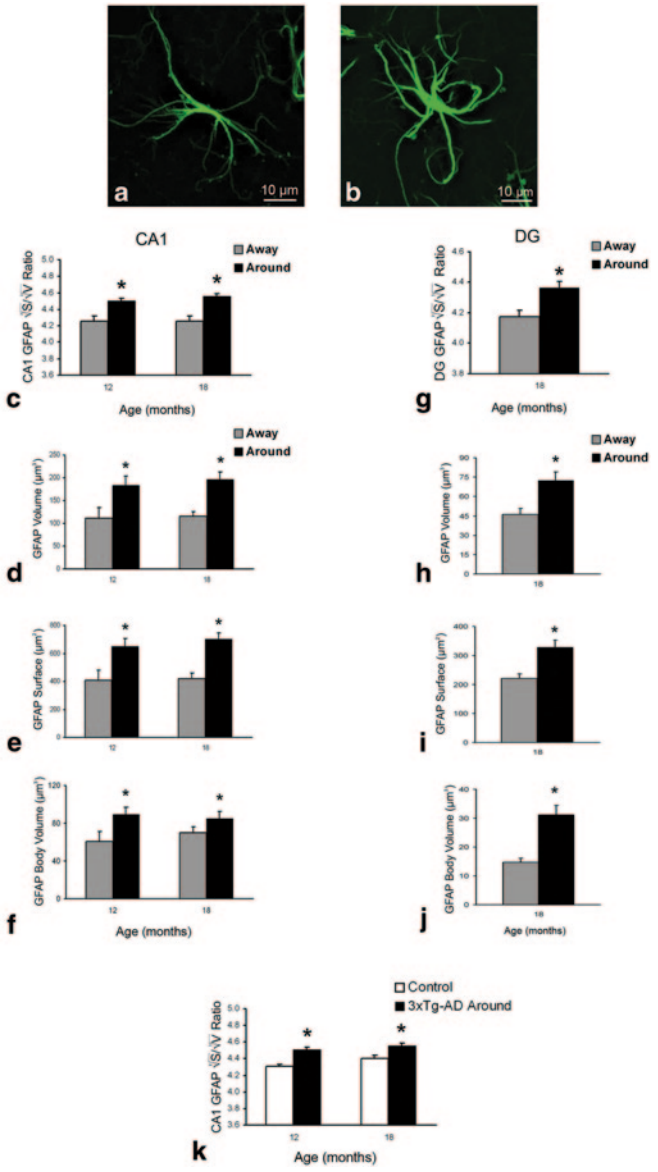

Fig. 12.7 Confocal single labeling micrographs of dual labeled immunohistochemistry illustrating the cytoskeleton alterations between astrocytes away (**a**) and around (**b**) plaques. Bar graphs showing GFAP positive astrocytic surface (**c**), volume (**d**), $^{2}\sqrt{S}/^{3}\sqrt{V}$ ratio (**e**) and body volume (**f**) differences between those astrocytes located around the amyloid plaques (Aβ) and those distant to the plaques in the CA1 of 3xTg-AD animals. $^{2}\sqrt{S}/^{3}\sqrt{V}$ ratio representation of astrocytic located around the amyloid plaques when compared to non-Tg control mice astrocytes at 12 and 18 months of age (**k**). Similar astrocytic surface (**g**), volume (**h**), $^{2}\sqrt{S}/^{3}\sqrt{V}$ ratio (**i**) and body volume (**j**) differences are observed in the DG at 18 months of age. Bars represent mean ± SEM.* = $p < 0.05$. (Modified from Olabarria et al. (2010) with permission.)

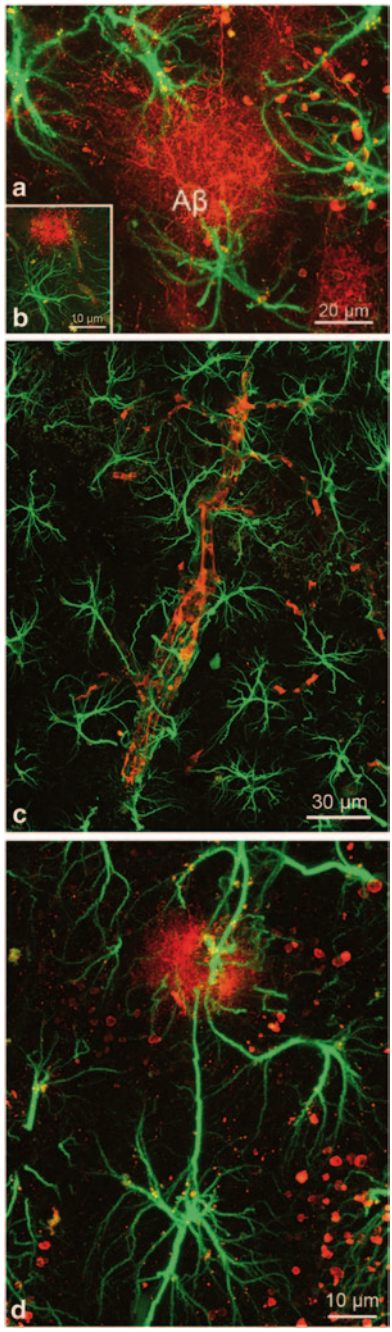

Fig. 12.8 Confocal dual labeling images (GFAP in *green* and Aβ in *red*) in 3xTg-AD mice showing the concentration of astrocytes around the Aβ accumulations (**a**–**d**). Astrocytes surround Aβ plaques (**a**, **b**) and Aβ loaded blood vessel (**c**), undergo astrogliosis including in some cases Aβ intracellular accumulation (**a**, **b**). Occasionally, some distant astrocytes send reactive processes towards a plaque (**d**). (Reproduced from Olabarria et al. (2010) with permission.)

In the above mentioned 3xTg-AD (Oddo et al. 2003), reduction in GFAP-positive profiles have been found in several brain regions (Olabarria et al. 2010; Yeh et al. 2011; Kulijewicz-Nawrot et al. 2012). These atrophic changes were quantified by decreased surface area and volume of GFAP-positive profiles, decreased volume of cell somata, decreased number of primary processes and reduction in the number of primary processes (Fig. 12.9). The total number of GFAP-positive astrocytes, however, remained stable in the hippocampus, entorhinal and prefrontal cortices of AD mice at all ages from birth to senescence (1–24 month of age) (Olabarria et al. 2010; Yeh et al. 2011; Kulijewicz-Nawrot et al. 2012). Similar atrophic changes were observed in hippocampal astrocytes from another AD animal model, the mutant APP (PDAPP-J20) mice carrying the Swedish and Indiana APP human mutations (Beauquis et al. 2013).

In the 3xTg-AD animals, reduced astroglial profiles appeared very early (at 1 months of age) in the entorhinal cortex, somewhat later in the prefronatal cortex (~6 months) and substantially later in the hippocampus (~9–12 months) (Figs. 12.10, 12.11) (Olabarria et al. 2010; Yeh et al. 2011; Kulijewicz-Nawrot et al. 2012). This atrophy of GFAP-positive profiles preceded β-amyloid deposition and formation of senile plaques. The reduction in GFAP profiles coincided with the reduced morphological presence of astroglial cells labeled with GS antibodies in the hippocampus and in the prefrontal cortex, but not in the entorhinal cortex (Olabarria et al. 2011; Yeh et al. 2013).

12.3.5 Astrodegeneration in AD: Loss of Homeostatic Support Defines Early Cognitive Impairments

Reduction in astroglial profiles, as evidenced by the morphometry of GFAP- and GS-positive cells, is indicative of a decrease in astroglial territories and hence in reduced astroglial coverage of the grey matter. This atrophy of astrocytes, which occurs early in the disease progression, may represent an important pathological stage in the disease progression. Atrophic astrocytes provide less synaptic coverage with deleterious consequences for synaptic transmission associated with compromised ion and neurotransmitter homeostasis and/or reduced local metabolic support (Verkhratsky et al. 2010; Rodriguez and Verkhratsky 2011). Astroglial degeneration, furthermore, affects the neuro-vascular unit and lessens neuroprotection. All these changes are likely to weaken synaptic transmission and affect synaptic plasticity, and thereby being responsible for initial cognitive deficiency observed during the early stages of AD.

These early cognitive deficits are the very first symptoms of AD, which start to develop years before the occurrence of specific histopathology (Terry 2000; Coleman et al. 2004). Weakening of cognitive abilities reflects in reduced synaptic connectivity due to decreased synaptic function and synaptic loss. Decrease in the number of synapses represents the earliest morphological changes in AD (Terry 2000), while the degree of synaptic loss correlates with the severity of dementia

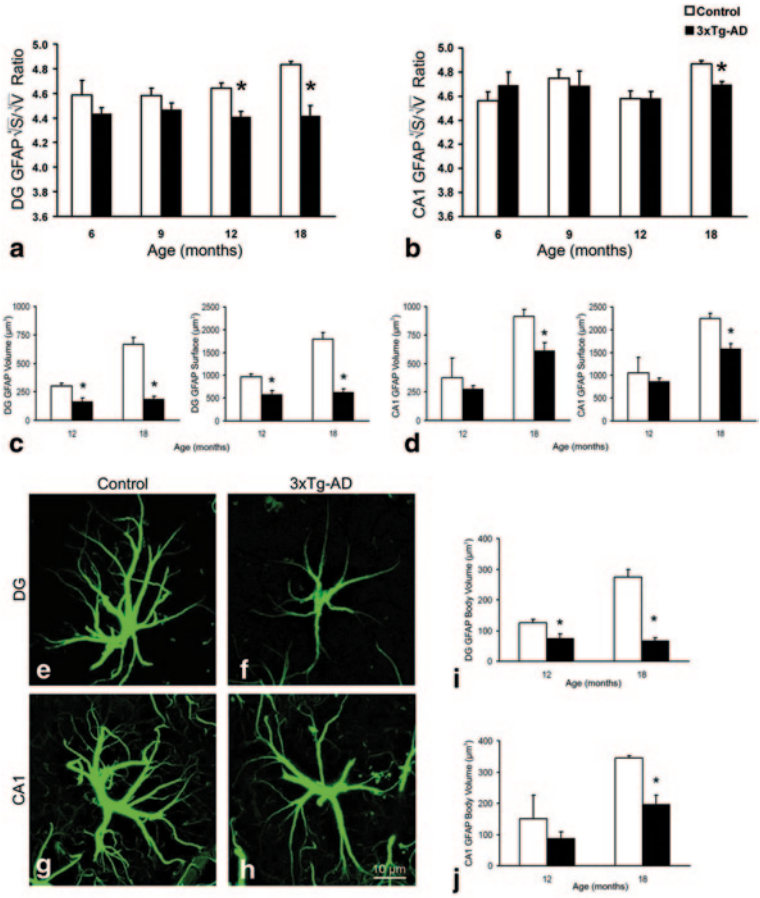

Fig. 12.9 Bar graphs showing a decreased GFAP surface, volume, $^{2}\sqrt{S}/^{3}\sqrt{V}$ ratio and body volume in both the DG (**a**, **c**, **i**) and the CA1 (**b, d, j**) of the hippocampus of the 3xTg-AD mice when compared to control animals. *Bars* represent mean ± SEM.* = $p < 0.05$. Confocal micrographs illustrating the astrocytic atrophy in 3xTg-AD mice in the DG (**f**) and CA1 (**h**) compared to control animals (**e** and **g**, respectively). (Modified from Olabarria et al. (2010) with permission.)

(DeKosky and Scheff 1990; Samuel et al. 1994). Early demise of synapses could be directly related to astrodegeneration and the resulting homeostatic failure. Astrocytes are critical for synaptogenesis and synaptic maintenance (Ullian et al. 2004; Eroglu and Barres 2010), whereas astroglial plasmalemmal transporters control local concentrations of ions and neurotransmitters, most notably glutamate, which, when not contained, causes local excitotoxicity. Astroglia also supports normal neuronal excitability and synaptic function through metabolic support accomplished by lactate shuttle (Magistretti 2006). Astrocytes are also critical for sustaining normal neurotransmission by supplying neurons with glutamine that is indispensable for

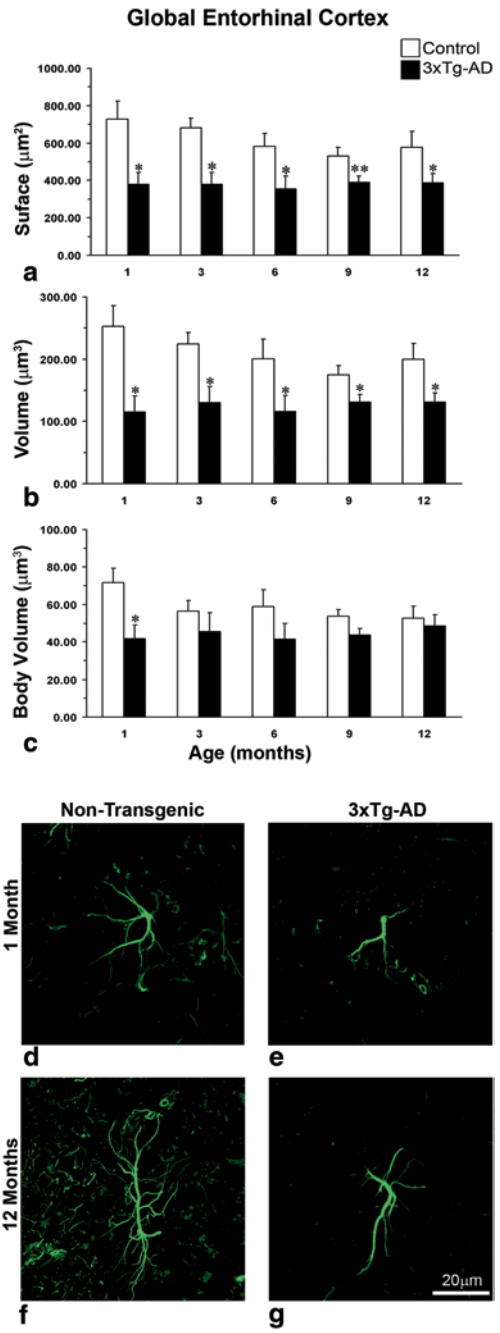

Fig. 12.10 Comparison of astrocytic GFAP surface and volume in the whole EC of non-Tg and 3xTg-AD animals across ages. Bar graphs showing comparison of GFAP (**a**) surface (**b**) volume and (**c**) body volume in global EC at the age of 1, 3, 6, 9 and 12 months between 3xTg-AD and non-transgenic animals. *Bars* represents mean ± SEM (*$p \leq 0.05$ compared with age matched non-transgenic control); Confocal micrograph showing astrocytic atrophy in 3xTg-AD at 1 month (**e**) and 12 months (**g**) compared with control animals (**d** and **f**). (Reproduced from Yeh et al. (2011) with permission.)

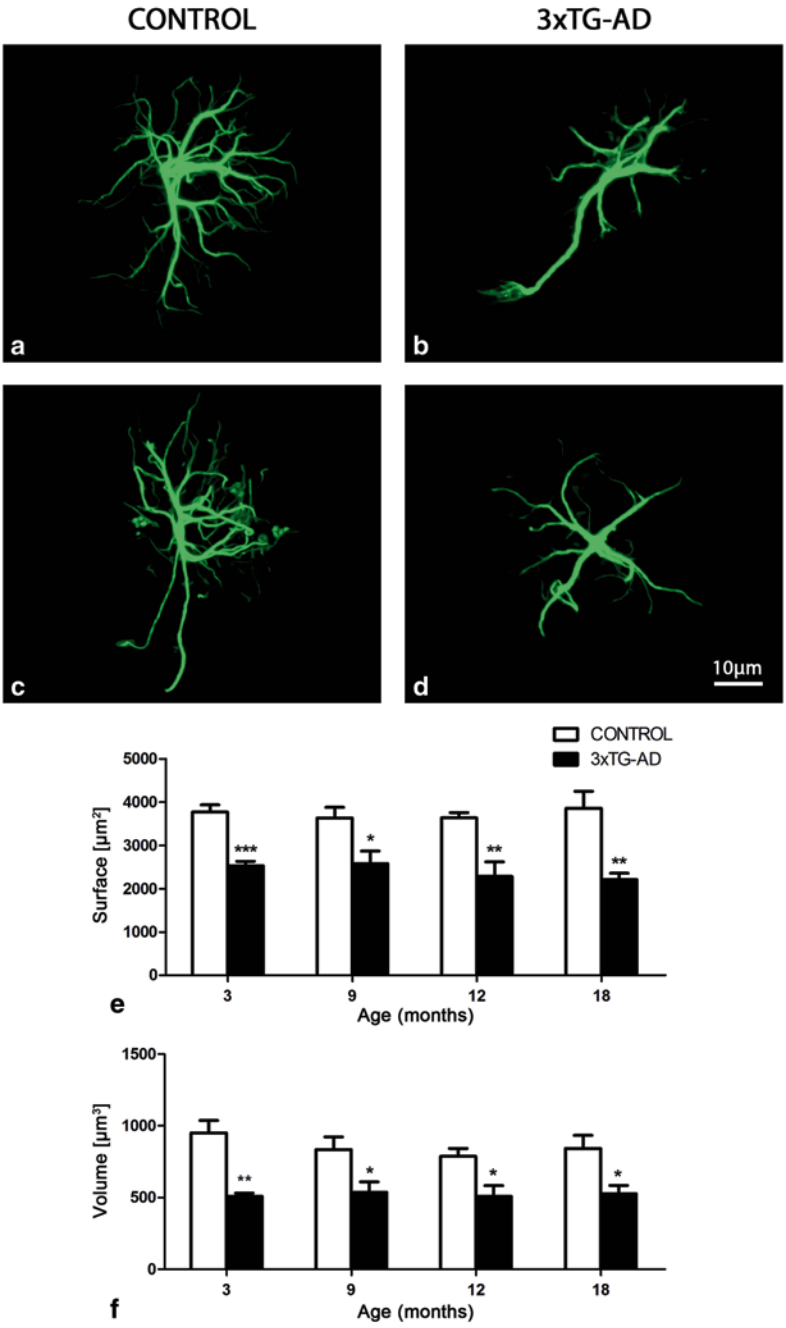

Fig. 12.11 Confocal images showing the classical morphology of GFAP-positive astrocytes in control non-Tg animals and astrocytic atrophy in the 3xTg-AD animals at 3 months (**a** and **b**, respectively) and 18 months (**c** and **d**, respectively) in the medial prefrontal cortex (mPFC). Bar graphs showing the decreases in the GFAP-positive surface area and volume throughout the whole extent of the mPFC (**e**–**f**) in 3xTg-AD mice when compared with control animals. *Bars* represent mean ± SEM. (Reproduced from Kulijewicz-Nawrot et al. (2012) with permission.)

glutamatergic and GABA-ergic pathways. Impairment of these critical functions associated with astrodegeneration can be a primary cause for distorted synaptic connectivity and early cognitive deficits in AD (Verkhratsky et al. 2010; Rodriguez and Verkhratsky 2011).

12.3.6 Astrodegeneration in AD: Dysfunctional Neuro-vascular Unit

AD pathology is often (if not always) associated with vascular deficiency. Blood flow is significantly reduced in the brains of patients with AD and especially in the early stages of the disease (see (Zlokovic 2008; Bell and Zlokovic 2009) for comprehensive review). These functional deficits reflect profound remodeling of vascularization in the diseased brains (Farkas and Luiten 2001). Brain microcirculation is controlled by both neuronal and astroglial inputs (Zonta et al. 2003; Iadecola and Nedergaard 2007; Attwell et al. 2010). Astrocytes are central elements of the neurovascular units that bridge neurons with local circulation. By releasing various factors, astrocytes target pericytes, vascular smooth muscle cells and endothelial cells, thus contributing to functional hyperemia and regulating blood-brain barrier (Zonta et al. 2003; Mulligan and MacVicar 2004; Takano et al. 2006). Astroglial atrophy, together with reactive rearrangement of neuro-vascular unit, may occur at both early and late stages of the disease and contribute to cognitive abnormalities and neuronal damage.

12.3.7 Astrodegeneration in AD: Deficits in Metabolic Support

Metabolic failure represents another common feature of AD. Progressive loss of glucose utilization has been observed in functional brain imaging in patients with different stages of AD; importantly, this metabolic stress is present in the early stages of the disease thus bearing a diagnostic significance (Mosconi et al. 2008). Experiments in vitro, in primary cultured astrocytes, demonstrated that treatment with β-amyloid affects cellular metabolism, although both decrease (Parpura-Gill et al. 1997; Soucek et al. 2003) and increase (Allaman et al. 2010) in glucose utilization were described. Similarly, both decrease (Blass et al. 2000; Liang et al. 2008) and increase (Bigl et al. 1999; Soucek et al. 2003) in the activity of glucose metabolism enzymes were reported in post-mortem AD brains.

12.3.8 *Astrodegeneration in AD: Paralysis of Astrogliotic Response Defines Susceptibility of Brain Tissue to AD Pathology*

Another important consequence of astroglial degeneration in AD is associated with the failure of their defensive function. In the 3xTg mice model, appearance of senile plaques as well as perivascular β-amyloid accumulation triggers astrogliotic response in hippocampal astrocytes, which become hypertrophic and upregulate GFAP expression (Olabarria et al. 2010, 2011). These hypertrophic astrocytes are specifically associated with β-amyloid deposits, whereas astrocytes distant to the plaques are generally atrophic (so in this sense, astroglial atrophy represents the early stage of AD progression and is complimented by astrogliosis at later stages, when specific lesions develop). In entorhinal and prefrontal cortices, however, astrocytic defense response appeared to be compromised because extracellular β-amyloid accumulation does not trigger astrogliotic response (Yeh et al. 2011; Kulijewicz-Nawrot et al. 2012). This protective failure may explain high vulnerability of entorhinal and prefrontal cortices to AD pathology.

12.4 Astrocytes as Therapeutic Targets in AD

Can astrocytes represent a new and potentially fundamentally important target for therapy in neurodegenerative disorders? Can astroglial atrophy and dysfunction be reversed or delayed, and can this affect the progression of AD or severity of cognitive deficits? These questions are of paramount importance for neurogliopathology. Only very recently astroglial cells begin to be considered as objects of treatment. Experiments on transgenic APP and 3xTg-AD mice have shown that chronic exposure of these animals to environmental stimulation (physical activity and/or enriched environment) reversed morphological atrophy of astrocytes, increased GFAP expression and normalized the appearance of GFAP-positive profiles (Fig. 12.12); these astroglia-specific changes were paralleled with a decrease in β-amyloid load (Beauquis et al. 2013; Rodriguez et al. 2013a). Chronic treatment of another AD model, the 5xFAD mice, which co-expresses the mutant forms of human APP (the Swedish mutation: K670N/M671L the Florida mutation: I716V; the London mutation: V717I) and presenilin-1 (M146L/L286V) transgenes (Oakley et al. 2006), with polyunsaturated fatty acid 2-hydroxy-docosahexaenoic acid, similarly reverted astroglial atrophy, restored adult neurogenesis and improved cognitive performance (Fiol-deRoque et al. 2013). Finally, genetic modification of astrocytes in APP/PS1 model of AD, in which astrocytes were virally transfected with a peptide that interferes with the immune/inflammatory calcineurin/nuclear factor of activated T-cells (NFAT) signaling cascades, ameliorated cognitive deficits and lowered β-amyloid burden (Furman et al. 2012). These all are of course very preliminary findings and yet they could signal new developments in astroglia-specific therapy of neurodegenerative diseases.

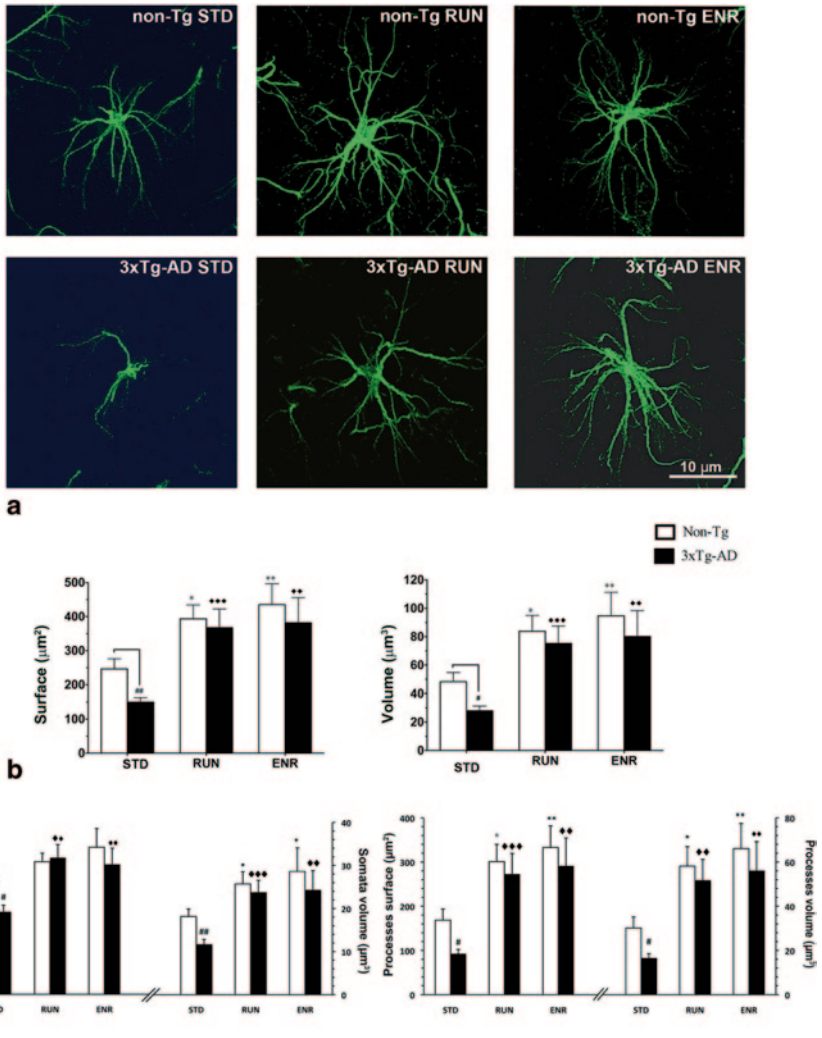

Fig. 12.12 GFAP-IR of astrocytes in the DG of non-Tg and 3xTg-AD animals housed in different conditions. **a** High magnification of representative confocal micrographs showing the astrocytic morphology in mice housed in standard conditions (STD;), RUN, and ENR. Scale bars, 10 µm. Note the morphological changes of the astrocytes from both genotypes induced by the different living conditions. **b** Histograms showing difference of surface area and volume of GFAP-positive astrocytes in the DG of non-Tg and 3xTg-AD mice housed under different housing conditions. **c** Histograms showing differences in surface area and volume of GFAP-IR astrocytic cell bodies and processes detected between non-Tg and 3xTg-AD mice housed under different housing conditions. *Bars* represent means ± S.E.M., #$p<0.05$, ##$p<0.01$ compared with non-Tg animals in same housing environment; *$p<0.05$, **$p<0.01$ compared with non-Tg mice housed under STD; $p<0.01$ and $p<0.001$ compared with 3xTg-AD mice housed under STD. (Reproduced from Rodriguez et al. (2013a) with permission.)

Conclusions

Astrocytes undergo complex alterations in AD, which are represented by atrophy and asthenia at the early stages and reactivity at the late stages of the disease, all these changes being region specific. These complex changes can be considered as pathologically relevant because they may define early cognitive deficits and later neurotoxicity. Targeting astroglia in neurodegeneration may result in new therapeutic strategies aimed at preventing and delaying the disease progression.

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Chapter 13
Possible Therapeutic Targets in Microglia

Mami Noda

Abstract In recent years, microglial cells are becoming to stand in the limelight. They may play a major role not only under physiological condition but also under many pathophysiological conditions. Manipulating their function may lead to cure or attenuation of neurological diseases, traumatic injury, psychiatric disorders, or even sleepiness or aging. In this chapter, the properties of microglial cells including receptors for neurotransmitters and neurohormones/neuromodulators, transporters, and other signals which are related to therapeutic possibilities are summarized.

Keywords Microglia · Inflammation · Pain · Neurodegenerative disorders · Psychiatric disorders · Microglia · Neurotransmitter receptor · Neuropeptide receptor · Endocrine system · Neurohormone · Cytokine · Chemokine · Neuropathic pain · Neurodegenerative diseases · ALS · Traumatic brain injury · LPS · Sleep loss · Aging · Obesity · Psychiatric disorder · Autism

13.1 Introduction

Microglial cells are immune cells in the central nervous system (CNS). Their contributions towards physiological and pathophysiological processes have been well documented (Kettenmann et al. 2011; Eyo and Dailey 2013; Verkhratsky and Butt 2013). In pathophysiological condition, microglial cells rapidly respond to any kind of damage signals accompanying poly-etiological insults to the CNS. Microglial responses in the CNS may occur as a result of trauma, ischemia, infection or neurodegenerative stimuli. Microglial cells have been implicated, to some extent, in the pathogenesis of all of the common neurodegenerative disorders involving protein aggregation such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). However, the precise role they play in the development of these pathologies remains unclear and it seems that they contribute to the pathological process in different ways in a disease-specific manner. A better understanding of their

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varied roles is essential if they are to be the target for novel therapeutic strategies (Gentleman 2013).

Activated microglia proliferate rapidly, migrate to the site of injury or infection and elicit defense response by phagocytosis of cell debris, production of cytokines, chemokines and reactive oxygen species, and presentation of antigens to other immune cells. In addition, microglia participate in tissue repair by producing neurotrophic factors. However, chronically activated microglia become neurotoxic to the surrounding CNS parenchyma.

Chronic activation of microglia has been shown to augment neurodegeneration in PD, AD, brain injury and number of other CNS pathologies. Identification of factors that control microglial activation, therefore, has become the major focus of recent research (Rangarajan et al. 2013). Microglial dysfunction may also contribute to genetic neurobehavioral disorders (Benarroch 2013). At the sites of injury or any pathological area, classical "activated" microglia, with an amoeboid morphology, show different molecular and genetic characteristics compared to those in resting microglia. Recently, this subdivision into two main types (resting and activated microglia) is regarded as oversimplification and more sophisticated microglial activity states throughout an activation process were proposed (Hanisch and Kettenmann 2007; Eyo and Dailey 2013).

Microglial activation is also regarded as a biomarker for traumatic brain injury (Hernandez-Ontiveros et al. 2013). During a cascade of secondary cell death and multiple inflammatory responses accompanying traumatic brain injury, inflammatory cytokines and chemokines spreads to normal brain areas juxtaposed to the core impacted tissue. Among immune cells involved, microglia is a key player in propagating inflammation to tissues neighboring the core site of injury.

Multiple sclerosis (MS) exhibits many of the hallmarks of an inflammatory autoimmune disorder including breakdown of the blood-brain barrier (BBB), the recruitment of lymphocytes, microglia, and macrophages to lesion sites. Inflammatory cascade is amplified by pro-inflammatory cytokines, compromising the BBB, recruiting immune cells from the periphery, and activating resident microglia (see review; Ortiz et al. 2013). Genome-wide microarray analysis comparing micro-dissected active cortical MS lesions with those of tuberculous meningitis (inflammatory control) showed that more than 80% of the identified MS-specific genes were related to T cell-mediated inflammation, microglia activation, oxidative injury, DNA damage and repair, remyelination and regenerative processes (Fischer et al. 2013).

Correlation between neurodegenerative diseases and oxidative stress is also suggested since mitochondrial dysfunction, i.e. cell energy impairment, apoptosis and overproduction of reactive oxygen species (ROS), is a final common pathogenic mechanism in aging and in neurodegenerative disease such as AD, PD and ALS (Emerit et al. 2004).

In this chapter, receptors, and other molecules, which are expressed or upregulated in activated microglia and possibly contributing to pathological conditions, are discussed as potential targets for medical intervention in microglia (Fig. 13.1).

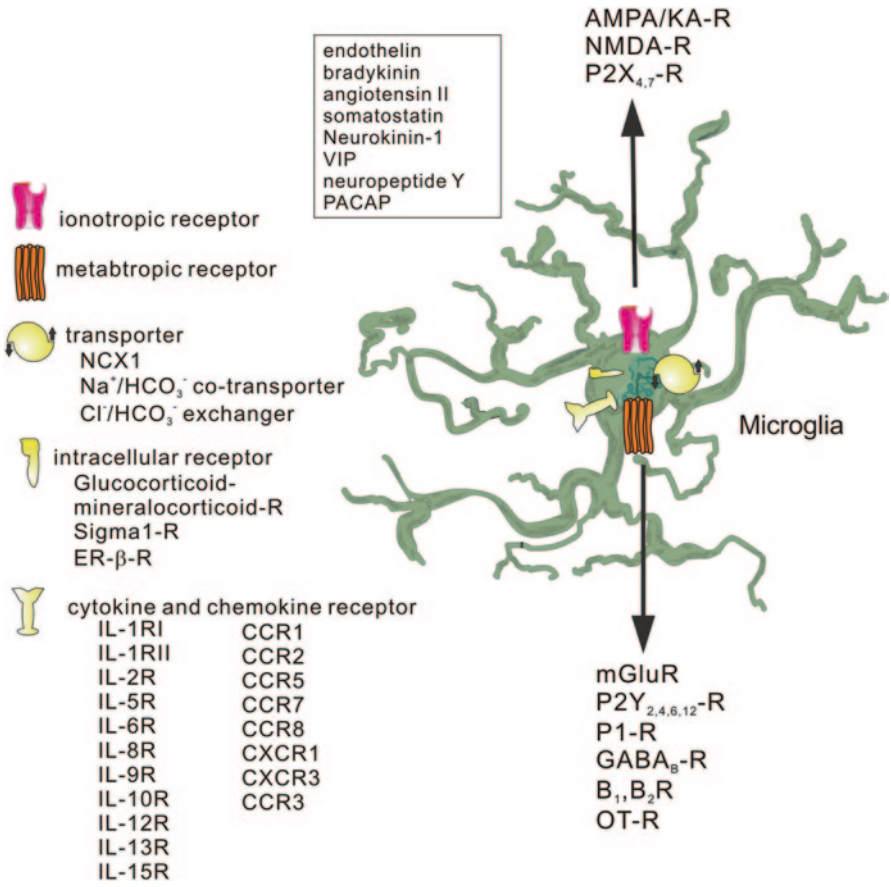

Fig. 13.1 Possible therapeutic targets in microglia. Neurotransmitter receptors, neuropeptide receptors, neuromodulatory receptors, cytokine and chemokine receptors, transporters, and various factors affecting microglial function

13.2 Neurotransmitter Receptors

Microglia express various types of neurotransmitter receptors (Kettenmann et al. 2011; Verkhratsky and Butt 2013; Kettenmann and Ransom 2013; Lee 2013), each of which can have a specific pathological role(s) which are discussed below.

13.2.1 Glutamate Receptors

13.2.1.1 AMPA Receptors

Microglia express functionally heterogeneous AMPA (α-amino-hydroxy-5-methyl-isoxazole-4-propionate)-type of receptors (Noda et al. 2000; Hagino et al. 2004) and

contribute to glutamate (Glu)-mediated neuron-glia interaction (Bezzi et al. 2001). Microglia express highly Ca^{2+} impermeable AMPA receptors due to the expression of GluA2 subunit. In addition, expression of plasmalemmal GluA2 subunits significantly increases upon activation of microglia, for example after treatment by lipopolysaccharide (LPS) (Beppu et al. 2013). Since a decreased expression of GluA2 was reported in some of the neurodegenerative diseases such as AD and Creutzfeldt-Jakob disease (Ferrer and Puig 2003; Carter et al. 2004), functional change in GluA2-deficient microglia was investigated, showing higher Ca^{2+}-permeability, consequently inducing significant increase in the release of pro-inflammatory cytokine, such as tumor necrosis factor-α (TNF-α). GluA2-deficient mouse brain also showed more neurotoxicity in response to kainic acid (Beppu et al. 2013). Therefore, involvement of microglia in glutamatergic synaptic transmission may be also important to understand the mechanism of some neurodegeneration in which low GluA2 is suggested (Noda and Beppu 2013).

In the hypothalamus and neurohypophysis of rats with streptozotocin (STZ)-induced diabetes, the expression of GluA2/3 was progressively down-regulated, being hardly detected at 4 months of STZ-diabetes, together with up-regulation of N-methyl D-aspartate receptor (NMDAR) subunit GluN1 and neuronal nitric oxide synthase (nNOS). In addition, both astrocytes and microglia appeared activated. Therefore, it was speculated that the glutamate receptors and NO are linked to overactivation of paraventricular and supraoptic nuclei leading ultimately to cell death (Luo et al. 2002). The lack of GluA2/3 in microglia may also contribute to the cell death.

13.2.1.2 Kainate Receptors

Microglia express kainate (KA) receptor subunits (Yamada et al. 2006; Noda et al. 2000) and KA-induced current was affected by application of concanavalin A which is a positive modulator selective for KA-preferring receptors (subunits GluR5–GluR7 and KA-1 or KA-2) (Huettner 1990; Partin et al. 1993; Wong and Mayer 1993), suggesting that only some of the KA-responsive cells express KA-preferring receptors and not only AMPA-preferring receptors (Noda et al. 2000). It was indicative of heterogeneous distribution of AMPA- and KA-preferring receptors among microglial cells; some cells express predominantly AMPA-preferring receptors, some cells express predominantly KA-preferring receptors, and some cells express both. The functional difference has not been identified yet.

13.2.1.3 NMDA Receptors

Although NMDA-induced membrane currents were not observed in primary cultured microglial cells (Noda et al. 2000), it was reported that cortical microglia express NMDAR subunits *in vitro* and *in vivo* and activation of the microglial NMDARs plays a pivotal role in neuronal cell death in the perinatal and adult brain

(Kaindl et al. 2012). Microglia have unique NMDAR subunit expression with a high level of the GluN2D subunit that is distinct from that found in neurons, indicating distinct NMDAR subunit assembly. The pathophysiologic relevance of microglial NMDARs is particularly high, as they confer sensitivity to excitotoxic cortical injury that is likely to be significant for a large variety of neurological diseases. For example, after stroke, glutamate released by damaged neurons in the infarct core can diffuse to nearby cells, and this can lead to an activation of microglia via their NMDARs and render them neurotoxic. Conversely, loss of function of the microglial NMDARs protects from gray matter damage.

13.2.1.4 Metabotropic Glutamate Receptors

Microglial cells also bear metabotropic glutamate receptors (mGluRs) (Biber et al. 1999; Farso et al. 2009; Taylor et al. 2002); mGluR1,5 (Group I) is linked to intracellular Ca^{2+} signaling, while mGluR2,3 (Group II) (Taylor et al. 2002) and mGluR4,6,8 (Group III) (Taylor et al. 2003) are coupled to adenosine 3′:5′ cyclic monophosphate (cAMP) and involved in regulation of TNF-α release and microglial cytotoxicity (Taylor et al. 2005). Since mGluRs is proposed as targets for multipotential treatment of neurological disorders (Byrnes et al. 2009), microglial mGluRs may be critical regulators in neuron to glia transmissions especially under pathological state.

13.2.2 Purinergic Receptors

The most widespread and possibly most functionally important receptors for microglia are purinoceptors (Verkhratsky et al. 2009b).

13.2.2.1 Ionotropic Purinoceptors (P2X Receptors)

The main type of ionotropic purinoceptors expressed in mature microglial cells are $P2X_4$ and $P2X_7$ receptors. The $P2X_4$ receptors are also constitutively expressed in microglia and contribute to microglial activation in particular in the context of neuropathic pain. Increased levels of $P2X_4$ receptors was found in activated microglia (Tsuda et al. 2003), whereas intrathecal injection of cultured microglia bearing $P2X_4$ receptors induced allodynia in the absence of peripheral nerve damage (Inoue and Tsuda 2009). Involvement of $P2X_4$ in KA-induced status epilepticus was also reported (Ulmann et al. 2013).

The $P2X_7$ receptors are involved in various neuropathologies (Franke et al. 2012) and contribute to various aspects of microglial reactions. $P2X_7$ receptors are abundantly present in immune cells and mediate many immune reactions, including the processing and the release of various cytokines. Microglial cells

constitutively express $P2X_7$ receptors and various brain lesions and neuropathologies (such as, for example, MS, ALS and AD) induce substantial up-regulation of $P2X_7$ receptors expression (Sperlagh et al. 2006; Verkhratsky et al. 2009a). Activation of $P2X_7$ receptors regulates multiple microglial processes from microglial activation to apoptotic death. Stimulation of $P2X_7$ receptors was reported to be necessary for microglial activation by amyloid-β (Aβ) protein (Sanz et al. 2009), and $P2X_7$ receptors control microglial secretion of pro-inflammatory factors (see (Kettenmann et al. 2011) and references therein). Incidentally, over-expression of $P2X_7$ receptors in microglia triggers their activation in the *in vitro* system in complete absence of any other exogenous factors (Monif et al. 2009). Recently $P2X_7$ receptor was reported to regulate microglial survival in living brain tissues (Eyo et al. 2013). Removal of extracellular Ca^{2+} or application of a potent $P2X_7$ receptor antagonist, protected microglial cell death. These pharmacological results were complemented by studies in tissue slices from $P2X_7$ receptor null mice, in which oxygen-glucose deprivation (OGD)-induced microglial cell death was reduced by nearly half. These results indicate that stroke-like conditions induce Ca^{2+}-dependent microglial cell death that is mediated in part by $P2X_7$ receptor. Increased neocortical expression of the $P2X_7$ receptor after status epilepticus and anticonvulsant effect of $P2X_7$ receptor antagonist were also reported (Jimenez-Pacheco et al. 2013). From a therapeutic standpoint, these findings could help direct novel approaches to enhance microglial survival and function following stroke and other neuropathological conditions.

13.2.2.2 Metabotropic Purinoceptors (P2Y Receptors)

Microglia express several metabotropic purinoceptors with predominant appearance of $P2Y_2$, $P2Y_6$, $P2Y_{12}$, and $P2Y_{13}$ receptors. Stimulation of these receptors as a rule triggers Ca^{2+} signals that often involve endoplasmic reticulum Ca^{2+} release and store-operated Ca^{2+} influx; overstimulation of P2Y pathways can produce a long-lasting activation of the latter that can contribute to various aspects of microglial activation (Toescu et al. 1998). The $P2Y_6$ receptors, distinctively sensitive to UDP, regulate microglial phagocytosis (Koizumi et al. 2007), whereas ADP-preferring $P2Y_{12}$ receptors are fundamental for acute microglial responses to pathological insults, for morphological activation, membrane ruffling and chemotaxis (Verkhratsky et al. 2009a). In addition $P2Y_{12}$ receptors are linked to integrin-β1 signaling, which regulates extension of microglial processes (Ohsawa et al. 2010; Eyo and Wu 2013). In the spinal cord $P2Y_{12}$ receptors are involved in the genesis of neuropathic pain (Inoue and Tsuda 2009).

As mentioned above, neuron-to-microglia purinergic signaling regulates microglial extension and retraction (see review (Eyo and Wu 2013)). In the event of neuronal injury, neurons release purines including ATP which can be degraded by endogenous enzymes into ADP and adenosine. Released purines diffuse in the extracellular space and activate P1 adenosine (A_3) and P2Y ($P2Y_{12}$) receptors on microglia that act in concert.

Recently, it was shown that soluble Aβ peptide 1–42 induced self-uptake in microglia through pinocytosis, a process involving activation of $P2Y_4$ receptors by autocrine ATP signaling. It demonstrates a previously unknown function of ATP as a "drink me" signal for microglia and $P2Y_4$ receptors as a potential therapeutic target for the treatment of AD (Li et al. 2013).

13.2.2.3 Adenosine (P1) Receptors

Adenosine receptors of P1 type are represented by classical seven-transmembrane spanning G protein-coupled receptors and are subclassified into adenosine A1, $A2_A$, $A2_B$, and A3 receptors with distinct pharmacological and functional properties. All four types of adenosine receptors were identified at the mRNA level in cultured rat microglia (Fiebich et al. 1996b). As mentioned above, purinergic activation leads to extension of microglial processes towards the injury site. Following microglial activation, adenosine can also activate $A2_A$ receptors that mediate microglial branch retraction. First, a role for the adenosine $A2_A$ receptor in microglial cytoskeletal rearrangements was suggested (Orr et al. 2009). Adenosine $A2_B$ receptors mediate an increase in interleukin (IL)-6 in human astroglioma cells (Fiebich et al. 1996a). Recently, there was a report that microglia also express A1 receptor which is upregulated upon ATP treatment. Moreover, selective stimulation of A1 receptors inhibits morphological activation of microglia, possibly by suppressing the Ca^{2+} influx induced by ATP. Microglial cells, pretreated with the A1 receptor agonist, exhibit lower capability to facilitate the nociceptive neurons, as compared with the cells treated with ATP alone (Luongo et al. 2014).

13.2.3 GABA (γ-Aminobutyric Acid) Receptors

Functional $GABA_B$ receptors were identified in a subpopulation of microglial cells in culture (Kuhn et al. 2004). The physiological role of microglial $GABA_B$ receptors are described in Kettenmann et al. (2011), but their pathophysiological role needs to be identified.

13.2.4 Cholinergic Receptors

The expression of neuronal α7 nicotinic receptors (nACh) was initially found in cultured mouse microglia (De Simone et al. 2005; Shytle et al. 2004). The activation of nACh receptors generally inhibits the immune response of microglial cells, thus representing endogenous "cholinergic anti-inflammatory pathway" (Shytle et al. 2004). On example is that nicotine was shown to inhibit $P2X_7$-mediated radical oxygen species (ROS) production in Aβ peptide 1–42 stimulated cultured microglial cells (Moon et al. 2008). Also, long-term (15 days) incubation of

corticostriatal organotypic slices with nicotine reduced the thrombin-dependent activation of microglia (Ohnishi et al. 2009). More recently, the α7 nACh receptor ligands reduced LPS-induced TNF-α release from microglia (Thomsen and Mikkelsen 2012).

13.2.5 Adrenergic Receptors

Accumulation of intracellular cAMP following stimulation of β2 receptors provided the first evidence for the expression of adrenergic receptors in microglia (Prinz et al. 2001). While β2 receptor stimulation suppressed LPS-induced release of IL-12p40 in cultured microglia (Prinz et al. 2001), stimulation of β1 receptors increased expression of IL-1β mRNA (Tanaka et al. 2002). Increasing intracellular cAMP suppressed proliferation of microglia (Fujita et al. 1998), inhibited ATP-induced release of TNF-α (Morioka et al. 2009) and modulate microglial migration and phagocytosis. Micoglial adrenergic receptors could be relevant in pathogenesis of AD as early degeneration of locus coeruleus in AD and depletion of adrenergic input to the brain affects the ability of microglia to provide for effective clearance of Aβ (Heneka et al. 2010). Recently, it became obvious that norepinephrine modulates the motility of resting and activated microglia via different adrenergic receptors; β_2 receptors mediate microglial process dynamics in resting cells whereas α_{2A} receptors do in activated cells. In addition, the presence of cross-talk between adrenergic and purinergic signaling in microglia was suggested (Gyoneva and Traynelis 2013). These data show that norepinephrine can modulate microglial motility, which could affect microglial functions in pathogenic situations of either elevated or reduced norepinephrine levels.

13.2.6 Dopamine Receptors

The $D_{1,2,3,4}$ (but not D_5) receptors were identified on translational level in microglia in culture and visualized by immunostaining in substantia nigra from Parkinsonian brains, suggesting that activation of microglia in PD triggers expression of dopamine receptors, which may explain an increase in microglia-related toxicity towards dopamine-producing neurons (Mastroeni et al. 2009). In PD, microglial cells in substantia nigra are activated and concentrated around dystrophic dopaminergic neurons (Rogers et al. 2007); the role of dopamine receptors in this specific activation and migration of microglia needs to be clarified further. The inhibition of D_4 receptors by specific agonist suppressed microglial activation in the spinal cord of superoxide dismutase 1 transgenic animal model of ALS; this reduced microglial activation coincided with slowing down the disease progression (Tanaka et al. 2008).

13.2.7 Serotonin Receptor

Microglia express 5-HT_2 serotonin receptors (Kettenmann et al. 2011; Kato et al. 2013b). There is a possibility that modulating microglia serotonin receptors may be a key target in the treatment of various psychiatric disorders, which likely will be a hot topic in the near future.

13.3 Neuropeptide Receptors

In addition to neurotransmitter receptors, microglia has receptors for a wide variety of neuropeptides, such as endothelin, bradykinin, angiotensin II, somatostatin, neurokinin-1, vasoactive intestinal peptide (VIP), neuropeptide Y (see (Kettenmann et al. 2011) (Kettenmann and Ransom 2013) for details). Bradykinin receptors, for example, have distinct effects on microglia from those on neurons. Inducible B_1 receptor stimulate microglial migration (Ifuku et al. 2007) and have rather neuroprotective effects against LPS-induced activation and inflammatory responses (Noda et al. 2006, 2007). Pituitary adenylate cyclase activating polypeptide (PACAP) activates a quinine-sensitive K^+ outward current and modulates activities in microglia (Ichinose et al. 1998), VIP and PACAP inhibit the MEKK1/MEK4/JNK signaling pathway (Delgado 2002b) and CREB binding protein (CBP)-nuclear factor (NF)-κB interaction (Delgado 2002a) in endotoxin-activated microglia. Neuropeptide receptors in microglia could become a strong therapeutic target, especially for AD.

13.4 Hormones and Hormone Receptors

It was indicated that glial cells may play a significant role in the link between the endocrine and nervous systems (Garcia-Segura et al. 1996; Chowen et al. 1996). Glial cells express nuclear receptors for both thyroid and steroid hormones and participate in the metabolism of these hormones, resulting in the production of neuroactive metabolites. Furthermore, glial cells synthesize endogenous neuroactive steroids, including pregnenolone and progesterone, from cholesterol. It is known that thyroid hormones, glucocorticoids, gonadal steroids, and neurosteroids affect glial functions. Under physiological conditions, hormonal effects on glia may have important consequences for neuronal development, metabolism, and activity and for the formation and plasticity of synaptic connections. In addition, glucocorticoids, gonadal steroids, and neurosteroids may affect regenerative processes in neurons by modulating glial responses after injury. These effects include the activation of microglia, which is regulated by glucocorticoids. Therefore, endocrine impairments affect microglial function as well as other cell types in the CNS.

13.4.1 Sex Hormones

Estrogen has been shown to be neuroprotective in stroke and other neural injury models. The presence of estrogen receptor-β (ER-β) in rat microglial cells was reported a decade ago (Mor et al. 1999). Estrogen-mediated neuroprotection is critically dependent on insulin-like growth factor (IGF)-1 signaling. Microglia appears as the source of IGF-1 and the locus of estrogen-IGF-1 interactions in stroke neuroprotection (Sohrabji and Williams 2013).

Microglial cell line BV-2, responded to hypoxia by enhanced pro-inflammatory cytokine secretion and reduced phagocytic activity, which is prevented by sex steroids resulting in a switch of microglial cells from a pro-inflammatory to a more anti-inflammatory phenotype. Anti-inflammatory effects of gonadal steroids might directly be mediated through hormone-microglia interactions in addition to known effects via astroglial regulation (Habib et al. 2013a, b). Estrogen is also neuroprotective in the MS model (Wisdom et al. 2013) and encephalomyelitis (Spence et al. 2013; Wu et al. 2013). Also, testosterone, being metabolized to estradiol and dihydrotestosterone, decreases reactive astroglia and reactive microglia after brain injury in male rats: role of its metabolites, estradiol and dihydrotestosterone (Barreto et al. 2007).

Neuronal plasticity is regulated by the ovarian steroids estradiol and progesterone in many normal brain functions, as well as in acute response to injury and chronic neurodegenerative disease. In a female rat model of axotomy, the estradiol-dependent compensatory neuronal sprouting is antagonized by progesterone (Bali et al. 2013).

In developing rat brain, microglia are essential to masculinization of brain and behavior (Lenz et al. 2013). Estradiol up-regulates the synthesis of prostaglandin E2 (PGE2), originally in neurons, and then activate microglia and stimulates their own release of PGE2. This feed-forward loop of PGE2 production is required for the masculinization of the preoptic area and therefore of sexual behavior (Welberg 2013). Microglial dysfunction may be also involved in abnormalities of brain development and sexual behavior.

13.4.2 Cortisol Receptor

Glucocorticoid- and mineralocorticoid receptors in microglia were reported (Tanaka et al. 1997). Corticosterone suppresses the proliferation of BV2 microglia cells via glucocorticoid, but not mineralocorticoid receptor (Nakatani et al. 2012). Limitaion of microglial activation was also reported in dopaminergic neurodegeneration and in acute stress model (Sugama et al. 2009; 2012). They suggest that glucocorticoids may serve as an important endogenous suppressive signal limiting neuroinflammation. Dexamethasone inhibits the Nox-dependent ROS production via suppression of MKP-1-dependent MAPK pathways in activated microglia, suggesting down-regulation of Nox-2 and overexpression of MKP-1 that regulate ROS and NO may form the potential therapeutic strategy for the treatment of neuroinflammation in neurodegenerative diseases (Huo et al. 2011).

13.4.3 Oxytocin Receptors

Oxytocin mediates social neuroprotection after cerebral ischemia. There is evidence for a direct suppressive action of oxytocin on cultured microglia, which is a key instigator in the development of neuroinflammation after cerebral ischemia (Karelina et al. 2011). Since oxytocin is important for social behavior (Eckstein and Hurlemann 2013), possible involvement of microglia via oxytocin needs to be investigated.

13.5 Cytokine and Chemokine Receptors

Microglial cells are in possession of several types of cytokine and chemokines receptors which regulate multitude of immune responses (see (Kettenmann et al. 2011; Kettenmann and Ransom 2013) for details). Microglia express multiple interleukin receptors (IL-1RI, IL-1RII, IL-5R, IL-6R, IL-8R, IL-9R, IL-10R, IL-12R, IL-13R, and IL-15R) (Lee et al. 2002) and the activation or cultured mouse microglia with LPS induced expression of IL-2 receptors (Sawada et al. 1995). Spinal cord microglia showed sensitivity to IL-6, connected with a signal transducer and activator of transcription (STAT) pathway, contributing to development of mechanical allodynia and neuropathic pain (Dominguez et al. 2008).

Among chemokine receptors (CCR) which are expressed in the cells of the CNS, microglial cells in particular express CCR1, CCR2, CCR7 and CCR5 (rodents) and CXCR1, CXCR3 and CCR3 (humans) (Boddeke et al. 1999a, b; Flynn et al. 2003). A major regulator of microglial activation and neurotoxicity in the CNS is the CX3CL1 (fractalkine, i.e. ligand)/CX3CR1 (fractalkine receptor) signaling axis (Zujovic et al. 2000; Cardona et al. 2006). On the other hand, it was suggested that CCR2/CCR5 antagonist has the potential for broad clinical use in neuropathic pain treatment (Padi et al. 2012) and decreased pain responses of CCR5 knockout mice to chemical or inflammatory stimuli was reported (Lee et al. 2013). In addition, another important chemokine, chemokine (C-C motif) ligand 1 (CCL-1), originally a well-characterized chemokine secreted by activated T cells, also plays an important role in neuropathic pain induced after partial sciatic nerve injury (Akimoto et al. 2013a). CCL-1 plays an important role in neuropathic pain induced by nerve injury and is also produced in various cell types in the CNS, especially in dorsal root ganglia. The specific receptor for CCL-1, CCR8, was found mainly in neurons but also in astrocytes and microglia. CCL-1 has multiple effects on microglia, i.e. increasing chemotaxis, motility, proliferation and phagocytosis. CCL-1 also activated microglia morphologically, promoted mRNA levels for brain-derived neurotrophic factor (BDNF) and IL-6, and increased the release of nitrite from microglia (Akimoto et al. 2013b). These indicate that CCL-1 may contribute to the development of neurological diseases, especially in neuropathic pain.

13.6 Other Receptors and Molecules

13.6.1 Toll Like-Receptors

Toll-like receptors (TLR1–9) are particularly diversified in microglia being involved in regulation of activation in response to multiple exogenous pathological factors. Activation of TLRs triggers several complex signaling cascades that often involve adaptor protein MyD88, transcription factors AP-1 and NF-κB (Hansson and Edfeldt 2005; Leulier and Lemaitre 2008). Different TLRs detect different infectious agents. Stimulation of TLRs initiates activation of microglia, and the type or receptor involved may define specific activation programs. It was recently reported that microglia works as key mediators of post-traumatic brain edema and was suggested that high-mobility group box protein 1 (HMGB1)-TLR4 signaling promotes neurovascular dysfunction after traumatic brain injury (Laird et al. 2014).

13.6.2 Sigma Receptor

Sigma-1 receptor (S1R), an endoplasmic reticulum-resident receptor with chaperone-like activity, has recently attracted great interest. S1R is involved in several processes leading to acute and chronic neurodegeneration, including ALS pathology. Pharmacological manipulation of S1R seems to be a promising strategy to cure ALS. It pointed to increased availability of growth factors and modulation of astrocytosis and of macrophage/microglia as part of the mechanisms (Peviani et al. 2013).

13.6.3 Ion Transporters

Microglial plasmalemmal transporters such as Na^+/Ca^{2+} exchanger (Ifuku et al. 2007), Na^+/HCO_3^- co-transporter and/or Na^+-dependent Cl^-/HCO_3^- exchanger (Schwab 2001) and Na^+/H^+ exchanger isoform 1 (Shi et al. 2013) are important in microglial migration.

13.6.4 Macrophage Colony Stimulating Factor

Increased levels of macrophage colony stimulating factor, increased microglia and microglial activation are found in many different CNS pathologies including tumors, neurodegenerative diseases and injury (reviewed in Charles et al. (2012); El Khoury and Luster (2008); Imai and Kohsaka (2002); Loane and Byrnes (2010)).

13.7 Pathological Conditions Relating to Microglial Activation

13.7.1 Sleep Deprivation

Deficit of sleep triggers changes in inflammatory signaling pathways in the brain and periphery. The TLR4 receptors activate inflammatory signaling cascades in response to endogenous and pathogen-associated ligands known to be elevated in association with sleep loss. TLR4 is therefore a possible mediator of some of the inflammation-related effects of sleep loss (Wisor et al. 2011a). In agreement to this observation, microglial changes in the cerebral response were observed after sleep loss, which was modulated by minocycline, a compound that attenuates microglial activation occurring in association with neuroinflammatory events (Wisor et al. 2011c). Actually changes in the sleep/wake cycle that occur subsequent to administration of D-methamphetamine are modulated by cerebral microglia; the effects of D-methamphetamine on sleep/wake cycles are mediated in part by actions on microglia, including possibly nNOS activity and cytokine synthesis (Wisor et al. 2011b). Recently more information about interaction between microglial activation and sleep or circadian rythm have been reported (Hayashi et al. 2013; Yang et al. 2013; Yu et al. 2013).

13.7.2 Obesity

In addition to neurodegenerative disorders, obesity is also considered to be associated with a state of chronic neuroinflammation. High-fat-diet exposure induces significant microglial activation not only in the hypothalamus (Yi et al. 2012) but also in the cerebellar white matter (Tapia-Gonzalez et al. 2011). Free fatty acid palmitate on its own induces activation of BV2 microglia cell line. Further, pre-exposure to palmitate changed the response of microglia to LPS, affecting the mRNA levels of the pro-inflammatory cytokines IL-1β and IL-6 (Tracy et al. 2013).

13.7.3 Aging

With aging, microglia change their morphology and may display diminished capacity for normal functions related to migration, clearance, and the ability to shift from a pro-inflammatory to an anti-inflammatory state to regulate injury and repair. This shift in microglia potentially contributes to increased susceptibility and neurodegeneration as a function of age (Harry 2013), though aging itself it not a disease. However, age-related macular degeneration, as well as neurodegenerative diseases such as AD, PD, share two characteristics in common: (1) a disease prevalence that increases markedly with advancing age, and (2) microglia-related neuroinflammation. These characteristics have led to the hypothesis that pathogenic mechanisms

underlying age-related neurodegenerative disease involve aging changes in microglia (Wong 2013).

As mentioned above, microglia have been implicated in disease progression for several age-related brain disorders (Lai et al. 2013). Also, hypothalamic inflammation and control of systemic ageing was reported. The hypothalamus is important for the development of whole-body ageing in mice, and that the underlying basis involves hypothalamic immunity mediated by IκB kinase-β, NF-κB and related microglia–neuron immune crosstalk (Gabuzda and Yankner 2013; Zhang et al. 2013).

Considering these evidence mentioned above, understanding and controlling microglial aging and age-associated microglial priming, being increased inflammatory status, may represent an opportunity for elucidating disease mechanisms and for formulating novel therapies (Wong 2013; Norden and Godbout 2013).

13.7.4 Psychiatric Disorders Including Autism Spectrum Disorder

Microglial cells, are more and more considered to contribute to multiple neuropathologies, including neuropsychiatric and developmental disorders. Growing evidence indicates a role for deregulation or malfunction of glial cells and their neuroinflammatory response in the brains of autistic patients (Theoharides et al. 2013).

Recently neurobiological basis of oligodendroglial abnormalities in schizophrenia and microglial activity in the context of the disease, in neonatal brain injury and in various experimental models of white matter damage were described (Chew et al. 2013). Microglia, have recently been also suggested to play important roles in neuropsychiatric disorders (Kato et al. 2013a) and possibility that modulating microglia may be a key target in the treatment of various psychiatric disorders has been proposed (Kato et al. 2013b). Future investigations to clarify the correlation between neuroendocrine factors and microglia may contribute to a novel understanding of the pathophysiology of neuropsychiatric disorders.

Conclusion

Microglial cells are fundamental elements of neuropathology. Microglial cells are constantly scanning the brain tissue for any signs of damage and when these latter occur microglial launch a defensive response generally known as microglial activation. Microglia express variety of receptors and respond to many signals. Identification of those signaling and manipulating microglial functions may lead to promising therapeutic strategy for neuropathological processes.

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Chapter 14
Novel Therapeutic Approaches to Malignant Gliomas

Vishnu Anand Cuddapah and Harald Sontheimer

Abstract Current treatment regimens for malignant gliomas are inadequate. Characterized by aggressive cell proliferation and migration, gliomas lead to a median patient survival of about 1 year despite medical intervention, including surgery, chemotherapy, and radiation. Numerous clinical trials are attempting to make headway; intraoperative visualization techniques, intensity-modulated radiation therapy, and inhibitors of growth factor pathways and angiogenesis are making incremental, although valuable, progress in our management of this devastating disease. Both current treatments and most clinical trials modulate traditional oncological features of cancers, such as DNA replication, cell proliferation, neovascularization, and necrosis. However, in this chapter we argue that for a sea-change in treatment to occur, unique characteristics of gliomas must be targeted. Blocking the association of glioma cells with the vasculature could contain tumor spread. Inhibiting ion channels on glioma cells prevents the shape and volume changes necessary for cell migration. And preventing assiduous glutamate release from glioma cells decreases excitotoxic neuronal death. To make a substantive impact on clinical management, novel therapies should focus on these neurodegenerative aspects of glioma biology.

Keywords Blood-brain barrier · Bradykinin · CaMKII · ClC-3 · Clinical trials · Chlorotoxin · Epilepsy · Glioblastoma Multiforme · Glioma · Icatibant · Invasion · Magnetic resonance imaging · Malignant glioma · Migration · Mitotic figures · Neoplasia · Neurodegeneration · Nuclear atypia · Pre-mitotic condensation · Scherer's structures · Seizures · Sulfasalazine · System xc- · Temozolomide · TM-601

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14.1 Characteristics of Malignant Gliomas

Gliomas constitute a diverse group of primary cancers affecting the brain and spinal cord. This devastating disease accounts for 80 % of all malignant tumors in the central nervous system, of which the most common subtype is called glioblastoma multiforme (GBM) (CBTRUS 2011). Approximately 5 per every 100,000 people in the United States have a malignant glioma every year (CBTRUS 2011), with exposure to ionizing radiation, hereditary syndromes, and increasing age being the greatest risk factors (Bondy et al. 2008). Pathologically, malignant gliomas are defined by nuclear atypia, elevated mitosis, increased cellularity, necrosis, and microvascular proliferation, traits common to other aggressive cancers of the body. Hence, current treatment regimens, including surgical resection, chemotherapy, and radiation therapy, have sought to solely target these features shared by other systemic cancers. However, the diffuse nature of malignant gliomas makes complete surgical resection essentially impossible, and combined with the chemo- and radio-resistance of glioma cells, explains the poor patient prognosis (CBTRUS 2011). Thus, there is a desperate need to understand the unique biology of malignant gliomas for the future development of more efficacious therapeutics.

14.1.1 Neoplasia in the Central Nervous System

In November 2007, 25 pathologists and geneticists assembled in Heidelberg, Germany on behalf of the World Health Organization (WHO) to standardize classification of tumors of the nervous system. They recommended that these tumors be sub-classified into seven categories: tumors of neuroepithelial tissue, tumors of the cranial and paraspinal nerves, tumors of the meninges, lymphomas/hematopoietic neoplasms, germ cell tumors, tumors of the sellar region, and metastatic tumors (Louis et al. 2007). Using the differentiation level of tumors, expressed on a scale of I to IV from differentiated to largely undifferentiated, these can be further subclassified and used to predict patient outcome. For example, most patients with WHO Grade II tumors usually survive for more than 5 years, while WHO Grade III and IV tumors lead to average survival times of 2–3 years and 1 year, respectively (Louis et al. 2007). Thus patient prognosis is strongly tied to the cellular and pathological composition of the tumor. This chapter is by and large concerned with the most malignant primary brain tumors and hence the term “malignant gliomas” specifically refers to GBM, which originate from neuroepithelial tissue and are the most common type of WHO Grade IV brain cancer.

The Central Brain Tumor Registry of the United States monitored 226,791 cases of brain tumors in the United States from 2004–2007 and found that gliomas composed 80 % of all malignant tumors (CBTRUS 2011). Males were 1.68 times more likely than females to have GBM, and Whites were 2.12 times more likely to have GBM as compared to Blacks (CBTRUS 2011). Age correlated with increased risk of GBM with the highest incidence in people 75–84 years old (CBTRUS 2011). Unfortunately, while GBM are the most common type of primary brain cancer,

they are also the most lethal as 1-year, 3-year, 5-year, and 10-year survival rates are 35%, 7%, 5%, and 3%, respectively (CBTRUS 2011). Given that these data were collected over the previous two decades, it is a reflection that the current standard of care for malignant gliomas is inadequate.

14.1.2 Risk Factors for Malignant Gliomas

Given the lethality, much research has been devoted to understanding the environmental and genetic risk factors associated with malignant gliomas. The Brain Tumor Epidemiology Consortium convened in 2008 to wade through contradictory results in the literature and identify key positive and negative risk factors associated with gliomas. High-dose ionizing radiation was identified as conferring increased risk of developing malignant gliomas (Bondy et al. 2008). This includes radiation exposure due to medically-indicated radiation therapy and nuclear fallout. However, non-ionizing radiation, including the radio frequency range used by mobile phones, does not correlate with an increased risk of malignant gliomas (Bondy et al. 2008). Atopic diseases, including eczema, allergic rhinitis, and asthma, correlate negatively with gliomas as demonstrated by a meta-analysis of 3450 patients (Linos et al. 2007). Mechanistically, this may result from elevated immune activity at baseline, increasing the ability of immune cells to identify and eliminate malignant cells.

Several genetic syndromes are associated with an increased risk of gliomas. However, only about 5% of patients with malignant gliomas have a family history of gliomas (Wen and Kesari 2008). Neurofibromatosis type 1 and type 2, caused by mutations in *NF1* and *NF2*, carry an increased predisposition for gliomas (Melean et al. 2004). Additionally, Tuberous Sclerosis Complex is an autosomal dominant disorder caused by mutation in the *TSC1* or *TSC2* gene and leads to gliomas in 10–15% of patients (Jozwiak et al. 2013). Germline mutations in the gene encoding p53 results in Li-Fraumeni Syndrome, an autosomal dominant disorder characterized by an increased incidence of malignant gliomas, breast cancer, leukemia, and sarcomas. Mismatch Repair Cancer Syndrome, also known as Turcot Syndrome, is characterized by gliomas, malignancies of the blood, and colorectal cancer secondary to mutations in the *MLH1*, *MSH2*, *MSH6*, or *PMS2* genes. Another rare disorder is Melanoma-Astrocytoma Syndrome, characterized by malignant melanomas and gliomas due to mutation in the *CDKN2A* gene (Azizi et al. 1995). These studies demonstrate that there are strong, although rare, genetic risk factors associated with the development of malignant gliomas.

14.1.3 Clinical Presentation and Diagnosis of Malignant Gliomas

Malignant gliomas typically present with headaches, nausea, and vomiting due to increased intracranial pressure, seizure in patients who have no recent head trauma or previous history of epilepsy, and/or focal deficits dependent on which areas of

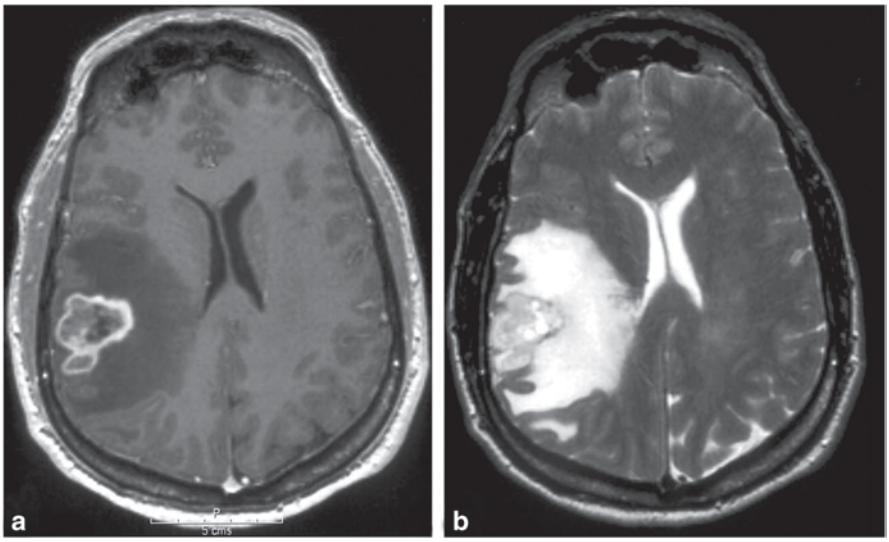


Fig. 14.1 Magnetic resonance imaging of a patient with malignant glioma. **a** Axial T_1-weighted image reveals water-containing tissue as hypo-intense. Central hypo-intense area is consistent with necrosis. **b** T_2-weighted MRI from the same patient allows water-containing tissue to be seen as hyper-intense. This signal is consistent with vasogenic edema. (Reproduced with permission from Pope 2005. Copyright American Society of Neuroradiology)

the cerebral hemispheres are affected. Affecting 50% of patients with malignant gliomas (DeAngelis 2001), headaches may be most severe in the morning and wake the patient from sleep. Seizures affect up to 80% of patients with malignant gliomas (van Breemen et al. 2009). Focal symptoms can include hemiparesis, visual field deficit, and aphasia. Subtle memory and personality changes may also present if the frontal and temporal lobes are compromised.

Upon suspicion of a malignant glioma, imaging with Magnetic Resonance Imaging (MRI) and contrast infusion is warranted. T_1-weighted MRI using spin echo and gadolinium contrast yields lipid-rich tissue as hyper-intense and water-containing tissue as hypo-intense. Thus a hypo-intense T_1-weighted image lacking well defined borders leads to further suspicion of a brain tumor (Fig. 14.1a). T_2-weighted MRI allows water-containing tissue to be seen as hyper-intense and lipid-rich structures as hypo-intense (Fig. 14.1b). Coupled with fluid-attenuated inversion recovery (FLAIR) sequence, which converts the free water (i.e. cerebrospinal fluid) signal to a hypo-intense signal, T_2-weighted images can be used to identify the edematous hyper-intense tissue associated with brain tumors. Widespread edema coupled with a heterogeneously enhancing mass and central necrosis is characteristic of malignant gliomas. While MRI imaging can raise suspicion for a brain tumor, definitive diagnosis requires pathological confirmation after stereotaxic biopsy or surgical resection of the tumor mass.

14.1.4 Pathological Features of Malignant Gliomas

Pathological examination of suspected neoplastic tissue is required for definitive diagnosis of malignant gliomas. This may sometimes lead to an underestimation of the degree of malignancy because of the heterogeneous nature of malignant gliomas (Rees et al. 1996). For example, a biopsy may be obtained from a more differentiated portion of the tumor, leading to an underestimation of disease severity. Nevertheless, identification of nuclear atypia, mitotic figures, endothelial cell proliferation, and necrosis are important for grading the level of malignancy. These features are not unique to malignant gliomas and are shared by other cancers of the body. Importantly, current therapies for malignant gliomas exclusively target these neoplastic features shared with other cancers as further detailed later in this chapter.

The WHO grading system for tumors rates the differentiation level of tumors on a scale of I to IV. WHO Grade I tumors typically have a favorable prognosis and are slow growing, not malignant, and lack nuclear atypia, mitotic figures, endothelial cell proliferation, and necrosis. WHO Grade II tumors are less differentiated and typically show nuclear atypia. WHO Grade III and Grade IV tumors are malignant and aggressive cancers showing signs of both nuclear atypia and mitosis. WHO Grade IV cancers in addition can contain areas of necrosis and microvascular proliferation and carry a poor prognosis. Most malignant gliomas are WHO Grade IV (CBTRUS 2011), reflecting the robustly de-differentiated state of individual glioma cells. And as with other WHO Grade IV cancers of the body, malignant gliomas require early and aggressive treatment to prevent uncontrolled growth and spread.

14.1.4.1 Nuclear Atypia and Mitotic Figures

Nuclear atypia, characteristic of WHO Grade II tumors and above, is abnormal nuclear morphology. The nuclei in these cells are pleomorphic, demonstrating variability in shape and size. Typically the nucleus to cytoplasm ratio is 1:4 or 1:6, but in tumor cells, including malignant gliomas, this ratio can approach 1:1. WHO Grade III cancers and above have larger numbers of mitotic figures, the mitotic spindles distinctive of actively dividing de-differentiated cells. Visualization of greater than 1 dividing cell per 10 high power microscopic fields correlates with a decrease in median survival from 5.5 years to 1 year in patients with malignant gliomas (Fulling and Garcia 1985).

14.1.4.2 Endothelial Cell Proliferation

Vascular endothelial cell proliferation is a common feature among aggressive cancers and portends a poor prognosis. Median survival decreases from 5.5 years to 3.5 years in patients with malignant gliomas when vascular endothelial cell proliferation is observed histologically (Fig. 14.2b) (Fulling and Garcia 1985). Typically

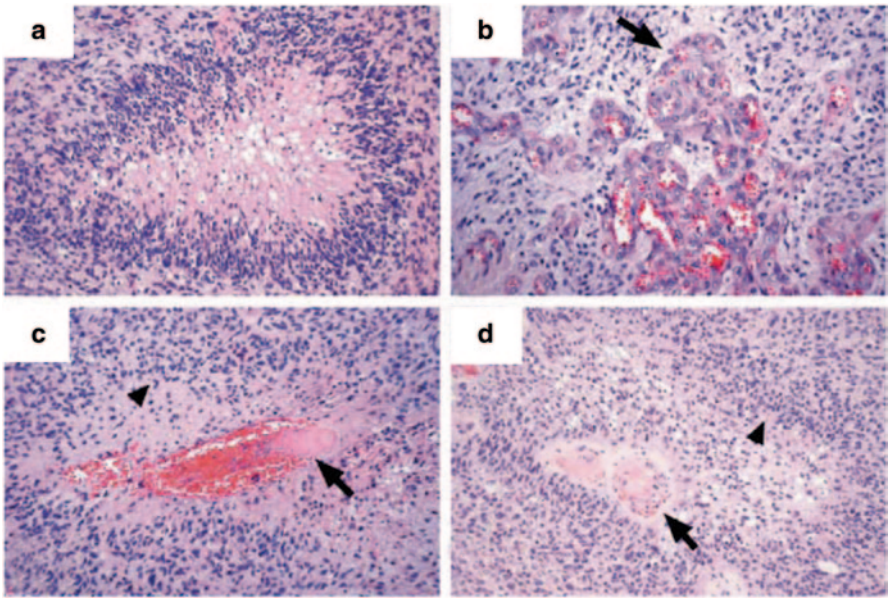


Fig. 14.2 Pathological features of malignant gliomas. **a** Central area of necrosis surrounded by glioma cells, characteristic of pseudopalisading necrosis. **b** *Arrow* points to glomeruloid tufts of densely packed vascular endothelial cells. **c**, **d** *Arrows* points to intravascular thrombosis, likely leading to pseudopalisades (*arrowheads*) as glioma cells move away from areas of necrosis. (Reproduced with permission from Brat and Van Meir (2004). Copyright Nature Publishing Group)

this aberrant proliferation appears as "glomeruloid tufts" of densely packed actively dividing endothelial cells (Jain et al. 2007) and is associated with WHO Grade IV tumors. Tumor cells classically drive endothelial cell proliferation and neovascularization by release of vascular endothelial growth factor (VEGF), the expression of which is induced by hypoxia (Plate et al. 1992; Shweiki et al. 1992).

Several recent studies have demonstrated that a portion of proliferating endothelial cells present in the tumor mass directly differentiate from glioblastoma stem-like cells. These endothelial cells contain the same mutations present in glioma cells, including amplification of the *EGFR* gene and chromosome 7 (Wang et al. 2010). In a separate study, injection of human glioblastoma stem-like cells into immunocomprimised mice lead to tumor vasculature partially composed of human endothelial cells (Ricci-Vitiani et al. 2010). Additionally, glioma cells were found to trans-differentiate into endothelial cells in a VEGF-independent manner (Soda et al. 2011). While mouse models demonstrate the importance of glioblastoma stem-like cells in the generation of endothelial cells, a follow-up study of primary human glioblastoma tissue samples showed that more than 90% of tumor vasculature is derived from endothelial cells and not glioblastoma stem-like cells (Rodriguez et al. 2012). Thus tumor-derived VEGF may play a more pivotal role in vascular endothelial cell proliferation leading to angiogenesis.

14.1.4.3 Necrosis

High cellular densities, mitotic figures, and nuclear atypia all indicate the robust proliferative ability of glioma cells. In areas of the tumor where cellular density overwhelms the available oxygen tension leading to hypoxia, neovascularization can be triggered secondary to VEGF released by glioma cells. However, these newly formed vessels are disorganized and dilated, hampering adequate perfusion of associated tissues (Fukumura et al. 2010). As compared to the peritumoral environment, total vascular density, functional vascular density, and total vascular perfusion is significantly reduced in the tumor core; at the center of large tumors, less than 25 % of vessels may effectively perfuse adjacent tissue (Vajkoczy and Menger 2000). Coupled with increased vascular permeability in tumor-associated vessels (Yuan et al. 1994) due to an impaired blood-brain barrier and increased susceptibility to vascular thrombosis (Fig. 14.2c, d) (Brat et al. 2004), tumor-associated vasculature is hypofunctional (Vajkoczy and Menger 2000).

Hypofunctional vessels in glioma tumor masses in turn lead to hypoxia-induced necrosis (Brat et al. 2004), a radiological and histological hallmark of Grade IV gliomas. Glioma cells surrounding necrotic tumor cores are up to 20 times more likely to undergo apoptosis and 50 times less likely to proliferate (Brat et al. 2004). These surrounding glioma cells are also more likely to migrate away from the necrotic core, as hypoxia increases the migratory ability of glioma cells (Brat et al. 2004). A central area of hypocellular necrosis surrounded by a rim of glioma cells is known as "pseudopalisading necrosis," because the migrating hypercellular glioma cells form a "palisade" around a hypoxic necrotic core (Fig. 14.2a). Pseudopalisading necrosis is distinctive of the most aggressive cancers and can be molecularly verified by upregulation of hypoxia inducible factor-1 (HIF-1), which is a transcription factor activated in cells exposed to low oxygen tension. HIF-1 controls the expression of a variety of substrates, including proteins which promote angiogenesis (e.g., VEGF and angiopoietin-1) and glioma cell migration (e.g., C-X-C chemokine receptor type 4 and matrix metalloproteinase-2) (Kaur et al. 2005; Zagzag et al. 2006). Thus dysfunctional vasculature associated with high-grade gliomas can lead to hypoxia and necrosis, promoting aggressive disease progression.

14.1.5 Molecular Insight Reveals the Heterogeneity of Malignant Gliomas

Recent work by The Cancer Genome Atlas Network has demonstrated that glioblastoma multiforme is a heterogenous disease that can be subdivided into four subgroups based on molecular abnormalities. The (1) Classical subtype is characterized by chromosome 7 amplification and chromosome 10 loss in 100 % of cases examined (Verhaak et al. 2010). About 97 % of cases also had elevated *EGFR* amplification, more so than the other three subtypes (Verhaak et al. 2010). There are also a high rate of *EGFR* mutation and a significantly lower rate of *TP53* mutation

(Verhaak et al. 2010). Other highly expressed genes in the Classical subtype were *NES*, *NOTCH3*, *JAG1*, and *SMO* and other genes typical of an astrocytic signature (Verhaak et al. 2010).

Loss of *NF1* were characteristic of the (2) Mesenchymal subtype (Verhaak et al. 2010). *NF1* mutations frequently occurred together with *PTEN* mutations (Verhaak et al. 2010). This subtype is so named because of expression of mesenchymal markers, including *CHI3L1* and *MET* (Verhaak et al. 2010). Higher rates of necrosis and inflammatory infiltrates, as well an astrocytic expression signature, characterize this subclass (Verhaak et al. 2010). It is also worth noting that immortalized cell lines most closely matched Mesenchymal tumors (Verhaak et al. 2010).

The (3) Proneural class is characterized by *IDH1* point mutations and alterations elevating *PDGFR* expression (Verhaak et al. 2010). *TP53* mutations were also common (Verhaak et al. 2010). Interestingly, genes upregulated in oligodendrocyte development, including *OLIG2*, *PDGFRA*, and *NKX2–2* were also upregulated in this subclass (Verhaak et al. 2010). Clinically, this subtype was most often found in younger patients and associated with a better prognosis (Verhaak et al. 2010). Finally, the (4) Neural subtype is identified by the presence of neuronal markers, including *NEFL*, *GABRA1*, *SYT1*, and *SLC12A5* (Verhaak et al. 2010).

While this subclassification was undertaken to gain a better understanding of genetic mutations that accompany this family of malignancies, the results provide some compelling reasons to speculate that different glioma subtypes arise from different cells of origin and that they may respond quite differently to treatment. For example, these studies have illustrated that the proneural subtype, similar to cells of an oligodendrocyte lineage, does not benefit from current treatment regimens consisting of radiation and chemotherapy, which yield significant decreases in mortality of the classical glioma subtype.

14.1.6 Treatment and Prognosis of Malignant Gliomas

The mainstay of therapy for malignant glioma is aggressive surgical resection, radiation therapy, and temozolomide/Gliadel wafers (Wen and Kesari 2008). Complete surgical resection of malignant glioma cells is nearly impossible given the extraordinary ability of glioma cells to infiltrate unaffected parenchyma and spread throughout the brain. Nevertheless, a retrospective study of 416 consecutive patients with malignant gliomas who were operated on at M. D. Anderson Cancer Center, Houston, Texas between 1993 and 1999 demonstrated that when greater than 98% of the tumor mass was resected, median survival increased to 13 months as compared to 8.8 months when less than 98% of the tumor mass was resected (Lacroix et al. 2001). Thus, aggressive surgical resection is the first clinical step to increasing median survival in patients with malignant gliomas.

Standard radiation therapy is delivered in 30 fractions over a 6-week period for a total dose of 60 Gy (Wen and Kesari 2008). A randomized study of 303 patients who underwent surgical resection of malignant gliomas demonstrated that radiation therapy can increase median survival from 3.2 months to 8 months (Walker

et al. 1978). More recently, a large randomized study of 573 patients at 85 different centers showed that when radiation therapy is combined with temozolomide, an oral chemotherapeutic agent that alkylates DNA, median survival can be extended from 12.1 months to 14.6 months (Stupp et al. 2005). Interestingly, the efficacy of temozolomide inversely correlates with the activity of O^6-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme that removes alkylated DNA. In patients who have epigenetic silencing of MGMT via methylation, median survival increased to 21.7 months upon co-administration of radiation therapy and temozolomide (Hegi et al. 2005). In lieu of temozolomide, Gliadel wafers can be inserted into the resection cavity to extend median survival from 11.6 to 13.8 months (Westphal et al. 2006). Gliadel wafers are biodegradable discs containing carmustine, an alkylating agent that can be placed inside the cranium to maximally target unresected tumor cells.

Beyond targeting of the tumor mass, several other comorbidities are treated symptomatically. Seizures, fatigue, cognitive deficits, hyper-coagulability, and depression can all arise in patients suffering from malignant gliomas. Although seizures are treated with anti-epileptic drugs (AEDs), prophylactic administration of AEDs in patients with malignant gliomas is not indicated because of a risk of toxic interactions with chemotherapeutic drugs (Glantz et al. 2000). Methylphenidate, typically prescribed for attention-deficit hyperactivity disorder, can improve fatigue and cognitive deficits in patients with malignant gliomas (Meyers et al. 1998). Quite significantly, up to 94% of patients with malignant gliomas reported symptoms consistent with depression 3 months after surgery, and given that depression correlates with decreased survival and increased medical complications (Litofsky et al. 2004), a holistic treatment regimen is essential.

14.2 Appreciating the Uniqueness of Gliomas

Although sharing significant histopathological and genetic features with other cancers as detailed in the previous section, malignant gliomas are unique cancers. Unlike other solid cancers of the body, which use intravascular hematogenous routes to metastasize to unaffected organs, malignant gliomas rarely metastasize outside of brain. Instead, glioma cells actively migrate on the abluminal surface of cerebral vessels for tumor expansion. Initially, gliomas are chemotactically drawn to vascular endothelial cells, thereby disrupting the native cytoarchitecture comprising the blood-brain barrier. After vascular engagement, individual gliomas cells migrate away from the tumor core. This dynamic process of cellular migration requires coordinated changes in shape and volume, a process dependent on flux of osmotically-active ions through ion channels and transporters expressed by gliomas.

Also unlike most solid tumors of the body, the growth and expansion of malignant gliomas occurs in an unexpandable space confined by the cranium. Therefore, to create an area of expansion for the growing tumor, glioma cells release glutamate in large concentrations through system xc-, a highly expressed cystine-glutamate

exchanger, encoded by the *SLC7A11* gene in humans. For every molecule of cystine imported through the channel, one molecule of glutamate is released, raising the concentration of extracellular glutamate to supra-physiological levels. This glutamate acts in an autocrine manner to promote migration of glioma cells, but also induces excitotoxicity in peritumoral neurons, culminating in neuronal death and room for tumor spread. Importantly, this accumulation of large concentration of extracellular glutamate leads to a collection of neurological sequelae strikingly similar to other neurodegenerative diseases. Despite accumulating evidence that malignant gliomas result in similar downstream effects as neurodegenerative diseases, all Food and Drug Administration (FDA)-approved treatment options target features of gliomas shared by other solid cancers of the body. Targeting unique features of gliomas may lead to an improved patient prognosis.

14.2.1 Glioma-Vasculature Interactions

As already mentioned, unlike other solid cancers of the body, malignant gliomas rarely metastasize out of the brain. Less than 2% of malignant gliomas metastasize outside of the central nervous system, usually occurring late in the disease course (Smith et al. 1969; Beauchesne 2012). Several hypotheses have attempted to explain why glioma cells poorly spread to extracranial organs. Metastasis typically occurs through three routes: vascular circulation, the lymphatic system, and contact by adjacent structures in a body cavity. To hematogenously spread to other organs, glioma cells must be able to intravasate into the vasculature; however, studies using C6 rat glioma cells demonstrate that glioma cells cannot penetrate the basement membrane associated with cerebral vessels to enter circulation (Bernstein and Woodard 1995). Alternatively, even if glioma cells entered the vasculature, it is possible that extracranial organs do not contain the milieu of chemoattractants and growth factors necessary to support satellite tumor formation. Also, given that gliomas form in an immuno-privileged environment, movement of glioma cells into systemic circulation may elicit an immune reaction preventing growth outside of the brain (Mourad et al. 2005). From an anatomic perspective, the dearth of lymphatic vessels in the central nervous system, coupled with dense connective tissue protecting dural veins and a lack of connection between intracranial circulation and the extracranial lymphatic system, prevents the extracranial egress of glioma cells.

Nevertheless, glioma cells are extraordinarily adept at migration and invasion, forming diffuse and expansive intracranial tumor masses. More than 70 years ago, Hans Joachim Scherer, a German neuropathologist, microscopically examined 100 brains of patients with brain tumors and outlined the most common routes of intracranial glioma cell migration and invasion, now known as “Scherer’s structures”(Scherer 1940). Scherer demonstrated that glioma cells in the grey matter congregate around cerebral blood vessels, especially capillaries and other small diameter vessels (Scherer 1940). This collection of glioma cells around vessels is known as “perivascular cuffing” and allows the growing tumor to coopt existing vasculature for its own support. As early as 1 week after implantation, glioma cells

injected into rat cerebrum encircled existing vasculature, forming a well-perfused tumor relatively early in the disease course (Holash et al. 1999). Thus vascular cooption allows the growing tumor to meet its energy demands early in the disease course before the onset of angiogenesis. In white mater, Scherer showed that glioma cells surround myelinated nerve fascicles (Scherer 1940). More recent studies have corroborated Scherer's pioneering findings and have explored the molecular mechanisms by which the abluminal surface of cerebral vessels and nerve fascicles function as conduits, along which glioma cells migrate away from the tumor core promoting tumor expansion.

14.2.1.1 Glioma Cells Migrate Along Cerebral Vasculature

To migrate along the vasculature, glioma cells must first contact individual vessels. Studies of transplanted C6 rat glioma cells into rat forebrain suggest that migrating glioma cells come into contact with the vasculature in less than 24 h (Farin et al. 2006). One mechanism by which glioma cells chemotactically migrate towards the vasculature is by responding to bradykinin, a kinin released by vascular endothelial cells (Montana and Sontheimer 2011). Bradykinin binds to the B2 receptor constitutively expressed by human glioma cells (Montana and Sontheimer 2011). When human glioma cells were placed on murine brain slices, in 2 h bradykinin induced nearly 85% of glioma cells to contact blood vessels; this interaction was reduced to 30% when the B2 receptor was selectively knocked down in human glioma cells (Montana and Sontheimer 2011). Bradykinin application also increased the total number of migrating glioma cells, and increased the speed and distance travelled by individual glioma cells (Montana and Sontheimer 2011), further emphasizing the importance of this molecular interaction.

Once contacting the vasculature, glioma cells migrate in a saltatory manner at a speed of about 6–25 μm/h (Farin et al. 2006; Turner and Sontheimer 2013; Watkins and Sontheimer 2011). Contact is maintained with the vasculature through activation of α-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) glutamate receptors on the leading edges of migrating glioma cells, which promote binding to extracellular matrix components, including type I and type IV collagen found in perivascular areas (Piao et al. 2009). Increased AMPA receptor expression correlated with enhanced expression of β1-integrins on the plasma membrane and increased numbers of focal adhesion complexes, all of which are involved in attachment of glioma cells to extracellular substrates, promoting glioma cell invasion (Piao et al. 2009).

14.2.1.2 Glioma Cells Disrupt Cytoarchitecture Associated with the Blood-Brain Barrier

The vasculature of the brain is unlike the rest of systemic circulation for at least two principal reasons: (1) tight junctions between vascular endothelial cells form

a blood-brain barrier, and (2) the "neurovascular unit," composed of vascular endothelial cells, astrocytes, and neurons, couples neuronal activity to vascular perfusion. Maintenance of the blood-brain barrier is important, as it prevents ions, molecules, and cells from non-selectively infiltrating the brain, where the extracellular milieu is tightly regulated for optimal neuronal functioning. One mechanism by which neuronal activity leads to enhanced vascular perfusion is through the spillover of glutamate from synapses onto adjacent astrocytes. This glutamate can activate astrocytic metabotropic glutamate receptors (mGluR), which through a cascade involving G-protein coupled signaling lead to downstream increases in intracellular [Ca^{2+}], which in turn leads to release of arachidonic acid metabolites and K^+, subsequently modulating of vascular tone (Drake and Iadecola 2007; Attwell et al. 2010). An increase in cerebral blood flow in response to increased neuronal activity is termed "functional hyperemia" and serves to enhance delivery of O_2 and glucose to and remove waste metabolites from hyper-metabolic brain regions.

As glioma cells migrate along the vasculature, the native cytoarchitecture is disrupted, opening the blood-brain barrier. Implantation of C6 rat glioma cells into the cerebrum of rats demonstrated that the processes and cell bodies of migrating glioma cells intercalate between vascular endothelial cells and astrocytic endfeet (Farin et al. 2006). Tumor cells actively "lift up" astrocytic processes from the vasculature as revealed by transmission electron microscopy (Zagzag et al. 2000). After movement of the astrocytic processes, glioma cells can directly attach to the perivascular basement membrane and cause the perturbed astrocytes to become "reactive" (Nagano et al. 1993), which in turn can lead to downstream ramifications including loss of functional hyperemia and impairment of neuronal function. Incidentally, the association of glioma cells with the vasculature also seems to promote proliferation; more than 60 % of glioma cells that divided were less than 20 μm from a vascular branch point (Farin et al. 2006).

14.2.2 Glioma Ion Channels Promote Aggressive Migration and Proliferation

As glioma cells migrate along the vasculature, they must move through the narrow extracellular spaces of the brain. To fit through these constrained spaces, glioma cells must undergo coordinated shape and volume changes, which are facilitated by ion channels and transporters expressed by glioma cells. Ion channels and transporters promote these morphological changes by moving osmotically-active ions across the plasma membrane, thereby osmotically driving water into and out of the cell. Given that approximately 70 % of the mass of a cell is water, this leads to robust changes in cellular volume, allowing glioma cells to navigate the tortuous extracellular environment of the brain.

Analogously, ion channel-mediated morphological changes are also important for glioma cell proliferation. The division of glioma cells is preceded by a short period of volume contraction known as pre-mitotic condensation. Pre-mitotic condensation is associated with the cell rounding and DNA condensation that occurs

in a mother cell before dividing into two daughter cells (Habela et al. 2008). This volume condensation is necessary for cell division, as its inhibition leads to an increase in glioma cell senescence.

14.2.2.1 Ion Channels Promote Hydrodynamic Shape and Volume Changes in Migrating Cells

Migrating human glioma cells shrink in volume by 33 % to move through narrow extracellular spaces irrespective of the size of the cell or spatial constraint (Watkins and Sontheimer 2011). This dramatic decrease in cellular volume is accomplished through the efflux of Cl^- ions into the extracellular space, leading to the obligated release of cytoplasmic water (Watkins and Sontheimer 2011). Glioma cells accumulate large concentrations of Cl^-, up to 100 mM, in the cytoplasmic space (Habela et al. 2009), likely due to the activity of NKCC1, a Cl^- cotransporter highly expressed by human glioma cells (Haas and Sontheimer 2010). The high intracellular $[Cl^-]_i$ provides a large driving force for Cl^- efflux, thereby moving water across the plasma membrane.

ClC-3, a voltage-gated Cl^- channel, has been identified as a key mediator of Cl^- efflux by human glioma cells. ClC-3 is highly expressed on the plasma membrane (Olsen et al. 2003) and is activated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)(Huang et al. 2001; Cuddapah and Sontheimer 2010), a Ca^{2+}-sensitive kinase. CaMKII-dependent activation of ClC-3 is critical for glioma cell migration, as its inhibition either though pharmacological or genetic manipulation prevents glioma cell movement through spatial barriers (Cuddapah and Sontheimer 2010; Cuddapah et al. 2013a). ClC-3 mediated Cl^- flux is coupled to movement of K^+ into the extracellular space through 2 Ca^{2+}-activated K^+ channels: $K_{Ca}3.1$ and $K_{Ca}1.1$ (Cuddapah et al. 2013a; Weaver et al. 2006). Coupling of Cl^- to K^+ movement may allow migrating cells to maintain electroneutrality.

Intracellular $[Ca^{2+}]$ in gliomas increases secondary to a variety of ligands and channels, including glutamate, bradykinin, epidermal growth factor (EGF), lysophosphatidic acid (LPA), or transient receptor potential (TRP) channels, all of which enhance migration and invasion (Lyons et al. 2007; Ishiuchi et al. 2002; Montana and Sontheimer 2011; Bomben et al. 2011; Cuddapah et al. 2013a, 2013b; Manning et al. 2000). Once Ca^{2+} increases, ion channels as well as cytoskeletal elements are regulated to promote migration. Specifically, three integral components of migration, including protrusion, attachment, and retraction, are activated. Protrusion involves the lamellipodium, rich with polymerizing actin filaments, which pushes the plasma membrane forward (Fig. 14.3a, b). Through a well-established treadmilling model, actin monomers away from the lamellipodium are depolymerized and actin monomers in the lamellipoium are polymerized, pushing the leading edge forward. This highly coordinated process requires Ca^{2+} signaling; Ca^{2+} signaling acts on Rho GTPases leading to downstream regulation of actin depolymerizing factor/cofilin family of proteins (Zheng and Poo 2007). Additionally, focal adhesions, which bind migrating cells to the substratum and provide contact points to build traction, are regulated by CaMKII (Easley et al. 2008). Thus Ca^{2+} dynamics are involved in

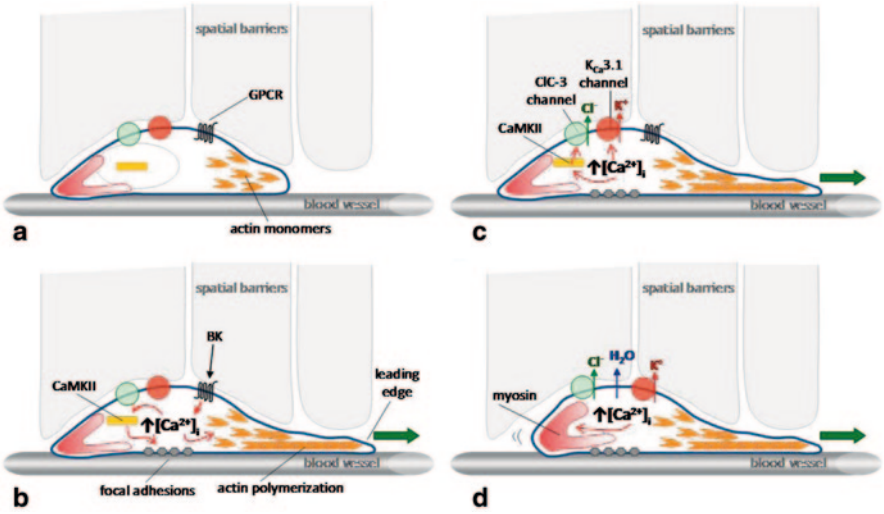


Fig. 14.3 An updated model of cell migration: Ion channels facilitate motility. **a** Glioma cells migrate along the abluminal surface of blood vessels and encounter spatial barriers requiring modulation of cellular shape and volume. **b** Ligands such as bradykinin can bind to G-protein coupled receptors (GPCRs) to increase $[Ca^{2+}]i$, leading to CaMKII-mediated regulation of focal adhesions and Ca^{2+}-mediated regulation of actin depolymerization via the actin depolymerizing factor/cofilin family of proteins. Actin polymerization on the leading edge pushes the leading edge forward. **c** $[Ca^{2+}]i$ increases also activate CaMKII-dependent ClC-3 channels leading to Cl^- efflux, and KCa3.1 channels leading to K^+ efflux. **d** Efflux of osmotically-active K^+ and Cl^- ions leads to loss of cytoplasmic water and cell shrinkage, facilitating movement through narrow spaces. Additionally, Ca^{2+}-dependent myosin contraction on the lagging edge pushes the cell forward

the protrusion of the lamellipodium and attachment to the substratum (Fig. 14.3b). Importantly, increases in intracellular $[Ca^{2+}]$ also activate ion channels, which flux osmotically-active ions to move cytoplasmic water across the plasma membrane (Fig. 14.3c), allowing the motile cell to change shape and volume. For example, bradykinin-mediated Ca^{2+} elevations can lead to K_{Ca} 3.1 and CaMKII-dependent ClC-3 channel activation in human glioma cells (Cuddapah et al. 2013a). This ion channel activation leads to shape and volume changes, and if inhibited, blocks migration (Watkins and Sontheimer 2011). At the lagging edge myosin II contracts pushing the cell forward (Yang and Huang 2005; Conrad et al. 1993) (Fig. 14.3d). Given that a variety of ligands enhance glioma cell migration through an increase in Ca^{2+}, this may be a convergent pathway common to multiple upstream signaling mechanisms.

14.2.2.2 Pre-mitotic Condensation is Necessary for Glioma Cell Proliferation

Similar to glioma cell migration, the division of glioma cells requires coordinated shape and volume changes. As glioma cells enter mitosis and prepare to divide

into two daughter cells, cellular shape changes from bipolar to round (Cuddapah et al. 2011; Habela and Sontheimer 2007; Habela et al. 2008). The importance of a round morphology seems manifold, including allowing the cellular membrane and organelles to split symmetrically, and stabilizing the mitotic spindle for equal distribution of genomic content (Rosenblatt 2008). This cellular rounding is associated with a simultaneous decrease in cytoplasmic volume by approximately 40% termed pre-mitotic condensation (Habela and Sontheimer 2007; Habela et al. 2009). Importantly, CaMKII activation of ClC-3 has been identified as a mechanism that promotes pre-mitotic condensation in human glioma cells. Dividing glioma cells have a large Cl^- conductance mediated by CaMKII activation of ClC-3 (Habela et al. 2008; Cuddapah et al. 2011). As in the context of migration, the large Cl^- conductance in dividing human glioma cells is due to efflux of osmotically-active Cl^- ions, which is sufficient to condense cytoplasmic volume by 33% (Habela et al. 2009). Inhibition of ClC-3 or CaMKII significantly increases the time required for cytoplasmic condensation, thereby decreasing glioma cell proliferation by 35% (Cuddapah et al. 2011).

This hydrodynamic process of pre-mitotic condensation may be coupled to other Ca^{2+}-dependent processes to ensure robust proliferation in human glioma cells. During the first phase of mitosis, prophase, cyclin B binds to Cdk1 to promote cell division (Fig. 14.4a). The replicated chromosomes then begin to condense inside an intact nuclear envelope. Additionally, the replicated centrosomes begin to migrate to opposite poles of the cell. Between prophase and metaphase is prometaphase, which includes several steps preceding formation of the metaphase plate (Fig. 14.4b). The nuclear envelope breaks down, and the replicated chromosomes attach to microtubules through their kinetochores. The microtubules, in turn, are attached to centrosomes, forming the mitotic spindle. Ca^{2+} and calmodulin levels increase around the centrosome and microtubules (Welsh et al. 1979; Wolniak et al. 1983) to stabilize tubulin polymerization and depolymerization. As the cell transitions into metaphase, both centrosomes, already located at opposite poles of the cell, pull on the chromosomes (Fig. 14.4c). This tension generated by the opposing centrosomes pulls the chromosomes to the center of the cell forming the metaphase plate. The symmetric microtubule tension also drastically changes the cytoarchitecture leading to cellular shape changes. The metaphase cell begins to become round characterizing the process known as mitotic cell rounding. Towards the end of metaphase, intracellular $[Ca^{2+}]$ spikes for about 20 s (Ratan et al. 1988; Poenie et al. 1986), potentially activating CaMKII and ClC-3 on the plasma membrane. As detailed above, because there is a large driving force for Cl^- efflux (Habela et al. 2009), opening of ClC-3 channels leads to Cl^- loss, subsequently driving the osmotic loss of cytoplasmic water. This cytoplasmic condensation leads to a shrinking of cellular volume, bridging kinases with their substrate proteins. After the intracellular $[Ca^{2+}]$ spike returns to baseline, daughter chromatids are separate to opposite ends of the cell towards the centrosomes, characterizing anaphase (Fig. 14.4d). As during prometaphase, Ca^{2+} and calmodulin levels remain elevated around the mitotic spindle, facilitating tubulin polymerization and depolymerization. This model suggests that Ca^{2+} is a master regulator and drives several necessary events between

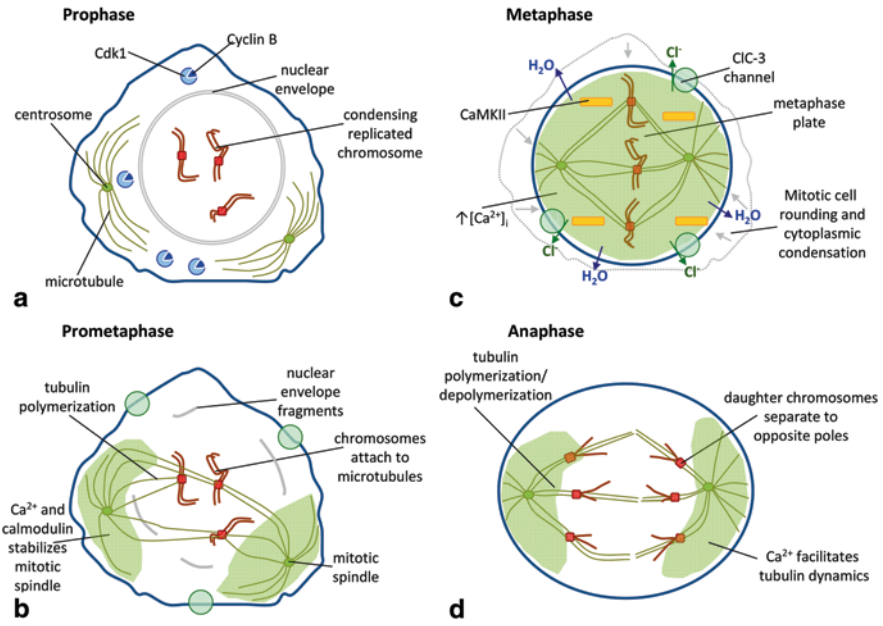


Fig. 14.4 An updated model of cell proliferation: Ion channels facilitate mitosis. **a** During prophase, cyclin B binds and activates Cdk1. The nuclear envelope is still intact, and the duplicated centrosomes begin to migrate to opposite ends of the cell. Replicated chromosomes begin to condense. **b** During prometaphase the nuclear membrane disintegrates, allowing microtubules to connect to kinetochores on the condensed chromosomes. On the other end, microtubules are attached to centrosomes, forming the mitotic spindle. Ca^{2+} and calmodulin stabilize tubulin polymerization on the mitotic spindle. **c** The dividing cell undergoes dramatic morphological changes during metaphase. The chromosomes align along the metaphase plate due to tension built by the mitotic spindle. The cell undergoes mitotic cell rounding. At the end of metaphase, $[Ca^{2+}]i$ spikes, potentially leading to activation of CaMKII then ClC-3. Activation of ClC-3 leads to Cl^- efflux, osmotic water loss, and cytoplasmic condensation. **d** By anaphase, the daughter chromosomes are migrating to opposite poles of the cell. Once again, Ca^{2+} and calmodulin promote tubulin polymerization/depolymerization in the mitotic spindle

the metaphase-anaphase transition, and if disrupted, hinders mitotic progression. Ion channels play a critical role in mitosis by facilitating the dynamic shape and volume changes characteristic of dividing cells.

14.2.3 Glutamate: Motogen, Mitogen, and Neuron Killer

An important feature that distinguishes gliomas from other solid cancers is that tumor growth occurs in an unexpandable space, a space confined by the cranium. To overcome this spatial constraint, human glioma cells kill native brain tissue to create room for tumor expansion. One of the principal mechanisms by which glioma cells kill neurons is by release of large amounts of glutamate, the principal

excitatory neurotransmitter of the nervous system. Glioma cells can increase the extracellular concentration of glutamate to 100 μM in a space 1,000-fold larger than cellular volume within 5 h (Ye and Sontheimer 1999). This is due to (1) a deficiency in glutamate uptake by glioma cells and (2) glutamate release by system xc-, a cystine-glutamate exchanger (Ye et al. 1999). Large extracellular glutamate concentrations in turn induce hyperexcitability and even excitotoxicity in neurons, causing neuronal death. Glutamate also acts in an autocrine manner, promoting glioma migration and growth by binding to Ca^{2+}-permeable AMPA receptors/channels expressed by human gliomas.

14.2.3.1 Glutamate as a Motogen and Mitogen

One of the key functions of astrocytes is to take up glutamate from the extracellular space to dampen neuronal excitability. This glutamate uptake is primarily achieved through plasma membrane Na^{+}-dependent glutamate transporters including GLT-1 and GLAST (in rodents; in human, EAAT2 and EAAT1, respectively). In contrast, gliomas, the malignant counterpart of astrocytes, fail to take up glutamate from the extracellular space (Ye and Sontheimer 1999). Na^{+}-dependent glutamate uptake is up to 100-times less in human glioma cells, secondary to decreased GLT-1 expression and mislocalization of GLAST to the nucleus (Ye et al. 1999). Instead, glutamate is persistently released through the system xc- cystine-glutamate exchanger. Cystine enters the cell and is converted to glutathione, an antioxidant critical for the absorption of free radicals formed by gliomas in hypoxic environments (Ogunrinu and Sontheimer 2010). Glutamate released in the extracellular space (1) acts in an autocrine loop on gliomas to increase tumor aggressiveness, and (2) induces hyperexcitability in peritumoral neurons and eventual excitotoxic cell death.

Human glioma cells lack the GluA2 subunit in endogenously expressed AMPA receptors, rendering the channels Ca^{2+}-permeable (Ishiuchi et al. 2002, 2007; Lyons et al. 2007). Therefore, when glutamate is released from the system xc-, it then acts in an autocrine/paracrine manner to increase Ca^{2+} signaling in glioma cells, which correlates with increased migration (Ishiuchi et al. 2002; Lyons et al. 2007). Inhibition of glutamate release from the system xc- or inhibition of Ca^{2+}-permeable AMPA receptors decreased glioma cell migration and reduced overall tumor growth (Lyons et al. 2007). Additionally, conversion of AMPA channels from Ca^{2+}-permeable to Ca^{2+}-impermeable by overexpression of the GluA2 subunit significantly decreased tumor volume by inhibiting glioma proliferation and invasiveness and increasing glioma cell apoptosis (Ishiuchi et al. 2002). Mechanistically, Ca^{2+} permeation through AMPA receptors may promote proliferation and migration through the activation of Ca^{2+}-sensitive ion channels that facilitate hydrodynamic shape and volume changes (Cuddapah et al. 2011, 2013a). Alternatively, Ca^{2+} influx may activate Akt, thereby enhancing signaling cascades that lead to proliferation and migration (Ishiuchi et al. 2007).

14.2.3.2 Gliomas Induce Seizures and Excitotoxicity Through Release of Glutamate

Beyond autocrine/paracrine signaling, glioma-released glutamate acts on neurons to induce hyperexcitability and eventual neuronal death. Human gliomas implanted into mouse cerebrums induced epileptic activity within 14–18 days, as measurable by electroencephalogram (Buckingham et al. 2011). This hyperexcitability was due to glutamate release from the system xc- acting at peritumoral neurons (Buckingham et al. 2011). Importantly, from a clinical perspective, pharmacological inhibition of the system xc- reduced neuronal hyperexcitability and seizure activity (Buckingham et al. 2011; Campbell et al. 2012).

As extracellular glutamate concentrations increase in the peritumoral environment, neuronal hyperexcitability snowballs into excitotoxicity. Abnormal glutamate activation of ionotropic glutamate receptors leads to excessive Ca^{2+} influx, leading to the activation of phospholipases and proteases associated with excitotoxicity. Incubation of neurons in media exposed to human glioma cells is sufficient to cause sustained Ca^{2+} increases in neurons, characteristic of excitotoxicity (Ye and Sontheimer 1999). This leads to neuronal death and can be reversed by blocking Ca^{2+}-permeable N-methyl-D-aspartate (glutamate) receptors with MK-801 or by inhibiting glutamate release from glioma cells (Ye and Sontheimer 1999).

14.2.4 Malignant Gliomas Are a Neurodegenerative Disease

As detailed above, glutamate release through the system xc- by gliomas leads to excitotoxicity and neuronal death. Neuronal death is a hallmark of neurodegeneration, an umbrella term for diseases that lead to the malfunctioning or loss of neurons. The term "neurodegeneration" is typically applied to Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), and trauma/injury to the central nervous system, all diseases which involve a progressive decrease in neuron and synapse counts. While the cause of neuronal loss can be variable, including protein misfolding, protein aggregates, astrogliosis, or loss of antioxidants, the endpoint is the same. Given that malignant gliomas lead to progressive neuronal loss, it too can be considered as a neurodegenerative disease. Appreciating gliomas as a form of neurodegeneration fosters more of a neurocentric approach. Approaching gliomas from a purely oncological perspective, as has historically been the case, does not take into account the unique brain microenvironment and its effects on disease progression.

14.2.4.1 Vasculature Disruption in Neurodegeneration

Cerebral vascular abnormalities typify many neurodegenerative diseases, and malignant gliomas certainly meet this criterion. Neurodegeneration, including neuronal loss and deficits in memory and learning, can directly result from poor

microvascular circulation secondary to ablation of pericytes, an important component of the neurovascular unit (Bell et al. 2010). Additionally, mutations in superoxide dismutase 1 (SOD1), which can lead to ALS, cause disruptions in the blood-spinal cord barrier by inducing downregulation of several endothelial tight junction proteins including ZO-1 and occludin (Zhong et al. 2008). Interestingly, the breakdown of the blood-spinal cord barrier precedes motor neuron loss in the ventral horn that is typical of the disease (Zhong et al. 2008), suggesting the vascular breakdown plays a causative role in neurodegeneration. Alzheimer's disease is also associated with disrupted vascular tone as amyloid beta deposits lead to disorganization of vascular smooth muscle cells (Christie et al. 2001). When gliomas are implanted in to mice overexpressing amyloid beta, tumors are less vascularized, demonstrating the inherent antiangiogenic properties of amyloid beta (Paris et al. 2010). Similar to other neurodegenerative conditions, gliomas are characterized by a malfunctioning blood-brain barrier and poor vascular perfusion. Glioma cells migrate along the vasculature, disrupting native cytoarchitecture, and induce angiogenesis, leading to the growth of leaky and hypo-functional vessels. Dysfunctional vessels may lead to a loss of coupling between neuronal firing and vascular perfusion and yield focal symptoms associated with gliomas.

14.2.4.2 Disruption of Glutamate Buffering in Neurodegeneration

The loss of plasma membrane glutamate transporter function in malignant gliomas coupled to assiduous glutamate release leads to neuronal hyperexcitability, excitotoxicity, and progressive neuronal loss. Since the 1990s it has been appreciated that GLT-1 is significantly downregulated in the brains and spinal cords of patients with ALS (Rothstein et al. 1995) leading to deficient glutamate uptake (Dunlop et al. 2003). This precedes the characteristic motor neuron loss on the ventral horn, suggesting a causative relationship between glutamate transport and neuronal survival (Howland et al. 2002; Guo et al. 2003). A similar deficiency in glutamate transport was observed in frontal cortex of patients with Alzheimer's disease (Masliah et al. 1996) and may explain an increased incidence of seizures in patients (Scarmeas et al. 2009). And models of Huntington's disease demonstrate that mutant huntingtin reduces astrocytic glutamate uptake, leading to excitotoxicity (Shin et al. 2005). These shared features validate the consideration of malignant gliomas as a neurodegenerative disease.

14.3 Expanding Conventional Therapies for the Treatment of Malignant Gliomas

Despite the unique features of malignant gliomas outlined in the previous section, current therapies target characteristics shared by other neoplasms. The present formula of treatment for malignant gliomas is maximal surgical resection, radiation

therapy, and chemotherapy, and most ongoing clinical trials and advancements in treatment regimens are making incremental progress using this same formula. In this section, we outline these selected advancements made in surgical care, chemotherapy, and radiation therapy. From a surgical perspective, intraoperative dyes and MRI, as well as neuronavigation, are enabling neurosurgeons to resect more neoplastic cells. Intensity-modulated radiation therapy and brachytherapy are emerging as potential alternatives to traditional external beam radiation therapy. In addition, alternative dosages of temozolomide may lead to improved patient outcomes. We also discuss several molecular inhibitors designed to interrupt glioma signaling cascades currently under clinical trials.

As of May 2013 90% (841/931) of active clinical trials for malignant gliomas were in Phase 0, I, or II (clinicaltrials.gov). The small number of trials that make it to Phase III demonstrate that most drugs being tested for malignant gliomas have poor efficacy or toxic side-effects. The majority of Phase III trials are related to alternative dosing of currently approved therapies, including temozolomide and radiation therapy. This focus on modulating doses of existing therapies may only lead to incremental success; novel therapies that dramatically alter glioma progression may result only after targeting unique features of gliomas and moving beyond a purely oncological perspective.

14.3.1 Advancements in Surgical Care

Complete surgical resections of Grade IV malignant gliomas are nearly impossible. The robust ability of individual glioma cells to migrate along blood vessels and white matter tracts allow gliomas to quickly spread throughout the brain. While neurosurgeons can debulk the majority of a tumor mass, individual cells intercalated into functional parenchyma cannot be resected. After resection and radio- and chemo-therapy, most malignant gliomas recur within 2–3 cm of the border of the original lesion (Hou et al. 2006). Nevertheless, maximal resection of the tumor mass does portend a better prognosis (Lacroix et al. 2001).

Intraoperative imaging of tumor borders has provided neurosurgeons with some success in identifying and resecting malignant tissue. 5-aminolevulinic acid is a compound in the porphyrin synthesis pathway. When it is applied to certain cells, including glioma cells, it leads to the intracellular creation of protoporphyrin IX, a fluorescent compound (Kennedy and Pottier 1992). Thus glioma tissue can be visualized intraoperatively using a microscope equipped with violet-blue light for fluorescence excitation. A multicenter Phase III study assessing 322 patients with malignant gliomas using 5-aminolevulinic acid found that the ability of surgeons to visualize and extract the tumor mass significantly increased from 36–65% (Stummer et al. 2006). This led to an increase of 6-month progression-free survival from 21–41% (Stummer et al. 2006; Pichlmeier et al. 2008).

Intraoperative neuronavigation and MRI also hold promise in enabling maximal tumor resections. Neuronavigation can provide helpful localization information

to the surgeon, especially for deep tumors extending to the skull base (Sure et al. 2000). Intraoperative MRI was evaluated in a randomized trial of 58 patients; the trial demonstrated that complete resection of tumor could be increased from 68–96% with its use (Senft et al. 2011).

Because the blood-brain barrier prevents lipophilic chemotherapeutic agents from reaching the tumor mass, several surgical techniques have attempted circumvent this barrier and directly infuse drugs into the cerebral parenchyma. For example, Gliadel wafers placed in the surgical cavity release carmustine to target unresected glioma cells (Westphal et al. 2006). Additionally, convection-enhanced drug delivery uses intracranial catheters to infuse chemotherapeutic agents into the brain under pressure, again circumventing the blood-brain barrier (Ferguson and Lesniak 2007; Kunwar et al. 2007).

14.3.2 Advancements in Radiation Therapy

Part of the mainstay of treatment for malignant glioma is external beam radiation therapy, involving positioning of the radiation source on the surface of the body closest to the tumor mass. Two alternate forms of radiotherapy currently being investigated for the treatment of malignant glioma are intensity-modulated radiotherapy and brachytherapy. Intensity-modulated radiation therapy attempts to minimize exposure of unaffected tissue to radiation by applying x-ray beams of different wavelengths at different locations, thereby tracing the 3-dimensionality of the tumor with radiation. It can reduce radiation exposure to the spinal cord, optic nerves, eye, and brainstem (Narayana et al. 2006). Brachytherapy involves the placement of a radiation source into the body adjacent to the tumor site, and like intensity-modulated radiation therapy, reduces the radiation exposure of eloquent tissue. The GliaSite Radiation Therapy System is an inflatable balloon catheter that can be inserted into the tumor resection cavity after surgical resection. The balloon is then filled with a solution containing iodine-125 for 3–6 days and can deliver 40–60 Gy to adjacent tissues (Tatter et al. 2003). When combined with standard external beam radiation therapy to give a total dose of 110 Gy, brachytherapy can increase survival by 3 months (Welsh et al. 2007).

14.3.3 Advancements in Medical Therapies

For patients diagnosed with malignant glioma, medical therapy includes temozolomide and drugs for symptomatic relief. A review of the drugs used for symptomatic treatment, including anti-epileptic drugs, corticosteroids, non-steroidal anti-inflammatory drugs, anti-coagulants, and anti-depressants, are outside the scope of this chapter. Below we review selected advancements in drugs used to target gliomas.

14.3.3.1 Alkylating Agents

Clinical trials assessing dosage of temozolomide, or combination therapies including temozolomide, are among the most common studies. Several groups have sought to determine if dosing temozolomide at lower doses for longer time, or higher doses for shorter time, decreases patient morbidity and increases patient survival. A Phase II randomized study of 85 patients demonstrated that while both dosing regimens were tolerated, higher doses for shorter time increased the 1-year survival rate (Clarke et al. 2009). Given that the efficaciousness of temozolomide is tied to the activity of MGMT, other groups have sought to modulate MGMT activity. O^6-benzylguanine inhibits MGMT activity in malignant gliomas, as determined by a Phase I study (Quinn et al. 2005) and promotes the anti-neoplastic properties of temozolomide. When administered in addition to temozolomide in a subsequent Phase II study, O^6-benzylguanine increased temozolomide sensitivity in patients with temozolomide-resistant anaplastic glioma, but not in patients with glioblastoma multiforme(Quinn et al. 2009).

14.3.3.2 Molecular Targets

Receptor tyrosine kinases are a large family of cell surface receptors, including EGF receptors (EGFR), platelet-derived growth factor receptors (PDGFR), and VEGF receptors (VEGFR), that respond to a variety of ligands. Signaling through this family of receptors is upregulated in malignant gliomas and is linked to cell proliferation, migration, and angiogenesis (Chi and Wen 2007). While there are no approved receptor tyrosine kinase inhibitors currently used for the treatment of malignant gliomas, several have demonstrated some clinical promise. Erlotinib (Tarceva®) is a small molecule, EGFR inhibitor used to treat lung and pancreatic neoplasms. A Phase II trial demonstrated an increase in patient survival by 5 months when erlotinib was combined with temozolomide and radiation therapy (Prados et al. 2009), while another similar trial demonstrated no significant effect (Brown et al. 2008). Vaccination of patients against EGFR variant III, a mutation expressed by 31 % of malignant gliomas (Heimberger et al. 2005), increased overall survival in a cohort of 18 patients (Sampson et al. 2010). And imatinib (Gleevec®), a tyrosine kinase inhibitor, showed minimal effects when administered as a monotherapy (Wen et al. 2006).

14.3.3.3 Inhibitors of Angiogenesis

There is currently one approved inhibitor of angiogenesis for the treatment of malignant gliomas. Bevacizumab (Avastin®) is a monoclonal VEGF antibody that was granted FDA approval in 2009 for the treatment of glioblastoma multiforme, and several studies have demonstrated its effectiveness (Vredenburgh et al. 2007a, 2007b). Cediranib (also known as AZD2171; *Recentin™)*, a VEGFR inhibitor,

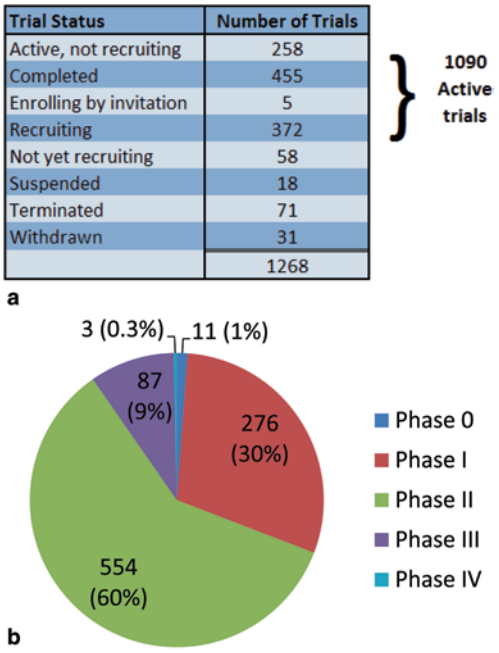
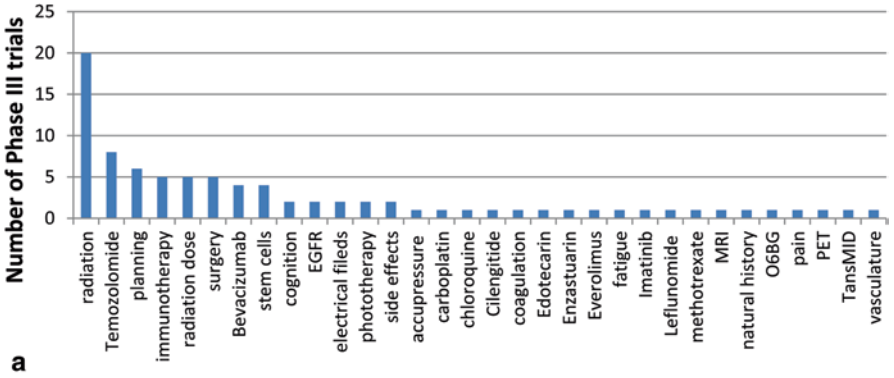


Fig. 14.5 All glioma clinical trials as listed on ClinicalTrials.gov as of May 2013. a We searched for "glioblastoma", "glioma", and "glioma (subependymal)" and found 1268 trials listed. Of these trials, 1090 or 86% were active. b Of the 1090 active trials, the vast majority (90%) were in Phase 0, I, or II

increased progression-free 6 month survival from 15–26% (Batchelor et al. 2010). In addition, it reduced edema and decreased vessel permeability as determined by MRI (Batchelor et al. 2007). Approved for colorectal cancer, aflibercept (Eylea®) is a scavenger protein targeted to VEGF with minimal effect on 6-month progression free survival when administered as a monotherapy upon glioma relapse (de Groot et al. 2011).

14.3.4 Current Clinical Trials for the Treatment of Malignant Gliomas

In May 2013, we searched for all registered clinical trials in the United States on ClinlicalTrials.gov in the following categories: "glioblastoma", "glioma", and "glioma (subependymal)." Of the 1,268 trials listed, 1,090 (86%) were active trials with a status of "active, not recruiting," "completed," "enrolling by invitation," or "recruiting" (Fig. 14.5a). Of the active trials, the vast majority (841/931 = 90%) were in Phase 0, I, or II (Fig. 14.5b). Only 9% of active trials had reached Phase III, and just three trials were in Phase IV (Fig. 14.5b). Thus the vast majority of



a

Current Phase IV trials for malignant gliomas	ClinicalTrials.gov identifier
1. Post-approval Study of NovoTTF-100A in Recurrent GBM Patients	NCT01756729
2. Correlation Between SV2A Expression in Tumour Tissue and Efficacy of Levetiracetam in Glioma Patients With Epilepsy	NCT00454935
3. Standard Temodal (Temozolomide) Regimen Versus Standard Regimen Plus Early Postsurgery Temodal for Newly Diagnosed Glioblastoma Multiforme (Study P05572)	NCT00686725

b

Fig. 14.6 All glioma Phase III and Phase IV trials on ClinicalTrials.gov as of May 2013. **a** All "active" Phase III glioma clinical trials were sub-categorized into distinct types of treatment. **b** All "active" Phase IV glioma clinical trials are listed with corresponding ClinicalTrials.gov identifier.

trials scrutinizing new therapies for malignant gliomas are either not reaching therapeutic significance or of intolerable toxicity. Another unfortunate possibility is that promising results are not developed into large multi-center Phase III trials because of a lack of funding (Watkins and Sontheimer, 2012).

Further analysis of current Phase III trials of gliomas reveals that most trials are focused on varying dosages or combinations of existing therapies. For example, 33/86=38% of Phase III trials are focused on varying regimens of temozolomide and/or radiation therapy (Fig. 14.6a). While these trials are worthwhile and may lengthen patient survival, it is unlikely they represent a sea-change in clinical management of gliomas. About another 10% of Phase III trials are focused on treatment of symptoms and side effects secondary to the glioma or its treatment, including cognitive disturbances, pain, and fatigue. Also currently in Phase III trials are O^6-benzylguanine, bevacizumab, EGFR inhibitors, and imatimib.

The current Phase IV trials are most likely to have a clinical impact in the near future (Fig. 14.6b). The NovoTTF™-100A System from Israel received FDA approval in April 2011 for the treatment of recurrent glioblastoma multiforme in adults. The medical device applies electric fields of alternating direction (AC fields) to the cranium through surface electrodes placed on the scalp. These AC fields are thought to disrupt the mitotic spindle assembly of dividing cells or disturb plasma membrane integrity, and thereby inhibit glioma cell proliferation (Kirson et al. 2004).

This data is based on the principle that cells are composed of charged molecules that can be reoriented in the presence of an AC field (Kirson et al. 2007). A randomized Phase III trial in 2012 demonstrated that the NovoTTF™-100A System may be as effective as chemotherapy in increasing survival (Stupp et al. 2012).

A second active Phase IV trial is determining if there is an association between expression of synaptic vesicle glycoprotein 2A (SV2A), a protein on synaptic vesicles, and the effectiveness of levetiracetam (Keppra®) in treating glioma-associated seizures. Levetiracetam is an antiepileptic drug that binds to SV2A to prevent seizures (Lynch et al. 2004) and is effective in a subset of glioma patients, so the study hopes to determine if expression of SV2A correlates with drug efficacy. The usefulness of levetiracetam is to treat seizures, an important comorbidity of gliomas, and not to modify glioma progression.

14.3.5 Moving Beyond a Purely Oncological Perspective

In this section, we outlined several promising up-and-coming therapies for the treatment of malignant gliomas. New therapeutic approaches, or dosage modulation of existing therapies, are deemed successful when median survival is increased on the order of a few months. Given that the new therapies of the next decade are currently in Phase III and IV trials, it is unlikely based on preliminary data that we will meaningfully change patient prognosis anytime soon. A paradigm shift in approaching malignant gliomas is desperately needed.

In the following section, we argue that novel approaches to gliomas must be taken. Most therapeutic strategies, including Phase III trials, approach gliomas from an oncological perspective. Perhaps new strategies should seek to disrupt the neurodegenerative aspects of the disease.

14.4 Novel Therapeutic Approaches to Gliomas: The Need for a Neurocentric Perspective

Several features of malignant gliomas are reminiscent of neurodegenerative diseases. Gliomas cells migrate along the vasculature and disrupt the blood-brain barrier as they do so. Additionally, due to (1) an inability to "buffer" extracellular glutamate and (2) aberrant release of glutamate through system xc-, human malignant gliomas induce seizures and excitotoxicity, eventually culminating in neuronal death. Similar to migrating neuroblasts along the rostal migratory stream (Turner and Sontheimer 2013), ion channels expressed by glioma cells promote cellular invasion by facilitating hydrodynamic volume changes. These volume changes also promote the robust rate of cell proliferation associated with malignant gliomas.

Novel therapies should target these unique features of gliomas instead of solely focusing on traditional oncological approaches. For example, preventing the association of glioma cells with the vasculature may prevent expansion of the tumor

mass and/or maintain integrity of the blood-brain barrier. Preclinical and clinical evidence suggest that inhibition of ion channels expressed by glioma cells reduces glioma cell migration and proliferation. And prevention of glutamate release from glioma cells may decrease disease aggressiveness and normalize neuronal excitotoxicity and cell death, thereby providing less space for tumor expansion.

14.4.1 Using Icatibant to Reduce Glioma Cell Association with the Vasculature

Dating back to the work of Scherer, it has been appreciated that glioma cells hijack cerebral vasculature as a substratum for migration. Bradykinin, a peptide byproduct of the action of kallikrein on high-molecular-weight kininogen, normally functions in the body to induce vasodilatation and vascular permeability. In the context of glioma biology, it is thought to be released from vascular endothelial cells. Glioma cells endogenously express bradykinin 2 receptors (B2R) and are chemotactically attracted to bradykinin (Montana and Sontheimer 2011; Cuddapah et al. 2013a), and in doing so, make contact with the vasculature. Knockdown of the B2R on glioma cells reduces migration and decreases the number of cells associated with the vasculature (Montana and Sontheimer 2011).

In 2011 the FDA granted icatibant (Firazyr®), a specific B2R antagonist, orphan drug status for the treatment of hereditary angioedema. Preclinical data suggest that icatibant may be effective at preventing the association of glioma cells and vasculature, thereby limiting glioma spreading (Montana and Sontheimer 2011). Icatibant significantly limited *in situ* tumor glioma growth in mouse brain slices (Cuddapah et al. 2013a). Inhibition of B2R with icatibant also decreases the activation of downstream ion channels, including ClC-3 and KCa3.1 channels, leading to impaired glioma cell migration (Cuddapah et al. 2013a). Future preclinical studies should pursue the use of this drug for the treatment of gliomas by administering icatibant to glioma-bearing mice and measuring mouse survival and the ability of gliomas to associate with the vasculature and spread in the brain.

14.4.2 Preventing Glioma Migration and Proliferation Through Inhibition of Glioma Ion Channels

As depicted in Figs. 14.3 and 14.4, ion channels promote shape and volume changes in proliferating and migrating cells. If volume changes are inhibited, glioma cell migration and proliferation is significantly reduced (Watkins and Sontheimer 2011; Habela and Sontheimer 2007). Cl^- channels play an integral role in controlling glioma cell volume, because of the large driving force for Cl^- movement out of the cell (Watkins and Sontheimer 2011; Habela et al. 2009). Therefore triggering glioma Cl^- channels may be a promising therapeutic strategy.

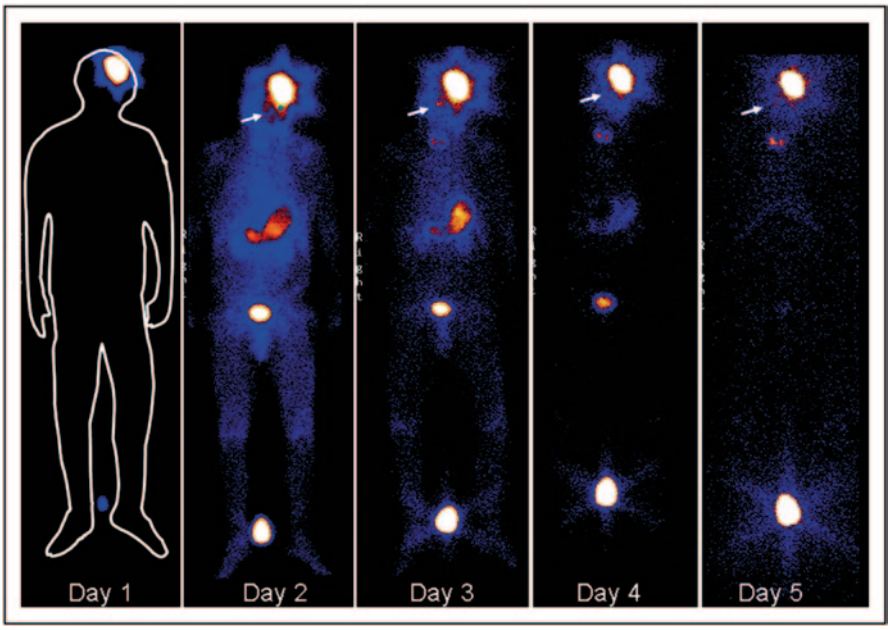


Fig. 14.7 Whole-body gamma radiation scans show specific localization and retention of 131I-TM-601 to malignant mass in Phase I study. Intracavitary administration of 131I-TM-601 demonstrated specific binding and retention to glioma mass at least 5 days later. Radiation standard placed next to patient's feet. (Reproduced with permission from Mamelak et al. (2006). Copyright American Society of Clinical Oncology)

Indeed, glioma Cl^- channels have already been targeted in Phase I/II trials for the treatment of malignant glioma (trial # NCT00040573). Cl^- channels can be inhibited by chlorotoxin, a toxin in the venom of the deathstalker scorpion (*Leiurus quinquestriatus*), which in turn can lead to an inhibition of cell migration (Soroceanu et al. 1999; McFerrin and Sontheimer 2005). Chlorotoxin binds to matrix metalloproteinase-2, which is upregulated in gliomas and is not endogenously expressed in the brain (Deshane et al. 2003). It specifically bound to 38/38 malignant gliomas as compared to 8/47 control specimens (Lyons et al. 2002).

In a Phase I study, TM-601, a synthetic compound modeled after chlorotoxin, was conjugated to iodine-131 (^{131}I-TM-601) and administered to 18 patients with recurrent gliomas (Mamelak et al. 2006). ^{131}I-TM-601 specifically bound to gliomas, was well tolerated, and potentially had anti-tumoral effects in patients (Fig. 14.7) (Mamelak et al. 2006). This anti-tumoral effect can be attributed to an inhibition of glioma Cl^- channels (Soroceanu et al. 1999; McFerrin and Sontheimer 2005; Lui et al. 2010), leading to a decrease in the migration and proliferation of glioma cells. Because of the binding specificity, ^{131}I-TM-601 has been successfully used to delineate the extent of glioma infiltration (Hockaday et al. 2005).

Because of its specificity of binding glioma cells, chlorotoxin is being used as a delivery system of both labeling and cytotoxic compounds. Chlorotoxin bound to

Cy5.5, a fluorescent marker with a near infrared emission, can be used to identify malignant tissue (Veiseh et al. 2007). To kill glioma cells, nanoparticles (Fu et al. 2012) and doxorubicine-loaded liposomes (Xiang et al. 2011) conjugated to chlorotoxin have also been created.

Upstream of both Cl^- channels, ligand-mediated $[Ca^{2+}]_i$ increases in glioma cells seem to converge upon inositol 1,4,5-trisphospate receptor subtype 3 (IP_3R_3) activation (Kang et al. 2010). Inhibition of IP_3R_3 with caffeine in a preclinical model blocked EGF- and bradykinin-mediated $[Ca^{2+}]_i$ increases and decreased *in vivo* tumor size (Kang et al. 2010). By blocking $[Ca^{2+}]_i$ increases, caffeine may be preventing downstream Ca^{2+}-sensitive Cl^- channel activation, leading to a less severe disease course.

14.4.3 Use of Sulfasalazine to Limit Glioma Growth, Seizures, and Neurotoxicity

Glioma growth occurs in an unexpandable space confined by the cranium. To make room for the growing tumor, glioma cells release glutamate, elevating the concentration of glutamate above 100 μM (Marcus et al. 2010) as compared to 1–3 μM in normal brain. This leads to hyperexcitability of peritumoral neurons (Buckingham et al. 2011) and eventual neuronal death due to N-methyl-D-aspartate (glutamate) receptor-mediated excitotoxicity (Ye and Sontheimer 1999).

Sulfasalazine (Azulfidine®), an FDA-approved sulfa drug used to treat rheumatoid arthritis, ulcerative colitis, and Crohn's disease, has been demonstrated to inhibit glutamate release from human glioma cells (Chung et al. 2005; Buckingham et al. 2011). Its mechanism of action is inhibition of system xc-, the glutamate-cystine exchanger, thereby preventing both glutamate release and cystine uptake (Chung and Sontheimer 2009). The lack of cystine in glioma cells in turn leads to decreased production of glutathione, an antioxidant, leading to glioma cell death (Chung and Sontheimer 2009).

Sulfasalazine inhibits both the autocrine function of glutamate as well as peritumoral hyperexcitability. Glutamate released by system xc- acts in an autocrine and paracrine manner at glioma Ca^{2+}-permeable AMPA receptors, leading to Ca^{2+} influx and enhanced migration and tumor expansion (Lyons et al. 2007; Ishiuchi et al. 2002). Intraperitoneal injection of sulfasalazine into glioma-bearing mice inhibited tumor growth (Lyons et al. 2007). In addition sulfasalazine significantly reduced hyperexcitability in peritumoral neurons and epileptiform activity in glioma-bearing mice (Buckingham et al. 2011). These promising preclinical results lead to the recent creation of a pilot study to determine the effect of sulfasalazine on glutamate concentration in patients with glioma, as measured by Magnetic Resonance Spectroscopy (clinical trial # NCT01577966).

In summary, we propose that in addition to ongoing clinical trials focusing on traditional glioma therapies, including surgical resection, chemotherapy, and radiation therapy, novel therapeutic avenues must be explored for a dramatic shift in patient care to occur. Studying glioma pathophysiology as a neurodegenerative

disease leads to an appreciation of the unique microenvironment of the brain, and with that comes a focus on glutamate excitotoxicity, the blood-brain barrier, and ion channel physiology. Our hope is that identifying a number of proteins that are uniquely upregulated in malignant gliomas will lead to more effective pharmacological and genetic strategies in the future to generate much needed alternative therapeutic approaches.

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Chapter 15
Hepatic Encephalopathy: A Primary Neurogliopathy

Sharon DeMorrow and Roger F. Butterworth

Abstract Hepatic encephalopathy (HE) and brain edema are serious central nervous complications of both acute and chronic liver failure. HE starts as a neuropsychiatric syndrome beginning with changes in personality and sleep disturbances progressing through cognitive dysfunction and motor symptoms to stupor and coma. In acute liver failure (ALF) progression may be very rapid, sometimes of the order of days (Butterworth and Vaquero, The liver; biology and pathobiology, pp. 600–617, 2009). Since the appearance of overt signs of encephalopathy in liver failure generally heralds a poor prognosis with a significant impact on clinical management options, on liver transplant outcome and on health-related quality of life, rational effective therapies are urgently required. Such therapies require knowledge of the precise mechanisms implicated in the pathogenesis of HE.

Keywords Hepatic encephalopathy · Liver failure · Cirrhosis · Alzheimer type 2 astrocytosis · Brain edema · Microglial activation · Minocycline · Chemokines

15.1 Neuroglial Pathology in Liver Failure

Pathologically, HE is characterized primarily by neuroglial alterations, the nature and extent of which relate to the type of liver failure (acute or chronic) and the severity of the liver injury. Terminal stages of ALF frequently result in brain herniation caused by intracranial hypertension resulting from brain edema and electron microscopic studies reveal that the brain edema in ALF is primarily cytotoxic (due to cell swelling) rather than vasogenic (due to breakdown of the blood-brain barrier) in nature (Kato et al. 1992). The cell manifesting the most severe swelling in ALF is the astrocyte (Fig. 15.1).

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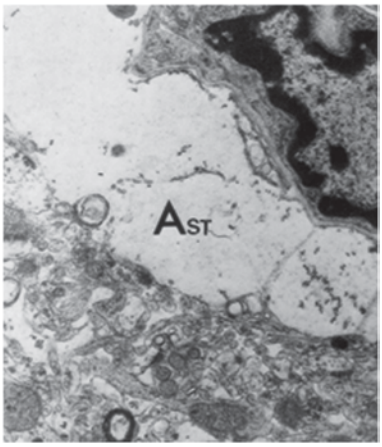

Fig. 15.1 Acute hyperammoniemia resulting from liver failure results in astrocyte swelling. A representative electron micrograph showing swelling of a perivascular astrocyte (Ast) from a patient with acute liver failure who died of brain herniation. (Figure adapted from Felipo and Butterworth (2002) and reproduced with permission from Elsevier)

In contrast to ALF, end-stage chronic liver failure (cirrhosis) results in characteristic pathological changes to the astrocyte; these changes are known as Alzheimer type 2 astrocytosis where the cell nuclei take on a characteristic shape and pattern consisting of marked pallor and swelling with a prominent nucleolus, margination of the chromatin pattern and glycogen deposition (Fig. 15.2). The nuclei may take on an array of shapes and forms depending upon the brain structure examined. These forms range from the classical spherical shape in the cerebral cortex to irregular lobulated forms in some basal ganglia structures; the appearance of multiplets has been reported to suggest an element of hyperplasia (Norenberg 1987).

The occurrence of multiple episodes of grade 4 (or stage IV) HE (coma) and/or a single prolonged period of coma in a patient with end-stage chronic liver failure may result in a condition known as "acquired non-Wilsonian hepatocerebral degeneration", the neuropathological features of which include Alzheimer type 2 astrocytosis. In addition, varying degrees of neuronal cell loss occur in cerebral cortex, cerebellum and basal ganglia structures (Butterworth 2007). A related condition known as "Parkinsonism in cirrhosis" appears to result from manganese deposition in substantia nigra of these patients (Butterworth 2013b).

Evidence of discrete alterations of the blood-brain barrier in HE have been proposed based upon studies of barrier permeability in an animal model of ALF (Nguyen 2010) but these findings were not confirmed by others (Bemeur et al. 2010a). Swelling of cerebrovascular endothelial cells has been demonstrated using electron microscopy in material from ALF patients (Kato et al. 1992) again suggesting that, if barrier changes do occur in HE, they are discrete in nature.

Microglial activation and its role in the pathogenesis of HE was first described in the brains of animals with ALF resulting from hepatic devascularisation (Jiang et al. 2006). Subsequent studies in this model revealed that activation of microglia was accompanied by grade 4 HE (coma) and brain edema as well as by increased expression of genes coding for pro-inflammatory cytokines in brain (Jiang et al. 2009a, b)

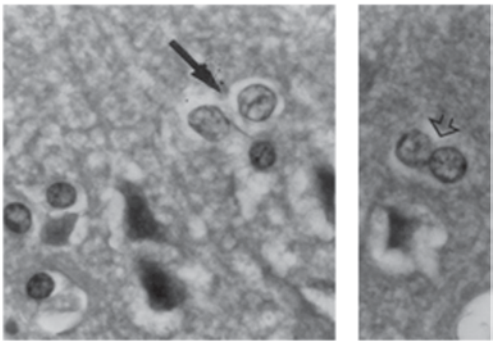

Fig. 15.2 Chronic hyperammoniemia resulting from liver cirrhosis results in Alzheimer type II astrocytes. Alzheimer type II cells (*arrow, left-hand panel*) show enlarged nuclei and margination of chromatin in this hematoxylin and eosin stained section of frontal cortex from a 51-year-old cirrhotic patient who died in hepatic coma. Doublets suggestive of a proliferative response are frequently seen (*right-hand panel, arrow head*). (Figure adapted from Felipo and Butterworth (2002) and reproduced with permission from Elsevier)

(Fig. 15.3). Microglial activation has subsequently been confirmed using various techniques and cellular markers in an animal model of ALF resulting from toxic liver injury (McMillin et al. 2012b; Rangroo Thrane et al. 2012). Activation of microglia has also been reported in material from a patient with ALF resulting from viral hepatitis (Butterworth 2011), in a material from cirrhotic patients who died in hepatic coma (Zemtsova et al. 2011) and increased signals in positron emission tomography (PET) studies in HE patients was attributed to microglial activation (Cagnin et al. 2006). Microglial activation has also been described in an animal model of biliary cirrhosis (D'Mello et al. 2009).

The subject of activation of microglia in HE and its relationship to pro-inflammatory mechanisms and the role of cytokines/chemokines in the pathogenesis of the CNS complications of liver failure are covered in-depth in a later section of this chapter.

15.2 Abnormalities of Neuroglial Function in Liver Failure

In addition to frank neuroglial pathology, both acute and chronic liver failure result in a wide range of alterations of neuroglial function including deficits in neuroglial cell volume regulation and neuroglial-neuronal metabolic trafficking of key intermediates as well as neuroinflammatory changes. Some of these changes appear to relate to exposure of neuroglia *in situ* to ammonia and manganese, two substances known to accumulate to toxic concentrations in brain in liver failure.

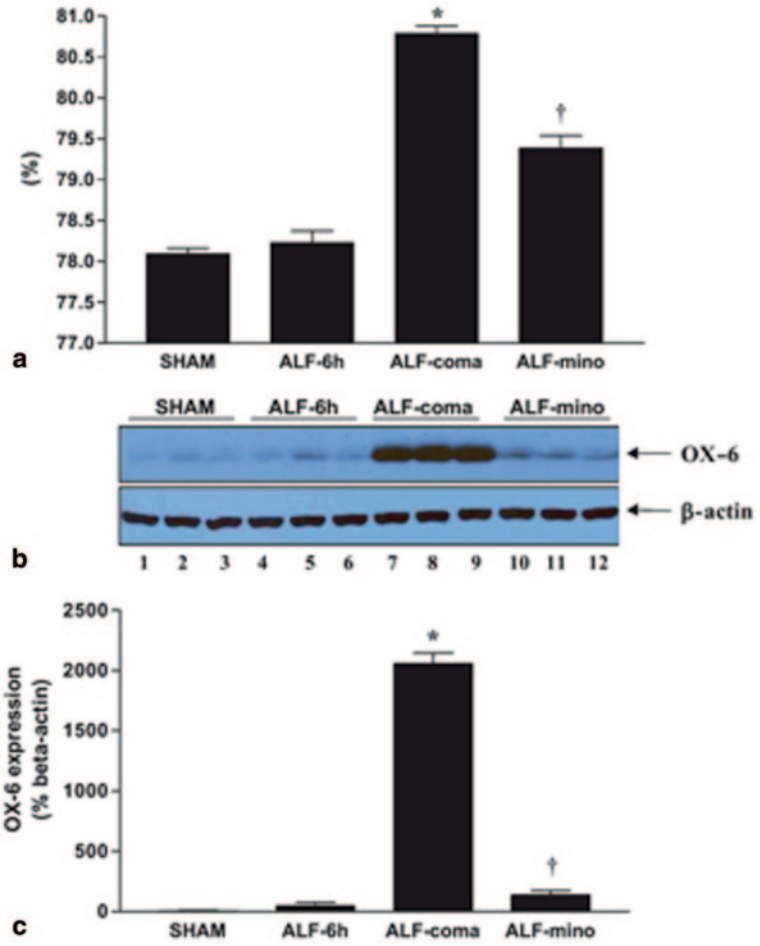

Fig. 15.3 Western blot analysis of OX-6 expression in acute liver failure (ALF) rats reveals that inhibition of microglial activation by minocycline treatment is correlated with attenuation of brain edema. **a** Percentage of brain water content of cerebral cortex from sham-operated controls (sham), ALF rats 6 h post- hepatic artery ligation/HAL (ALF-6 h), ALF rats at coma stage of encephalopathy (ALF-coma) and in ALF rats treated with minocycline (ALF-mino); **b** OX-6 protein expression in cerebral cortex from sham-operated controls (lanes 1–3), ALF rats 6 h post-HAL (lanes 4–6), ALF rats at coma stage of encephalopathy (lanes 7–9) and in ALF rats treated with minocycline (lanes 10–12); **c** Histogram representation of OX-6 expression in the various treatment groups. Data represent mean ± SEM of $n = 10$ animals per treatment group. Significant differences indicated by $^*p < 0.01$ versus shan-operated controls and ALF-6 h; $\dagger p < 0.001$ versus ALF-coma (ANOVA with post hoc Tukey's test). (Figure reproduced from Jiang et al 2009a, with permission from Wiley)

15.2.1 Neuroglial Cell Volume Regulation

In ALF, it is generally considered that failure of cell volume regulation in astroglia underpins the phenomenon of cytotoxic brain edema that so frequently leads to increased intracranial pressure, brain herniation and patient death. Various mechanisms have been proposed to explain this failure of neuroglial cell volume regulation in ALF that include as described in two subheadings below.

15.2.1.1 Osmotic Effects of Increased Intracellular Glutamine

Being devoid of a functional urea cycle, brain relies upon glutamine synthesis for the removal of excess ammonia and the enzyme responsible, glutamine synthetase (GS) has a predominantly, if not exclusively, astroglial localization. Brain and cerebrospinal fluid (CSF) ammonia and glutamine concentrations correlate well with severity of HE in both ALF (Clemmesen et al. 1999) and in end-stage cirrhosis (Laubenberger et al. 1997). Based upon studies in cultured cortical astrocytes exposed to ammonia as well as in animal models of pure hyperammonemia (in the absence of liver failure), it has been proposed that the accumulation of glutamine in brain in liver failure leads to an osmotic gradient that contributes to cell swelling. This hypothesis is supported by a report describing a protective effect of the GS inhibitor methionine sulfoximine on cell swelling and brain edema in hyperammonemic animals (Brusilow et al. 2010). However, although inhibition of glutamine synthesis was beneficial under these conditions, the use of 13C-nucler magnetic responence spectroscopy failed to show a correlation between *de novo* synthesis of glutamine in brain with either the severity of HE or with the presence of brain edema in animals with ALF (Chatauret et al. 2003) suggesting the presence of additional/alternative factors.

15.2.1.2 Neuroglial Proteins Involved in Cell Volume Regulation

Alterations in expression of genes coding for key neuroglial proteins with suggested roles in cell volume regulation have been reported in experimental models of HE.

Aquaporin-4 (AQP-4) is highly expressed in astroglia, particularly in the endfeet that ensheath brain capillaries where it mediates transmembrane movement of water. An important role for the protein has been suggested to contribute to the pathogenesis of brain edema in a range of clinical situations. In relation to the cerebral complications of liver failure, increased AQP-4 expression was reported in cultured cortical astrocytes exposed to millimolar concentrations of ammonia that led to significant cell swelling (Rama Rao et al. 2003). However, results in animal models of liver failure have so far given conflicting results; whereas HE and brain edema were accompanied by increased concentrations of AQP-4 in one model of ALF (Eefsen et al. 2010), no such changes were reported in a subsequent study (Wright et al. 2010).

In contrast to the equivocal nature of AQP-4 in relation to HE, progressive decreases in expression of the astroglial marker protein, glial fibrillary acidic protein (GFAP) have consistently been reported in both experimental animal models of liver failure and in patient material. In rats with ALF resulting from hepatic devascularisation, GFAP expression was decreased as a function of the increase in brain water (Belanger et al. 2002). Selective decreases of GFAP were previously reported in several brain structures from cirrhotic patients with HE; these structures included cerebral cortex, thalamus and basal ganglia (Sobel et al. 1981) whereas no such changes were subsequently reported in cerebellum of similar patients (Kril et al. 1997). There is evidence to suggest a role of ammonia in the pathogenesis of the loss of GFAP in liver failure, a proposal that is based upon the report of a loss of expression of GFAP in cultured astrocytes exposed to ammonia which resulted in significant cell swelling and destabilization of the GFAP molecule (Neary et al. 1994). It was proposed that loss of expression of GFAP, given its importance as a cytoskeletal protein in astroglia, could lead to altered visco-elastic properties of the cell thus favouring cell swelling (Belanger et al. 2002).

Significant increases in expression of the astroglial 45 kDa isoform of the facilitative glucose transporter GLUT-1 were reported in an animal model of ALF (Belanger et al. 2006) and, as for GFAP, a role for exposure to ammonia was suggested. In addition to its established role in the transport of glucose, it has been proposed that GLUT-1 acts as a water channel leading to the suggestion that upregulation of the protein in ALF may contribute to (or result from) the appearance of brain edema (Belanger et al. 2006).

One report described significant losses in expression of Kir 4.1, an inwardly-rectifying potassium channel expressed in astroglial endfeet that may have a function, in part, in cell volume regulation, has been described in a rat model of ALF (Obara-Michlewska et al. 2011).

15.2.2 Neuroglial Amino Acid Transporter Proteins

High affinity transport of amino acids is a major function of astroglia. Such transport mechanisms exist to maintain the supply of key intermediates required for cellular energy metabolism, synthetic processes and for the termination of action of neurotransmitters such as glutamate and γ-aminobutyric acid (GABA) that form the basis of the so-called “glial-neuronal metabolic interactions “. Astroglial *glutamate transporters* are essential components of the glutamate-glutamine cycle and are responsible for the removal of excess glutamate from the synaptic cleft. Expression of the sodium-dependent, high affinity glutamate transporters EAAT-1 and EAAT-2 (in human; in rodents GLAST and GLT-1, respectively) have been reported in animal models of both acute and chronic liver failure (Knecht et al. 1997; Suarez et al. 2000) resulting in increases in extracellular brain glutamate concentrations (Vaquero and Butterworth 2006). It has been suggested that limitations in the availability of glutamate in astroglia could limit

ammonia-removal capacity since glutamate is the obligate precursor for GS, and, if so, could limit the synthesis of glutamine.

Decreases in expression of the high affinity *glycine transporter* (GLYT-1) have been reported in cerebral cortical extracts from rats with ALF resulting from hepatic devascularisation (Zwingmann et al. 2002). Given the predominantly astroglial localization of GLYT-1 in cerebral cortex, it was proposed that these changes might relate to increases in availability of extracellular glycine with the potential to activate the glycine neuromodulatory site on the N-methyl-D aspartate (NMDA) subclass of glutamate receptor. NMDA receptor activation has been proposed to explain the hyperexcitability and nitrosative stress that occur in ALF (Vaquero and Butterworth 2006).

Following the cloning and characterization of high affinity *glutamine transporters* (small neutral amino acid transporters or SNATs) and given the consistent finding of increased brain glutamine in HE, a study was undertaken to assess SNATs in an animal model of ALF (Desjardins et al. 2012). Coma/edema stages of encephalopathy were accompanied by a selective decrease in expression of SNAT-5. Given the astroglial localization of SNAT-5 (Cubelos et al. 2005) it was proposed that down-regulation of transporter expression in liver failure could result in effective "trapping" of glutamine in the cell, an action that is consistent with cell swelling due to glutamine accumulation in the astrocyte as had previously been widely proposed. Moreover, since astroglial glutamine functions as immediate precursor of releasable (transmitter) glutamate, a limit upon its availability following decreased release from the astroglial cell has the potential to result in impairment of glutamatergic transmission an action that could result in excessive inhibition that is also characteristic of HE.

15.2.3 Neuroglial Translocator Protein

Translocator protein (TSPO), previously known as the "peripheral-type benzodiazepine receptor" is a mitochondrial protein responsible for the transport of cholesterol into the mitochondrion. The protein is expressed predominantly by neuroglia with both astroglia and microglia exhibiting high levels of expression in mammalian systems. Increased expression of TSPO has been reported in a wide range of hyperammonemic disorders including urea cycle enzymopathies, ALF, animals with end-to-side portacaval anastomoses and in patients with end-stage chronic liver failure (see (Ahboucha and Butterworth 2008) for review). In one study in humans using PET and the TSPO ligand 11C-PK11195, increased signals were observed in anterior cingulate cortex where the magnitude of the increased signals correlated with the degree of cognitive impairment (Cagnin et al. 2006). The origin of these increased signals was considered to relate to microglial activation. Interest in TSPO in HE relates to the well-established relationship of the protein to the GABA system. Activation of neuroglial TSPO results in increased transport of cholesterol into the mitochondrion followed by increased synthesis of so-called "neurosteroids", one of

which, allopregnanolone is a very high affinity agonist for the neuronal GABA-A receptor. Increases in concentration of allopregnanolone were reported in autopsied brain tissue from patients who died in hepatic coma (Ahboucha and Butterworth 2005). Based upon these findings, it was proposed that the increase in "GABAergic tone" resulting from activation of TSPO sites and the subsequent increase in synthesis of allopregnanolone could also contribute to the excessive neuroinhibition in HE.

15.3 Evidence for Microglial Activation in the Pathogenesis of HE

As stated above, microglial activation has been demonstrated to be a key feature in the pathogenesis of HE due to both ALF and chronic liver cirrhosis. Clinically, indirect evidence for microglial activation has been demonstrated by an upregulation of the microglial marker Ionized calcium binding adaptor molecule 1 (Iba-1) in post mortem cortical brain tissue from patients with liver cirrhosis and HE, when compared to cirrhotic patients without HE (Zemtsova et al. 2011). In addition, data from a comprehensive gene expression profile analysis demonstrated an upregulation of markers for both the pro-inflammatory M1 and anti-inflammatory M2 microglial phenotypes, suggesting that both subpopulations of microglia may be present in patients with HE due to cirrhosis (Gorg et al. 2013). Furthermore, increased [11C]-PK11195 binding to the TSPO in patients with proven cirrhosis and minimal HE was suggested to be a reflection of microglial activation in these patients (Cagnin et al. 2006). Taken together, these clinical data indirectly support a role of microglia activation in HE.

In contrast, evidence for a direct role for microglia activation in the neurological consequences of both ALF and liver cirrhosis is more striking in many animal models of these diseases. Furthermore, in many of the models used, treatment modalities shown to inhibit microglia activation also alleviated or prevented the cognitive impairment and neurological decline observed during HE. Specific details are described below.

15.3.1 Toxic Liver Injury

A range of hepatotoxic agents have been used to uncover basic mechanisms responsible for the CNS complications of liver failure. This topic was reviewed by a panel of experts nominated by The International Society for Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN) who, after careful deliberation, recommended two toxic models based upon the extent of the characterization. The two models of ALF were the azoxymethane (AOM) mouse model and the thioacetamide (TAA) rat model (Butterworth et al. 2009). The AOM mouse model of ALF exhibits many of

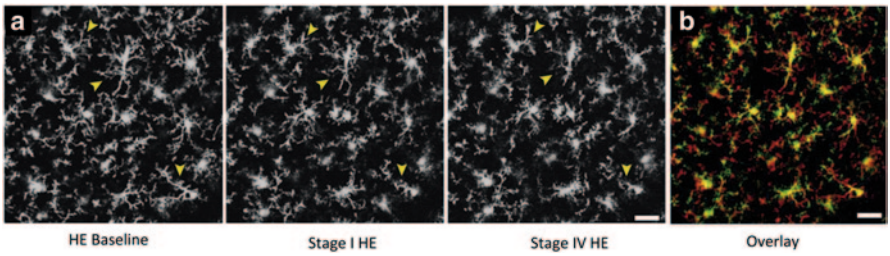

Fig. 15.4 The terminal coma stage of HE is associated with microglial activation. **a** Representative 2 photon laser scanning microscopy images from transgenic mice expressing green fluorescence protein under the control of the CX3CR1 promoter (expressed only in microglia) following AOM administration at 0 h (baseline), stage I and stage IV of HE (collapsed Z-stacks). **b** Overlay of representative images from HE at 0 h (*red*) and stage IV (*green*). *Yellow* represents the overlapping staining. During the latter stage, microglia appear more ameboid and activated. Scale bar represents 15 μM. (Figure adapted from Rangroo Thrane et al. (2012) and reproduced with permission from Elsevier)

the pathophysiological characteristics of human HE due to acute liver failure. These features include (1) a clear pattern of neurological behaviors starting with the prodromal phase due to liver failure, where neurological symptoms are not yet evident followed by a number of distinct phases of neurological decline that rapidly progress to stupor andcoma; (2) the presence of cerebral edema; and (3) high levels of ammonia in the blood and brain. Very elegant and detailed analyses of the morphological changes in microglia and real-time analysis of microglial dysmotility after AOM have been demonstrated (Rangroo Thrane et al. 2012). Both microglia activation (as demonstrated by an ameboidal phenotype) and motility (as demonstrated by analysis of the turnover rate) were shown to be altered in the cerebral cortex at late stages of HE when severe neurological symptoms were evident, coinciding with the appearance of brain edema (Fig. 15.4). Increased OX-42/CD11b immunoreactivity was also demonstrated in the cerebral cortex of AOM-injected mice (Chastre et al. 2012), which was attenuated by treatment with the tumor necrosis factor (TNF)-α neutralizing molecule etanercept. Furthermore, there was a concomitant attenuation of AOM-induced liver injury and decreased expression of neuroinflammatory molecules in the brain after etanercept treatment (Chastre et al. 2012).

15.3.2 Ischemic Liver Failure

Ischemic liver failure, although uncommon, is encountered clinically. Experimental ALF can be induced by the performing an end-to-side portacaval anastomosis followed by hepatic artery ligation. Rats undergoing this surgery exhibit key clinical features of HE, including cerebral edema and hyperammonemia, which ultimately result in grade 4 HE (hepatic coma). An increase in the number of OX-42/CD11b positive microglia has been demonstrated in the frontal cortex, thalamus and

hippocampus starting 6 h after surgery (early stage HE) and worsening at the time of coma/edema (Jiang et al. 2009a, b), which could be alleviated by either mild hypothermia (Jiang et al. 2009b) or minocycline (Jiang et al. 2009a), with a concomitant attenuation of the progression of HE and brain edema.

15.3.3 Portal-Systemic (Bypass) Encephalopathy

In a related, more subtle model of HE induced by end-to-side portacaval shunt surgery alone, without subsequent hepatic artery ligation, rats develop mild cognitive impairment over the following 3–4 weeks. Associated with this mild form of HE, currently referred to clinically as "minimal HE", is a change in the morphology of major histocompatibility complex class II (MHCII)-positive microglia to a more ameboid, activated phenotype (Agusti et al. 2011). Curiously, these changes were restricted to cerebellum. Chronic infusion of a p38 mitogen-activated protein kinase inhibitor reversed the morphological changes observed in microglia and prevented the cognitive impairment (Agusti et al. 2011).

15.3.4 Biliary Cirrhosis

Obstruction of the common bile duct induces a reproducible model of biliary cirrhosis in rats. Bile duct-ligated (BDL) animals have liver failure, developing jaundice, portal hypertension, portal-systemic shunting, bacterial translocation and immune system dysfunction. BDL rats are hyperammonemic but show only low-grade encephalopathy (decreased locomotor activities) (Butterworth et al. 2009). Using this model, microglia are activated predominantly in the cerebellum, with only traces of activation in the striatum and thalamus (Rodrigo et al. 2010) (Fig. 15.5a). Interestingly, no microglia activation was evident in the frontal cortex. Treatment with ibuprofen reduced the microglia activation and reversed the concomitant cognitive impairments observed (Rodrigo et al. 2010). Similarly, microglia activation has been shown after BDL in mice, as demonstrated by morphological changes in Iba-1 positive microglia (D'Mello et al. 2009). However, in contrast to the rat model, the activation of microglia was localized to the cerebral cortex rather than the cerebellum (Fig. 15.5b). The cause of these region-and species-selective changes remains unknown. As discussed in a separate section of this chapter, the activation of microglia in BDL mice is thought to subsequently recruit monocytes to the brain that contribute to the cognitive impairment observed (Kerfoot et al. 2006).

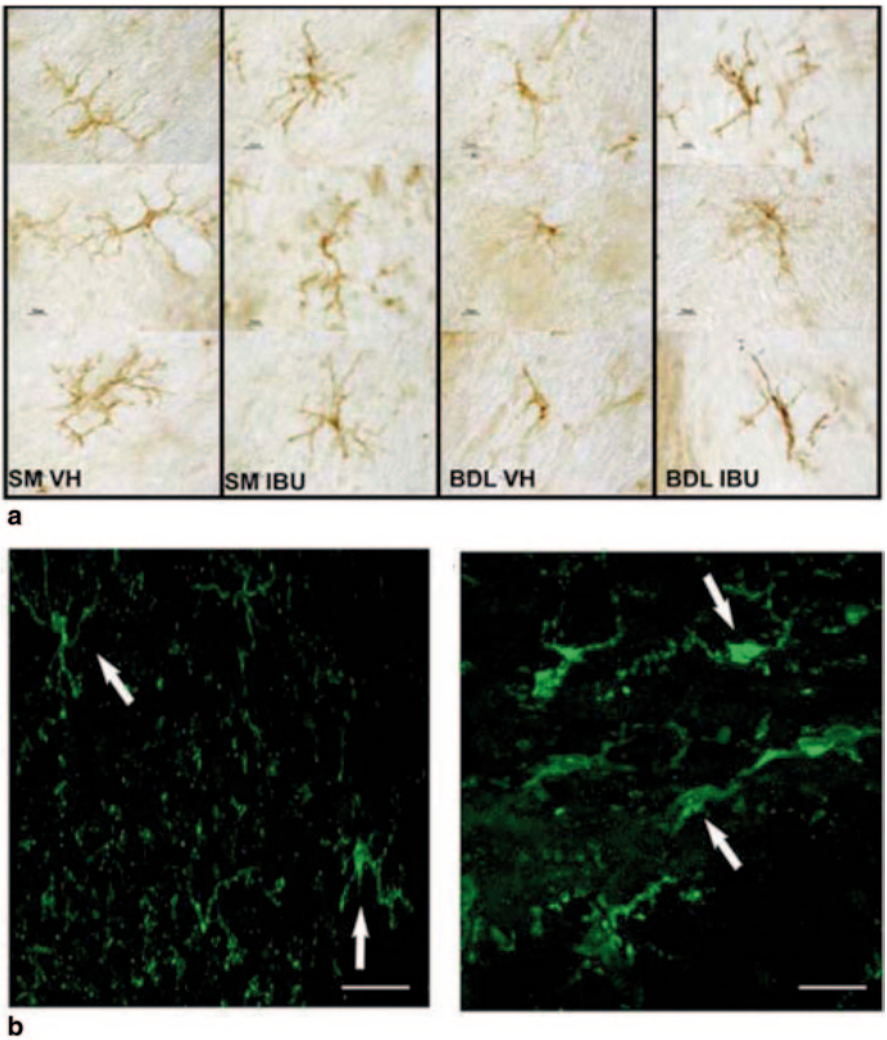

Fig. 15.5 Experimental models of biliary cirrhosis and mild HE are associated with microglial activation. **a** Rats underwent bile duct ligation (*BDL*) or sham (*SM*) surgery and were subsequently treated with either treated with vehicle (*VH*) or Ibuprofen (*IBU*). Microglial activation was assessed using MHCII expression. Microglia in the cerebellum of BDL rats showed an activated, ameboid phenotype. (Figure adapted from Rodrigo et al. (2010), and reproduced with permission from Elsevier.) **b** Immunohistochemistry staining for Iba-1-positive microglia in the cortex of BDL mice. Microglia in mice undergoing sham surgery (*left panel*) have resting ramified morphology and longer processes, while after BDL, microglia are more rounded with retracted and thicker processes indicative of microglia activation. (Figure adapted from D'Mello et al. (2009) and reproduced with permission from the Society for Neuroscience)

15.3.5 Ammonia Neurotoxicity

It has long been accepted that ammonia plays an important role in the neurological symptoms of HE. Hyperammonemia has been demonstrated in the blood and cerebral cortex at all stages of HE in both ALF and in cirrhosis. Furthermore, injection of a bolus dose of ammonium acetate into rats in the absence of liver failure results in a transient comatose state, supporting a role for ammonia in the neurological decline of HE. In contrast, rats fed an ammonium-containing diet for up to 28 days display cognitive deficits more indicative of minimal HE (Felipo et al. 1988). However, the role of ammonia toxicity in the activation of microglia and subsequent neuroinflammatory response is not as clearly defined. Treatment of primary microglial cultures with ammonia results in microglia swelling, migration, alterations in filipodia length, and Iba-1 expression (Zemtsova et al. 2011; Lachmann et al. 2013). Similarly, increased Iba-1 immunoreactivity and expression was demonstrated in the cerebral cortex of the *in vivo* rat model of ammonia intoxication (Zemtsova et al. 2011). In contrast, injection of an acute bolus dose of ammonium acetate into mice had no effect on the morphology or turn-over rate of microglia (Rangroo Thrane et al. 2012).

Microglial activation was evident in the cerebellum of chronic ammonium-fed rats, as demonstrated by in-depth morphometric analyses of MHCII positive cells (Rodrigo et al. 2010). In support of a role for microglia activation in the cognitive deficits observed in this chronic hyperammonia model, treatment with ibuprofen reduced the microglia activation and reversed the concomitant cognitive impairments observed (Rodrigo et al. 2010).

A note of caution: It should be borne in mind that experimental preparations, either *in vitro* or *ex vivo* in which mammalian cells in culture or experimental animals with intact livers are exposed to very high doses of ammonia alone are satisfactory preparations only for the study of mechanisms related to ammonia toxicity in normal animals. Given the multiple other factors that are known to affect brain function and that are generated in liver failure such as manganese accumulation, hyponatremia, certain fatty acids and inflammation, extrapolation of findings from ammonia-treatment paradigms to the liver failure clinic should be made with extreme caution.

15.4 Neuroinflammatory Cytokines Associated with Microglial Activation in HE

It is now commonly accepted that the development and progression of HE in liver failure shares a strong relationship with neuroinflammatory processes. Indeed, in patients and in animal models of HE, systemic inflammation causes worsening of the encephalopathy, and it has been proposed that proinflammatory signals may act synergistically with ammonia toxicity to bring about the neurological complications of acute and chronic liver failure (Shawcross et al. 2004; Butterworth 2011, 2013a).

Inflammatory molecules such as TNFα, interleukin (IL)-1β and IL-6 levels were increased in patients with ALF as well as in animal models (Wright et al. 2007; Bemeur et al. 2010b). In addition to the proinflammatory cytokines, neuroinflammation can be regulated by chemokines, or chemotactic cytokines. These molecules are involved in cell-cell communication, effecting a directional migration and activating various cell types of the immune system including microglia. A number of chemokines, such as chemokine ligand 2 (CCL2), have been shown to be upregulated in liver failure patients and in animal models of HE (D'Mello et al. 2009; McMillin et al. 2012b). A summary of our current knowledge of the inflammatory cytokines and chemokines in HE can be found in Table 15.1. Key inflammatory molecules and their reported effects on microglial activation and/or function are summarized below:

15.4.1 TNFα

TNFα is a potent proinflammatory cytokine. Circulating levels of TNFα are increased as a function of the severity of HE in both patients (Odeh 2007)and experimental animals (Jiang et al. 2009a) with liver failure. Moreover, the presence of TNFα gene polymorphisms is known to influence the clinical outcome in patients with ALF (Bernal et al. 1998). In experimental models of ALF, mice lacking the TNF receptor 1 (TNFR1) gene had a delayed onset of encephalopathy and an attenuation of brain edema (Bemeur et al. 2010b). TNFα has been shown to activate microglia in a number of experimental models of neuroinflammation (Lambertsen et al. 2012; Rubio-Perez and Morillas-Ruiz 2012). With respect to HE, as mentioned above, systemic levels of TNFα are increased in the AOM model of ALF (Chastre et al. 2012). Inhibition of TNFα signaling by systemic treatment with etanercept reduced systemic inflammation, attenuated the neurological decline and prevented microglial activation in the cerebral cortex (Chastre et al. 2012). These data support the hypothesis that peripherally-derived TNFα, at least in part, contributes to the microglial activation and subsequent neurological decline in liver failure. In support of this concept, neurological complications occurring in the BDL model of biliary cirrhosis were shown to be the consequence of monocyte recruitment in response to TNFα signaling and occurred via microglial activation. Specifically, peripheral TNFα signaling stimulates microglia to produce CCL2, which subsequently mediates monocyte recruitment into the brain (D'Mello et al. 2009). These findings were suggested to constitute a novel immune-to-brain communication pathway with the potential to result in altered neuronal excitability and neurological complications in cholestatic liver disease. A schematic diagram of this concept can be found in Fig. 15.6.

Table 15.1 Known inflammatory markers in HE

Inflammatory modulator	Model and species	Tissue assessed	Mechanism	Reference
TNFα	Hepatic devascularization in rats	Frontal cortex/serum/CSF	Specific role for TNFα not assessed; however, treatment with the anti-inflammatory minocycline or hypothermia inhibited microglia activation and cognitive deficits	Jiang et al (2009a, b)
	Bile duct resection in mice	Cerebral cortex, whole blood	Shown to be upregulated in the circulation, Thought to be responsible for microglia activation (TNFR1 knockout mice have dampened microglia response after bile duct resection)	D'Mello et al. (2009)
	Azoxymethane model of acute liver failure in mice	Frontal cortex	Onset of severe encephalopathy (coma) and brain edema was delayed in mice lacking the TNFR	Bemeur et al. (2010b)
	Portacaval shunt in rats	Cerebellum and plasma	Specific role for TNFα not assessed; however, treatment with specific p38 MAPK inhibitor inhibited microglia activation and cognitive deficits	Agusti et al. (2011)
	Azoxymethane model of acute liver failure in mice	Plasma	TNF antagonist etanercept reduced systemic inflammation, microglial activation and time to coma after AOM treatment	Chastre et al. (2012)
	Acetaminophen model of acute liver failure in mice	Liver/whole brain/serum	Used as a marker for inflammation	Villano et al. 2012
IL-1β	Hepatic devascularization in rats	Frontal cortex/serum/CSF	Specific role for IL-1β not assessed; however, treatment with the anti-inflammatory minocycline or hypothermia inhibited microglia activation and cognitive deficits	Jiang et al (2009a, b)
	Azoxymethane model of acute liver failure in mice	Frontal cortex	Onset of severe encephalopathy (coma) and brain edema was delayed in mice lacking the IL-1 receptor	Bemeur et al. (2010b)
	BDL and chronic hyperammonemia in rats	Cerebellum	Specific role for IL-1β not assessed; however, treatment with the non-steroidal anti-inflammatory drug ibuprofen inhibited microglia activation and cognitive deficits	Rodrigo et al. (2010)

Table 15.1 (continued)

Inflammatory modulator	Model and species	Tissue assessed	Mechanism	Reference
	Portacaval shunt in rats	Cerebellum	Specific role for IL-1β not assessed; however, treatment with specific p38 MAPK inhibitor inhibited microglia activation and cognitive deficits	Agusti et al. (2011)
IL-6	Hepatic devascularization in rats	Frontalcortex/serum/CSF	Specific role for IL-6 not assessed; however, treatment with the anti-inflammatory minocycline or hypothermia inhibited microglia activation and cognitive deficits	Jiang et al (2009a, b)
	Azoxymethane model of acute liver failure in mice	Plasma and cerebral cortex	Specific role for IL-6 not directly assessed; however, treatment with the TNF antagonist etanercept reduced systemic inflammation, microglial activation and time to coma after AOM treatment	Chastre et al. (2012)
	Acetaminophen model of acute liver failure in mice	Liver/whole brain/serum	Used as a marker for inflammation	Villano et al. (2012)
IL-10	Acetaminophen model of acute liver failure in mice	Liver/whole brain/serum	Used as a marker for inflammation. Upregulation attenuated in brains from transgenic mice expressing the human Serpin B3	Villano et al. (2012)
CCL2	Bile duct resection in mice	Cerebral cortex, whole blood	CCL2 expression increased in activated microglia in response to TNFα signaling. Causes recruitment of CCR2-expressing monocytes in the brain	D'Mello et al. (2009)
	Azoxymethane model of acute liver failure in mice	Cerebral cortex	CCL2 expression increased predominantly in neurons. Correlated to increased microglia activation. Specific inhibitors of CR2 and CCR4 delays neurological decline and time to coma	McMillin et al. (2012b)
	Human autopsy samples of liver cirrhosis	Frontal cortex	CCL2 mRNA and immunoreactivity increased in patients with liver cirrhosis with HE but not in cirrhotics without HE	Bradley et al. (2013)

Table 15.1 (continued)

Inflammatory modulator	Model and species	Tissue assessed	Mechanism	Reference
TGFβ	Azoxymethane model of acute liver failure in mice	Liver/serum/ cerebral cortex	TGFβ is thought to be derived from the periphery. Systemic treatment with a TGFβ specific neutralizing antibody attenuates the microglia activation and delays neurological decline	McMillin et al. (2012a, 2013)

AOM azoxymethane, *BDL* bile duct ligation, *CCL2* chemokine ligand 2, *CCR* chemokine receptor, *CSF* cerebrospinal fluid, *HE* hepatic encephalopathy, *IL* Interleukin, *MAPK* mitogen-activated protein kinase, *TGFβ* transforming growth factorβ, *TNFα* tumor necrosis factor α, *TNFR1* TNF receptor 1

15.4.2 IL-1β

IL-1β is a potent proinflammatory cytokine that is predominantly expressed in activated macrophages (peripherally) and microglia (centrally) (Deng et al. 2011; Lambertsen et al. 2012). In the brain, rather than regulating microglia activation, it is proposed that IL-1β plays an important role in the downstream consequences of microglial activation on brain function, injury and repair (Lambertsen et al. 2012). Increased cortical IL-1β expression has been demonstrated in animal models HE due to ALF or cirrhosis (Jiang et al. 2009b; Bemeur et al. 2010b; Rodrigo et al. 2010). Furthermore, mice specifically lacking the IL-1β receptor had a delayed onset of encephalopathy and an attenuation of brain edema (Bemeur et al. 2010b). Increased expression and secretion of IL-1β in the brain during HE may then exert deleterious effects on astroglial function. Indeed, treatment of astrocyte cultures with recombinant IL-1β increased the expression of the astrocyte proteins, GFAP, AQP-4, hemoxygenase 1 and inducible nitric oxide synthase (Chastre et al. 2010) and led to induction of the mitochondrial permeability transition complex (Alvarez et al. 2011).

15.4.3 TGFβ

The role of transforming growth factor β (TGFβ) in the inflammatory response is largely context dependent. Specifically, TGFβ has both anti-inflammatory and pro-inflammatory effects on various immune cells in the body, including microglial activation. Increased levels of TGFβ have been demonstrated in the liver and serum in the AOM model of ALF (McMillin et al. 2012a). The authors demonstrated that peripheral TGFβ inhibits the expression of the protective factor Gli1 in the cerebral cortex during both acute and chronic liver damage, and speculated that this has implications on microglial activation (McMillin et al. 2013). Systemic treatment

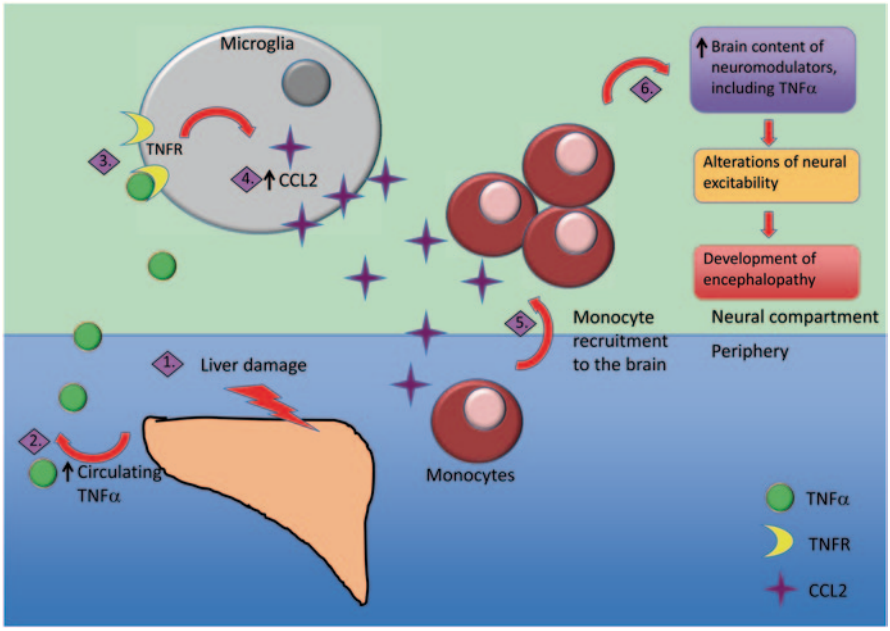

Fig. 15.6 Possible mechanism whereby systemic inflammation leads to microglial activation and monocyte recruitment into the brain as key factors in the pathogenesis of HE in liver failure. Upon damage to the liver (*1*) there is an increase in circulating TNFα (*2*). It is proposed that this increase in TNFα is then able to activate TNFR located on microglia (*3*) to bring about their activation. Activated microglia then produce increased amounts of the chemokine CCL2 (*4*), which signals for the recruitment of peripheral monocytes to the brain (*5*). Together, the activated microglia and increased infiltration of monocytes leads to and increased brain content of neuromodulators including TNFα, alterations in neural excitability and ultimately the development of encephalopathy (*6*)

of mice with a neutralizing anti-TGFβ antibody delayed the neurological decline observed in AOM-induced ALF (McMillin et al. 2013), and attenuated the morphological changes in Iba-1 positive microglia (McMillin et al. 2013). However, whether TGFβ is acting directly on microglia to regulate the neuroinflammatory response in these models of HE, or whether the changes in microglial activation are an indirect effect of the protective effect of anti-TGFβ neutralizing antibodies remains to be established.

15.4.4 CCL2

The chemokine ligand 2 (CCL2 or monocyte chemotactic protein-1) and its receptors CCR2 and CCR4 have been implicated in a number of neuropathologies ranging from traumatic brain injury to autoimmune disease (Yao et al. 2012). CCL2 can be produced by a number of cell types in the brain, including astrocytes and

microglia (Yao et al. 2012). Neurons have also been shown to release CCL2 after brain ischemia or lipopolysaccharide administration (Kim et al. 2012). The consequences of CCL2 expression are varied and context-dependent. For example, CCL2 has been shown to activate microglia as well as increase the infiltration of circulating macrophages (Yao et al. 2012). Furthermore, while CCL2 expression is upregulated in a number of neuropathies, the consequences are detrimental in some disease states and protective in others (Conductier et al. 2010).

CCL2 immunoreactivity has recently been demonstrated to be increased in the frontal cortex of autopsy samples from cirrhotic patients with HE but not in cirrhotic patients who died with no neurological symptoms (Bradley et al. 2013). As mentioned above, increased CCL2 expression has also been demonstrated in microglia in the cerebral cortex in the animal model of biliary cirrhosis. It is postulated that the release of this chemokine is responsible for triggering the recruitment of circulating monocytes to the affected brain region, which contributes to the subsequent neurological decline (D'Mello et al. 2009) (Fig. 15.6). Similarly, in the AOM model of HE, CCL2 mRNA and protein expression is upregulated in the cerebral cortex (McMillin et al. 2012b). However, in this model CCL2 immunoreactivity appeared to be predominantly neuronal (McMillin et al. 2012b). Pretreatment of mice with specific inhibitors of CCR2 and CCR4 attenuated the increase in microglia activation (demonstrated by morphological changes in Iba-1 positive microglia) and subsequently delayed the neurological decline (McMillin et al. 2012b), supporting the hypothesis that during ALF, neurons produce and secrete the chemoattractant CCL2 that may cause the recruitment, proliferation and activation of microglia. Whether CCL2 also causes a recruitment of circulating monocytes in ALF, in a similar manner to that observed in chronic liver cirrhosis is unknown.

Conclusion

Hepatic encephalopathy and brain edema are serious neurological complications of liver failure that are characterized by neuroglial pathology involving primarily swelling of astrocytes and activation of microglia. In addition to frank pathology, significant alterations of neuroglial function are encountered in liver failure. Such changes include cell volume regulatory deficiencies leading to cytotoxic brain edema and its complications, reductions in expression of astroglial amino acid transporters and the increased expression of the mitochondrial translocator protein. Activation of microglia are revealed using a range of cell-specific markers and are a predominant feature of liver failure independent of the cause of liver damage. Increases in expression of genes coding for pro-inflammatory cytokines (TNFα, IL-1b, IL-6) and chemokines (CCL2) occur in brain as a consequence of microglial activation leading to the currently well-accepted notion that neuroinflammation (inflammation of the brain *per se*) is a major feature of HE in liver failure. Possible mechanisms responsible for activation of microglia in liver failure include ammonia neurotoxicity, brain lactate accumulation and liver-brain pro-inflammatory signalling. A

series of studies in experimental liver failure demonstrate that anti-inflammatory treatments using a range of approaches including proinflammatory cytokine gene deletion, neutralizing anti-cytokine antibodies such as etanercept, CCL2 receptor antagonists and minocycline result in prevention or significant attenuation of the severity of HE and brain edema in liver failure. Translation of these interesting leads into the clinic has the potential to provide novel therapeutic opportunities for the management and treatment of HE and brain edema in liver failure.

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Chapter 16
Astroglia and Severe Mental Illness: A Role for Glutamate Microdomains

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Abstract Postmortem studies of schizophrenia have historically been focused on abnormalities of neurons, with only a small but growing body of work focused on astroglia. The limitations of this approach are reflected by the recent failed efficacy of several neuron-centric pharmacological treatments. In this chapter, we will review glutamate neurochemistry and present a novel hypothesis related to astrocyte dysfunction in schizophrenia. We posit that plasma membrane glutamate transporters expressed on astrocytes help partition extrasynaptic regions, where pools of extracellular glutamate may be tightly regulated. These so-called "glutamate microdomains" may impact excitatory neurotransmission via modulation of extrasynaptic glutamate receptors. This hypothesis is supported by structural, biochemical and electrophysiological evidence, which suggests that the fidelity of these domains could be altered in severe mental illness.

Keywords Schizophrenia · Psychosis · Glutamate transporter · Microdomain · Glutamate spillover

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16.1 Introduction

The study of severe mental illness includes materials/data obtained from living patients, animal models, and postmortem studies using brain tissues from afflicted patients. This review will primarily focus on postmortem studies that inform the hypothesis that remodeling of astroglial processes leads to glutamate spillover in glutamate circuits. We also argue that there are specialized extracellular regions, or microdomains, where glutamate levels are tightly regulated and contribute to astrocyte-neuron interactions via modulation of extrasynaptic glutamate receptors. We will present data that argue for an abnormality of extracellular glutamate microdomains secondary to astroglial deficits found in the brain in schizophrenia.

The focus of this chapter on astrocyte-associated changes in schizophrenia represents an important extension of the glutamate hypothesis of schizophrenia beyond the N-methyl-D-aspartate (NMDA) receptor signaling complex. For decades, the prevailing hypotheses of schizophrenia were centered on neurotransmitter receptor dysfunction, first with D2 dopamine receptors, and more recently NMDA subtype of glutamate receptors. Studies focused on these hypotheses explored how changes in neurotransmitter receptors, localized to the postsynaptic density (PSD) or presynaptic structures on neurons, contributed to the pathophysiology of severe mental illness (reviewed in (McCullumsmith et al. 2004a)). In general, the interpretation of the data in these studies is biased towards the view that observed changes in variables such as measures of receptor subunit gene expression reflect a primary deficit in pre- or postsynaptic neurons. In the sections below, we seek to develop a more balanced view of the role(s) of astroglia in the pathophysiology of this often devastating illness.

16.2 Glutamate Neurotransmission

Glutamate Release and Reuptake The process of release, activity as a ligand, and reuptake of glutamate involves three distinct cell types: the astrocyte, the presynaptic neuron and the postsynaptic neuron (Salt et al. 1996). In the presynaptic neuron, glutamine can be converted to glutamate by the enzyme glutaminase, and packaged into vesicles by a family of vesicular glutamate transporters (VGLUT1–3) for release into the synapse (Bellocchio et al. 2000; Takamori et al. 2000). Glutamate is released into the synapse and may occupy and activate ionotropic [NMDA, α-amino-3-hydroxy-5-methyl-isoxazole propionate (AMPA), and kainate] or metabotropic (mGluR1-8) glutamate receptors on both neurons and astrocytes (Hollmann and Heinemann 1994; Hollmann et al. 1994; Salt et al. 1996). Glutamate is rapidly removed from the synapse by a family of plasma membrane excitatory amino acid transporters localized to postsynaptic neurons and astrocytes (Masson et al. 1999b). Recovered glutamate may enter the tricarboxylic acid cycle via conversion to alpha-ketoglutarate by glutamate dehydrogenase, be converted to glutamine by glutamine

synthetase and transported back into the synapse, or be released into the extracellular space by a variety of mechanisms (Malarkey and Parpura 2008), including the cystine/glutamate antiporter. In astrocytes, glutamate may also be added to this cycle via *de novo* synthesis of glutamate in a pathway involving pyruvate carboxylase and transaminases (Brainard et al. 1989; Patel et al. 2001; McKenna 2011). Recovered glutamate can also contribute to formation of lactic acid. Lactate production is favored in astrocytes, while lactate breakdown is favored in neurons (Stobart and Anderson 2013). Lactate may be efficiently shuttled from astrocytes to neurons, suggesting that lactic acid may be a preferred energy source for neuronal structures enveloped by astrocytic processes (Stobart and Anderson 2013). Finally, several families of novel glutamate receptor and transporter associated molecules regulate glutamate release and reuptake through intracellular signaling mechanisms (Jackson et al. 2001; Lin and Maiese 2001; Marie et al. 2002; Watanabe et al. 2003).

Glial Plasma Membrane Glutamate Transporters The excitatory amino acid transporters (called EAATs in human) are expressed in the plasma membranes of neurons and glia throughout the brain in a region and cell specific manner (Arriza et al. 1993; Utsunomiya-Tate et al. 1996). EAATs mediate glutamate transport by an electrogenic exchange of 3 Na+, 1 H+, and 1 glutamate (monovalent anion at conditions in the brain) molecule into the cell and 1 K+ ion out of the cell, with the net inward movement of positive charges (Zerangue and Kavanaugh 1996; Levy et al. 1998). EAATs are likely homomers comprised of 2–3 non-covalently linked subunits that have 6–10 transmembrane domains (Danbolt 2001). The transporters have specific patterns of cellular localization. EAAT1 and EAAT2 have primarily been localized to astroglia. EAAT3–4 and EAAT5 are primarily localized to neurons and the retina, respectively (Arriza et al. 1997; Danbolt 2001). In the prefrontal cortex, glial transporters (EAAT1 and EAAT2) are predominately expressed in discrete subsets of astrocytes which account for approximately 90 % of synaptic glutamate reuptake (Regan et al. 2007). The glial transporters are localized to perisynaptic processes facing the synaptic cleft (Tzingounis and Wadiche 2007). In the rodent, activation of the promoters and expression of EAAT1 (called GLAST in the rodent) and EAAT2 (GLT-1 in rodent) is generally nonoverlapping (Regan et al. 2007). In addition, the GLAST, but not GLT-1, promoter was activated and EAAT1 expressed in oligodendrocytes, suggesting that EAAT1 has a role in myelination and CNS connectivity (Regan et al. 2007). The functional importance, perisynaptic localization, and heterogeneity of expression of the glial glutamate transporters suggests that examination of the expression and function of these molecules may be a high yield target for studies of neuropsychiatric illnesses that involve alterations in glutamate transmission.

EAAT Expression, Processing, and Trafficking EAATs are synthesized in the endoplasmic reticulum and have extensive posttranslational modification in the Golgi, including N-linked glycosidation of at least two sites that are important for homomultimer formation (Kalandadze et al. 2004). EAATs are then trafficked to the plasma membrane where, localization and clustering are regulated by protein-protein interactions and phosphorylation (Conradt and Stoffel 1997; Figiel and Engele

2000; Gamboa and Ortega 2002; Schluter et al. 2002; Figiel et al. 2003). Ultrastructural studies indicate that most EAAT1-2 expression is in the plasma membrane (Chaudhry et al. 1995). EAATs may be removed from the plasma membrane via endocytosis and shuttled back to the cell surface via recycling endosomes, or be targeted for degradation in lysosomes (Nakagawa et al. 2008).

Extracellular Glutamate Glutamate released at the presynaptic terminal diffuses out of the synaptic cleft and may be transported into the astrocyte by EAATs or spillover to extrasynaptic areas (Masson et al. 1999a; Bridges et al. 2012). Extrasynaptic glutamate may also originate from astrocytes via vesicular release, the cystine/glutamate antiporter (also known as the system X_c^-), or other less prominent mechanisms (Montana et al. 2004; Haydon and Carmignoto 2006; Bridges et al. 2012). The cystine/glutamate antiporter releases glutamate and transports cystine into the astrocyte for glutathione synthesis. Glutamate levels in the extracellular milieu are postulated to be tightly regulated, as activation of extrasynaptic glutamate receptors has potent effects. For example, activation of extrasynaptic NMDA receptors promotes initiation of NMDA spikes, while long-term potentiation (LTP) and long-term depression (LTD) can be readily induced in the adult cortex by activation of extrasynaptic GluN2B containing NMDA receptors (Massie et al. 2008; Chalifoux and Carter 2011).

Removal of glutamate from the synaptic cleft may be conceptualized as a two-step process, involving first the high affinity binding of glutamate by perisynaptic transporters, and second the transport of bound glutamate by the transporter across the plasma membrane (Tong and Jahr 1994; Tzingounis and Wadiche 2007). Once bound, glutamate may be "unbound" or released, or alternatively, transported across the plasma membrane (Tong and Jahr 1994; Tzingounis and Wadiche 2007). The "capture efficiency" of the EAATs is defined as the ratio of the rate of unbinding of glutamate to rate of transport, which is reported to be about 0.5 (Tzingounis and Wadiche 2007). The relatively low rate of transport of bound glutamate compared to the capture efficiency suggests that the EAATs first act as buffers for released glutamate (Tzingounis and Wadiche 2007). Thus, glutamate molecules may bounce from one transporter binding site to another, until transported, limiting glutamate spillover from the synaptic cleft.

Glutamate Spillover The density of perisynaptic glutamate transporter protein, the amount of glutamate released, and the rate of glutamate transport determine, in part, the kinetics of glutamate diffusion away from the synaptic cleft. While several regions have well characterized glutamate spillover between excitatory synapses (such as the cerebellum and hippocampus), there is ongoing debate regarding the extent of glutamate diffusion in other regions, including the frontal cortex, where spillover of glutamate may detrimentally lead to loss of input specificity and activation of cell death pathways (Kullmann and Asztely 1998; Hardingham and Bading 2002; Hardingham et al. 2002; Lozovaya et al. 2004a; Tsvetkov et al. 2004; Marcaggi and Attwell 2007; Leveille et al. 2008). Under physiologic conditions, release of glutamate may exceed the capacity of cortical synapses to remove glutamate from the cleft (Weng et al. 2007; Drew et al. 2008). Mathematical models suggest

that glutamate may diffuse and activate NMDA receptors within a radius of 0.5 μm from the release point (Rusakov and Kullmann 1998). Thus, the spatial arrangement of glutamate synapses, their glutamate transporter buffering zones, and extrasynaptic glutamate receptors will determine the extent and effect of glutamate spillover (Lehre and Danbolt 1998; Sem'yanov 2005; Weng et al. 2007).

Glutamate Microdomains We posit that glutamate microdomains are formed by specialized protein clusters on the membranes of astrocytic processes apposed to extrasynaptic glutamate receptors expressed on specialized regions of neuronal membranes (Fig. 16.1) (Grosche et al. 1999; Genda et al. 2011). Diffusion of glutamate between domains or domains and synapses would be limited by the dense expression of glutamate transporters between these specialized structures (Lehre and Danbolt 1998).

Extrasynaptic Glutamate Receptors The G protein-linked mGluRs have a central role in regulating synaptic glutamate. mGluRs are expressed perisynaptically, and activation of mGluR2/3 receptors decreases presynaptic glutamate release (Schoepp 2001). Thus, activation of mGluRs may serve as a brake on glutamate spillover, preserving input specificity by diminishing synaptic glutamate levels (Huang and Bergles 2004). This mechanism has recently been exploited to develop a highly selective mGluR2 agonist that decreases glutamate release (Patil et al. 2007). This development suggests a role for the pharmacological modulation of glutamate spillover as a treatment for schizophrenia and other illnesses where psychosis is a central feature.

16.3 The Schizophrenia Syndrome

Schizophrenia is most properly considered to be a syndrome, as its specific causes are unknown, and the diagnosis is based on the presence of a heterogeneous collection of signs and symptoms. There is no blood test, specific genetic marker, or cognitive battery that can predict who will develop this illness. The schizophrenia phenotype is characterized by the presence of specific clinical findings, often divided into positive, negative, and cognitive symptoms (Buchanan et al. 2000). Positive symptoms include hallucinations, which are often auditory. Patients report that they hear voices that are clearly located outside of their heads, most often engaged in a running commentary on their thoughts and behaviors (Kay 1990; Badcock 2010). Other common positive symptoms include paranoid delusions and disorders of thought processes (Kay 1990; Badcock 2010). Much more debilitating are the negative symptoms, which are associated with the diminution of normal social behaviors, and include withdrawal, decreased speech, diminished eye contact, decreased or muted facial expression and vocal inflection, and diminished spontaneous movement (Buchanan et al. 2000; Fleischhacker 2000). Cognitive impairments in this illness include, but are not limited to, deficits in verbal fluency, executive function, and working memory (Rajji and Mulsant 2008; Szoke et al.

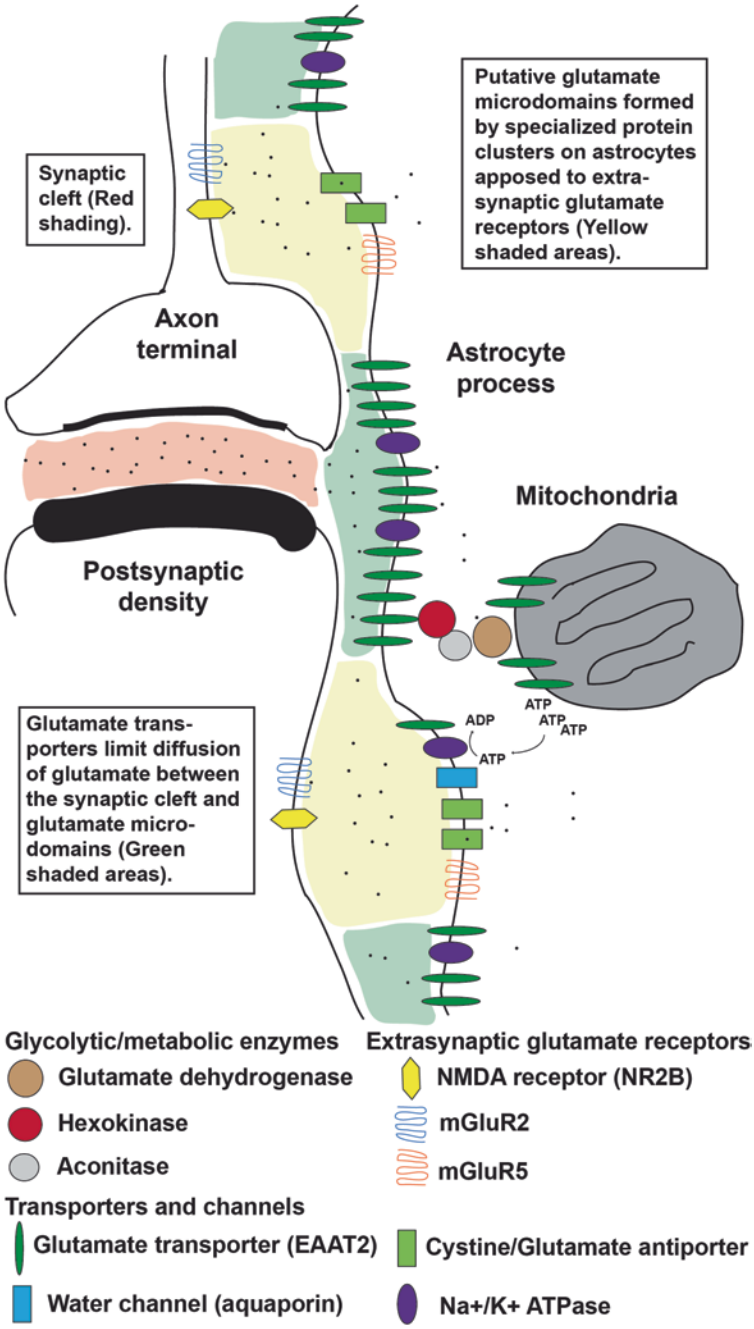

Fig. 16.1 Glutamate microdomains may be formed by specialized protein complexes found on plasma membranes in extrasynaptic regions where astrocytic and neuronal membranes are apposed to one another. Glutamate is released into the synapse (*red shading*) where it may bind and activate glutamate receptors. Plasma membrane glutamate transporters localized to perisynaptic astroglial processes bind and transport glutamate (*green shading*). Glutamate levels in extrasynaptic regions (*yellow shading*) may be regulated by glutamate spillover from synapses, release of glutamate from astrocytes, as well as reuptake of glutamate by glutamate transporters

2008; Wobrock et al. 2008; Potkin et al. 2009; Zanello et al. 2009). Few individuals suffering from schizophrenia have all of these symptoms, but the persistence of several characteristic symptoms, like auditory hallucinations, must be present in order for someone to be diagnosed with this disorder (Buchanan et al. 2000).

16.4 Prevailing Hypotheses of Schizophrenia

For decades, scientists have focused on the dopamine hypothesis of schizophrenia, which postulates that dysregulated dopaminergic neurotransmission is a key feature of the pathophysiology of the illness. The dopamine hypothesis is based on the observation that antipsychotics block D_2 receptors, and antipsychotic affinity for these receptors correlates with the ability to attenuate psychotic symptoms. Although numerous studies point to dopaminergic abnormalities in schizophrenia, dopamine dysfunction cannot completely account for all of the symptoms observed, since neuroleptics typically are effective only for the positive symptoms of the illness while negative symptoms and cognitive deficits are relatively refractory to treatment with typical antipsychotics (Joyce and Meador-Woodruff 1997; Laruelle et al. 1999). Consequently, alternative hypotheses that may help explain the pathophysiology of schizophrenia have been sought.

While the dopaminergic neurotransmitter system was implicated due to the effects of antipsychotic drugs, this system does, of course, not act in isolation. Dopamine receptors are found throughout the brain, where they modulate excitatory and inhibitory neurotransmission via G-protein signaling pathways. Blockade of dopamine receptors in corticolimbic circuits directly alters release of other neurotransmitters including glutamate and γ-aminobutyric acid (GABA). Not surprisingly, extensive postmortem studies have found changes in glutamatergic and GABAergic systems in this illness (Alda et al. 1996; Lewis et al. 2004; Deep-Soboslay et al. 2011; McCullumsmith and Meador-Woodruff 2011). However, evidence for involvement of glutamate receptor dysfunction, in particular the NMDA-subtype glutamate receptor, suggests a prominent role for glutamatergic abnormalities. NMDA receptor antagonists (but not GABA receptor antagonists) can induce both the positive and negative symptoms of schizophrenia, including cognitive deficits (Javitt and Zukin 1991; Tamminga 1999). Moreover, these compounds can exacerbate both positive and negative symptoms in schizophrenia (Lahti and Tamminga 1995). Chronic administration of phencyclidine (PCP)-like compounds induces a persistent psychotic symptomatology (Tsai and Coyle 2002) and reduces frontal lobe blood flow and glucose utilization, which is remarkably similar to the "hypofrontality" described in schizophrenia (Hertzmann et al. 1990).

Despite these observations, the complexity of schizophrenia is not readily explained by a static neurochemical model. The onset of schizophrenia is typically in late adolescence or early adulthood (Alda et al. 1996). The onset of positive and negative symptoms in a previously normally functioning person, coupled with a lifetime of waxing and waning symptoms, accompanied by the possibility of a steady

decline in social, occupational, and cognitive functioning, has led to longitudinal neurodevelopmental models that take into account genetic and environmental factors (Marenco and Weinberger 2000). Data supporting the neurodevelopmental hypothesis include studies suggesting that schizophrenia is associated with late winter births in urban environments, as well as a number of other prenatal, perinatal, and postnatal events (Marenco and Weinberger 2000; Lewis and Levitt 2002). Schizophrenia is perhaps best considered a disorder of neuroplasticity (McCullumsmith et al. 2004a). Plasticity refers to the ability of a system to affect reversible, long term changes in response to stimuli. Molecular correlates of learning and memory, including LTP and LTD, likely facilitate neuroplasticity in the brain. These processes are significantly impaired in severe mental illnesses, including schizophrenia (Malenka and Nicoll 1999; McCullumsmith et al. 2004b; Talbot et al. 2009). Glutamate transmission is a central component of LTP and LTD and, hence, has a central role in plasticity.

16.5 Glutamatergic Abnormalities in Schizophrenia

A number of studies have evaluated glutamate neurotransmission is schizophrenia using different approaches. In this section, we first discuss data from magnetic resonance spectroscopy (MRS), a technique which is largely focused on measuring glutamate, glutamine, and associated metabolites in specific brain regions of living patients. A strength of these studies is that the data are collected from afflicted individuals relatively close in time to the onset of the illness, while postmortem studies are examining brain tissues from more aged individuals who have had a lifetime of psychiatric illness. Next, we will briefly discuss data from postmortem studies, with a particular focus on extrasynaptic glutamate receptors and astroglial transporters.

Magnetic Resonance Spectroscopy Findings in Schizophrenia The balance of studies using MRS to examine glutamate, glutamine, N-acetylaspartate (NAA), and other metabolic intermediates have yielded mixed results (Bartha et al. 1997; Deicken et al. 1997; Theberge et al. 2002; Hutcheson et al. 2012; Kraguljac et al. 2012b). While a couple of studies have found changes in glutamate in schizophrenia, one large meta-analysis found decreases in the glutamate metabolite NAA in the basal ganglia and frontal lobe (Kraguljac et al. 2012a, b). Changes in NAA levels suggest abnormalities of glutamate synthesis and/or cycling in schizophrenia (Clark et al. 2006). A different meta-analysis found decreased glutamate and decreased glutamine in the medial frontal cortex in schizophrenia, suggesting that glutamate neurotransmission is diminished in this illness (Marsman et al. 2011). One interesting finding from these studies is the loss of correlation between NAA and glutamate levels in subjects with schizophrenia, compared to disease-free control subjects (Hutcheson et al. 2012; Kraguljac et al. 2012a). Taken together with the meta-analyses, these data suggest a significant abnormality in the glutamate/glutamine cycle in limbic circuits in schizophrenia. One limitation of the MRS approach

is that it measures all glutamate, glutamine or NAA without regard for it/them being intra or extracellular. For example, there may be a global increase in glutamine in the anterior cingulate cortex, with a large increase in intracellular pools and a small decrease in extracellular levels.

Abnormalities of Glutamatergic Enzymes in Schizophrenia There are several key enzymes involved in the glutamate/glutamine cycle as well as the synthesis or break-down of glutamate. Changes in enzymes levels may impact the amount of glutamate available for release from neurons and glial cells. Several studies have found decreased expression of carboxypeptidase II (binding and activity), glutaminase (mRNA), and glutamine synthetase (mRNA and protein) in limbic regions in schizophrenia (Burbaeva et al. 1999; Goff and Coyle 2001; Laruelle et al. 2003; Bruneau et al. 2005). Other studies have found increases in glutaminase (mRNA and activity) (Gluck et al. 2002; Bruneau et al. 2005). While these data support the hypothesis that glutamate synthesis and cycling may be impaired in schizophrenia, all of these studies were done at the regional level, and thus fail to capture the complexity of glutamate synapses at the cellular or subcellular level. For example, there may be diminished expression of glutamate enzymes in astrocytes, but increased expression in pyramidal neurons. Finally, one of the most interesting findings is a decrease in the dipeptidase glutamate carboxypeptidase II (GCP II; also known as NAALADase) activity in the frontal cortex and hippocampus in schizophrenia. GCPII catabolizes N-acetylaspartyl glutamate (NAAG) to glutamate and NAA (Ghose et al. 2004). These findings are consistent with the MRS data discussed above, which found decreased levels of NAA. NAAG antagonizes NMDA receptors, and increased levels (secondary to diminished GCP II activity) might contribute to NMDA receptor hypofunction. One strength of this study is that the authors measured enzyme activity, and not just transcript or protein levels, a technically demanding approach (Tsai and Coyle 2002).

Glutamate Receptor Abnormalities in Schizophrenia The observation that PCP may cause a schizophreniform psychosis in persons without a prior diagnosis of schizophrenia led to investigation of ionotropic glutamate receptor expression in schizophrenia. Initial hypotheses were focused on the idea that a loss or hypofunction of NMDA receptor activity would be reflected by diminished expression of NMDA receptor subunits as well as NMDA receptor binding sites. However, on balance, studies of NMDA receptor expression in the postmortem brain in schizophrenia have no clear or consistent pattern of findings (McCullumsmith et al. 2012). For example, there are over 18 studies of NMDA receptor subunit expression in the frontal cortex alone. Other than some changes in binding site expression, the hypothesis that there is deficient NMDA receptor expression stands largely unproven (McCullumsmith et al. 2012). Similar to NMDA receptors, AMPA and kainate receptor studies generally do not have a consistent pattern of abnormalities other than perhaps changes in AMPA receptor GluA2 subunit expression in the hippocampus (Tamminga 1999; Meador-Woodruff et al. 2001a; Harrison 2004). Interestingly, administration of PCP, which blocks the NMDA receptor channel, leads to increased glutamate release, which may lead to spillover of glutamate from

the synaptic cleft to extrasynaptic areas, activating extrasynaptic (non-NMDA) glutamate receptors. This mechanism may simulate/reflect the condition in schizophrenia, where impaired astrocyte function leads to diminished glutamate buffering and reuptake.

Metabotropic Receptor Expression in Schizophrenia While there are fewer postmortem studies of mGluRs, compared to ionotropic receptors, the data are no less contradictory. For example, mGluR3 protein expression has been reported as increased, decreased and unchanged in the frontal cortex (Gupta et al. 2005; Corti et al. 2007; Ghose et al. 2009; Shan et al. 2012). Genetic linkage studies suggest that mGluR5 is involved in schizophrenia, and increased mGluR5 and mGluR1 transcript and mGluR1 protein expression have been found in prefrontal cortex in this illness (Ohnuma et al. 1998; Devon et al. 2001; Gupta et al. 2005; Volk et al. 2010).

Abnormalities of Glutamate Transporters in Schizophrenia Several studies have reported region-level changes in the expression of the glial glutamate transporters EAAT1 and EAAT2 in schizophrenia. EAAT1 protein expression was decreased and EAAT1 glycosylation altered in the dorsolateral prefrontal cortex (DLPFC) (Bauer et al. 2008, 2010). Decreased EAAT1 and EAAT2 protein was found in the superior temporal gyrus, while only EAAT2 protein was decreased in the hippocampus (Shan et al. 2013). In contrast to these protein studies, increased levels of EAAT1 mRNA were found in the anterior cingulate cortex and thalamus (Smith et al. 2001; Bauer et al. 2008; Rao et al. 2012), suggesting a compensatory response to diminished glutamate reuptake capacity. Alterations in EAAT2 mRNA have also been reported in the hippocampus (decreased) and neocortex (increased, decreased and unchanged) in schizophrenia (Ohnuma et al. 1998, 2000; Matute et al. 2005; Lauriat et al. 2006; Bauer et al. 2008; Rao et al. 2012).

The neuronal transporters have also been studied. We have previously reported increased expression of EAAT3 protein and mRNA in the anterior cingulate cortex, while other studies have measured EAAT3 mRNA expression in the frontal cortex (increased), DLPFC (no change) and striatum (decreased) (McCullumsmith and Meador-Woodruff 2002; Lauriat et al. 2006; Nudmamud-Thanoi et al. 2007; Bauer et al. 2008; Horiuchi et al. 2012; Rao et al. 2012). No changes in EAAT3 protein levels were detected in the superior temporal gyrus or hippocampus (Shan et al. 2013). These conflicting data for neuronal glutamate transporters mirror the findings of glutamate receptor subunit expression, and are limited by the likelihood that glutamate transporter expression changes may be cell-specific, and change in different directions in different populations of cells. These findings have contributed to reformulation of the glutamate hypothesis of schizophrenia, with the idea are changes in glutamate receptor and/or transporter expression in schizophrenia is not a problem of too much or too little protein expression, but a problem with protein trafficking or signaling. Localization and activity of astroglial-localized glutamate transporters is mediated, in part, by protein-protein interactions. Expression of some of these EAAT-interacting proteins has been assessed in severe mental illness.

EAAT-Interacting Proteins in Schizophrenia Several glutamate transporter-interacting molecules have been identified, including G-protein suppressor pathway 1 (GPS1), JWA, ARHGEF11, and KIAA0302 (also called beta III spectrin). These molecules can affect glutamate transport function through trafficking, anchoring, phosphorylation, glycosylation, and degradation of transporters in the brain. For example, GPS1 decreases EAAT2-mediated glutamate reuptake through a direct protein-protein interaction, and levels of GPS1 protein are elevated in the frontal cortex in schizophrenia. These data suggest that there may be normal levels, but decreased activity, of a specific transporter due to modulation of transporter function or localization to the plasma membrane (Bauer et al. 2008).

In summary, changes in glutamate neurotransmission in schizophrenia point towards complex abnormalities of protein localization and function, contributing to the molecular neuropathology that underlies the schizophrenia phenotype.

16.6 Glutamate Spillover in Schizophrenia

Glutamate Spillover in Schizophrenia As outlined above, several postmortem studies have found changes in EAAT expression in schizophrenia, as well as changes in the molecules that regulate EAAT localization and activity (Ohnuma et al. 1998, 2000; Smith et al. 2001; McCullumsmith and Meador-Woodruff 2002; Lauriat et al. 2006; Bauer et al. 2008). In general, these changes in gene expression are consistent with diminished regional expression of astroglial (but not neuronal) glutamate transporter expression and activity. Further, a recent genetic study analyzing copy number variants reported a subject with schizophrenia with a deletion of several EAAT1 exons (Cook and Scherer 2008). Finally, characterization of the complete GLAST (EAAT1) knockout found changes consistent with behavioral endophenotypes associated with schizophrenia, including locomotor hyperactivity and abnormal social behavior (Karlsson et al. 2008, 2009). Several of these abnormal behavioral findings were reversed by administration of antipsychotic medication or mGluR2/3 receptor agonist administration, which decreases presynaptic glutamate release. These data suggest that there are region-specific deficits in EAAT reuptake capacity in schizophrenia, which could lead to glutamate spillover.

Chronic Glutamate Spillover May Lead to Remodeling of Synapses In the prefrontal cortex (PFC) in schizophrenia, there are changes in the structure, composition, and numbers of excitatory synapses (Broadbelt et al. 2002; Lewis et al. 2003, 2008). Increased packing density, decreased numbers of dendritic spines and diminished expression of structural proteins suggest significant alterations of synapses in this region (Selemon et al. 1995; Rajkowska et al. 1998, 2002). Several reports have found specific alterations in layers III and IV of the PFC, including abnormalities of pyramidal cells and interneurons (Hashimoto et al. 2003; Dong et al. 2005; Huang and Akbarian 2007; Huang et al. 2007; Lewis et al. 2008). One well-replicated finding is a decrease in parvalbumin-positive interneurons in the middle cortical layers

(Lewis et al. 2001; Hashimoto et al. 2003). A lamina-specific deficit in inhibitory tone could lead to increased release of glutamate, which ,combined with diminished reuptake capacity, could lead to increased glutamate spillover.

Accumulating evidence from postmortem gene expression studies suggests neurochemical alterations consistent with spillover. We have found increased mGluR2/3 protein in the PFC (Gupta et al. 2005), which may be interpreted as an attempt by synapses to decrease spillover by decreasing presynaptic release. Expression of the cystine/glutamate antiporter catalytic subunit (xCT) was also increased in the DLPFC (Baker et al. 2008). This molecule is expressed on glia and releases glutamate into the extrasynaptic space in exchange for uptake of cystine, which is required for glutathione synthesis. The effect of increased xCT protein expression on glutamate release is not known, because it is the activity, and not expression level, of this molecule that determines the rate of glutamate release. However, changes in the expression of xCT minimally suggest abnormalities of the regulation of extracellular glutamate levels (Baker et al. 2008). Finally, a number of studies have also described changes in ionotropic glutamate receptor binding site expression, suggesting a change in NMDA and AMPA receptor stoichiometry in the frontal cortex in schizophrenia (Akbarian et al. 1996; Healy et al. 1998; Ibrahim et al. 2000; Meador-Woodruff et al. 2001b; Beneyto and Meador-Woodruff 2006; Beneyto et al. 2007; Harney et al. 2008). Interestingly, preclinical studies have shown that glutamate spillover is associated with alterations in ionotropic receptor subunit composition and function (Lozovaya et al. 2004b; Harney et al. 2008).

We propose that there is remodeling of glutamate synapses in schizophrenia secondary to glutamate spillover (Fig. 16.2). Glutamate spillover may be secondary to increased release (in a misguided attempt to activate "sick" NMDA receptors), as well as deficits in glutamate reuptake capacity. In this setting, we would predict that perisynaptic localization of glutamate transporters on the plasma membrane of astrocytes is diminished, either as a primary deficit in transporter localization, as a compensation for increased extrasynaptic glutamate release, or both. Redistribution of glutamate transporters on astrocytes would lead or contribute to increased spillover, causing excitotoxicity and loss of input specificity. Further, we postulate that these deficits are initially relatively subtle but chronic in nature, leading to inappropriate remodeling of excitatory synapses, which do not function normally. This idea is supported by the phenotype of the GLAST/EAAT1 knockout mice, which have moderate cognitive and behavioral impairment, but no morbidity or mortality associated with seizures (Watase et al. 1998; Karlsson et al. 2008, 2009).

16.7 A Role for Glutamate Microdomains

Glutamate Microdomains Critical to cellular function of proteins is subcellular locality or microenvironment, in which proteins cluster and interact with numerous others. These biologically and morphologically discrete microdo-

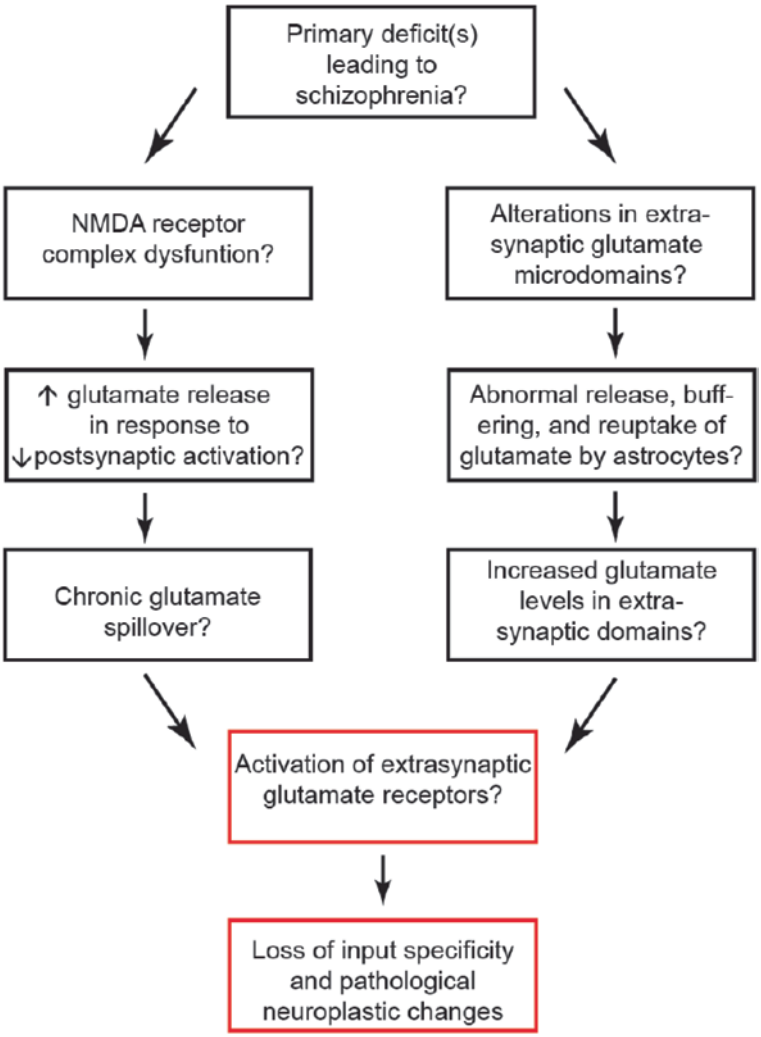

Fig. 16.2 There are likely a number of primary causes, which yield the constellations of symptoms found in schizophrenia. Proximate causes might include NMDA receptor dysfunction or abnormalities of glutamate microdomains. The *red boxes* may be a final common pathway that contributes to the phenotype of this syndrome

mains require tightly regulated trafficking of component proteins and thus organize the intermolecular environment for proteins and their interactions (Grosche et al. 1999; Hemler 2003; Füllekrug and Simons 2004; Tzingounis and Wadiche 2007; Farr et al. 2009; Newpher and Ehlers 2009). Evidence for cortical glutamate microdomains is based on several observations: (1) extracellular glutamate levels may vary between 0.2–7 μM in the extrasynaptic space in a region- and milieu-dependent manner; (2) a large body of work has described localization of

functional glutamate receptors outside of synapses; (3) the localization and buffering/transport properties of glutamate transporters strongly suggest partitioning of non-synaptic extracellular spaces; (4) several recent studies have characterized the functional coupling of protein complexes, structural proteins, organelles, and signaling pathways that converge on cellular processes involving glutamate; and finally, (5) glial cells may form electronically independent morphological structures that ensheath neuronal structures of unknown function (Grosche et al. 1999, 2002; Szabadkai and Rizzuto 2007; Spat et al. 2009; Genda et al. 2011). Taken together, these data suggest that glutamate microdomains are formed by specialized regions of astrocytic processes and neuronal membranes (Fig. 16.1) (Grosche et al. 1999; Genda et al. 2011). Glutamate levels within these domains may be determined by spillover from synapses or release from astrocytes (Fig. 16.1) (Lehre and Danbolt 1998).

The composition and function of specialized protein clusters in astrocytes has recently been investigated in rodent brain tissues. Using immunoisolation, one study found a complex comprised of GLT-1 (the rodent isoform of EAAT2), Na^+/K^+-ATPase, hexokinase, and mitochondria (Genda et al. 2011), while another found Na^+/K^+-ATPase, the water channel aquaporin 4, and mGluR5 (Fig. 16.2) (Illarionova et al. 2010). Other studies have found functional coupling of glutamate reuptake, cytosolic and mitochondrial sodium exchange, and glucose utilization in astrocytes (Hertz 2011; Skytt et al. 2012). Interacting partners and ultrastructural localization have not been determined for the cystine/glutamate antiporter.

Evidence for Abnormalities of Glutamate Microdomains in Schizophrenia: Structural Abnormalities In the frontal cortex in schizophrenia, there are changes in volume and cell density suggesting significant alterations in the spatial arrangement of synapses and microdomains in this illness (Selemon et al. 1995; Rajkowska et al. 1998, 2002; Broadbelt et al. 2002; Lewis et al. 2003; Lewis and Gonzalez-Burgos 2008). Specifically, there is thinning of cortical gray matter accompanied by decreased density of astrocytes as well as a loss of neuropil, while the balance of studies has typically found no changes in the number of neurons (Selemon et al. 1995; Rajkowska et al. 1998, 2002; Broadbelt et al. 2002; Lewis et al. 2003; Lewis and Gonzalez-Burgos 2008). These findings suggest a marked abnormality in the ultrastructural elements that account for the large volume of gray matter not occupied by cell bodies or synapses.

Abnormalities of Mitochondria A few studies have assessed the density of mitochondria in schizophrenia. One study found a decrease in the number of mitochondria per synapse in the striatum in treatment-responsive subjects with schizophrenia, while another found decreased volume fraction and area density of mitochondria in subjects with duration of disease more than 21 years (Uranova et al. 2001; Somerville et al. 2011, 2012). In addition, decreased expression of transcripts for a mitochondrial proton transporter were found in the frontal cortex in schizophrenia, and association of the glycolytic enzyme hexokinase 1 with mitochondria was decreased in the parietal cortex in this illness (Gigante et al. 2011; Regenold et al. 2012). These data suggest an abnormality of mitochondrial coupling in schizophrenia, which may

reflect an alteration in the association of mitochondria with proteins that comprise these domains.

Changes in Glutamate Receptor and Transporter Expression The changes in glutamate receptor and transporter expression detailed above are consistent with diminished glutamate reuptake capacity, indicating increased diffusion of glutamate between synapses and microdomains. In addition, changes in extrasynaptic expression of the cystine/glutamate antiporter (which releases glutamate from astrocytes) indicate an increased capacity for extrasynaptic release of glutamate (Kristiansen and Meador-Woodruff 2005; Baker et al. 2008). Both of these mechanisms could lead to increased activation of extrasynaptic glutamate receptors in extracellular glutamate microdomains.

Conclusions

We propose that there is increased activity of extrasynaptic glutamate receptors in schizophrenia secondary to increased levels of extrasynaptic glutamate (Fig. 16.2). Increased extrasynaptic glutamate may be due to increased presynaptic release and spillover (in a misguided attempt to activate sick NMDA receptors in the PSD), increased diffusion of glutamate out of the cleft secondary to deficits in glutamate buffering and reuptake capacity, and/or increased release of glutamate from astrocytes. In this setting, we would predict that integrity of glutamate microdomains is disrupted, either as a primary deficit in the assembly and localization of these domains, as a compensation for increased synaptic glutamate release and spillover, or both. Regardless of the mechanism, we hypothesize that the composition and localization of astrocytic proteins in glutamate microdomains are abnormal in schizophrenia, leading to pathological neuroplastic changes in the structure and function of glutamate circuits in schizophrenia.

Interestingly, several promising trials of various glutamate receptor modulators have failed to yield new pharmacological treatments for schizophrenia, including NMDA receptor glycine-site agonists (targeting NMDA receptors in the PSD), AMPA receptor modulators, called AMPAkines (targeting AMPA receptors in the PSD), and more recently mGluR2 modulators (targeting presynaptic release of glutamate). The challenges of developing effective glutamatergic drugs may be harder to surmount because the pathosphysiology of excitatory neurotransmission in schizophrenia has generally been viewed in a neuron-centric manner. Viewing abnormalities of glutamate transmission as a problem of astrocyte dysfunction may yield fresh insights for developing pharmacological targets to treat this devastating illness.

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Chapter 17
Emerging Roles for Glial Pathology in Addiction

Kathryn J. Reissner and Peter W. Kalivas

Abstract Addiction to drugs of abuse is a debilitating chronic disease, for which long-term treatment success rates are low. Because of the human and financial cost of addiction, considerable effort is dedicated toward understanding the processes that initiate and sustain substance abuse disorders. Among these processes, growing evidence indicates an integral contribution of glial cells, including astrocytes, oligodendrocytes, and microglia. For example, decreased white matter content and integrity in the prefrontal cortex is correlated with impaired prefrontal cognitive control, a cardinal feature of addiction pathology. In addition, decreased astroglial plasma membrane glutamate transport in the nucleus accumbens is a critical mediator of seeking for multiple classes of drugs of abuse. The following chapter introduces addiction neurocircuitry, and goes on to describe drug-dependent changes in glial cells that have been identified within this circuitry. Special emphasis is given to drug-induced impairments in the physiological relationships between neurons and glia. Lastly, evidence for the therapeutic potential of glial-specific targets is discussed.

Keywords Addiction · Astrocyte · Cocaine · Oligodendrocyte · Microglia · Dopamine · Glutamate

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17.1 Introduction: The Neurocircuitry and Cell Biology of Addiction

Despite the fact that glial cells outnumber neurons in the brain, they have historically been largely considered as a secondary source of trophic support to neurons (Barres 2008). Over the past several years, however, seminal roles for neuron-glial interactions in nervous system function have become increasingly clear. There is now extensive evidence for a functional relationship between neurons and glia in a wide variety of nervous system processes, including development, synaptic plasticity, injury response, vascular function, sleep, and others (Chung and Barres 2012; Parpura et al. 2012). As discussed in detail in this edition, there is also a considerable and growing body of data that indicate important roles for glial cells in pathological processes, representing a target of intervention for conditions among epilepsy, encephalopathy, cancer, neurodegenerative diseases, and psychiatric conditions (Parpura et al. 2012).

Among the psychiatric conditions in which glial dysfunction is implicated is addiction to drugs of abuse (Haydon et al. 2009; Cooper et al. 2012). Drug-induced changes in morphology, content, and function of glial cells have been shown at the levels of functional magnetic resonance imaging and molecular and cellular physiology in both human addicts and in preclinical animals models, and in response to drugs across chemical categories including psychostimulants, opiates, nicotine, and ethanol. In advance of an in depth discussion of the drug-induced changes in gliobiology, it is worthwhile to consider the neurocircuitry engaged by the rewarding and pathological actions of drugs abuse. This circuitry provides the framework of brain nuclei where drug-related adaptations have been observed.

Cellular effects of drugs of abuse have been thoroughly described in brain regions within reward centers of the brain, supporting the hypothesis that addiction is a disease of reward learning and associations (Hyman et al. 2006; Lalumiere and Kalivas 2007; Mameli and Luscher 2011). Acute reinforcing effects of many drugs of abuse are mediated by robust release of dopamine (DA) from the ventral tegmental area (VTA) onto structures of the mesocorticolimbic system, including prefrontal cortex (PFC), nucleus accumbens (NAc), dorsal striatum, amygdala, and hippocampus (17.1) (Di Chiara and Imperato 1988; Koob and Nestler 1997; Martinez and Narendran 2010). Similarly, DA release also occurs in association with natural rewards, and constitutes an important mechanism of association between salience and environment or adaptive behaviors. However, in contrast to drug-induced DA signaling, DA responsiveness declines following multiple exposures to natural rewards, and it is the repeated release of dopamine that commandeers the normally adaptive process of reward learning (Lalumiere and Kalivas 2007).

The intensity and mechanisms of DA release varies depending on the addictive drug, but nonetheless represents a common theme of reinforcement. Following multiple exposures, changes in cellular physiology, morphology, and gene expression patterns are observed within the DA-ergic axon terminal fields. Thus, the hyperdopaminergic state induced by chronic drug exposures leads to long-term changes in

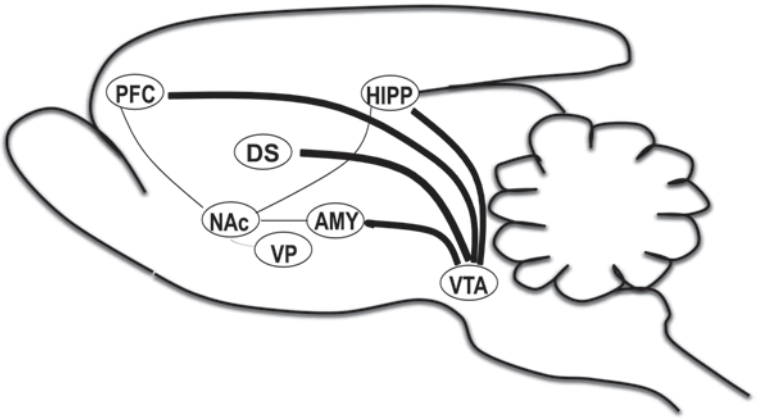

Fig. 17.1 Reward neurocircuitry engaged by chronic drug abuse. *AMY* amygdala, *DS* dorsal striatum, *HIPP* hippocampus, *NAc* nucleus accumbens, *PFC* prefrontal cortex, *VP* ventral pallidum, *VTA* ventral tegmental area. Dopaminergic projections from the VTA are depicted by a *heavy line*. Glutamatergic and γ-aminobutyric acid (GABA)ergic projections, the latter from the NAc to VP, are depicted by *thin lines*. (For additional information, see (Feltenstein and See 2008; Koob and Volkow 2010; McGinty et al. 2011; Reissner and Kalivas 2013))

the cells of the VTA and in those receiving innervation from the VTA, as well as in synaptic connections downstream, in particular in the glutamatergic projection from the PFC to the NAc (Fig. 17.1) (Kalivas et al. 2005; Mameli et al. 2009; Mameli and Luscher 2011). Thus, it is generally believed that understanding the long term cellular changes induced within the mesocorticolimbic reward pathways depicted in Fig. 17.1 by chronic exposure to drugs of abuse will not only inform understanding of the fundamental biology of adaptive and maladaptive reward learning, but will also illuminate candidate targets for pharmacotherapies for addiction.

A growing appreciation of enduring changes in cellular dynamics within the reward circuitry following chronic drug exposure has fueled the belief that cellular targets of addiction may lie within deficits in executing goal-directed behaviors (Nestler and Aghajanian 1997; Chen et al. 2010; Kalivas and Volkow 2011; Van den Oever et al. 2012). The appreciation has contributed to a growing shift in the understanding and treatment of addiction as a chronic disease based on cellular pathology following protracted drug abuse (Leshner 1997; Dackis and O'Brien 2005; Chandler et al. 2009; Courtwright 2012). This shift has in turn fueled study of the relationship between drug-dependent cellular adaptations and addiction-related behaviors. Accordingly, within the last 10 years, changes in glial content, structure, and function have been revealed. The following sections provide an overview of studies from human and preclinical animal models across levels of analysis from whole brain imaging to cell biology and electrophysiology.

17.2 Astroglial Expression of Receptors and Transporters

In considering the effects of drugs of abuse on glial cells, and on glial contributions to mechanisms of addiction, it is important to appreciate to what degree relevant classes of receptors and transporters are expressed on these cells within the brain reward circuitry. Because the relationship between astrocytes and neurons are the most thoroughly studied in this context, astrocytes will be the predominant glial cell type considered in this section. It has for some time been appreciated that receptors for many transmitter systems are expressed on astrocytes, including glutamate, γ-aminobutyric acid (GABA), serotonin, opioids, purines, and others (Porter and McCarthy 1997; Abbracchio et al. 2006; Shigetomi et al. 2012). More recently, receptor expression has also been demonstrated for endocannabinoid CB1 receptors, and stimulation of astroglial CB1 receptors by neuronal endocannabinoid release leads to synaptic potentiation via astroglial-derived glutamate transmission (Navarrete and Araque 2008, 2010). Of particular relevance to this chapter, recent evidence has accumulated showing expression of multiple types of DA receptors in a range of glial cell types in vivo, particularly astrocytes. For example, DA receptors of the subtypes D1, D3–5 (Miyazaki et al. 2004) and D2 (Khan et al. 2001; Shao et al. 2013) have been reported.

Astrocytes also express a wide range of plasma membrane transporters for amino acids, nucleosides, glucose, monocarboxylates, GABA, monoamines, and more (Morgello et al. 1995; Inazu et al. 2003; Pierre and Pellerin 2005; Nagasawa et al. 2007; Fuente-Martin et al. 2012; Kittel-Schneider et al. 2012). Although astroglial expression of DA transporters has been found in some cases (Takeda et al. 2002; Karakaya et al. 2007), but not others (Dahlin et al. 2007; Kittel-Schneider et al. 2012), functional uptake of both DA and serotonin in astrocytes has been reported (Kimelberg and Katz 1985; Hirst et al. 1998; Inazu et al. 1999). Of relevance to this chapter, expression of glutamate transporters on astrocytes has been demonstrated to be of particular importance for protection against excitotoxicity and in maintaining fidelity in synaptic communication. Greater than 90% of glutamate uptake occurs via high affinity glutamate transporters expressed on astrocytes (Anderson and Swanson 2000; Danbolt 2001). A deficit in astroglial glutamate uptake has been widely reported following exposure to multiple drugs of abuse, and is discussed in greater detail below.

Functionality of astroglial G protein-linked receptors has also been well described (Bradley and Challiss 2012). Considerable evidence both *in vitro* and *in vivo* indicates that neurotransmitters released from neurons (as well as neuromodulators and hormones) can stimulate astroglial receptors, leading to a Ca^{2+} signal which triggers release of gliotransmitters and in turn evoked Ca^{2+} signals in neurons (Bezzi and Volterra 2001; Agulhon et al. 2008; Lu et al. 2009; Zorec et al. 2012), as well as local and long-distance Ca^{2+} waves that are propagated through networks of astrocytes via gap junctions (Goldberg et al. 2010). *In vivo*, sensory stimulation to a mouse whisker leads to an astroglial Ca^{2+} response that is dependent upon metabotropic glutamate receptors (mGluRs) (Wang et al. 2006; Halassa and Haydon 2010).

Signaling from neurons to astrocytes also leads to reciprocal modulation of synaptic and neuronal function by astrocytes themselves (Lu et al. 2009). This bidirectional nature of communication between neurons and astrocytes and its role in shaping synaptic function has led to the concept of the tripartite synapse (Araque et al. 1999), which is further supported by electron microscopy and reconstruction studies illustrating the intimate and intricate physical relationship between neurons and astrocytes (Genoud et al. 2006; Witcher et al. 2007; Belanger et al. 2011).

Because changes in synaptic strength and signaling within the reward neurocircuitry have been heavily implicated in the cellular pathophysiology of addiction, in particular to cocaine (Stuber et al. 2010; Luscher and Malenka 2011), the morphological and functional relationships between neurons and astrocytes indicate the potential for involvement of astrocytes in mechanisms of drug dependence. Accordingly, the following sections will in more detail discuss evidence for drug-dependent changes in glial cell biology in both human addicts and animal models of addiction, with consideration for how these changes relate to changes in synaptic function.

17.3 Effects of Drug Administration on White Matter Volume and Glial Content

Human brain imaging studies have revealed a great deal of information about neural differences between drug-dependent individuals and healthy, abstinent controls, particularly within regions of the PFC (Goldstein and Volkow 2011). Brain matter is largely characterized as white and gray matter; white matter is primarily comprised of glial cells and myelinated axons, in contrast to neuronal cell bodies, which are the major constituent of gray matter (Fowler et al. 2007). Many studies have documented deficits in both volume of both white and gray matter, as well as in metabolic activity in the PFC, of human drug abusers (for review see (Fowler et al. 2007)).

For example, a number of studies have revealed significant differences in white matter content between drug addicts and healthy controls (Schlaepfer et al. 2006). Further, while frontal and temporal white matter volume in humans continues to increase until the mid-40s, compared to normal control subjects, cocaine dependent subjects do not exhibit an age-dependent increase in white matter volume (Bartzokis et al. 2002). Relatedly, deficits in frontal white matter integrity also appear with chronic drug abuse (Lim et al. 2002, 2008). Integrity is a measure revealed by techniques including diffusion tensor imaging, which quantifies directional diffusion of molecules along a fiber tract, providing an indicator of fiber structure and connectivity (Le Bihan et al. 2001). White matter integrity across the brain is significantly correlated with treatment outcome for cocaine dependence (Xu et al. 2010); relatedly, frontal lobe white matter integrity at the start of treatment is a predictor of alcohol relapse 6 months later (Sorg et al. 2012). Collectively, these studies indicate that structural deficits in white matter, composed largely of glia, correlate with cortical structural deficits in addicted individuals.

Other studies have reported decreased gray matter measured in a variety of brain regions within cocaine-dependent subjects (Bartzokis et al. 2000; Franklin et al. 2002; Sim et al. 2007). Prolonged cocaine abuse has been postulated to represent an accelerated aging process with respect to reduction in gray matter volume (Bartzokis et al. 2000; Ersche et al. 2013). Indeed, the rate of loss of cortical and subcortical gray matter volume in cocaine-dependent subjects is almost twice that of healthy age-matched subjects (Ersche et al. 2013) and accelerated age-dependent loss of gray and white matter has also been reported in chronic alcoholics (Pfefferbaum et al. 1992).

What is the implication of a prefrontal white matter deficit? Deficient prefrontal cognitive control is a hallmark feature of addiction (Goldstein and Volkow 2002; Kalivas and Volkow 2005; George and Koob 2010). Impairments of this nature are correlated with increased measures of impulsivity and loss of executive control; for example, decreased frontal and parietal cortex white matter integrity of cocaine-dependent subjects correlates with impaired performs on the Iowa Gambling Task, suggesting that white matter integrity may well be functionally related to functional impairments in decision making (Lane et al. 2010). However, it is difficult using human studies to dissociate correlation with causation in the relationship between white matter content and integrity with impaired executive function. Furthermore, in all human imaging studies, changes identified between drug-dependent and healthy controls cannot be differentiated between drug-related changes and pre-existing difference which might serve a correlative or causative relationship. As such, preclinical animal models serve a valuable function to allow comparison between naïve baseline and changes which develop with drug experience. Moreover, human studies are confounded by complexity of single drug versus polydrug abusers, length and severity of drug abuse, and other environmental factors and individual differences. These confounds underscore the importance of animal models in understanding the cellular mechanisms responsible for addiction-related behaviors.

In a more limited scope of studies, these principles have been recapitulated in preclinical animal models. For example, diffusion tensor imaging of chronic cocaine treated rats has revealed a significant decrease in white matter integrity of the corpus callosum compared to saline controls; a decrease in myelin-associated protein in the same region was also observed (Narayana et al. 2009). In a separate study, rats trained to self-administer cocaine demonstrated impairment in a working memory task, which was correlated with decreased counts of both neurons and oligodendrocytes in the PFC (George et al. 2008). Moreover, drug seeking by rats trained to self-administer cocaine who choose drug seeking despite an adverse consequence (foot shock) was reversed by stimulation of prefrontal cortical activity (Chen et al. 2013), providing an analog of the hypofrontality observed in human addicts. Future studies will be necessary to further ascertain the relationship between drug abuse, white matter content, and compulsive drug seeking. If then, chronic drug use leads to deficiencies in white and/or gray matter, it becomes important to understand the cellular mechanism(s) by which this may occur, and whether targeting these mechanisms may represent a means of treatment intervention for addiction.

17.4 Effects of Drug Administration on Properties of Astrocytes

17.4.1 Astroglial Inflammation

While functional imaging studies provide important insight into changes at the level of circuitry and anatomy, changes in expression and function of specific proteins provides additional information on glial consequences of drug exposure. A number of studies in preclinical animal models of drug abuse and addiction have demonstrated increased measures for astrocyte activation. For example, increased glial fibrillary acidic protein (GFAP) expression within the reward neurocircuitry has been observed following exposure to cocaine (Fattore et al. 2002; Bowers and Kalivas 2003), methamphetamine (Guilarte et al. 2003; Friend and Keefe 2013), morphine (Beitner-Johnson et al. 1993; Song and Zhao 2001), and alcohol (Fletcher and Shain 1993; Goodlett et al. 1993; Franke 1995; Tagliaferro et al. 2002; Vongvatcharanon et al. 2010). Increased GFAP expression is a hallmark feature of inflammation (or activation) of astrocytes (Pekny and Nilsson 2005; Sofroniew 2009; Sofroniew and Vinters 2010) and hence may reflect enduring cellular inflammation initiated by drug exposure. In some cases, however, long-term alcohol exposure has also been associated with a decline in GFAP expression and astrocyte complexity (Franke 1995; Rintala et al. 2001). Detailed histological analysis on postmortem alcoholic human brains have revealed enlarged cell bodies but also patch-like losses in GFAP-positive cells (Cullen and Halliday 1994; Miguel-Hidalgo 2005), indicating a complexity of cellular effects. Nonetheless, many studies have revealed that exposure to drugs of abuse leads to glial inflammation and activation of innate immune response (for reviews see (Watkins et al. 2005, 2009; Crews et al. 2011)).

17.4.2 Blood-Brain Barrier Integrity

Psychostimulant abuse has also been well documented to lead to breakdown of the blood brain barrier (BBB)(Kousik et al. 2012). The BBB is a physical barrier to the central nervous system (CNS) formed by brain endothelial cells interacting with pericytes, basement membrane of the vasculature, and astrocytes (Banerjee and Bhat 2007; Abbott et al. 2010; Krueger and Bechmann 2010; Kousik et al. 2012). The tight association of astrocyte end-feet with vasculature is a critical component, and astroglial inflammation can lead to perturbation of BBB integrity. Permeability of the BBB for any reason leads to vulnerability to CNS exposure to a variety of chemical and biological insults, from which it is normally protected. A good example is the fact that mounting evidence indicates increased vulnerability of the nervous system to HIV infection in psychostimulant abusers (Rippeth et al. 2004; Buch et al. 2012). HIV infection within the brain in turn leads to an increased incidence of neuro-AIDS, a condition characterized by generalized neurologic deficits secondary to HIV infection (Buch et al. 2012; Hauser et al. 2012). The abuse of

psychstimulants such as cocaine and methamphetamine leads to glial inflammation and permeability of the BBB, enhanced vulnerability to HIV infection and other opportunistic viruses and pathogens.

17.4.3 Astroglial Glutamate Transport: A Critical Mediator of Drug Seeking

Among the most widely reported astroglial changes following self-administration of drugs of abuse is downregulation of the high affinity glutamate transporter EAAT2/GLT-1 (Reissner and Kalivas 2010). Downregulation of GLT-1 is frequently associated with activated astrocytes, particularly following injury (Binns et al. 2005; Pekny and Nilsson 2005; Cata et al. 2006; Tawfik et al. 2008). GLT-1 is downregulated in the NAc following self-administration and withdrawal from cocaine (Knackstedt et al. 2010; Fischer-Smith et al. 2012), nicotine (Gipson et al. 2013), and heroin (Shen et al. personal communication). Results following alcohol exposure are more complex; however, in one study 24 h after 7 days alcohol exposure, higher basal extracellular glutamate levels and corresponding decrease in glutamate uptake was reported in the NAc (Melendez et al. 2005), which is consistent with other studies reporting chronic increases in basal extracellular glutamate levels (Mann et al. 2008). Moreover, chronic self-administration in alcohol-preferring rats results in decreased GLT-1 protein levels in the NAc; treatment with ceftriaxone reversed this decreased, as well as measures of alcohol seeking in alcohol-preferring rats (Qrunfleh et al. 2013; Sari et al. 2013).

GLT-1/EAAT2 is a member of the EAAT family of high affinity glutamate transporters (Danbolt 2001), and rapid glutamate uptake is important for the tight regulation of synaptic glutamate concentrations and the prevention of synaptic excitotoxicity (Danbolt 2001; Jiang and Amara 2011). In addition, impaired clearance of glutamate from the synaptic cleft can impact decay currents of ionotropic glutamate receptors, indicating that at sub-excitotoxic levels, changes in glutamate concentrations resulting from altered transporter function can shape neuronal currents (Tzingounis and Wadiche 2007). Importantly, restored expression of GLT-1 is associated with decreased measures of cocaine seeking (Baker et al. 2003; Knackstedt et al. 2010; Reissner et al. 2014).

Interestingly, besides following drug abuse, EAAT2/GLT-1 levels and transporter-dependent glutamate uptake are also decreased in aged rodent brains (Wheeler and Ondo 1986; Najlerahim et al. 1990; Saransaari and Oja 1995; Vatassery et al. 1998; Potier et al. 2010) as well as in aged brains in a transgenic mouse model of Huntington's Disease (Behrens et al. 2002) and in postmortem brains from Alzheimer's Disease (AD) patients (Masliah et al. 1996; Jacob et al. 2007; Simpson et al. 2010; Woltjer et al. 2010). Moreover, heterozygous-deficient GLT-1 (+/−) mice crossed with a double transgenic AD model mouse demonstrated an earlier onset of memory deficits compared with the AD model mouse with a wild-type GLT-1 genotype (Mookherjee et al. 2011). These findings collectively indicate that

impaired GLT-1 expression and function may contribute to age-dependent synaptic and cognitive impairment, and is supportive of the idea that cocaine-dependent changes may accelerate the aging process (Ersche et al. 2013).

While synaptic glutamate levels are predominantly controlled by GLT-1, basal extracellular glutamate levels are correspondingly largely controlled by the action of the cystine-glutamate exchanger, system xc^- (Baker et al. 2002a, b). System xc− is composed of a small, catalytic subunit, xCT, and a large subunit, NF2 (Lim and Donaldson 2011; Lewerenz et al. 2013). While some neuronal expression of system xc^- has been reported, it is predominantly astroglial. Hence, extracellular glutamate levels are controlled by release of glutamate via system xc−, and uptake by EAATs (largely GLT-1 in adult rodents). Interestingly, while expression of both xCT and GLT-1 are decreased following cocaine self-administration and extinction training (Knackstedt et al. 2010), net basal extracellular glutamate levels are decreased, indicating that expression of xc^- may be the more dominant toward controlling basal extracellular levels measured via microdialysis (Baker et al. 2003). This has led to a model in which basal levels are decreased by suppressed xCT, but synaptic levels are increased following glutamate release, due to the compromised synaptic uptake cause by impaired GLT-1 function and expression (Fig. 17.2) (McFarland et al. 2003).

A consequent dysregulation of synaptic and extrasynaptic glutamate levels following suppression of xCT and GLT-1 has considerable consequences on cellular function (Kalivas 2009). Decreased basal levels of extracellular glutamate (due to decreased xCT) lead to decreased tone on presynaptic inhibitory mGluR2/3 receptors, which results in increased glutamatergic transmission. Activity of glutamatergic PFC innervation of medium spiny neurons of the NAc is a driving force of reinstatement to numerous drugs of abuse, and pharmacological agonism of these presynaptic mGluR2/3 receptors (or antagonism of postsynaptic excitatory glutamate receptors) can block reinstatement (Moussawi and Kalivas 2010; Reissner and Kalivas 2010). Hence, the decreased expression of xCT and GLT-1 leads to a combinatorial effect on the cellular controls designed to regulate synaptic input to the NAc.

Extracellular glutamate levels appear not only to mediate tone on neuronal receptors, but can also stimulate Ca^{2+} oscillations in astrocytes via stimulation of mGluR receptors, as discussed in the preceding section. This Ca^{2+}-dependent glutamate transmission from astrocytes leads to N-methyl D-aspartate receptors (NMDARs)-mediated slow inward currents (SICs) on medium spiny neurons in the NAc (D'Ascenzo et al. 2007). What is the behavioral consequence of changes in glutamate transmission from astrocytes? One clue comes from transgenic mice engineered to express a dominant negative vesicle protein in astrocytes; these mice demonstrate impaired cocaine-induced reinstatement of conditioned place preference, as well as cue-induced reinstatement in a self-administration paradigm (Turner et al. 2013). These findings are consistent with a model in which glutamatergic gliotransmission may mediate the cellular pathology of addiction. However, more studies will be required to fully understand the consequences of impairments of gliotransmission following drug abuse.

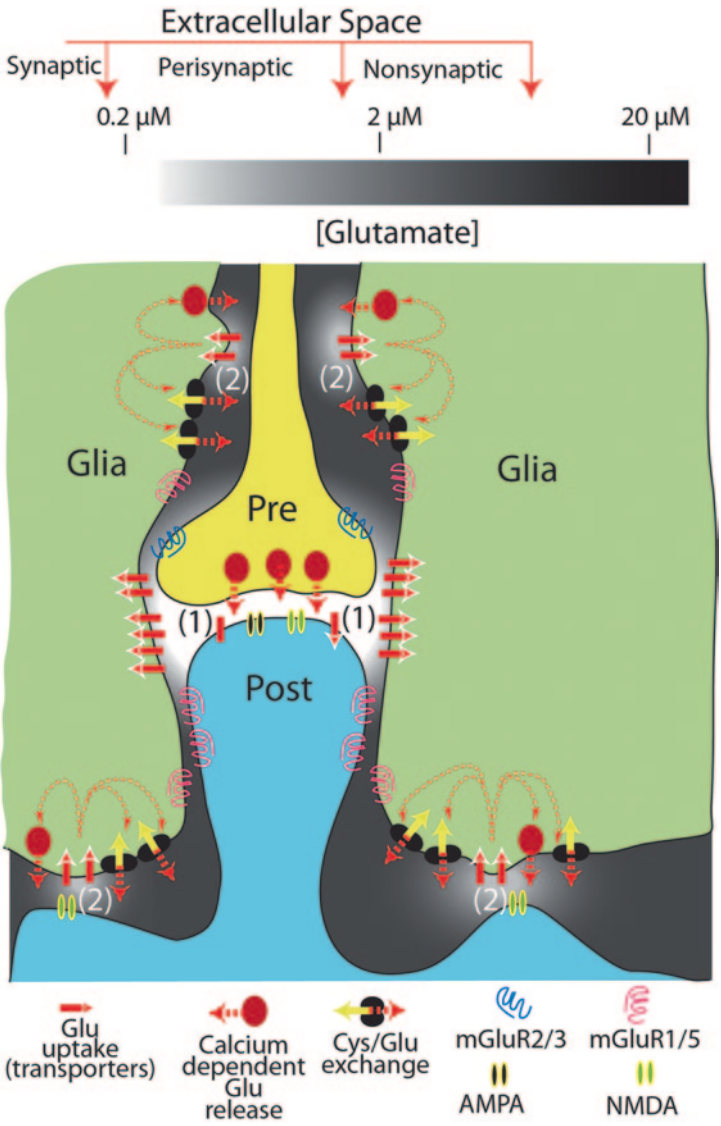

Fig. 17.2 Cystine/glutamate exchange and glutamate uptake regulate synaptic and extrasynaptic glutamate levels. Both the glutamate transporter GLT-1 and glutamate exchanger catalytic subunit xCT are reduced following chronic self-administration and extinction. Ca^{2+}-dependent glutamate release from astrocytes is also documented, although cystine/glutamate (Cys/Glu)exchange is the primary source (Baker et al. 2002a; Haydon et al. 2009). Synaptic glutamate levels (*1*) are tightly controlled within the sub-micromolar range at rest (during synaptic transmission glutamate can raise to ~1.1 mM), whereas extrasynaptic levels (*2*) can approach the 20 µM range. Reduced expression of xCT will lead to decreased basal extrasynaptic levels, and hence reduced tone of presynaptic inhibitory mGluR2/3 receptors. This leads to increased glutamate release (and hence, synaptic levels) within the NAc during cocaine seeking, which is exacerbated by reduced GLT-1 expression. Inotropic glutamate, AMPA (α-amino-3-hydroxy-5-methyl-isoxazole propionate) and NMDA, receptors are shown. (Figure reproduced with permission from (Moussawi et al. 2011))

17.5 Beyond Astrocytes: Effects of Drug Administration on Oligodendrocytes and Microglia

In addition to cellular adaptations within astrocytes, drug-dependent changes in oligodendrocytes and microglia have been reported. Withdrawal from chronic cocaine administration is associated with decreased expression of white matter proteins in the NAc including myelin basic protein, proteolipid protein (PLP), myelin oligodendrocyte glycoprotein, and myelin associated glycoprotein, which is prevented by treatment with the β-lactam antibiotic ceftriaxone (Kovalevich et al. 2012). As mentioned above, ceftriaxone is a known inducer of GLT-1 expression (Rothstein et al. 2005), and is effective at preventing reinstatement of cocaine (Sari et al. 2009; Knackstedt et al. 2010; Fischer et al. 2013). Interestingly, postmortem gene expression profiling in NAc tissue from human addicts has revealed a decrease in mRNA coding for myelin-associated proteins, including myelin basic protein and PLP1 (Albertson et al. 2004; Bannon et al. 2005). In a separate study, a decrease in PLP1 was confirmed by in situ hybridization in the ventral caudate, putamen, and internal capsule of postmortem tissue from human cocaine abusers (Kristiansen et al. 2009).

As stated in the preceding section, it is well documented that activation of glial cells occurs in response to opioid exposure. However, in contrast to the analgesic effects of opioids that are mediated through opioid receptors, activation of glial cells appears to occur via toll-like receptors (TLRs) and mediate nonanalgesic effects of opioids (Hutchinson et al. 2011). TLRs are microbe receptors expressed on immune cells, named for homology to the *Drosophila* Toll protein (Anderson et al. 1985; Lemaitre et al. 1996). The signaling of TLRs is a critical component in activating an immune response (Hanke and Kielian 2011). Within the CNS, TLRs are expressed predominantly on microglia, but are found on activated astrocytes as well, and on oligodendrocytes and neurons to a lesser degree (Hanke and Kielian 2011). TLR4 serves as the receptor for lipopolysaccharide, which has allowed for elucidation of a complex downstream signaling cascade leading to increased transcriptional regulation by nuclear factor kappa-light-chain-enhancer of activated B cells (Kawai and Akira 2006). Activation of the TLR4 receptor in response to opioid exposure *in vivo* opposes the analgesic effects and is responsible for opioid-induced hyperalgesia, or hypersensitivity to pain that often occurs after long term opioid use and abuse and in fact oppose the analgesic effects mediated through opioid receptor (Ossipov et al. 2005; Buchanan et al. 2010). Effects of opioid versus TLR actions can be dissociated using (−) and (+) stereoisomers of opioid agonists and antagonists; while TLRs are nonstereoselective, opioid receptors only bind to (−)-isomers. Genetic deletion or pharmacological inhibition of TLR4 receptors with (+)-naloxone impair opioid-induced CPP and reduce opioid self-administration (Hutchinson et al. 2012).

17.6 Modulation of Neuronal Cell Biology by Glial-derived Factors

17.6.1 GDNF, A Negative Regulator of Drug Reward

In the preceding section, we introduced the concept of glutamatergic gliotransmission, and how the regulation of glutamate release and uptake by astrocytes may contribute to disruptions in glutamate homeostasis and drug-induced cellular pathologies (Cali et al. 2009). Importantly, glial cells and in particular astrocytes secrete many proteins and molecules in addition to glutamate which may also be adaptive or inflammatory in response to a protracted drug history. For example, glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor-βfamily of growth factors that, despite its glio-centric name, is expressed in a variety of cell types including astrocytes, microglia, and neurons (Schaar et al. 1993; Pochon et al. 1997). GDNF is predominantly expressed within the basal forebrain, substantia nigra, and striatum, and was initially isolated on the basis of its ability as a component of astrocytes to selectively protect DA neurons of the substantia nigra in culture (O'Malley et al. 1992; Schaar et al. 1993; Duarte et al. 2012). A number of studies collectively indicate that GDNF action opposes drug taking, as well as the effects of chronic exposure on seeking of multiple drugs of abuse (Pierce and Bari 2001; Carnicella and Ron 2009; Ghitza et al. 2010). However, manipulation of GDNF expression and activity in the VTA promotes the incubation of cocaine craving (Lu et al. 2009) but not heroin craving (Airavaara et al. 2011), indicating the different roles may exist in difference brain regions and at different times following drug exposure.

17.6.2 Gliotransmitters: Beyond Glutamate

D-serine is a naturally occurring amino acid transmitter mainly released by astrocytes (and neurons in some brain regions), and is a co-agonist for NMDA receptors (Ben Achour and Pascual 2012; Radzishevsky et al. 2013). Stimulation of NMDARs by D-serine is necessary for hippocampal NMDA-dependent long-term potentiation. Relatedly, D-cycloserine (DCS) is a broad spectrum antibiotic and partial agonist at the glycine site of NMDA receptors, with functions as a cognitive enhancer and anticonvulsant (Ohno and Watanabe 1996; Wlaz et al. 1996; Myers and Carlezon 2012), and is under investigation as a treatment for substance use disorder (Olive et al. 2012).

A growing body of evidence suggests that administration of either D-serine or DCS may reduce drug seeking. Reduced D-Serine levels are observed in the NAc following cocaine administration (Curcio et al. 2013). Further, perfusion of D-Serine in the bath of NAc core slice electrophysiology restored the ability to induce long-term potentiation and depression. Relatedly, microinjection of D-Serine in the NAc blocked behavioral sensitization to cocaine. D-Serine administered

systemically prior to or immediately after extinction training sessions significantly reduces the reinstatement of cocaine seeking (Kelamangalath and Wagner 2010). Similarly, systemic administration of D-serine or DCS blocks expression of cocaine conditioned place preference (CPP) (Yang et al. 2013). DCS facilitates extinction of cocaine CPP (Botreau et al. 2006). Moreover, the effect of DCS can be long-lasting and reduce reinstatement of the CPP behavior (Paolone et al. 2009; Thanos et al. 2009). A Phase 2, "D-Serine for Cocaine Dependence Pilot" clinical study is currently underway (http://www.clinical trials.gov NCT01715051).

Besides glutamate and D-Serine, ATP is an additional gliotransmitter released by astrocytes. Extracellular adenosine is largely derived from astroglial ATP, acting on neuronal adenosine receptors. Evidence exists for a role of adenosine receptors, in particular A2A receptors, in the rewarding effects of drugs of abuse, and in reinstatement to drug seeking (Soria et al. 2006; Brown et al. 2009; O'Neill et al. 2012).

17.7 Glial Cells: An Emerging Pharmacotherapeutic Target for Addiction

The study described above illustrates the potential for astrocyte-derived D-serine as pharmacotherapy for substance abuse disorder. D-serine is one of a number of examples of therapies based in glial cell biology, and adaptions in neuron-glial signaling (Cooper et al. 2012). For example, as stated previously, at least three compounds known to induce expression of GLT-1 are also under investigation as addiction therapies. N-acetylcysteine, ceftriaxone, and propentofylline are all compounds of separate classes which upregulate GLT-1, and suppress reinstatement to cocaine(Baker et al. 2003; Sari et al. 2009; Knackstedt et al. 2010; Reissner et al. 2014). Human clinical trials indicate safety and efficacy of N-acetylcysteine against drug use for nicotine, cocaine, and cannabis (LaRowe et al. 2007, 2013; Knackstedt et al. 2009; Gray et al. 2012; Berk et al. 2013). Further, the restored expression of GLT-1 is necessary in order for propentofylline to effectively block reinstatement to cocaine (Reissner et al. 2014). Thus, while the field of gliobiology in drug abuse is in a relatively early stage, credible evidence already exists to support the hypothesis that targeting glial physiology affects neuronal cell biology and drug craving.

Conclusions and Perspectives

There is no question that both acute and chronic exposure to drugs of abuse leads to changes in astrocytes, oligodendrocytes, and microglia, within regions heavily implicated in reward neurocircuitry. However, our understanding of the effects of drug-dependent changes in glial structure and activity is at an early stage. Important questions remain to be addressed, such as: what are the relationships between changes in expression of myelin-related genes, deficits in white matter volume and

integrity, and compulsive drug seeking? What are the relationships between glial inflammation and changes in white matter content, or between drug-dependent changes in astrocyte physiology and neuronal synaptic communication? These questions will be central toward future progress in understanding and treating addiction as a brain disease.

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Chapter 18
Astroglial Connexins as Elements of Sleep-Wake Cycle Regulation and Dysfunction

Xinhe Liu and Christian Giaume

Abstract Sleep is fundamental for survival of animals and humans. Minor perturbations in sleep duration or timing can lead to cognitive impairments. Patients with sleep disorders suffer from high health risks and poor quality of life. Mechanisms of sleep function and their dysfunction are still far from being understood and concepts based on novel hypotheses are required. Discovery of dynamic neuronal-glial interaction has offered the opportunity to perceive sleep-wake cycle from another angle. Here, we summarize data indicating how astrocytes can contribute to the regulation of sleep, with a focus on the roles of astrocytes in adenosine homeostasis and metabolism. Also, we discuss evidence indicating that several treatments known to affect sleep-wake cycle have an impact on connexin-mediated astroglial networking. We further hypothesize that sleep homeostasis will be affected by pharmacological or genetic modifications of connexin channel functions in astrocytes. Taken together, accumulating knowledge on astrocyte functions in sleep-wake cycle implicates the glial cells as promising new therapeutic targets for sleep disorders treatment.

Keywords Sleep cycle · Sleep-wake cycle · Sleep disorders · Connexin · Astrocytes · NREM · SWS · REM · EEG · Astrocytic adenosine · Homeostatic · Brain function · Neuronal function · Circadian regulation · Sleep factors

18.1 Introduction

Sleep is of fundamental importance for animal and human survival. Indeed, even a minor restriction in total sleep time can lead to significant cognitive impairments. Sleep disorders are highly prevailing in modern society and represent a heavy burden on individual life and social economy. It is estimated that at least 10 % of the population is affected (Ram et al. 2010). Among the most prevalent sleep disorders are insomnia, narcolepsy, obstructive sleep apnea syndrome and restless legs syndrome (DeMartinis et al. 2009; for a detailed classification of sleep disorders, see

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Voderholzer and Guilleminault 2012). Taking as an example, insomnia is a sleep disorder characterized by an inability to obtain adequate amounts or quality of sleep that has a negative impact at both individual and societal levels. For instance, most of the studies find a higher rate of work accidents in insomniacs with severe consequences in direct and indirect costs. It was demonstrated that a long working week, exposure to vibrations, and "having to hurry" are the main risk factors for sleep disturbances (Ribet and Derriennic 1999). Also, shift work and night work are probably the major precipitating factors of insomnia at the workplace (Metlaine et al. 2005). Sleep disturbances are highly prevalent in neurodegenerative and psychiatric disorders where they worsen the quality of life and possibly the clinical development (Dauvilliers 2007; Wulff et al. 2011). Finally, sleep restriction has recently been identified as an important risk factor for metabolic disorders, such as diabetes and obesity (Spiegel et al. 2009).

Intensive investigations into the mechanism controlling sleep have been performed over the last decades. However, sleep functions, as well as mechanisms of its impairment, are still not fully understood and new concepts based on novel hypotheses are required. The evidence of dynamic interaction between neurons and glial cells, obtained during the last two decades, offers the possibility to address the understanding of sleep-wake cycle regulation under a new angle.

18.2 From the Neurocentric to the Neuroglial View of Sleep-Wake Cycle

Sleep is a behavioral state characterized by reduced movement and responsiveness to sensory stimuli. Using the electroencephalography (EEG) and electromyography (EMG) recordings, sleep is broadly divided into two stages: non-rapid eye movement (NREM) sleep or slow wave sleep (SWS) sleep, characterized by low frequency/high amplitude EEG activity and reduced muscle tone, and rapid eye movement (REM) sleep, characterized by high frequency/low amplitude EEG activity and a complete loss of muscle tone. Until recently, most of the studies explained sleep-wake physiology based solely on neuronal function, as in the research on regulation of slow oscillations in NREM sleep. NREM sleep is characterized by global cortical oscillations of synchronized neuronal activity (Steriade 2006; Steriade et al. 1993). Major components of this activity are slow oscillations (< 1 Hz), which can also be observed under some forms of anesthesia. The slow oscillation is a fundamental network phenomenon that organizes other sleep rhythms (Steriade 2006) and it has been suggested to play a role in sleep-dependent memory consolidation (Huber et al. 2004; Marshall et al. 2006). Importantly, this slow rhythm activity is enhanced by sleep deprivation (SD) and has been proposed to reflect the homeostatic regulation of sleep in both rodents and humans (Borbely et al. 1981, 1984). Slow oscillations have been proposed to arise from (i) the intrinsic bistability of certain neurons (Amzica and Steriade 1995; Cunningham et al. 2006), (ii) the interaction between local excitatory and inhibitory networks (Shu et al. 2003), and (iii) the action of neuromodulators (Compte et al. 2003; Steriade et al. 1993).

For the last few decades, a growing amount of research on astrocytes has led to the recognition that these glial cells are indispensable participants in synaptic transmission, brain metabolism and homeostasis, and that many brain functions result from dynamic interaction between neurons and astrocytes (Haydon and Carmignoto 2006; Theodosis et al. 2008; Verkhratsky 2010). Astrocytes are tightly associated with pre- and post-synaptic elements (Halassa et al. 2007; Ventura and Harris 1999). In rodents, an astrocyte domain is estimated to cover 20,000–120,000 synapses, while this number reaches ~2 million in humans (Oberheim et al. 2009). Astrocytes modulate neuronal activity through the release of active molecules, termed "gliotransmitters", such as ATP, glutamate and D-serine (Halassa and Haydon 2010; Newman 2003; Oliet and Mothet 2009; Perea and Araque 2010). For instance, glia-derived D-serine acts as a co-agonist of the N-methyl-D-aspartate (NMDA) glutamate receptor and controls synaptic plasticity (Panatier et al. 2006), whereas astroglia-released ATP accumulates as adenosine in the extracellular space and suppresses synaptic transmission through activation of neuronal A1 receptors (Pascual et al. 2005). More recently, Halassa and Haydon have provided direct evidence for the involvement of neuroglial interactions in sleep modulation (Halassa et al. 2009). However, apart from the single cell level, at the so called "tripartite synapse" (Araque et al. 1999; Perea et al. 2009), neuroglial interactions should also be considered at a more integrated level between neuronal circuits and astroglial networks (Giaume et al. 2010). This is due to the fact that astrocytes are also organized into communicating networks *via* gap junction channels formed by connexins (Cxs), similar to neuronal networks formed *via* synapses (Giaume et al. 2010). In the following of the chapter, we will present evidence from latest advances in sleep-wake physiology research on how astrocytes could be involved in sleep regulation and how their malfunctions could contribute to sleep pathology, thus representing novel treatment targets.

18.3 Astrocytic Adenosine Contributes to Sleep-Wake Cycle Regulation

18.3.1 Adenosine and Sleep Homeostasis

There are two modes of regulation of sleep: circadian regulation, which controls the timing of sleep during the day, and which will not be discussed here, and homeostatic regulation, which controls the depth and amount of sleep depending on the duration of prior wakefulness (Borbely 1982). The SWS of NREM sleep, as a marker of sleep pressure, is highest during the initial hours of natural sleep and is further increased by SD (Dijk et al. 1990). The mechanism underlying homeostatic regulation is largely attributed to action of sleep factors. A sleep factor accumulates in the brain proportionally to prior waking time and decreases with sleep. Also the sleep factor is expected to induce sleep upon administration. One of the most studied sleep factors is adenosine. *In vivo* measurements in cats and other species have

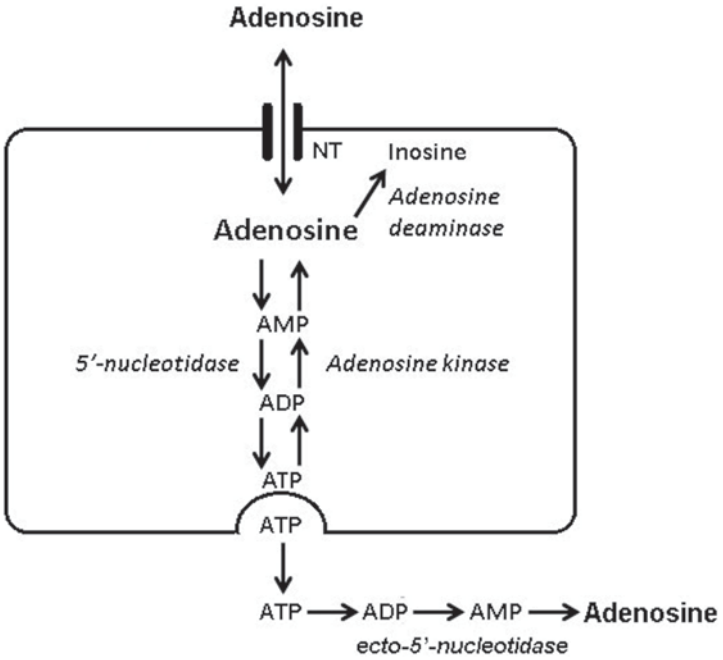

Fig. 18.1 Metabolic pathway of adenosine. See text for details; *NT*, nucleoside transporter

shown that extracellular concentration of adenosine is higher during spontaneous wakefulness than during sleep in various brain regions involved sleep-wake controls (Porkka-Heiskanen et al. 2000). Depending on the brain region, adenosine acts on different adenosine receptors, predominately A1 and A2a receptors (A1R, A2aR), to regulate sleep. Generally, adenosine induces sleep by inhibiting wakefulness-promoting neurons or/and stimulating sleep-promoting neurons *via* A1R or/and A2aR (Bjorness and Greene 2009; Porkka-Heiskanen and Kalinchuk 2011). In contrast to spontaneous sleep, adenosine has been found to steadily increase during SD only in the basal forebrain and the cortex (Porkka-Heiskanen et al. 2000; Basheer et al. 1999; Wigren et al. 2007; Murillo-Rodriguez et al. 2004). In particular, destruction of the cholinergic neurons in the basal forebrain abolishes the increase of adenosine during SD and subsequent recovery sleep, while basal forebrain stimulation *via* NMDA receptor activation is required to induce the increase of adenosine and increase in recovery sleep (Kalinchuk et al. 2008; Wigren et al. 2009).

18.3.2 Astrocytes as a Regulator of Extracellular Adenosine

The major metabolic pathway of adenosine is summarized in Fig. 18.1. Adenosine is mainly formed from AMP, catalyzed intracellularly by 5′-nucleotidase and extracellularly by ecto-5′-nucleotidase. Adenosine is converted back to AMP by

adenosine kinase (AK), which in the CNS is localized to the cytoplasm of astroglial cells. The transmembrane transport of adenosine is accomplished by two families of nucleoside transporters (NT); the equilibrative transporters (ENTs) and concentrating transporters (Geiger and Fyda 1991); among them the ENT1 staining has been found in astrocytes (Alanko et al. 2006).

Both neurons and glia can influence extracellular adenosine concentration. Direct evidence demonstrating that astrocytes are a source of extracellular adenosine in the brain through ATP release comes from the work of Haydon and colleagues. They generated an inducible transgenic mouse line, termed the dnSNARE mouse, with astrocyte-specific expression of a dominant-negative domain of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) (Pascual et al. 2005). This mouse has disrupted vesicular release of gliotransmitters, and extracellular ATP and adenosine concentration in brain slices of these mice is found to be significantly reduced (Pascual et al. 2005; Schmitt et al. 2012). Consequently, dnSNARE mice exhibit attenuated adenosine-mediated presynaptic inhibition, which can be reconstituted by application of exogenous ATP (Pascual et al. 2005). These results support the idea that astroglial ATP vesicular release is a major source of extracellular adenosine. In addition, astrocytes can also contribute to extracellular adenosine through ATP release *via* Cx hemichannels and pannexin channels, especially in pathological conditions (Kang et al. 2008; Garré et al. 2010; Iglesias et al. 2009; Orellana et al. 2011). Given also the fact that AK is almost exclusively expressed in astrocytes in the adult mouse brain (Studer et al. 2006), these glial cells are likely to be a key regulator of extracellular adenosine levels by an adenosine cycle involving release of ATP, uptake of extracellular adenosine degraded from ATP via NTs and conversion of intracellular adenosine to AMP by AK (Boison 2008).

18.3.3 Astrocytes and Sleep Homeostatic Regulation

Considering their importance in regulating adenosine levels, it is logical to speculate that astrocytes are involved in adenosine-dependent sleep regulation. Indeed, EEG/EMG recordings revealed that the dnSNARE mouse has reduced SWS during basal NREM sleep and significantly attenuated increase in SWS and recovery sleep after SD (Halassa et al. 2009). Intracerebroventricular infusion of an A1R antagonist produced similar phenotype in wild type mice suggesting that astrocytic adenosine acts on A1R to modulate sleep pressure (Halassa et al. 2009). In agreement with this view, both conditional knockout of A1R (Bjorness et al. 2009) and overexpression of AK that reduces extracellular adenosine (Palchykova et al. 2010) cause sleep homeostasis phenotypes similar to those of the dnSNARE mice.

There are several lines of evidence suggesting that adenosine might be involved in some sleep disorders. For example, adenosine increase during SD and subsequent sleep recovery is attenuated in old rats (Wigren et al. 2009), and binding of adenosine receptor decreases in aged humans, possibly due to loss of A1R (Meyer et al. 2007). Also, it is found that rats exposed to sleep fragmentation, a likely cause

of excessive daytime sleepiness in patients of sleep apnea and other sleep disorders, exhibit elevated sleepiness and show rise of adenosine in the basal forebrain (McKenna et al. 2007). Studies of humans have shown that a single polymorphism of the adenosine deaminase gene leads to lower enzymatic activity of adenosine deaminase and presumably higher adenosine levels. Subjects with this polymorphism have enhanced slow wave sleep and sleep pressure (Bachmann et al. 2012). Finally, Hines and colleagues, using the dnSNARE mouse showed that astrocyte signaling to A1R is required for 12 h SD-induced alleviation of depressive-like behaviors in mouse model of major depression, and that the effect can be mimicked by administration of an A1R agonist (Hines et al. 2013). Considering the high co-occurrence of sleep disorders and depression (Nutt et al. 2008), their study implicates that astrocyte-derived adenosine might be involved in sleep disorders.

Taken together, malfunction of astroglial adenosine signaling and metabolism could be as yet unidentified causes of certain primary sleep disorders or secondary sleep problems associated with other diseases. Thus, the cellular machinery of the astrocyte signaling and metabolic pathways could represent potential therapeutic targets that should be worthy of research attention.

18.4 Neuroglial Metabolic Coupling and Sleep

18.4.1 Sleep and Metabolism

Sustenance of brain functions is energy expensive; 20% of the total energy produced by the body is consumed by the brain, which represents only 2% of the body mass (Pellerin and Magistretti 2012). One of hypothetic functions of sleep is to restore energy spent during wakefulness (Frank 2010). In agreement with this view, overall metabolic rate of the cerebral cortex is reduced during NREM sleep (Maquet 1995). Interestingly, the prime energy source of the brain, i.e. extracellular glucose, increases during NREM sleep and decreases during wakefulness and REM sleep (Netchiporouk et al. 2001; Dash et al. 2013). Moreover, sleep impairments are associated with higher risks of pathological metabolism, such as obesity, diabetes, hypertension, etc. (Porkka-Heiskanen et al. 2003; Spiegel et al. 2009). Apart from peripheral conditions, functional imaging also revealed a variety of brain areas that undergo abnormal metabolic states in patients of major sleep disorders including insomnia and catalepsy (Desseilles et al. 2008).

18.4.2 Astrocytes and Brain Metabolism

The level of neural activity is tightly coupled with brain metabolic rate. Both glucose utilization and blood flow increase with neural activity, i.e. during cognitive tasks. For the past decades, intense research efforts have demonstrated that

astrocytes play a central role in neuro-vascular metabolic coupling. Astrocytes are structurally adapted and perfectly positioned to support synaptic activity, which constitutes the largest brain energy expenditure (Bélanger et al. 2011). Indeed, astrocytes extend perisynaptic processes closely contacting synapses (Ventura and Harris 1999) which contain neurotransmitter receptors and transporters to sense synaptic activity. On the other hand, astroglial vascular processes, called endfeet, enwrap blood vessels and express glucose transporters to control blood flow and uptake of glucose (Allaman et al. 2011). How astrocytes respond to neuronal activity and supply energy needs of neurons is theorized by the astrocyte-neuron lactate shuttle hypothesis (Pellerin and Magistretti 1994; 2012). Briefly, synaptic activity increases extracellular glutamate, which is taken up by astrocyte plasmalemmal glutamate transporters, and results in an increase in $[Na^+]_i$ stimulating Na^+/K^+ pump and increasing astroglial consumption of ATP. This in turn triggers astroglial glucose uptake from circulation and glycolysis, that produces lactate which is released and taken up by neurons as the energy substrate. This model has been supported by numerous *in vitro, ex vivo* and *in vivo* studies (Barros et al. 2009; Cholet at al. 2001; Voutsinos-Porche et al. 2003; Chuquet at al. 2010; Lin et al. 2010; Rouach et al. 2008), indicating that neuronal activation is accompanied by sustained production of lactate by astrocytes and that lactate, instead of glucose, is the preferred energy substrate of neurons.

18.4.3 Astrocyte-Derived Lactate and Sleep

It has been observed that cortical lactate concentration rises upon waking and maintains elevated during continuous wakefulness and that it persistently declines during NREM sleep (Naylor et al. 2012). Also, cerebral SWS decreases the rate of glycolysis, which produces lactate (Wisor et al. 2012). Moreover, the NREM-associated decline of lactate reflects sleep pressure (Dash et al. 2012). Lactate levels are not only correlated with sleep-wake changes, but there is also evidence that lactate affects excitability of the orexin (wakefulness-promoting) neurons in the lateral hypothalamic nuclei. These neurons fire during wakening and are silent during sleep, moreover, loss of orexin neurons or orexin receptors is an established cause of narcolepsy (Lin et al. 1999; Chemelli et al. 1999). Orexin neurons are essentially astroglial lactate sensors. They specifically utilize lactate released by astrocytes, their spontaneous firing rate is correlated with lactate concentration and lactate increases their sensitivity to excitatory inputs (Parsons and Hirasawa 2010). The effects of lactate on orexin neuron behavior, thus, indicate a possible mechanism by which astrocyte-derived lactate could influence sleep regulation.

So far, we have only focused on lactate, which is only one of the possible links between neuroglial metabolic coupling and sleep. There are many other players of astroglial metabolism, including glucose, ATP, etc., that may also influence this process. Considering the vital roles of astrocytes in brain metabolism, it is tantalizing to hypothesize that astrocytes may be indirectly involved in sleep physiology and pathology *via* their metabolic functions, although this remains largely unclear and needs investigation.

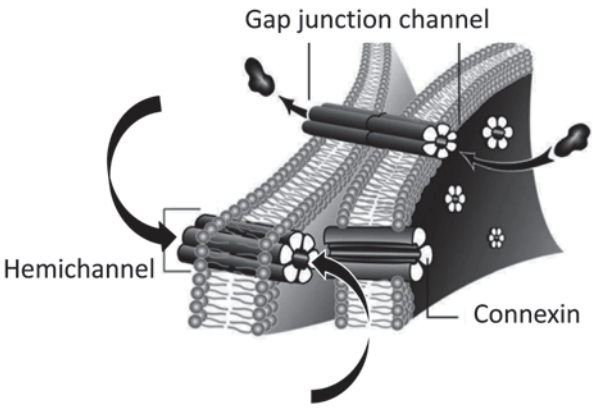

Fig. 18.2 Connexin-based gap junction channels and hemichannels. The *upper arrows* indicate the direct intercellular exchanges of ions, low molecular weight signaling molecules and metabolites through a gap junction. The two *lower arrows* indicate that the permeants though hemichannels can be either taken up or released by astrocytes

18.5 Astroglial Connexins and Sleep

18.5.1 Connexins, Hemichannels, Gap Junction Channels and Communicating Networks of Astrocytes

Astrocytes are organized in networks of cells connected by gap junction channels. These astroglial networks are involved in the homeostasis of ions, glutamate and second messengers and in the removal of toxic substances (Giaume et al. 2010). They contribute to the intercellular trafficking of energy metabolites to neurons and sustain synaptic activity (Rouach et al. 2008). In the brain, astrocytes are the cell population that exhibits the highest level of Cx expression. Although a minor expression of a third Cx, Cx26, cannot be excluded in sub-populations of astrocytes (Mercier and Hatton 2001; Nagy et al. 2001, 2011; but see Filippov et al. 2003), the two prevalent astroglial Cxs are Cx30 and Cx43. This has been confirmed using mice double knock out of Cx30 and Cx43 in which gap junctional communication assessed by dye coupling in hippocampal astrocytes is totally abolished (Wallraff et al. 2006; Rouach et al. 2008).

Connexins aggregate at junctional plaques and form hexameric rings of Cxs, termed connexons or hemichannels (Fig. 18.2). Hemichannels were initially assumed to remain closed, however, functional Cx hemichannels were later demonstrated to exist in astrocytes enabling astrocytes to play new roles in neuroglial interaction (Bennett et al. 2003; Giaume et al. 2013). Connexin hemichannels are permeable to glucose and represent an alternative pathway for the uptake and the release of energy metabolites (Retamal et al. 2007).They are also permeable to neurotransmitters such as glutamate (Yé et al. 2003) and ATP (Kang et al. 2008), thus playing a role in autocrine and paracrine brain communication. In diverse pathological situations, the pattern of Cx expression, the extent of gap junctional communication and the hemichannel activity are modified in astrocytes, as recently reviewed (Giaume et al. 2010, 2013). These changes depend on the type and severity of insult,

the distance from the lesion site and the time post-injury. As a consequence, both deleterious and protective effects have been reported, in particular after ischemia, stroke or trauma (Giaume et al. 2010).

18.5.2 Astroglial Connexins Could Be Involved in Sleep-Wake Regulation

Several findings support that possible changes in Cx channel-mediated communication could be related to sleep regulation. General anesthetics (Mantz et al. 1993) and oleamide, a sleep-inducing lipid (Guan et al. 1997), have been reported to inhibit gap junctions in primary cultures of astrocytes. *GJB6* gene (Cx 30) expression is under circadian control; it peaks during the dark period corresponding to the spontaneous waking period in mice (Maret et al. 2007). Sleep deprived mice exhibited an increase in expression of *GJB6* but not of *GJA1* (Cx43) compared to control animals (Liu, Petit, Magistretti and Giaume unpublished data). In addition to setting regulations directly related to sleep, glucose- and lactate-permeable astroglial gap junctions have been demonstrated to provide the basis for metabolic networks that regulates brain energy homeostasis and that sustains neuronal activity in hypoglycemic conditions (Rouach et al. 2008). Moreover, Cx hemichannels in astrocytes have been shown to be permeable to glucose (Retamal et al. 2007). Therefore, the reported alteration in the astrocyte-specific glycogen metabolism when wakefulness is prolonged instrumentally or pharmacologically (Petit et al. 2010) may be associated with changes in astroglial Cx-based channels since astroglial gap junctions provide the support for metabolic networking (Rouach et al. 2008). Based on these observations, astrocytic Cx30 could be a candidate that exhibits changes in expression and function when the sleep-wake cycle is perturbed. As neuronal activity is altered through different phases of the sleep-wake cycle, this working hypothesis is strengthened by evidence demonstrating that Cx30 is regulated by neuronal activity. This is established at transcriptional level since *GJB6* expression is increased in mice exposed to an enriched environment (Rampon et al. 2000) and at the functional level since gap junctional communication mediated by Cx30 channels is controlled by neuronal activity in the glomerular layer of the olfactory bulb (Roux et al. 2011).

18.5.3 Drugs Affecting Sleep-Wake Status Have Differential Impacts on Cortical Astroglial Networks

One approach to investigate whether Cx gap junction channels are involved in sleep homeostasis is to determine whether Cx expression and function are affected by molecules that impact sleep and/or treat sleep disorders. One of the most prescribed molecules is modafinil, a eugeroic, which following administration in humans and

mice suppresses both NREM and REM sleep. Compared to REM sleep and waking, a significant decline of energy metabolism is observed in cortical areas during NREM. This is shown using glucose uptake measurement in many animal species as well as in human (Ramm and Frost 1986; Vyazovskiy et al. 2008; Nofzinger et al. 2002). Since NREM represents more than 85 % of the total sleep time in B6 mouse, it can be assumed that neurometabolic requirements are larger in modafinil-treated mice than in control mice (i.e. sleeping mice). Recently, this compound was tested for its effects on the expression of Cx43 and Cx30 at the mRNA and protein levels (Liu et al. 2013). This study performed in the cortex of adult mouse demonstrated that Cx30 levels increased, while those of Cx43 were not affected, after modafinil administration. In addition, in acute cortical slices, modafinil increases dye coupling in a dose-dependent manner and this effect is abolished following tetrodotoxin (TTX) treatment in order to suppress neuronal activity (Liu et al. 2013). Interestingly, these observations are in line with a previous work carried out in thalamocortical slices demonstrating that gap junction-mediated electrotonic coupling is increased in neurons by similar concentration (150 μM) of and exposure time (30 min) to modafinil (Urbano et al. 2007). As neurons express Cxs, Cx36 being a prevalent type (Söhl et al. 2005), these findings demonstrated that, in the somatosensory cortex, the general effect of modafinil on Cx-based intercellular communication is to enhance Cx-based networking in astrocytes as well as in neurons. Consequently, it was suggested that the wakefulness-promoting action of modafinil might be linked to an increase in neuroglial interactions. In addition, the effects of modafinil on gap junctional communication are prevented by carbenoxolone, an extensively used gap junction blocker (Beck et al. 2008). In this study carried out in freely moving postnatal day 13 rats, the modafinil-induced increase of the sleep state-dependent, auditory-evoked potential amplitude was also blocked by carbenoxolone administration. In contrast, in acute cortical slices gamma-hydroxybutyric acid (GHB), a compound that has an effect on sleep regulation opposite to modafinil, has an inhibitory action on astroglial gap junctions, an effect which is not tetrodotoxin sensitive (Liu et al. 2013). This GHB inhibition observed with experiments carried out in acute cortical slices is in agreement with previous works performed in cultured astrocytes showing that sleep-inducing oleamide decreases gap junctional communication (Guan et al. 1997).

However, it cannot be generalized that drugs maintaining wakefulness increase coupling while those inducing sleep decrease coupling. As illustrated in Fig. 18.3a, Ritalin® (methylphenidate), another molecule known to have a wakefulness-promoting action, inhibits gap junctional communication (Liu and Giaume unpublished data). This discrepancy is likely due to different mechanisms of action. A similar remark is also true for the different sensitivity of the modafinil and GHB effects to tetrodotoxin.

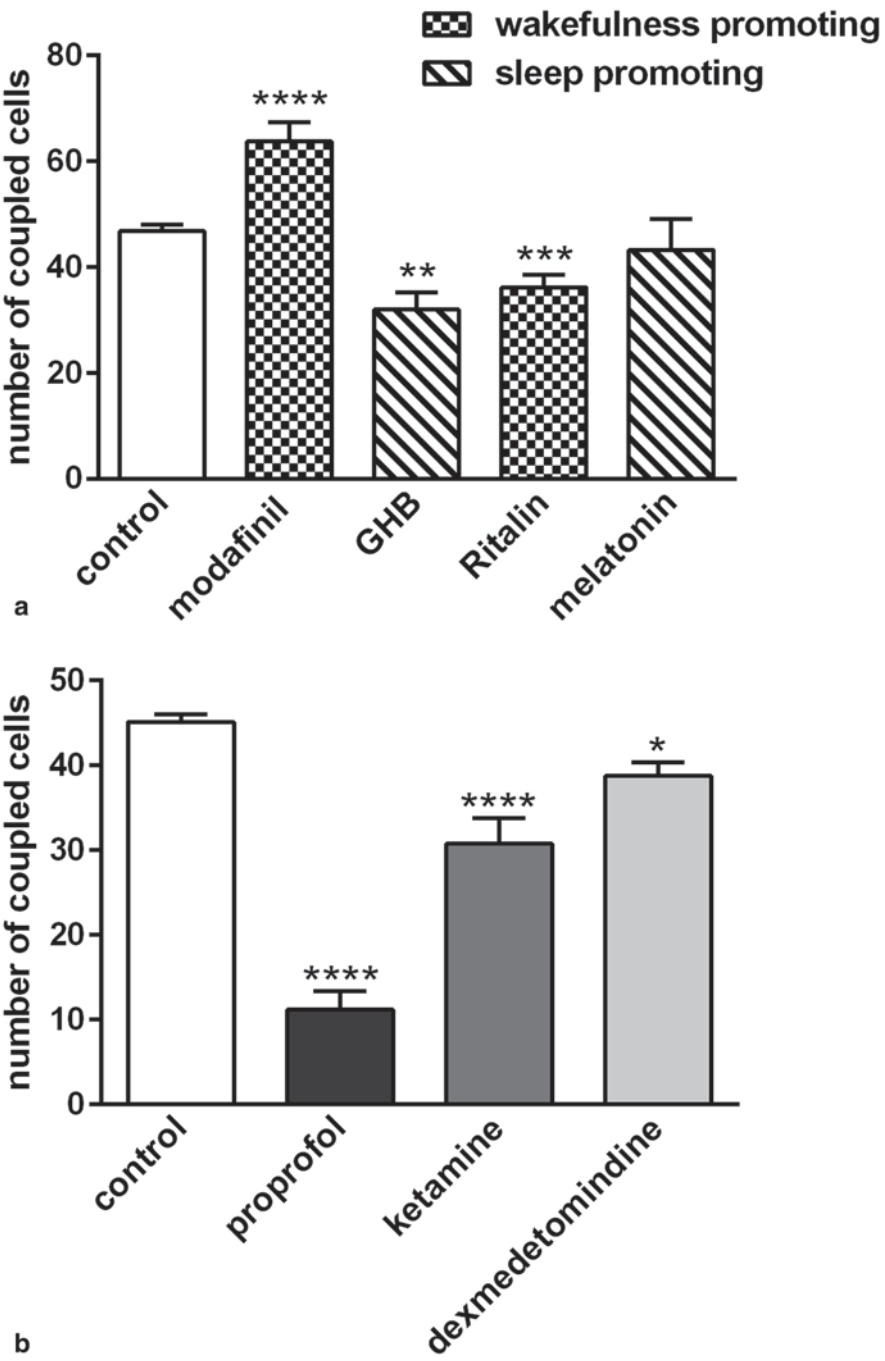

Fig. 18.3 Effects of drugs affecting the sleep-wake cycle and anesthetics on gap junctional communication between astrocytes in acute cortical slices. Gap junctional communication in astrocytes was assessed as previously described (Liu et al. 2013). **a** Effect of wakefulness-promoting

18.5.4 General Anesthetics Inhibit Astrocytic Gap Junctional Communication and Hemichannel Activity in Acute Cortical Slices

Some general anesthetics, such as halothane and enflurane, have for a long time been considered as broad inhibitors of gap junctions (see Rozental et al. 2001). Indeed, a screening of several classes of anesthetics has been performed using confluent primary cultures of mouse astrocytes (Mantz et al. 1993). This initial study revealed that the intravenous general anesthetics, propofol and etomidate, as well as halogenated anesthetics, halothane, enflurane and isoflurane, induced significant and dose-dependent inhibitions of gap junctional communication. In contrast, diazepam, morphine, ketamine, thiopental and clonidine were ineffective. We investigated whether their inhibitory action on gap junction channels could also be observed in acute cortical slices that are a more integrate model to study astrocyte properties. As illustrated in Fig. 18.3b using three different anesthetics, propofol, ketamine and the more recently developed dexmedetomindine, we also observed differences in the inhibitory potency when these drugs were used at clinically relevant concentrations (150, 300 and 1 μM, respectively). Indeed, as in cultured astrocytes propofol was found to be the most efficient, while ketamine had a significant effect in contrast to what was observed in culture, and dexmedetomindine had a weak but significant inhibitory effect (Liu and Giaume unpublished data). Again as stated previously for molecules affecting sleep status, there is not a general feature for the potency of effect of the tested anesthetics, although they all induce an inhibition of gap junctional communication in cortical astrocytes.

Concluding Remarks

An active and dynamic role is played by glial cells in many brain functions and pathologies (see Kettenmann and Ransom 2013). Among glial cells, astrocytes are certainly one cell type that influences neuronal function and survival in both healthy and diseased conditions (see Giaume et al. 2007; Rossi and Volterra 2009; Verkhratsky et al. 2012; Parpura and Verkhratsky 2012). We have largely focused this chapter on a single feature of astrocytes: the high expression level of Cxs. Astroglial Cxs are now associated with the modulation of synaptic activity and plasticity (Pannasch et al. 2011), neuronal death (Froger et al. 2010; Orellana et al. 2011) as well as many other brain pathologies (see Giaume et al. 2010). This is also the case for sleep-wake cycle regulation and its dysfunction, in which Cx channels could

drugs (200 μM modafil and 20 μM Ritalin®) and sleep inducing drugs (1 mM GHB and 4 μM melatonine) on the number of coupled cells after the whole-cell recording of a cortical astrocyte. **b** Differential inhibitory effect on astrocyte coupling exerted by three different classes of anesthetics used at clinical relevant concentration (150 μM for propofol, 300 μM for ketamine, 1 μM for dexmedetomidine)

play a role. As neuronal behavior is characterized by synchronous network activities during sleep, it is expected that neuroglial interactions also involve interactions at an integrated level between neuronal circuits and astroglial networks. We have here discussed evidence indicating that several treatments known to affect sleep homeostasis have an impact on astroglial networking. In the near future we expect that evidence will emerge to demonstrate that sleep-wake cycle regulation is reciprocally affected by pharmacological or genetic modifications of Cx channel expression and/or functions in astrocytes. Hopefully, a definitive validation of the role of astroglial networks in sleep-wake cycle will contribute to the identification of valuable alternative therapeutic targets for the treatment of sleep disorders.

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Chapter 19
Experimental Treatment of Acquired and Inherited Neuropathies

Robert Fledrich, Ruth M. Stassart and Michael W. Sereda

Abstract Peripheral neuropathies belong to the commonest neurological diseases and underlying causes are multiple, comprising a variety of acquired and hereditary factors. However, clinical symptoms are often similar, rendering differential diagnosis difficult, with no unambiguous etiological assignment in approximately 25 % of all cases (Berlit et al., Guidelines German society for neurology, 2012). This has substantially hampered the development of therapeutic strategies and leaves many patients without any treatment option until now.

Most commonly, a neuropathy patient will present with distally pronounced symmetric muscle weakness, walking disabilities and sensory impairment. However, rarer forms are characterized by asymmetric nerve affection, or by an involvement of the autonomic nervous system (Dyck and Thomas, Med Clin North Am 52:895–908, 2005). While hereditary neuropathies usually manifest during childhood or young adulthood, acquired forms may peak at advanced age (Dyck and Thomas, Med Clin North Am 52:895–908, 2005). Diagnosis includes careful history taking, in particular with regard to underlying diseases (e.g., diabetes, alcohol) and family history, a clinical examination, analysis of the cerebral spinal fluid, electrophysiological testing of the peripheral nerves and, if required, a sural nerve biopsy.

By means of electrophysiology, peripheral neuropathies are classically subdivided into axonal and demyelinating forms (Dyck and Thomas, Med Clin North Am 52:895–908, 2005). In general, a reduction of the compound muscle action potentials (CMAP), and normal nerve conduction velocity (NCV) implies purely axonal neuropathies, while a slowing of the NCV suggests a demyelinating neuropathy (Dyck and Thomas, Med Clin North Am 52:895–908, 2005). However, mixed forms are known. Axonal neuropathies are defined by a primary damage to the neuronal

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body or the axon, which results histologically in a Wallerian-type of axonal degeneration (e.g., fragmentation of the nerve fiber in acute degeneration) and subsequent axonal loss (Dyck and Thomas, Med Clin North Am 52:895–908, 2005). In contrast, demyelinating neuropathies are caused by acquired or hereditary damage to Schwann cell function (Dyck and Thomas, Med Clin North Am 52:895–908, 2005). Histologically, features of demyelination comprise abnormally thin myelin sheaths, and, classically, onion bulb formation (axons surrounded by concentric layers of multiple Schwann cell membranes) (Dyck and Thomas, Med Clin North Am 52:895–908, 2005). Clinically more relevant, however, is the inability of affected Schwann cells to maintain axonal integrity. Consequently, axonal degeneration also occurs in demyelinating neuropathies, secondary to demyelination and Schwann cell impairment (Dyck and Thomas, Med Clin North Am 52:895–908, 2005).

Importantly, the degree of axonal loss and the subsequent denervation of the target tissue (muscle or sensory organs) cause the extent of clinical impairment in both, demyelinating and axonal neuropathies (Dyck and Thomas, Med Clin North Am 52:895–908, 2005). Therefore, the view has emerged that axonal loss marks the final common pathway of all (demyelinating and axonal) neuropathies. Whether the same pathomechanisms underlie this pathway has to be clarified in future. Targeting the final common pathway would provide a promising therapeutic option which would be applicable to a large number of affected patients, independent of the primary disease cause.

Up to now, only limited therapies are available for immune mediated acquired neuropathies, whereas hereditary forms remain largely untreatable (Li, Semin Neurol 32:204–214, 2012; Nobile-Orazio, Revue Neurol 169:S33–S38, 2013). The following chapter will focus on therapeutic approaches for acquired and hereditary neuropathies and a special emphasize will be given to experimental strategies in various animal models.

Keywords Inherited neuropathies · Guillain-Barré-syndrome · GBS · Neurological diseases · Axonal · Demyelinating · Inflammatory demyelinating neuropathy · CIDP · Lewis-Sumner-syndrome · Diabetic neuropathy · Charcot Marie Tooth disease · CMT

19.1 Neuropathies—Clinical Presentation and Pathophysiology

19.1.1 Acquired Neuropathies

Acquired demyelinating neuropathies may be immune mediated, metabolic, or less frequently caused by toxic substances. Most of them are characterized by a slowly progressive, chronic disease, despite the existence of acute forms like the immune-mediated Guillain-Barré-syndrome (GBS).

19.1.1.1 Immune-mediated Demyelinating Neuropathies

The most common immune-neuropathy is the chronic inflammatory demyelinating neuropathy (CIDP), which is distinguished from GBS by a disease course evolving at least over 2 months (Vallat et al. 2010; Dalakas 2011). Classically, CIDP patients suffer from a symmetric proximal and distal muscle weakness as well as from sensory symptoms (Dalakas 2011). Occasionally, an affection of cranial nerves is observed. Most patients display a monophasic disease course with a slow, continuous disease progression, yet, a subset of patients develops a relapsing-remitting form (Vallat et al. 2010; Dalakas 2011). Besides the "classic" CIDP, several disease variants, like pure motor or sensory neuropathies exist (Vallat et al. 2010; Dalakas 2011). In addition, related immune-mediated disorders, especially the multifocal acquired demyelinating sensory and motor neuropathy (MADSAM) and the Lewis-Sumner-syndrome are often summarized as CIDP sub-entities. Though, it remains controversial which forms should better be ranked as distinct demyelinating neuropathies (Meyer Zu Hörste et al. 2007a; Vallat et al. 2010; Berlit et al. 2012). Mainly, the multifocal motor neuropathy (MMN) and the anti-myelin-associated glycoprotein neuropathies are considered as distinct diseases (Meyer Zu Hörste et al. 2007a; Berlit et al. 2012).

The clinical heterogeneity of CIDP renders diagnosis often difficult, and influenced the development of appropriate diagnostic criteria (Vallat et al. 2010; Dalakas 2011). Long-time, narrowly defined research oriented diagnostic criteria were used, which appeared, however, to be insufficient for clinical practice (too low sensitivity) (England et al. 2009; Vallat et al. 2010; Van den Bergh et al. 2010). Within the last years, improved guidelines have been developed, with a better relation between sensitivity and specificity (Van den Bergh et al. 2010). These are composed of mandatory electrophysiological and clinical criteria but also include supportive features [e.g., cerebrospinal fluid (CSF) protein, clinical improvement after immunomodulatory treatment and nerve biopsy findings] aiming at the diagnosis of a definite, probable, possible or atypical CIDP (Van den Bergh et al. 2010).

The diagnostic challenges along with the large spectrum of possible clinical symptoms may explain why the estimated incidence of CIDP varies between 1 to 1.9 per 100,000, with a maximum of 6.7 per 100,000 around the age of 70 to 80 years (Lunn et al. 1999; Mygland and Monstad 2003; Rajabally et al. 2009). Likewise, the incidence has been supposed to be underestimated, with up to 20% of all neuropathy patients without clear etiological assignment suffering de facto from CIDP (Latov 2002).

A firm diagnostic identification of CIDP patients is especially important with regard to therapeutic consequences. CIDP was initially discovered as a steroid sensitive polyneuropathy in the 1950s (Austin 1958). Indeed, CIDP is regarded as an autoimmune disease, although no specific trigger or autoantigen has been identified so far (Hughes et al. 2006; Vallat et al. 2010; Dalakas 2011). That CIDP most probably constitutes an autoimmune disease is derived from a variety of evidences obtained from studies on CIDP patient material, combined with lessons from experimental animal models.

Interestingly, histopathological examinations of sural nerve biopsies from CIDP patients demonstrate usually only minor or no endoneurial T-cell infiltration and a moderate increase in macrophages which often form small clusters around endoneurial blood vessels (Vallat et al. 2010; Dalakas 2011; Weis et al. 2011). Macrophages are thought to represent the antigen-presenting cells in the disease and to be the final effector cells, mediating the destruction of the myelin sheath (Hughes et al. 2006; Dalakas 2011) (Fig. 19.1a). Endoneurial macrophages express the human leukocyte antigen (HLA)-DR and are able to express the CD1 receptor family, hence allowing the presentation of either conventional or non-protein antigens to T-cells (Van Rhijn et al. 2000; Hughes et al. 2006). In addition, different studies on CIDP patients revealed the expression of inflammatory cytokines, chemoattractant proteins and co-stimulatory proteins for T-cell activation by macrophages (Hughes et al. 2006; Vallat et al. 2010; Dalakas 2011) (Fig. 19.1a).

Furthermore, Schwann cells and endothelial cells may contribute to antigen presentation and T-cell stimulation in CIDP (Hughes et al. 2006). Both cell types are in principle able to express major histocompatibility complex class II (MHC-II) molecules, and Schwann cells can present myelin basic protein to responsive T-cell lines and induce T-cell proliferation *in vitro* (Wekerle et al. 1986; Argall et al. 1992a, b; Atkinson et al. 1993; Lilje and Armati 1997; Hughes et al. 2006).

Activated T-cells infiltrate the nerve in response to chemokines and cell adhesion molecules produced by endothelial cells (Hughes et al. 2006). The T-cells detected in sural nerve biopsies of CIDP patients demonstrate no clonal T-cell response but a heterogeneous Vß gene usage (Stienekemeier et al. 1999; Hughes et al. 2006). Whether these observations argue against a limited number of antigens, or are rather the result of epitope spreading remains unclear (Hughes et al. 2006).

A role for humoral factors has been suggested because of the therapeutic benefit of plasmapheresis in a part of CIDP patients (Dalakas 2011). However, plasmapheresis does not only eliminate putative autoantibodies but also removes other inflammatory associated molecules, which may cause clinical improvement (Dalakas 2011). Indeed, various studies reported different chemokines, cytokines and metalloproteinases to be increased in blood, cerebrospinal fluid or nerves of CIDP patients (Hughes 2010; Dalakas 2011).

In order to support the role of potential antibodies in CIDP, immunoglobulin G (IgG) from CIDP patients sera has been injected intraneurally into the sciatic nerve of rats, which indeed induced demyelination (Yan et al. 2000). Also, the intraneural injection of antibodies against myelin antigens alone results in demyelination in laboratory animals (Hughes et al. 1985).

In experimental auto-immune neuritis (EAN), immunization with the myelin proteins P0, P2 or PMP22 emulsified with Freund's adjuvant is used in order to induce an inflammatory demyelinating disease (Hughes et al. 2006). However, the disease course is mainly acute and monophasic, thus resembling more to GBS than CIDP. A biphasic disease with demyelination mainly in the spinal roots and signs of epitope spreading has been established in a different rat strain, the Dark Agouti rat (Jung et al. 2004). Histologically, EAN is characterized by prominent T-cell infiltrates and macrophages within the endoneurium (Powell et al. 1983), and a passive transfer of T-cells induces the disease (Linington et al. 1984, 1992), supporting the

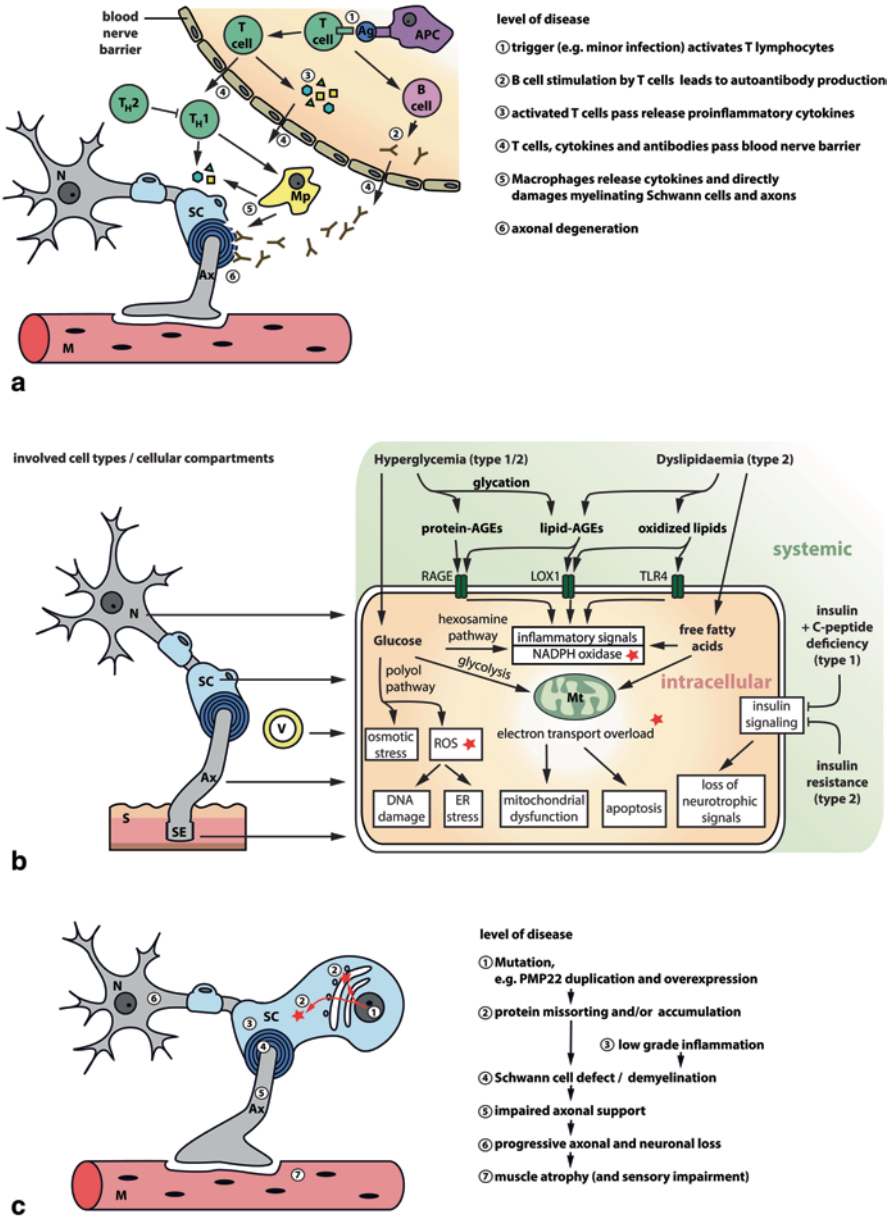

Fig. 19.1 Pathomechanisms in acquired and inherited peripheral neuropathies. **a** Hypothetical model of the pathological mechanisms of autoimmune disease in the peripheral nervous system. In the systemic immune compartment, autoreactive T lymphocytes (T cell) become activated by antigen-presenting cells (APC) (*1*). The stimulation of B-cells by autoreactive T-cells leads to the production of autoantibodies, which pass the blood nerve barrier (BNB) (*2*). In addition, T-cells release proinflammatory cytokines (*3*), cross the BNB and enter the peripheral nervous system (*4*). Here, T-cells differentiate into proinflammatory T-helper cells 1 (Th1) and antiinflammatory Th2 cells, as well as into Th17 cells. Infiltrating macrophages function as APCs and effector cells as they release cytokines, toxic mediators and directly damage myelinating Schwann cells and

importance of T-cells in these experimental models. However, in EAN in mice, CD4 and CD8 T-cells as well as B-cells are not needed for disease induction, although disease severity is altered in animals lacking CD4/CD8 T-cells (Zhu et al. 1999; Zhu 2002).

Numerous studies tried to identify potential autoantibodies in CIDP, but results continued to be inconsistent (Hughes and Willison 2012). Initial attempts focused on myelin proteins, especially on P0 but also on P2 and PMP22, i.e. on those which are predominantly expressed in the peripheral nervous system (PNS) (Hughes and Willison 2012). The most promising study revealed antibodies to P0 in around 28 % of CIDP patients (in numbers: 6 out of 21 patients) (Yan et al. 2001). Other approaches, searching for glycolipid antibodies like for galactocerebroside remained negative, and more recent studies reported antibodies against proteins of the axoglia junction like for neurofascin in only a very low number of patients (5 out of 119 CIDP patients) (Hughes and Willison 2012; Ng et al. 2012). Possibly, the development of new techniques allowing a broader search for both glycolipid and protein antigens as well as the recognition of their physiological domains may lead to more positive results in future (Hughes and Willison 2012). However, observations from NOD mice (a non-obese spontaneous diabetes mouse model) lacking the costimulatory molecule B7.2 (CD86), pointed to the significance of the regulatory immune system which may challenge the search on single autoantigens (Salomon et al. 2001; Hughes et al. 2006). These mice do not develop diabetes but instead demonstrate a chronic inflammatory polyneuropathy (Salomon et al. 2001; Hughes et al. 2006). The costimulatory molecule CD86/B7.2. expressed by antigen-presenting cells is required for T-cell activation by interacting with CD28 and CTL4 receptors on T-cells (Hughes et al. 2006). Also, the overexpression of CD86 in transgenic mice resulted in an inflammatory demyelinating disease of the central nervous system (CNS) and spinal root. Mice lacking the costimulatory CD28 show a strongly attenuated EAN (Zhu et al. 2001). Another study furthermore demonstrated that a depletion of CD25+CD4+ regulatory T-cells induced an autoimmune neuropathy (Setoguchi et al. 2005). Thus, a loss of regulatory autoimmune responses, potentially caused by a non-specific stimulus, may generate an autoimmune disease of

axons (*5*). The ultimate clinical impairment is caused by the degree of axonal loss (*6*). **b** Factors linked to type 1 diabetes and/or type 2 diabetes cause DNA damage, endoplasmic reticulum stress, mitochondrial complex dysfunction, apoptosis, and loss of neurotrophic signaling. This cell damage can occur in neurons (N), axons (Ax), sensory end-organs (SE) of the skin (S), Schwann cells (SC) and vascular endothelial cells (V), all of which can lead to neuropathological axonal loss. The relative importance of the pathways indicated in this network varies with cell type, disease profile and time (*AGE* advanced glycation end products, *ROS* reactive oxygen species (*red star*), *ER* endoplasmic reticulum, *LOX1* oxidized LDL receptor 1, *RAGE* receptor for advanced glycation end products, *TLR4* toll-like receptor 4). **c** Genetic defects in myelinating Schwann cells (*1*) can lead to missorting or accumulation of mutated/overexpressed proteins (*red star*) (*2*). Besides subsequent demyelination, malfunctioning Schwann cells (*3*) fail to sustain axonal support (*4*) which then leads to progressive axonal and neuronal loss (the final common pathway) (*5*). The clinical phenotype is ultimately determined by neurogenic muscle atrophy (*6*). *Ax* axon, *SC* Schwann cell, *N* neuron, *M* muscle

the PNS (Hughes et al. 2006). However, a direct link between these observations in experimental models and the human CIDP disease still awaits to be proved. In some patients, an expression of costimulatory molecules has been found in nerve biopsies (Kiefer 2000; Murata and Dalakas 2000). In addition CD4+CD25+FoxP3+ regulatory T-cells are reduced in blood of CIDP patients (Chi et al. 2008). Other studies detected higher levels of Th17 cells and interleukin 17 (IL-17), which are thought to be involved in several autoimmune diseases, in blood and CSF of CIDP patients (Chi et al. 2010).

In summary, a variety of different studies on patients and animal models strongly argues for CIDP as an autoimmune disease. The precise contribution of the diverse immune components to the pathomechanism of CIDP has to be further determined. Here, especially the complex interaction between effector cells, T-cells and the immunoregulatory system as well as the role and nature of autoantibodies remain key issues of future research.

19.1.1.2 Diabetic Neuropathy

The prevalence of diabetes mellitus worldwide has been estimated to be 2.8 % in 2000 and 4.4 % in 2030 (Wild et al. 2004). Diabetes is referred to as type 1 if an autoimmune disease leads to the destruction of insulin-producing pancreatic beta cells (insulin dependent type), resulting in a lack of insulin. The much more frequent type 2 diabetes (>90 %), on the contrary, specifies acquired decreased insulin sensitivity in peripheral tissues (insulin independent type, insulin resistance) (NIDDK 2011). Importantly, 30–50 % of diabetic patients eventually develop neuropathic symptoms during their disease, rendering diabetes with 25–35 % by far the commonest identified cause for peripheral neuropathy (Maser et al. 1989) (Johannsen et al. 2001). Diabetic neuropathies are usually of predominant axonal origin, but frequently show mixed pathology accompanied by demyelination (Wilson et al. 1998; Herrmann et al. 2002; Valls-Canals et al. 2002). Purely primary demyelinating forms, however, are rare (Stewart et al. 1996).

Diabetes can impact the PNS in many ways, e.g. by distal symmetrical polyneuropathy (DSP), predominant small fiber neuropathy, autonomic neuropathy, radiculoplexopathy (diabetic amyotrophy), mononeuritis multiplex and mononeuropathy, of which DSP is the most frequent presentation (Callaghan et al. 2012). Neurological symptoms caused by DSP comprise distally pronounced and proximally spreading sensory and motor impairment (Dyck and Thomas 2005). In DSP, sensory disorders are much more frequent than motor deficits and patients display hyperalgesia and allodynia (increased and painful sensation to innocuous stimuli) (Daousi et al. 2004). Neuropathic pain, indeed, is one of the most burdening symptoms for patients with DSP and is present in 10–20 % of all diabetic patients (Galer et al. 2000; Daousi et al. 2004; Barrett et al. 2007; Abbott et al. 2011). Muscular symptoms like weakness in the lower limbs are rare but wasting of intrinsic hand muscles may occur. Motor impairments like unsteady gait in patients with diabetic neuropathy are

rather the result of sensory disturbances (Dyck and Thomas 2005). Occasionally, diabetic patients with a small fiber type of neuropathy display an involvement of the autonomic nervous system which may lead to gastroparesis, constipation, urinary retention, erectile dysfunction and cardiac arrhythmias (Callaghan et al. 2012). Degenerating small fibers also trigger foot ulceration ("diabetic foot") and neuropathic osteo-arthropathy (Said et al. 1983; Shun et al. 2004). Finally, there is also a substantial number of diabetic patients who have nonsymptomatic neuropathy (Miralles-García et al. 2010).

Histological observations reported in diabetic neuropathy include both (either mixed or solely), primary axonal degeneration with secondary demyelination and, much less frequent, primary demyelination with secondary axonal breakdown. Regeneration associated events like axonal sprouting and Schwann cell proliferation are evident and the occurrence of onion bulbs (multiple promyelinating Schwann cells surrounding a single axon) and Schwann cell basal lamina hypertrophy pinpoint neuropathological processes (Brown et al. 1976; Behse et al. 1977; Llewelyn et al. 1991; Dyck and Thomas 2005; Said 2007).

Pronounced axonal atrophy and characteristic nodal and paranodal aberrations that (next to demyelination) impair nerve conduction velocity are frequent findings in type 1 but are almost absent in type 2 diabetes. The progressive character of axonal atrophy generally is milder in type 2 diabetes (Greene et al. 1992; Forsblom et al. 1998; Sima et al. 2004; Sima and Kamiya 2006). However, initial predominant affection of small myelinated and nonmyelinated somatosensory fibers allows the use of skin biopsies and the quantification of intraepidermal nerve fiber densities as an early marker of type 2 diabetes (Shun et al. 2004; Umapathi et al. 2007).

Although the precise mechanisms underlying diabetic neuropathy remain unclear, hyperglycemia is widely considered to constitute a causative key factor (Sugimoto et al. 2008; Tomlinson and Gardiner 2008; Callaghan et al. 2012). In addition, endoneurial microvascular abnormalities have been demonstrated to impair nerve perfusion, and to cause hypoxic or ischemic nerve damage (Cameron et al. 2001). Insulin itself has been reported to harbor neurotrophic properties, although it is not involved in neuronal glucose uptake (Xu et al. 2004; Toth et al. 2006). The insulin deficiency in type 1 and the insulin resistance in type 2 diabetes are therefore discussed to directly contribute to the genesis of neuropathy (Kim and Feldman 2012). Other factors may contribute with respect to the specific type of diabetes, e.g., in the insulin-resistant type 2 diabetes, dyslipidaemia is thought to play a major role (Vincent et al. 2009).

Hyperglycemia is believed to be especially deleterious when persisting over longer time periods. Systemic overload and intracellular excess of glucose leads to increased activity of cellular glucose metabolizing pathways (Fig. 19.1b). Increased glycolysis, for example, overcharges the mitochondrial electron transport chain, thereby leading to the generation of reactive oxygen species (Vincent et al. 2004). Moreover, oxidative stress is also provoked when glucose passes the polyol pathway which increases cellular osmolarity and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) levels (Oates 2002; Obrosova 2005).

Finally, metabolic turnover of glucose via the hexosamine pathway can trigger inflammatory responses (Vincent et al. 2011). Next to metabolic pathway overload, hyperglycemia leads to the generation of advanced glycation end products (AGEs) (Sugimoto et al. 2008). Overglycation of proteins, lipids and nucleic acids impairs their biological function (Duran-Jimenez et al. 2009), and extracellular binding of AGEs to their cognate receptor (RAGE) triggers inflammatory responses and oxidative stress (Vincent et al. 2007).

Furthermore, dyslipidaemia is evidently linked to type 2 diabetes and patients display elevated blood triglycerides and altered composition of circulating lipoproteins (Clemens et al. 2004; Wiggin et al. 2009) (Fig. 19.1b). Although the precise pathophysiological significance remains unclear, several studies pointed to a role of dyslipidaemia in oxidative stress (Vincent et al. 2007, 2009; Nowicki et al. 2010). Here, the activation of different receptors like LDL receptor LOX1, toll-like receptor 4 and RAGE by oxidized or glycated plasma lipoproteins was reported to induce oxidative stress.

The C-peptide, which connects both insulin subchains, is reduced in patients with type 1 diabetes, whereas it is unaltered in type 2 diabetes (Webb and Bonser 1981). Although initially considered to be biologically inert, C-peptide is bioactive and its depletion contributes to diabetic neuropathy probably via functional impairment of the Na^+-K^+-ATPase and endothelial nitric oxide synthase enzyme activities (Ido et al. 1997; Sima 2004; Ekberg and Johansson 2008). In line, the therapeutic replacement of C-peptide in type 1 diabetic patients resulted in improved peripheral nerve function (Ekberg and Johansson 2008).

The abovementioned molecular mechanisms result in manifold damages to various cell types. Mitochondrial dysfunction, endoplasmatic reticulum stress, DNA damage and apoptosis may occur in neurons, Schwann cells and endothelial cells of the microvasculature (Fig. 19.1b) (Callaghan et al. 2012). Eventually, these pathological processes lead to dysfunction or even death of peripheral nerve fibers, underlying the clinical symptoms as reviewed above. In summary, diabetic neuropathy compromises quality of life to a considerable extent and, given the frequent incidence, has an enormous socio-economic impact (Williams et al. 2002). Therapeutic options are therefore highly demanded and are under intense research as discussed below.

19.1.2 Hereditary Demyelinating Neuropathies

Hereditary demyelinating neuropathies are clinically referred to as Hereditary Sensory and motor neuropathies (HMSN), or by geneticists to Charcot-Marie-Tooth (CMT) disease.

CMT diseases comprise the most frequent inherited disorders of the PNS with a prevalence of up to 1 in 2500 (Skre 1974; Emery 1991). Affected humans develop slowly progressive, distally pronounced atrophic muscle weakness, subsequent walking disabilities and sensory impairments. The CMT disease onset

and progression are strikingly variable, ranging from clinically asymptomatic to wheelchair-bound, and a therapy is not available (Pareyson and Marchesi 2009; Reilly et al. 2011; Schenone et al. 2011; Siskind and Shy 2011)

Around 50 years ago, prior to genetic insight, the first classification of seven CMT subtypes was introduced, demonstrating the wide clinical heterogeneity of the disease (Dyck 1968). Today, this classification still provides the basis for the clinical categorization of CMT and the electrophysiological value of the motor NCV is used to distinguish the demyelinating CMT type 1 (CMT1) with strongly reduced NCV (<38 m/s) from the axonal CMT type 2 (CMT2) with normal or slightly reduced NCV (Harding and Thomas 1980). Severely affected children were classified as patients with congenital hypomyelinating neuropathies (CHN) or Déjerine Sòttas syndrome (DSS) (Reilly and Shy 2009). Genetically determined neuropathies with primary sensory or autonomic impairments were termed as hereditary sensory and autonomic neuropathies, whereas purely motor forms were referred to as distal hereditary motor neuropathies (Reilly and Shy 2009).

From the 1990s on, rapid progress in genetics led to the identification of a large number of genomic loci associated with CMT. Importantly, different genetic defects in various genes result in a very similar clinical phenotype and disease genes alone are not sufficient to meet the requirements of a practical CMT categorization. Therefore, the currently applied classification system incorporates both, genetic and clinical characteristics.

In the mid-1990s, the first genetically modified animal models with a CMT-like pathology and clinical phenotype were generated (Martini et al. 1995; Huxley et al. 1996; Magyar et al. 1996; Sereda et al. 1996). Before, only naturally occurring mouse mutants such as the Trembler mice were available (Suter et al. 1992b). Numerous CMT rodent models have been created since then and the knowledge about the nature of CMT disease has greatly expanded in the last 10 years, as discussed in recent reviews (Siskind and Shy 2011; Fledrich et al. 2012b; Li 2012). Importantly, several recent human therapeutic trials with ascorbic acid (vitamin C) have failed despite promising data derived from a CMT mouse model, leaving the urgent need for treatment unresolved (Burns et al. 2009; Micallef et al. 2009; Verhamme et al. 2009; Pareyson et al. 2011; Lewis et al. 2013).

So far, more than 900 mutations in 60 genes have been identified to cause CMT and animal models for the most common forms of human CMT are now available (Fledrich et al. 2012b). Disease genes can be expressed ubiquitously as well as solely in Schwann cells or neurons. A regularly updated list can be found on http://www.molgen.ua.ac.be/CMTMutations. Rodents with aberrations in the genes of the peripheral myelin protein 22 kDa (PMP22), gap junction protein beta 1 (GJB1), also known as connexin 32, and myelin protein zero (MPZ) have been most extensively studied and constitute models for the commonest subtypes of human Charcot Marie Tooth disease, the demyelinating neuropathies CMT1A/E, CMT1X and CMT1B, respectively.

19.1.2.1 Charcot Marie Tooth disease type 1A and 1E are PMP22-related neuropathies

The underlying genetic defect for the most prevalent CMT subtype 1A (CMT1A) is an intrachromosomal duplication on chromosome 17p11.2 (Lupski et al. 1991; Raeymaekers et al. 1991). The *PMP22*gene is located within the duplicated region and its increased gene dosage is causative for the disease (Palau et al. 1993; Sereda et al. 1996; Suter and Scherer 2003). PMP22 is a small hydrophobic transmembrane protein, which is, next to ubiquitous expression, located in the compact myelin of Schwann cells in the PNS (Snipes et al. 1992). Patients with CMT1A exhibit approximately 1.7 fold *PMP22* mRNA overexpression in Schwann cells (Yoshikawa et al. 1994), although this is not a consistent finding (Hanemann et al. 1994). Demyelination, concentric layers of multiple promyelinating Schwann cells around naked axons (onion bulbs) and secondary axonal loss are typical histopathological features of peripheral nerves in patients with CMT1A (Gabreëls-Festen and Wetering 1999). Several *Pmp22* transgenic mouse lines (Huxley et al. 1996, 1998; Magyar et al. 1996; Robertson et al. 2002) and one line of *Pmp22* transgenic rats ('CMT rat') (Sereda et al. 1996) have been generated by the integration of extra copies of the cloned *Pmp22* gene. CMT rats carry approximately three copies (hence "low copy") of the wildtype mouse *Pmp22* gene resulting in an approximately 1.6 fold mRNA overexpression in peripheral nerves (Sereda et al. 1996, 2003). CMT rats display abnormalities already during development like dysmyelination and Schwann cell hyperproliferation (Grandis et al. 2004; Fledrich et al. 2012a). Older CMT rats, however, demonstrate demyelination, onion bulb formation, reduction in mean axon size as well as axonal loss. Moreover, *Pmp22* transgenic rats suffer from progressive muscle atrophy resulting in grip strength reduction and gait impairment (Sereda et al. 2003; Meyer Zu Hörste et al. 2007b). Although CMT rats were derived from one founder, they recapitulate a striking disease variability after being kept on an outbred background for numerous generations (Fledrich et al. 2012a). High disease variability has been reported for humans affected by CMT1A within the same family and even among monozygotic twins (Kaku et al. 1993; Garcia et al. 1995). Hence, the CMT rat models important molecular, histological and phenotypical hallmarks of patients with CMT1A particular well,rendering it an adequate animal model for testing therapeutic compounds. When bred to homozygosity, *Pmp22* transgenic rats display a severe amyelinating phenotype resulting in limb paralysis and adolescent death (Sereda et al. 1996; Niemann et al. 2000), resembling CHN andDSS in patients. Next to the CMT rat, various transgenic mouse models have been generated, many of which carry a high number of additional genomic *Pmp22* copies and may thus be less appropriate rodent models for CMT1A.

In humans, *PMP22* point mutations account for only 2.5 % of all CMT cases and mostly underlie the severe forms of demyelinating peripheral neuropathies, CHN and DSS (Szigeti et al. 2006). Neuropathies caused by *PMP22* point mutations were historically referred to as CMT1A but are now termed CMT1 type E (Schenone et al. 2011). Interestingly, a family with a rare axonal CMT form (CMT2) was

recently described carrying a dominant point mutation in the *PMP22* gene (Gess et al. 2011). The spontaneous, dominantly inherited mutations in the *Pmp22* gene result in leucine-to-proline (L16P) or glycine-to aspartic acid (G160D) replacements in the PMP22 protein of the *Trembler*-J (*Tr J*) or *Trembler* (*Tr*) mouse, respectively (Suter et al. 1992a, b; Dyck and Thomas 2005). An identical *Tr J* single point mutation in a severely affected CMT disease type 1E (CMT1E) family (Valentijn et al. 1992) allows the use ofthese mice as models for the corresponding human disease. The pathomechanism leading to myelination defects in these mutants remains poorly understood. However, for both strains, *Tr* and *Tr J*, altered PMP22 protein folding, trafficking and accumulation in Schwann cells were described and the differences in the phenotype may be due to different susceptibility to form protein aggregates containing mutant PMP22 (Naef and Suter 1999; Notterpek et al. 1999; Colby et al. 2000; Fortun et al. 2006) (Fig. 19.1c).

19.1.2.2 Animal Models of Myelin Protein Zero (MPZ) Related Neuropathies Resemble Patients with CMT1B

Myelin Protein Zero (MPZ or P_0) is an immunoglobulin-related adhesion molecule and constitutes the most abundant myelin protein in peripheral nerve (Greenfield et al. 1973; Filbin and Tennekoon 1993). More than 100 different mutations in the *MPZ* gene have been identified in patients suffering from autosomal demyelinating hereditary neuropathies and engineered animal models are available for some of them (Shy 2006a). Interestingly, there is a growing number of *MPZ* mutations which lead to axonal CMT (Warner et al. 1996; De Jonghe et al. 1999; Misu et al. 2000; Auer-Grumbach et al. 2003; Li et al. 2006; Marttila et al. 2012). Mild forms are termed CMT type 1B, the third most common CMT form, whereas severe forms are referred to as CHN and DSS (Warner et al. 1996; Shy 2006a). Recently, two families with *MPZ* duplication and peripheral neuropathy have been reported (Høyer et al. 2011; Maeda et al. 2012). Cases with a deletion of an entire *MPZ* allele are so far not known. In mice, a heterozygous null allele causes a relatively mild demyelinating phenotype and mice are indistinguishable from wildtype littermates until postnatal week 4 (Martini et al. 1995). Mouse models which completely lack functional *Mpz* genes exhibit a progressive behavioural phenotype and fail to establish compact myelin in a large proportion of nerve fibers (Martini et al. 1995). Years before the recent reports of *MPZ* duplication cases in humans it has already been shown that transgenic overexpression of the *MPZ* gene is not tolerated and causes a severe CHN-like phenotype in mice (Wrabetz et al. 2000). However, *MPZ* mutations associated with severe forms of inherited neuropathy result in transcripts harbouring premature stop codons within terminal exons that are not subject to the nonsense mediated decay surveillance system (NMD) (Inoue et al. 2004). Therefore, erroneous mRNA is not degraded but translated into truncated proteins with potential dominant-negative activity and subsequent aggregation in the endoplasmatic reticulum (ER) (Inoue et al. 2004). Protein products of other *MPZ* mutations which also escape the NMD but are not retained in the ER can be integrated into the myelin sheath. A resulting mild CMT associated phenotype may likely be the consequence of loss of function mutations

in one *MPZ* gene which can be partially rescued by the intact allele, as underlined by the mild phenotype in heterozygous *Mpz* null mice (Martini et al. 1995; Khajavi et al. 2005). In humans, the deletion of *Mpz* serine 63 (S63del) or its mutation to *Mpz* cysteine 63 (S63C) exert demyelinating mild late onset CMT type 1B and severe early onset DSS, respectively (Hayasaka et al. 1993; Kulkens et al. 1993). The respective pathological mechanisms are apparently different; *Mpz*S83del is retained in the ER whereas *Mpz*S63C is misarranged into the myelin sheath (Wrabetz et al. 2006). Genetically, engineered mice harbouring mutated *Mpz*S63del come closest to human CMT1B, exhibiting distally pronounced demyelination, reduced NCV and signs of muscle atrophy (Wrabetz et al. 2006).

19.1.2.3 Mouse Mutants Carrying Mutations in the Gap Junction Protein Beta 1 (Gjb1) Gene Resemble Patients with CMT1X

The gap junction protein beta 1 (GJB1), historically termed connexin-32 (Cx-32), is located in the noncompact myelin of the PNS and CNS and mutations cause the second most common demyelinating CMT (CMT1X) (Suter and Scherer 2003). CNS signs and symptoms have been found in some patients with CMT1X (Abrams and Scherer 2012). More than 250 disease causing mutations have been described and the X-linked inheritance explains the fact that male patients are clinically affected and females show only subclinical signs, e.g., NCV slowing (Kleopa and Scherer 2006). Despite the high number of reported mutations in the *GJB1* gene causing CMT1X in humans, the resulting clinical severity appears to be relatively uniform, including those with a deleted gene. Hence, most mutants are thought to cause a loss of function (Hahn et al. 2000; Dubourg et al. 2001). Mice hemizygous for the *Gjb1* gene may therefore be practical to study human CMT1X (Young and Suter 2001). Mice lacking both *Gjb1* alleles as much as mice transgenically expressing mutant GJB1 (R142W) phenocopy human CMT1X patients and develop a late onset demyelinating neuropathy predominantly affecting motor fibers (Nelles et al. 1996; Anzini et al. 1997; Scherer et al. 1998; Jeng et al. 2006; Kleopa and Scherer 2006). Transgenic expression of *Gjb1* under the control of the Schwann cell specific *Mpz* promoter rescued the phenotype in mice which lack endogenous *Gjb1*, indicating that loss of Schwann-cell-autonomous expression of *Gjb1* causes demyelination in CMT1X (Scherer et al. 2005).

19.2 Treatment of Neuropathies

19.2.1 Treatment of Inflammatory Demyelinating Neuropathies

Clinically approved therapies in CIPD encompass several immunomodulatory and immunosuppressive substances. First line treatment includes immunoglobulins,

corticosteroids and plasmapheresis (Eftimov et al. 2009, 2012; Hughes and Mehndiratta 2012; Mehndiratta and Hughes 2012). In a retrospective investigation, two third of CIDP patients responded to these first line therapies (Cocito et al. 2010). Corticosteroids are known for their anti-inflammatory potential and are commonly used in many autoimmune disorders (Dalakas 2011). Plasmapheresis removes potential antibodies as well as other circulating immune molecules from the blood of CIDP patients and provides a well evidenced based treatment option although it is less safe and more invasive compared to other immune therapies (Dalakas 2011; Mehndiratta and Hughes 2012). Immunoglobulins are thought to exert a variety of effects on the immune system like on autoantibodies, the complement system, soluble immune factors as well as on adhesion molecules and receptors on macrophages, although the precise mechanism is not understood (Dalakas 2011). The intravenous application of immunoglobulins (IVIG) has been shown to be beneficial in the acute and long-term treatment of CIDP in placebo-controlled, randomized studies (Hughes et al. 2008; Eftimov et al. 2009; Hughes 2010). Interestingly, approximately half of the patients can discontinue the IVIG treatment after 6 months without clinical deterioration (Hughes et al. 2008; Dalakas 2011).

In addition to these first-line therapies, other immune therapies like interferon-ß (IFN-ß), azathioprine, methotrexate, cyclosporin A, cyclophosphamide or mycophenolate mofetil may be used in specific cases, but still need to be better assessed in randomized, controlled clinical trials (Vallat et al. 2010). Other new treatment options include, among others, the monoclonal antibody alemtuzumab, directed against CD52 expressed on lymphocytes and monocytes, as well as rituximab, a monoclonal antibody targeting the B-cell specific molecule CD20, which have been shown to be beneficial in small, uncontrolled studies (Marsh et al. 2010; Benedetti et al. 2011; Dalakas 2011). Additionally, the antibody natalizumab targeting α4β1 integrin (VLA4) on leukocytes, the immunomodulatory agent fingolimod (a sphingosin-1-phosphat-receptor agonist) as well as eculizumab, directed against complement C5 may provide future therapeutic options for CIDP (Dalakas 2012). However, beneficial effects have to be carefully contrasted with the risk of potential severe side effects and toxicity.

In experimental autoimmune neuritis (EAN) a multitude of different treatment approaches has been tested, of which a detailed description is beyond the scope of this chapter (for review see Meyer Zu Hörste et al. 2007a) . Therefore, a broad, incomplete overview of different strategies will be given. However, it is important to note that therapeutic approaches in EAN may in general be more related to GBS than to CIDP.

In EAN, plasma exchange, immuneadsorption and IVIGs all ameliorate disease severity in accordance with the current treatments used in CIDP patients (Meyer Zu Hörste et al. 2007a). In addition, a variety of other immunomodulatory and immunosuppressive substances have been shown to reduce or prevent EAN, some of which might be promising in CIDP (Hughes et al. 2006). One example is the immunomodulatory substance leflunomide (Korn et al. 2001); however, it has been reported to cause a neuropathy (Bonnel and Graham 2004; Hughes et al. 2006).

Other therapeutic approaches may help to better understand the pathomechanisms of the disease and several approaches aimed at the induction of tolerance by

applying myelin antigens (such as myelin homogenate, myelin peptides or derivatives) before disease induction (Gaupp et al. 1997; Meyer Zu Hörste et al. 2007a). While these approaches proved successful in EAN, a translation into patients seems unlikely (Meyer Zu Hörste et al. 2007a). In addition, an inhibition of autoreactive T-cells has been addressed with T-cell receptor antibodies (TCR) or TCR DNA vaccination (Jung et al. 1992; Araga et al. 1999; Stienekemeier et al. 2001; Meyer Zu Hörste et al. 2007a). The regulatory immune system provides another target in EAN, for example in a statin therapy which increases T-regulatory cells, reduces Th1/Th17 cytokines and alleviates the disease course (Li et al. 2011). Moreover, an agonistic antibody against the co-stimulatory molecule CD28 improved the disease (Schmidt et al. 2003). That an increase in regulatory T-cells and M2-macrophages ameliorates the disease was also shown by an experimental therapy with a plant-origin ligand of the glucocorticoid receptor (Zhang et al. 2009). The humoral immune response has not only been successfully targeted with immunoglobulins, but also with complement inhibitors in EAN (Feasby et al. 1987; Jung et al. 1995; Meyer Zu Hörste et al. 2007a). Further studies addressed the dysregulation of cytokines. Antibodies and antagonists against proinflammatory cytokines, like tumor necrosis factor (TNF) and IFN-γ improved the disease (Meyer Zu Hörste et al. 2007a). Vice versa, anti-inflammatory cytokines including interleukin 4 and 10, IFN-ß and transforming growth factor ß (TGF-ß) had beneficial effects in EAN (Meyer Zu Hörste et al. 2007a). For example, erythropoietin has been shown to ameliorate EAN by inducing TGF-ß in macrophages (Mausberg et al. 2011). Cyclooxygenase inhibitors improved EAN via reduction of eicosanoids in macrophages (Hartung et al. 1988a; Miyamoto et al. 1998). The inhibition of macrophages or macrophage associated factors showed improvement in several experimental EAN studies (Craggs et al. 1984; Jung et al. 1993; Zou et al. 1999; Nicoletti et al. 2005). In addition, endothelial adhesion and penetration of the blood nerve barrier (BNB) has been addressed by blocking adhesion molecules (VLA4 and LFA1), their receptors (VCAM1 and ICAM1) and L-selectin (Archelos et al. 1993, 1994; Meyer Zu Hörste et al. 2007a). Also, inhibiting MMP and TNFα attenuated EAN, possibly by preventing BNB damage (Redford et al. 1997). Next to therapies directly interacting with the immune system, neuroprotection provides an important approach, targeting the final common pathway of axonal loss in peripheral neuropathies. Destruction of the nerve structure has been alleviated by radical scavengers or inhibitors of nitric oxid synthesis (Hartung et al. 1988b; Zielasek et al. 1995). Furthermore, sodium and potassium ion channel blockers improved EAN, such as the sodium ion channel blocker flecainide, which prevented axonal degeneration in EAN (Bechtold et al. 2005; Meyer Zu Hörste et al. 2007a).

In summary, corticosteroids, IVIG and plasmapheresis provide evidence based beneficial therapies for acute and long-term CIDP treatment. However, only two thirds of all patients respond to these treatments, pointing out the need of new therapeutic proceedings. Here, not only immunomodulatory approaches, but also neuroprotective and remyelination promoting strategies are highly demanded. Moreover, although various promising treatments have been established in EAN, a successful translation to patients is widely lagging behind. In this regard, better animal models in closer relation to human CIDP would be advantageous.

19.2.2 Treatment of Diabetic Neuropathy

19.2.2.1 Glucose Control in Type 1 and Type 2 Diabetes has Emerged as a Modifiable Risk Factor for the Development of Neuropathy in Diabetic Patients

For type 1, seven studies have been performed over the past 30 years (Holman et al. 1983; Lauritzen et al. 1985; Dahl-Jørgensen et al. 1986; Jakobsen et al. 1988; Diabetes Control and Complications Trial (DCCT) 1993; Reichard et al. 1993; Linn et al. 1996). Up to 70 % reduction in neuropathy was reported in these studies and only one out of the seven (Lauritzen et al. 1985) showed no significant benefit of tighter glucose control. In sharp contrast, eight randomized controlled trials of patients with type 2 diabetes have produced by far less striking results with a maximum of 7 % neuropathy reduction (Azad et al. n.d.; Kawamori and Kamada 1991; Ohkubo et al. 1995; Tovi et al. 1998; UK Prospective Diabetes Study Group (UKPDS) 1998; Shichiri et al. 2000; Gaede et al. 2003; Duckworth et al. 2009; Ismail-Beigi et al. 2010). Among these trials, however, three out of four studies that investigated nerve conduction or quantitative sensory testing showed significant results in favor of glucose control (Kawamori and Kamada 1991; UK Prospective Diabetes Study Group (UKPDS) 1998; Shichiri et al. 2000). Therefore, despite the disparate impact on type 1 or type 2 diabetes, glucose control constitutes the only disease-modifying therapy available for diabetic neuropathy so far (Said 2007; Callaghan et al. 2012). However, the treatment of diabetes with insulin can by itself cause neuropathy (Gibbons and Freeman 2010). Treatment-induced neuropathy is usually associated with pain and autonomic dysfunctions, but symptoms may improve significantly with time (Gibbons and Freeman 2010).

As described above, a number of pathophysiological events occur upon diabetes and neuropathy likely results as a combination of direct axonal and/or Schwann cell injury due to hyperglycemia, dyslipidaemia, insulin deficiency/resistance and microvascular dysfunction leading to ischemia. Furthermore, perturbed metabolic pathways induce oxidative stress and the accumulation of toxic AGEs. The development and use of animal models of diabetes enabled preclinical therapeutic testing based on recognized steps in the diabetic pathophysiology (Singleton and Smith 2012). Although several promising results were derived from cell culture and experimental therapeutic approaches in animal models, largely no rational treatment has significantly proven effects at reversing or slowing neuropathy progression in patients (Singleton and Smith 2012). Multiple trials with vasodilatory agents showed no clinical response (Coppey et al. 2006), nor did nerve growth factor treatment (Apfel et al. 2000). Also trials with aldose reductase inhibitors, which prevent excessive glucose entry into the polyol pathway thereby reducing oxidative stress turned out to be negative in humans after promising results in animal models (Hotta et al. 2006; Chalk et al. 2007).

Alpha lipoic acid, acetyl-L carnitine and benfotiamine are three related drugs which act to reduce oxidative stress, a key component of diabetic neuropathy

(Singleton and Smith 2012). Treatment with alpha lipoic acid has been shown to promote neuropathic pain relief but not improvement in other neuropathy measures (Ziegler et al. 2004, 2006). Acetyl-L carnitine, however, another antioxidant, has shown significant improvement in sural nerve morphology and visual analog pain scale (VAS) in two parallel randomized, blinded controlled trials in diabetic patients (Sima et al. 2005). Strikingly, the antioxidant benfotiamine (*S*-benzoylthiamine *O*-monophosphate), a vitamin B1 derivative, showed neuropathic improvement in a clinical phase III trial (Stracke et al. 2008). Taken together, these trials underscore the impact of oxidative stress and antioxidants constitute promising drugs to stop or reverse pathological processes in diabetic neuropathy in the future.

19.2.3 Treatment of Hereditary Demyelinating Neuropathies

There is no specific treatment option available for any genetic neuropathy so far (Pareyson et al. 2011). Pharmacological approaches with ganglioside, creatine and very recently, oral administration of ascorbic acid showed no beneficial effects in patients with CMT subtype 1A (Young et al. 2008; Burns et al. 2009; Micallef et al. 2009; Verhamme et al. 2009; Pareyson et al. 2011). To date, next to genetic counseling for diagnosis, symptomatic therapy is restricted to physical therapies, orthopedic treatments (e.g., for foot deformity) and pain as well as fatigue management (Reilly and Shy 2009).

19.2.3.1 Symptomatic Treatment of Patients with CMT

Since there is no drug therapy for CMT available yet, symptomatic therapeutic management requires multidisciplinary approaches including, among others, neurologists, orthopedists, surgeons and physiotherapists (Pareyson and Marchesi 2009). Rehabilitative studies have shown that moderate exercise leads to improved walking ability and lower limb strengthening (Lindeman et al. 1995; Chetlin et al. 2004; El Mhandi et al. 2007; Young et al. 2008). Custom fitted ankle-foot orthoses can be of help and are commonly used to overcome foot drop, thereby facilitate walking (Burns and Ouvrier 2006; Carter et al. 2008).

Surgical interventions have especially been used to medicate skeletal deformities, in particular pes cavus deformity (Beals and Nickisch 2008; Ward et al. 2008). Possible treatments comprise soft-tissue surgery (tendon transfers and releases), osteotomies and joint fusions (Beals and Nickisch 2008; Ward et al. 2008). There are, however, still no clear guidelines available defining the indication for foot surgery in patients with CMT (Pareyson et al. 2011). Furthermore, 15–20 % of patients with CMT suffer from substantial scoliosis and, in most severe cases, require surgical treatment (Horacek et al. 2007; Karol and Elerson 2007).

Posture abnormalities and foot deformities as well as the neuropathy itself can additionally be causative for emerging pain (Carter et al. 1998; Padua et al. 2008)

which can be treated pharmacologically (Burns and Ouvrier 2006; Shy 2006b; Carter et al. 2008; Herrmann 2008). Reduced muscle strength and possibly impaired cardiopulmonary function can further lead to fatigue (Schillings et al. 2007; Kalkman et al. 2008). The treatment of four patients with CMT with the analeptic drug modafinil to address fatigue has shown some improvement, unfortunately associated with substantial side effects (Carter et al. 2006).

19.2.3.2 Antagonizing the Progesterone Receptor Lowers Toxic Pmp22 Overexpression and Ameliorates the Clinical Phenotype of CMT Rats

One obvious therapeutical strategy in CMT1A is lowering the toxic overexpression of the *PMP22* gene. This notion was derived from *Pmp22* transgenic rats which show high variability in the levels of *Pmp22* mRNA expression and the severity of the clinical phenotype (Sereda et al. 1996; Niemann et al. 1999). Importantly, the *Pmp22* mRNA expression in peripheral nerves of CMT rats does not correlate with the disease severity at a given time point. However, expression levels may play an important role early in the disease course as *Pmp22* mRNA serves as a prognostic marker in CMT rats and may be used to predict the future disease course (Fledrich et al. 2012a). In the quest for a target which may (co)regulate *PMP22* mRNA expression, we focused on the sexual hormone and neurosteroid progesterone. The progesterone receptor (PR) is expressed in Schwann cells and application of progesterone resulted in activation of the *Pmp22* gene *in vitro* (Désarnaud et al. 1998) and *in vivo* (Melcangi et al. 1999). We hypothesized that antagonizing the PR may in turn reduce *Pmp22* overexpression and therefore could positively influence CMT1A disease. Indeed, therapeutic application of the PR antagonist Onapristone over 7 weeks starting early postnatally ameliorated the neuropathic phenotype in male CMT rats by reducing the toxic overexpression of *Pmp22* mRNA (Sereda et al. 2003). Onapristone also prevented axonal loss in a long-term study using female CMT rats when treatment was started at 4 weeks of age, similar to the age when CMT1A patients present in the clinic in young adolescence (Meyer Zu Hörste et al. 2007b). Unfortunately, Onapristone displayed side effects in humans (e.g., liver function test abnormalities) and is not available. We are therefore currently examining the effect of a new PR antagonist which is safe for humans. Our studies with PR antagonists demonstrate that targeting the toxic overexpression of the *PMP22* gene is a promising rational to treat CMT1A.

19.2.3.3 Vitamin C Treatment Improves Pathology in Severely Affect Pmp22 Transgenic Mice but has no Therapeutic Effect in Patients with CMT1A

Vitamin C (ascorbic acid) is an antioxidant drug and is required for myelinating Schwann cells in order to form extracellular matrices and basal laminae *in vitro* (Podratz et al. 2004). In severely affected *Pmp22* transgenic mice (C22 het line),

weekly oral application of ascorbic acid reduced *Pmp22* mRNA overexpression, lowered the number of severely hypomyelinated axons, improved the motor performance and increased the life span of mice (Passage et al. 2004). These results from the *Pmp22* transgenic mouse model and the simple translation of ascorbic acid, an over the counter (OTC) drug, to patients with CMT1A launched a number of large scale multi-center trials (Burns et al. 2009; Micallef et al. 2009; Verhamme et al. 2009; Pareyson et al. 2011, Lewis et al. 2013). Unfortunately, none of these clinical trials reported beneficial effects of ascorbic acid to patients with CMT1A thus far. Nonetheless, small effects may have been missed due to insensitivity of outcome measures in CMT1A patients with CMT1A (Pareyson et al. 2011).

19.2.3.4 Addressing Axonal Survival in CMT1A

Loss of peripheral axons ultimately determines the disease severity in patients with CMT1A (Berciano et al. 2000). Rescue of axonal degeneration as well as supporting their regeneration constitutes a therapeutic strategy in CMT1A. This view is supported by the notion that *Pmp22* transgenic rats showed axonal preservation when crossbred to rats harboring the naturally occurring Wld^s (Wallerian degeneration slowing) mutation (Meyer Zu Hörste et al. 2011). Neurotrophin-3 (NT-3) has been reported to promote nerve regeneration after injury and the survival of Schwann cells (Meier et al. 1999). Therapeutic application of NT-3 in immune incompetent mice with xenograft transplants of sural nerve biopsies from CMT1A patients with CMT1A (as well as from *Trembler-J* mice) augmented axonal regeneration (Sahenk et al. 2005). Improved regeneration and remyelination after experimental acute nerve injury in *Trembler-J* mice, mostly of small caliber axons, was achieved after subcutaneous injection of recombinant NT-3 three times a week over 8 weeks. This observation was translated to patients with CMT1A patients and a clinical pilot study was performed (Sahenk et al. 2005). In a small group of 8 patients with CMT1A patients NT-3 treatment was well tolerated when performed three times weekly over a 6-month time period. Treated patients with CMT1A displayed an increase in myelinated fiber density, a reduction of the neurological impairment score (NIS) as well as improved sensory modalities when compared to placebo controls (Sahenk et al. 2005). However, considering the small number of patients, these results need to be confirmed in larger cohort studies.

19.2.3.5 Approaching Low Grade Inflammation as a Therapeutic Rational in CMT1A and CMTX

Experimental studies have demonstrated that macrophages and T lymphocytes are a pathological feature of mice carrying mutations in myelin proteins (Schmid et al. 2000; Berghoff et al. 2005; Kobsar et al. 2005; Fischer et al. 2008; Martini et al. 2008). Genetic suppression of T- and B-lympocyte function using RAG1-deficient mice ameliorated the demyelinating phenotype in CMT type X (GJB1 deficient)

mice whereas it worsened disease progression in CMT type 1B (MPZ deficient) mice (Kobsar 2003; Berghoff et al. 2005). Low copy *Pmp22* transgenic mice (C61 het line) display no pathological amelioration when cross bred with *Rag-1* mutants, indicating that lymphocytes are not disease modifying in this CMT1A model (Kohl et al. 2010b). However, ablation of the chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) as well as of the colony stimulating factor (CSF-1) in GJB1 deficient mice reduced macrophage abundance and ameliorated the neuropathological features in the respective double mutants (Groh et al. 2010, 2012). Thus, MCP-1 and CSF-1 may act as macrophage attractants which in turn may aggravate neuropathological processes in GJB1 deficient mice. Increased abundance of macrophages in the endoneurial compartment of peripheral nerve has also been reported in C61 het mice as well as in the CMT rat (Kobsar et al. 2005; Wessig et al. 2008; Kohl et al. 2010a). Accordingly, crossbreeding of the C61 het line to *Mcp-1* null mutants resulted in a reduced macrophage abundance in peripheral nerves and in a remarkable amelioration of the neuropathic phenotype (Kohl et al. 2010a).

19.2.3.6 Restoring Missorted Proteins in PMP22 Related Neuropathies

Under normal conditions, PMP22 protein folding is only moderately effective and approximately 80 % of the newly synthesized protein is degraded by the proteasome (Pareek et al. 1997; Notterpek et al. 1999; Sanders et al. 2001). In Schwann cells of *Trembler-J* mice the protein degrading pathways of the lysosome and proteasome are overloaded and abnormal cytosolic aggregates containing mutant (misfolded) PMP22 and ubiquitin are formed (Notterpek et al. 1999; Ryan et al. 2002). These aggregates (aggresomes) are found in both, *Pmp22* mutants carrying point mutations (*Tr-J*) and high copy number *Pmp22* overexpressing mice (C22 line) (Notterpek et al. 1999; Fortun et al. 2006). Autophagy emerged to play an important role in the removal of aggresomes (Fortun et al. 2003) and its induction, either by nutrient deprivation or via pharmacological activation by rapamycin, resulted in aggresome degradation in Schwann cells of *Pmp22* mutant and overproducing mice (Fortun et al. 2007; Madorsky et al. 2009; Rangaraju et al. 2010). A further promising therapeutic rational for protein misfolding disorders involves the enhancement of chaperone expression (Muchowski and Wacker 2005). Inhibition of heat shock protein 90 (HSP90) by geldenamycin effectively enhanced cytosolic chaperone levels and improved myelination, along with the trafficking of PMP22 in dorsal root ganglion explant cultures from C22 het neuropathic mice (Rangaraju et al. 2008). Supporting the correct protein folding and turnover of PMP22 may therefore constitute a promising therapeutic strategy upon strong *PMP22* overexpression. Unfortunately, dramatic caloric restriction is not suitable for patients with CMT. For both drugs, rapamycin and geldanamycin derivatives, preclinical therapy studies have not been performed yet.

Aggregates containing point mutated PMP22 have also been reported to be located and retained in the ER of the *Trembler-J* (CMT1E) mice which induces

Schwann cell death via apoptosis(Khajavi et al. 2007). Treatment with the curry spice compound curcumin may facilitate translocation of misfolded protein from the ER to the plasma membrane, subsequently reducing cytotoxicity (Egan et al. 2004; Teijido et al. 2004; Khajavi et al. 2005; Yang et al. 2005). Importantly, in *Trembler-J* mice oral administration of curcumin not only reduced ER retention and cytotoxicity of mutant PMP22 protein, but also mitigated their neuropathic phenotype in a dose dependent manner. On the histological level, axonal size and myelin sheath thickness were improved upon curcumin diet (Khajavi et al. 2007). Besides its effect of facilitating the release of ER retained proteins, curcumin may also support axonal survival via its potential as a neuroprotective agent (Cole et al. 2007). Given the low toxicity profile of curcumin, this treatment of CMT1E may be well translatable to affected patients.

19.2.3.7 Therapeutic Targets in MPZ Related Neuropathies

Similar to PMP22 mutant protein, ER retention and cytotoxicity was also reduced by curcumin treatment in HeLa cells when transfected with *S83del* mutant forms of *MPZ* (Khajavi et al. 2005). Furthermore, in ER stressed *MpzS63del* transgenic-mice Schwann cells display a consequential canonical unfolded protein response (UPR), including the expression of the transcription factor C/EBP homologous protein (CHOP), a protein previously reported to induce apoptosis in ER stressed cells (Pennuto et al. 2008; Zinszner et al. 1998; Rutkowski et al. 2006; D'Antonio et al. 2009). Importantly, genomic ablation of *Chop* in *MpzS63del* mice completely rescued motor impairments and reduced demyelination (Pennuto et al. 2008). The UPR in these mice is associated with detrimental attenuation of the translational machinery mediated by the phosphatase GADD34, a downstream effector of CHOP (D'Antonio et al. 2013). Genetic and pharmacological inhibition of GADD34 in mutant Schwann cells *in vitro* and *in vivo* leads to a reset of the perturbed translational homeostasis, ultimately resulting in striking amelioration of protein accumulation, demyelination and neurophysiological dysfunction in *MpzS63del* transgenic mice (D'Antonio et al. 2013). Therefore, targeting the UPR and the translational machinery may provide new possible therapeutic interventions for ER stress related inherited neuropathies.

19.2.3.8 Biomarkers Could Improve the Development of a Therapy for CMT

Despite its monogenetic cause, patients with CMT1A display a marked interindividual variability of disease severity. The underlying reason for this variability is largely unknown and epigenetic factors have been discussed (Pareyson et al. 2009). At present, the assessment of the individual disease severity in patients with CMT1A is performed solely by clinical and electrophysiological examinations. The CMT neuropathy score (CMTNS) is a nine item composite scale taking

into account sensory and motor symptoms (Shy et al. 2005) and is currently being applied as primary outcome measure in clinical trials (Reilly et al. 2010). The CMTNS ranges from 1 (good clinical performance) to 36 (severely affected) and was reported to increase only about 0.68 points per year in patients with CMT1A (Shy et al. 2005). An even slower progression was reported within a recent therapy trial with ascorbic acid (0.25 points per year) (Pareyson et al. 2011). In light of the slow disease progression, insensitive outcome measures may increase the risk of false negative results in clinical trials and biomarkers could add powerful tools to monitor therapeutic effects (Pareyson et al. 2011; de Visser and Verhamme 2011). Biomarkers may not only serve as a sensitive surrogate marker of clinical severity, but may also identify responders to a putative therapy. CMT rats recapitulate the striking disease variability observed in patients with CMT1A. In a proof of principle study we have demonstrated that the expression levels of selected genes in sciatic nerve and skin tissue can be utilized to measure and predict the disease severity in CMT rats. Importantly, we validated these disease severity markers in skin biopsies of 46 patients with CMT1A (Fledrich et al. 2012a). At the moment, these markers are examined with regard to disease progression within a large pan-European consortium. In the near future we hope to provide the clinical practice with applicable biomarkers which in turn may accelerate the development of a therapy for CMT1A.

19.3 Summary

Within the last decades, substantial progress has been made in the characterization and diagnosis of peripheral neuropathies. Especially, numerous new genes causing hereditary neuropathies have been described, with more than 900 identified mutations in total (Fledrich et al. 2012b). Clinical symptoms, however, may be similar, regardless of whether an immune-mediated process, diabetes or a genetic alteration is the underlying factor. Moreover, diseases can overlap, with for instance a superimposed CIDP or DNP in primarily hereditary neuropathies (Rajabally et al. 2000; Ursino et al. 2013). Hence, deciphering the right diagnosis remains challenging in many cases, pointing to the demand for new diagnostic tools such as biomarkers (Fledrich et al. 2012a, b).

Whereas the onset of different neuropathy types is related to the respective primary cause, the ultimate clinical deterioration is invariably caused by axonal loss. A shared final pathway, common for all neuropathies, may therefore implicate similar underlying mechanisms. Thus, identifying the molecular processes leading to axonal dysfunction and breakdown is one major challenge in the future, which would pave the way for new therapeutic strategies applicable for a large spectrum of neuropathy patients independent of the primary defect.

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Chapter 20
Satellite Glial Cells as a Target for Chronic Pain Therapy

Menachem Hanani and David C. Spray

Abstract Sensory ganglia are essential for the transmission of sensory signals from the periphery to the central nervous system, and abnormal electrical activity in sensory neurons is a major factor in chronic pain. The main type of glial cells in these ganglia are satellite glial cells (SGCs), which surround the sensory neurons tightly. Recently it became evident that SGCs are altered profoundly after nerve injury and that they contribute to chronic pain by influencing neuronal activity. We review the biochemical, pharmacological and physiological changes that SGCs undergo in a variety of pain models, which include: augmented expression of glial fibrillary acidic protein and of proinflammatory cytokines, increased gap junctions, and reduced expression of inward rectifying K^+ channels type K4.1. We discuss how some of these changes can lead to abnormal interactions between SGCs and neurons, and to chronic pain. We propose that these interactions may be utilized as targets for pain therapy. A most attractive option is to reduce the propagation of intercellular calcium waves in the ganglia by agents that inhibit signaling via gap junctions and purinergic P2 receptors.

Keywords Chronic pain · Satellite glial cells · Sensory ganglia · Gap junctions · Purinergic receptors · Cytokines · Glutamate · GABA · Pannexin1 · Pain · Sensory

20.1 Sensory Ganglia and Pain

Chronic pain is a major public health problem with a prevalence of about 20% in the adult population (Breivik et al. 2006). Under the term "chronic pain" there is a wide range of pain disorders such as sciatica, back ache, phantom limb pain,

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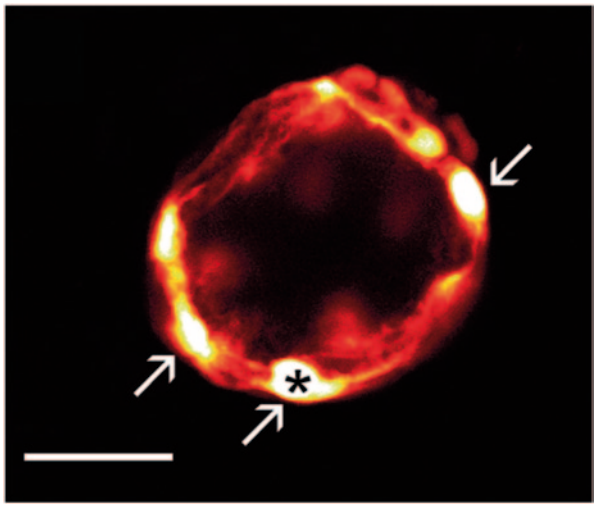

Fig. 20.1 Satellite glial cells (SGCs), shown by confocal microscopy. A single SGC (*) was injected with the fluorescent dye Lucifer yellow, and the dye passed via gap junctions to neighboring SGCs that make an envelope around an unlabeled sensory neuron (*center*). Some of the SGCs are indicated with *arrows*. Guinea-pig DRG. Calibration bar, 50 μm

postherpetic pain, migraine, irritable bowel, and trigeminal neuralgia. For many of the patients suffering from chronic pain analgesic treatment cannot be achieved, and the development of effective pain therapy is one of the main challenges in biomedicine.

An important station in the pain pathways are sensory ganglia, which contain the somata of sensory neurons, and satellite glial cells (SGCs) that tightly surround them (Fig. 20.1) (Hanani 2005, 2012; Huang et al. 2013; Jasmin et al. 2010; Pannese 2010). The best studied sensory ganglia are the dorsal root ganglia (DRG), which innervate most of the body, but considerable information is also available on trigeminal ganglia (TG), which innervate the face, mouth and head. In numerous studies on various pain models in animals it was found that neurons in sensory ganglia are hyperexcitable and are spontaneously active (ectopic activity). Also, there is strong evidence for ectopic firing in primary afferents in patients suffering from chronic pain (Devor 2006). Thus it has been concluded that the single most important factor in the generation and maintenance of chronic neuropathic pain are *changes in excitability of primary nociceptive afferents* (Devor 2006; Klusáková and Dubový 2009; Na et al. 2000; Sapunar et al. 2012; Scadding and Koltzenburg 2006). Therefore, sensory ganglia are a likely target for research and therapy of chronic pain, especially as, unlike the central nervous system (CNS), they are not protected by an equivalent of the blood brain barrier (Hanani 2005).

20.2 Satellite Glial Cells in Sensory Ganglia

Traditionally, pain therapy focuses on neurons (in particular in the spinal cord), but it has been argued that the frequent failure in the treatment of chronic pain is due to targeting the neurons, whereas a more suitable target might be glial cells (Milligan

and Watkins 2009). Indeed, there is strong evidence that microglia and astrocytes in the spinal cord are major contributors to chronic pain (Inoue and Tsuda 2012; Ji et al. 2013). Less is known regarding glial cells in sensory ganglia, but in light of the importance of sensory ganglia in pain, it is reasonable to assume that these cells, like their spinal cord counterparts, i.e. astrocytes, play a role in pain. The main type of glial cells in sensory ganglia are SGCs, which share many properties with astrocytes, including the expression of glutamine synthetase and various neurotransmitter transporters (for reviews see Hanani 2005; Huang et al. 2013; Jasmin et al. 2010; Takeda et al. 2009). A unique feature of SGCs, which distinguishes them from astrocytes, is that several of them usually form a sheath that surrounds individual sensory neurons completely, thus forming a neuron-SGC functional unit (Hanani 2005; Hanani and Spray 2013; Pannese 2010) (Fig. 20.1). SGCs within this unit are coupled by gap junctions (Cherkas et al. 2004; Hanani et al. 2002). The pharmacology of SGCs is largely unexplored, but it is known that they are endowed with functional receptors for ATP (Ceruti et al. 2008; Huang et al. 2013; Magni and Ceruti 2013; Weick et al. 2003), cytokines (Huang et al. 2013; Takeda et al. 2009), and endothelin (Feldman-Gorianchik and Hanani 2011). The presence of gap junctions and purinergic P2 receptors that respond to ATP is noteworthy as both have been shown to mediate the spread of intercellular calcium waves between astrocytes (Scemes and Giaume 2006) and also between SGCs (Suadicani et al. 2010). SGCs release ATP (Huang et al. 2010; Suadicani et al. 2010) and cytokines (Takeda et al. 2009; Zhang et al. 2007) and possibly other chemical messengers, which enable bidirectional communication between these cells and neurons. SGCs undergo major changes as a result of injury to peripheral nerves, and appear to contribute to chronic pain in a number of animal pain models (Dublin and Hanani 2007; Huang et al. 2010; Ohara et al. 2008; Thalakoti et al. 2007; Takeda et al. 2009).

20.3 Survey on Changes in SGCs After Injury

Tables 20.1, 20.2, 20.3 list changes that various injuries induce in SGCs in sensory ganglia. Although comprehensive, the tables are not complete, and for older literature the reader is referred to previous reviews (Hanani 2005; Pannese 1981). The main message of these tables is that a wide range of injury types leads to consistent effects in these cells. It should be emphasized that following injury, sensory neurons undergo major changes as well, but these are not included in the tables (for reviews see Devor 2006; Dib-Hajj et al. 2010). It is evident from the tables that: (1) Most of our knowledge on SGCs derives from work on rats and mice. These animals are indispensable for basic research, but information on higher species, and especially humans, is missing. As rodent pain models may not exactly reflect human disease, the conclusions from rodent work should be regarded with caution when attempting to extrapolate the conclusions to humans. (2) There is a clear pattern of changes in SGCs across the various models, which include somatic, orofacial, visceral pain, and pain in systemic disease. The most prominent change is the augmented

Table 20.1 Changes in satellite glial cells in DRG following somatic injury

Type of injury	Changes in SGCs	Comment	References
Sciatic N axotomy	GFAP, gap junction upregulation	Rats, mice	Woodham et al. (1989); Hanani et al. (2002)
Sciatic N axotomy	TGF-α and TGF-α receptor upregulation	Rats	Xian and Zhou (1999)
Sciatic N neuritis	Dye coupling increase, gap junctions increase	Mice	Ledda et al. (2009)
Skin scarification	Proliferation	Mice	Elson et al. (2004)
Sciatic N axotomy	Hypertrophy (requires months to develop), proliferation	Rats	Shinder et al. (1999)
Chronic DRG compression	Increased coupling, GFAP upregulation, reduced Kir currents	Rats	Zhang et al. (2009)
DRG inflammation	GFAP and cytokine upregulation	Rats	Siemionow et al. (2009)
Tetanic sciatic N stimulation	GFAP, P2X7 receptor upregulation	Rats	Liang et al. (2010)
Spine injury	GFAP and TNF-alpha upregulation	Rats	Miyagi et al. (2006);
Spinal N crush	GFAP and TNF-alpha upregulation	Rats; ipsi- and contralateral DRG affected	Hatashita et al. (2008)
Non-compressive disc herniation	GFAP upregulation	Rats; ipsi- and contralateral DRG affected	Li et al. (2011a)
Intervertebral disc injury	GFAP upregulation	Rats	Li et al. (2011b)
Plantar skin incision	GFAP upregulation	Mice	Romero et al. (2013)
Paw inflammation	P2X7 receptor upregulation	Rats	Chen et al. (2008)
Paw inflammation	Dye coupling increase	Mice	Dublin and Hanani (2007)
Sciatic N CCI	IL-6 upregulation	Rats, ipsi- and contralateral DRG affected	Dubový et al. (2010)
Periganglionic inflammation	COX-2 upregulation	Rats	Amaya et al. (2009)
Sciatic N ligation, root sections	p75 upregulation	Rats	Zhou et al. (1999)
Sciatic N axotomy	FGF-2 and S100β upregulation	Rats	De-Freitas Azevedo Levy et al. (2007)
Sciatic N crush	FGF-2 upregulation	Rats	Grothe et al. (1997)
Mild sciatic N compression	GFAP upregulation, macrophage infiltration	Rats; carpal tunnel syndrome model	Schmid et al. (2013)
Sciatic N crush	TNF receptor, TNF upregulation	Rats	Ohtori et al. (2004)
Trauma	P2X7 receptors upregulation	Human	Chessell et al. (2005)
Sciatic N. CCI	Cannabinoid type 2 receptor upregulation	Rats; ipsi- and contralateral DRG affected	Svízenská et al. (2013)

Table 20.1 (continued)

Type of injury	Changes in SGCs	Comment	References
Spinal N ligation	GFAP upregulation	Rats	Liu et al. (2012); Xie et al. (2009)
Spinal N ligation	MMP-2 upregulation	Rats	Kawasaki et al. (2008)
Spinal N ligation	HMGB-1 upregulation	Rats	Shibasaki et al. (2010)
Spared nerve injury	GFAP upregulation	Rats	Xie et al. (2009)
Bone cancer	GFAP upregulation	Mice	Peters et al. (2005)

DRG dorsal root ganglion, *ERK* extracellular signal-related kinases, *N* nerve, *p75* the low-affinity receptor for the neurotrophins, *HMGB-1* high mobility group box-1, *CCI* chronic constriction injury, *FGF* fibroblast growth factor, *TNF* tumor necrosis factor, *COX* cyclooxygenase, *IL* interleukin, *ERK* extracellular signal-related kinases,*GFAP* glial fibrillary acidic protein, *P2* purinergic receptor type 2, *LPS* lipopolysaccharide ERK, extracellular signal-related kinases; *MMP-2* matrix metalloproteinase 2 *TGF-a*, transforming growth factor α.

expression of GFAP, which is a well-established marker for astrocyte activation (Pekny and Nilsson 2005). GFAP immunostaining is particularly useful in the case of SGCs, because under resting conditions, GFAP level is very low, whereas it is greatly augmented after injury. GFAP belongs to the family of intermediate filament proteins, and has structural roles in astrocytes; however, the functional significance of its upregulation its obscure (for reviews see Gao and Ji 2010; Middeldorp and Hol 2011). It has been proposed that GFAP upregulation contributes to pain, but firm information is lacking. GFAP-null mice displayed higher degree of neuronal damage than normal mice, suggesting that GFAP has a protective role. A central question is whether GFAP is essential for glial activation, or whether it just accompanies this process. Chudler et al. (1997) commented that although changes in GFAP immunoreactivity in the TG and alterations in neuronal response properties were correlated, the causal relation between these phenomena is still unknown.

Another change in SGCs after a variety of injuries in mice is the increase in gap junctions among SGCs (Figs. 20.2 and 20.3). Much less work has been done on rats, and the only dye coupling study in rats was done on rat DRG (Zhang et al. 2009). There is also immunohistochemical evidence for an increase in connexins expression in rat TG after damage (Garrett and Durham 2009; Ohara et al. 2008). Increased cytokine synthesis is a common feature in SGCs following damage, which may have functional significance, as these mediators can contribute to neuronal excitation.

Our knowledge on the pharmacology of SGCs is limited, particularly in pathological states. SGCs display P2X and P2Y purinergic receptors. In two injury models in mice, it was found that the sensitivity of SGCs to ATP is increased 100-fold (Kushnir et al. 2011). In one of these models (submandibular inflammation) the P2 receptor population appears to switch from P2Y to P2X. Following in vitro inflammation, P2Y receptors are upregulated in SGCs (Ceruti et al. 2008). Katagiri et al. (2012) showed an increase in P2Y12 receptors in rat following lingual inflammation. All these studies were done in TG, and there is only a preliminary study showing an increase in responses to ATP in mouse DRG due to systemic inflammation (Blum et al. 2014). As SGCs are likely to communicate among themselves and also with neurons by chemical messengers, much more work needs to be done

Table 20.2 Changes in satellite glial cells in TG following orofacial injury

Type of injury	Changes in SGCs	Comment	References
Temporo-mandibular joint inflammation	Connexins 26,36,40 upregulation	Rats	Garrett and Durham (2009)
Capsaicin in temporo-mandibular joint	S100B and p38 expression increased	Rats; all divisions of TG affected	Thalakoti et al. (2007)
Submanibular inflammation	P2 receptors upregulation	Mice; calcium imaging study	Kushnir et al. (2011)
Whisker pad inflammation	Kir1.4 downregulation	Rats	Takeda et al. (2011)
Whisker pad inflammation	GFAP, IL-1β upregulation	Rats	Takeda et al. (2007)
Infraorbital N CCI	Connexin 43 upregulation	Rats	Ohara et al. (2008)
Infraorbital N CCI	Kir1.4 downregulation	Rats	Vit et al. (2008)
Infraorbital N CCI	Proliferation	Rats	Donegan et al. (2013)
Single tooth injury	GFAP upregulation in all parts of TG	Rats	Stephenson and Byers (1995)
Inferior alveloar N crush	GFAP upregulation	Rat	Chudler et al. (1997)
Partial infraorbital N ligation	Proliferation, GFAP upregulation	Mice	Xu et al. (2008)
Infraorbital N axotomy	Dye coupling increase, sensitivity to ATP increased	Mice	Cherkas et al. (2004)
Temporo-mandibular joint inflammation	ERK phosphorylation,	Rats	Bi et al. (2013)
Temporo-mandibular joint inflammation	GFAP upregulation	Rats; all divisions of TG affected	Villa et al. (2010a)
Infraorbital N axotomy	P2 receptor upregulation	Mice; calcium imaging study	Kushnir et al. (2011)
Tooth extraction	GFAP upregulation	Rats	Gunjigake et al. (2009)
Tooth pulp inflammation	GFAP upregulation	Rats	Matsuura et al. (2013)
Tooth movement	NGF upregulation	Rats	Kurata et al. (2013)
Lingual N crush	GFAP, P2Y12R upregulation	Rats; all divisions of TG affected	Katagiri et al. (2012)

TG trigeminal ganglion, *Kir* inward rectifying potassium channels, *N* nerve, *CCI* chronic constriction injury, *Kir1.4* inward rectifying potassium channel type 1.4, *IL* interleukin, *GFAP* glial fibrillary acidic protein, *P2* purinergic receptor type 2, *ERK* extracellular signal-related kinases,

on pharmacological changes in SGCs. Although the understanding of SGCs under normal and pathological conditions is rather limited, the available information provides several promising leads to possible pain therapies that could be based on SGC-neuron interactions. Several recent review articles on pain therapy present a rather deplorable situation: there is an extremely wide range of pain types, and there are dozens of mechanisms that seem to be involved (Gold and Gebhart 2010; Ren and Dubner 2010). For example, several types of voltage sensitive Na^+ channel are found in sensory neurons, and different human pain disorders are associated

Table 20.3 Changes in satellite glial cells in DRG or TG following miscellaneous types of injury

Type of injury	Changes SGCs	Comment	References
Chemotherapeutic drugs (systemic)	Dye coupling increase, GFAP upregulation	Mouse DRG	Jimenez-Andrade et al. (2006); Warwick and Hanani (2013)
Systemic LPS	Dye coupling increase, GFAP upregulation, P2 receptors upregulation	Mouse DRG	Blum et al. (2014)
Water avoidance stress	GFAP upregulation	Rat DRG	Golovatscka et al. (2012)
Aging	Dye coupling increased	Mouse DRG	Huang et al. (2006)
Intestinal inflammation or obstruction	Dye coupling increased	Mouse DRG	Huang and Hanani (2005); Huang et al. (2010)
In vitro bradykinin	P2Y receptor upregulation	Cultured mouse TG	Ceruti et al. (2008)
Diabetes model	Aldose reductase upregulation	Rats DRG	Jiang et al. (2006)
Acute morphine	GFAP, IL-1β upregulation	Mouse DRG; acute effects	Berta et al. (2012)
Simian immunodeficiency virus	Virus infection	Monkey DRG; a model for HIV	Burdo et al. (2012)
Antiretroviral drug	Chemokine upregulation	Rat DRG	Bhangoo et al. (2007)
Multiple sclerosis model	Dye coupling increase, GFAP upregulation	Mouse DRG	Warwick et al. (2014)
Diabetes model	GFAP upregulation	Rat, mouse DRG	Hanani et al. (2014)

DRG dorsal root ganglion, *TG* trigeminal ganglion, *IL* interleukin, *GFAP* glial fibrillary acidic protein, *P2* purinergic receptor type 2

with an increase in different Na^+ channels (e.g., Nav1.3), but paradoxically in some cases Na^+ channels are downregulated after injury (e.g., Nav1.7 and 1.8) (Berta et al. 2008; Dib-Hajj et al. 2010). For these types of Na^+ channels, evolution has favored mutations in certain rodents rendering Nav1.7 in the mole rat insensitive to acid (Smith et al. 2011) and Nav1.8 in the grasshopper mouse being inhibited rather than excited by scorpion toxin (Rowe et al. 2013). Numerous biochemical pathways are altered in pain states, and various studies emphasize a given pathway as being central to the pain syndromes, with the exclusion of others. The conclusion of the reviews is that there is little hope of making substantial progress in this field. These conclusions might be indeed justified, but perhaps we should look at this problem from a fresh angle. For example, there is growing evidence that glial cells in the spinal cord can serve as a therapeutic target (Ji et al. 2013). However, targeting the CNS has several limitations, the main one being the presence of the blood brain barrier, which limits the types of drugs that can be used. Secondly, the great structural and pharmacological complexity of the CNS is a serious obstacle. In contrast to the CNS, sensory ganglia are rather simple structurally, and display a very limited neuronal and glial diversity. These ganglia offer additional advantages: there is no blood-ganglion barrier, and as they are an early station in the pain pathways, targeting them is more efficient, as it can prevent the development of central changes.

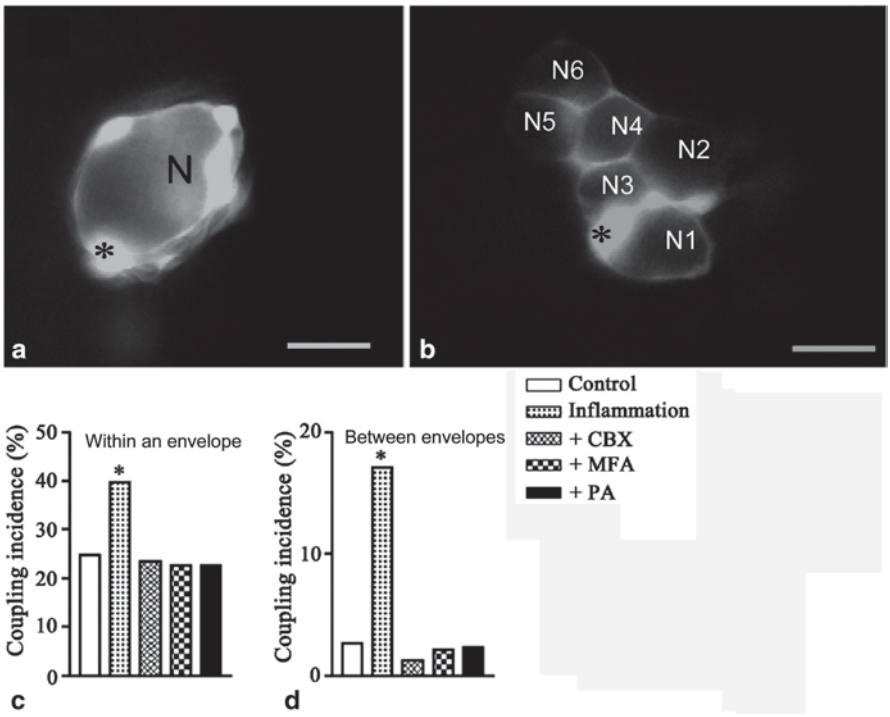

Fig. 20.2 Dye coupling in sensory ganglia is augmented by peripheral inflammation. The fluorescent dye Lucifer yellow (*LY*) was injected into SGCs of S1 DRG ganglia of mice to determine the degree of gap junction mediated coupling between the cells. Dinitrobenzoate sulfate (*DNBS*) was instilled into the large intestine to induce inflammation, and the ganglia were examined in vitro 10–12 days later. **a** LY-injected SGC is coupled to other SGCs only around the same neuron. **b** Dye coupling between SGCs around different neurons (N1-N5), observed in DNBS-treated mouse. The *asterisks* indicate the LY-injected SGCs. Scale bars, 20 μm. The histograms (**c**, **d**) show the effect of gap junction blockers: carbenoxolone (CBX, 50 μM), meclofenamic acid (MFA, 100 μM) and palmitoleic acid (PA, 30 μM), on the augmented coupling among SGCs after inflammation. **c** Incidence of coupling between SGCs around the same neuron. **d** Incidence of coupling between SGCs around different neurons; N = 81–129 for each of the experimental conditions, $p < 0.001$ compared with results obtained after DNBS in the absence of blockers. *$p < 0.01$, N = 17–29. Fisher's exact test. (Modified from Huang et al. 2010)

20.4 Selecting Therapeutic Targets in SGCs

Astrocytes usually share the neurotransmitters and receptors of their neighboring neurons (Verkhratsky 2009). This seems to hold also for SGCs. Thus, it is not easy to target SGCs chemically without also influencing the neurons. Still, some pharmacological differences between SGC and sensory neurons have been found and may be utilized to target SGCs selectively. Another strategy is to aim at abnormal SGC-neuron interactions. Below we list several such possibilities.

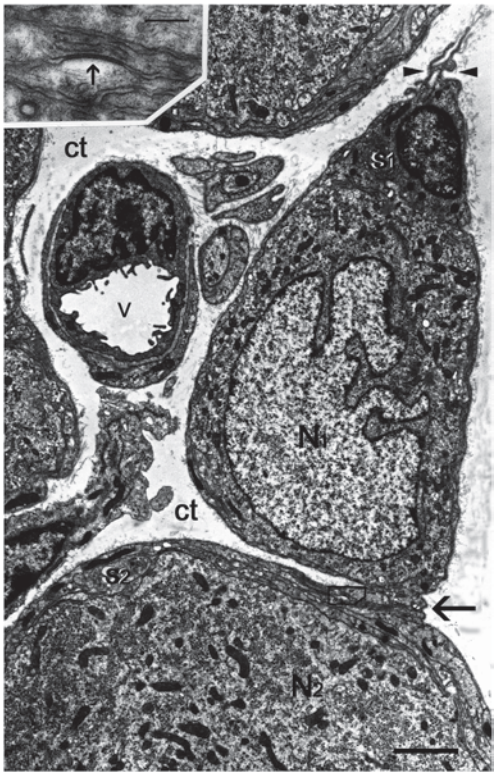

Fig. 20.3 Electron micrograph showing the effect of peripheral inflammation on SGCs. Neuritis of the sciatic nerve caused the formation of a bridge (*arrow*) connecting two SGCs (s1, s2), each part of the sheath around a different neuron (N1, N2). The inset is a high magnification of the boxed area, which is within the bridge formed by the two SGCs. The dark band (indicated by an *arrow*) is a gap junction. On the basis this micrograph alone it cannot be determined that the gap junction is between two different SGCs, because the gap junction can be between different processes of the same SGC. However, the dye coupling results strongly support the conclusion that injury increases SGC-SGC coupling by gap junctions. Minute protrusions (*arrowheads*) emerge from the outer contour of the SGC sheath (s1) of the neuron N1 and project into the connective tissue space (ct). v, blood vessel. Scale bar, 2 μm; inset: scale bar, 0.2 μm

20.4.1 Potassium Channels

The main K^+ channel in astrocytes is the inward rectifying (Kir)4.1 channel, which plays a key role in generating the membrane potential and in regulating the extracellular K^+ concentration, i.e. "K^+ spatial siphoning/buffering". K^+ that accumulates outside neurons during neuronal activity enters the astrocytes *via* Kir4.1 channels and is distributed through the cell (siphoning) or glial syncytium (buffering) to regions where K^+ concentration is low, thus reducing the danger of excessive

neuronal depolarization. These channels are ideally suited for this function because their conductance is larger in the inward than the outward direction and increases when the external K^+ concentration increases. We first identified Kir channels in SGCs from mouse TG (Cherkas et al. 2004), which were later identified as predominantly Kir4.1 (Takeda et al. 2011; Tang et al. 2010; Vit et al. 2008), and were found to be the most abundant channels in SGCs. Although direct evidence for K^+ buffering in SGC is still lacking, it is highly likely that SGCs, like astrocytes, can regulate the external K^+ concentration. Several studies have shown that Kir4.1 are downregulated in sensory ganglia following damage (Takeda et al. 2011; Vit et al. 2008), which is expected to lead to K^+ accumulation in the extracellular space and to neuronal depolarization, causing increased neuronal firing and possibly neuronal damage. It is noteworthy that Kir4.1 is found only in SGCs and not in sensory neurons. Moreover, it was found that these channels are downregulated in TG in two different pain models. In support of this, silencing of Kir4.1 using RNA interference led to spontaneous and evoked facial pain-like behavior in rats (Vit et al. 2008). Therefore manipulations that open these channels have a clear therapeutic value. To our best knowledge currently there is no compound that can open Kir4.1 channel, and a search for such a drug holds considerable promise.

As an aside it is interesting to mention that tricyclic antidepressants, which are known to block Kir4.1 (Su et al. 2007), have clear analgesic actions (O'Connor and Dworkin 2009), which demonstrates the complexity of identifying effective and selective analgesic drugs.

20.4.2 Gap Junctions

A change in SGC that has been found in all the pain models that were examined is an increase in gap junction-mediated coupling (see Tables 20.1, 20.2, 20.3). Several studies have shown that blocking gap junctions with gap junction blockers (e.g., Hanstein et al. 2010; Huang et al. 2010; Thalakoti et al. 2007; Warwick and Hanani 2013) or by neutralizing connexin43 by RNA interference (Ohara et al. 2008), reduced pain behavior in rodents. The mechanisms by which augmented gap junctions contribute to chronic pain are not clear, but it has been suggested that gap junctions, along with P2 receptors, are a major factor in the propagation of intercellular calcium waves, which can mediate the spread of excitation in the ganglia (see next section for further details). A problem with this approach is that gap junction blockers are not highly selective (Spray et al. 2002), and might have additional actions, for example they may act on neurons. A presumed gap junction blocker, tonabersat, was found to be effective in treating orofacial pain (Durham and Garrett 2009; Silberstein 2009), but its precise mode of action is not well established. Certainly, more effort to develop better gap junction blocking drugs is required.

20.4.3 P2 Purinergic Receptors

ATP is recognized as a pain mediator in both the central and peripheral nervous systems, and there have been several reports that blockers of P2 purinergic receptors have analgesic actions (Burnstock and Sawynok 2010; Jarvis 2010). The main P2 receptors in sensory neurons are P2X2,3 and their activation by ATP and related agonists leads to neuronal excitation. P2 receptors in glia appear to have a major role in chronic pain. In the central nervous system, P2X4R receptors in microglia seem to play an important role in chronic pain (Inoue and Tsuda 2012). SGCs are endowed with P2Y and P2X receptors (Ceruti et al. 2008; Villa et al. 2010b; Weick et al. 2003). Of the P2Y receptors, P2Y1,2,4,6,12,13 receptors are present in SGCs in TG. Of the P2X receptors, there is evidence for P2X7R receptors. We showed that in two orofacial pain models the sensitivity to ATP of SGCs in TG increased 100-fold (Kushnir et al. 2011), which appears to be due to a switch of the P2 receptor population from P2Y to P2X. The exact P2X receptor subtype that was augmented was not determined, but candidates are P2X2,4 and 5, but not P2X7R, which is present in SGCs from both pain models and controls. P2X7 receptors are upregulated in SGCs in human DRG after damage (Chessell et al. 2005), and it could be argued that these receptors are involved in neuropathic pain, but it was found that activation of P2X7R receptors in SGC in rat DRG leads to reduced expression of P2X2,3 receptors in the neurons, which will reduce ATP-mediated excitation of the neurons (Gu et al. 2010; Huang et al. 2013). Thus the contribution of these receptors is likely to depend on the balance between their two opposing effects (Huang et al. 2013). P2X7 receptors are of interest because they are associated with the opening of pannexin1 channels (see below).

Intercellular calcium waves, which are mediated by gap junctions and P2 receptors, are likely to be an important means for signaling in sensory ganglia (Suadicani et al. 2010). These two mechanisms are upregulated after injury, and therefore they are potentially ideal targets for pain therapy (see Fig. 20.4). Currently, no highly selective blockers for either gap junctions and to most P2 receptors are available, and further research in this direction is highly promising.

20.4.4 Glutamate Receptors

It has been long established that sensory neurons possess glutamate receptors (Puil and Spigelman 1988), and that SGCs have glutamate plasma membrane transporters (Hanani 2005). However, it has been reported only recently that glutamate receptors are also present in SGCs in both TG and DRG of rats (Kung et al. 2013). Laursen et al. (2014) reported that SGCs in rat TG contain glutamate and that depolarization evoked glutamate release from them was perhaps mediated by a reversal of the plasma membrane transporter as suggested by using transporter blockers, but see Wagner et al. (2014). They also reported that glutamate excited the neurons via

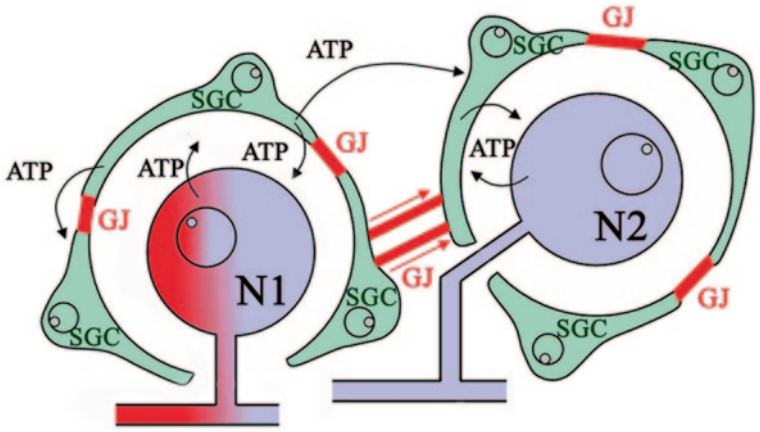

Fig. 20.4 Schematic diagram describing the structural and functional relations between SGCs and neurons in sensory ganglia, and the consequences of peripheral injury (axotomy, inflammation, etc). The axon of neuron 1 (N1) had been exposed to local injury, which causes various changes in the neuronal soma. One such change is increased firing of action potentials, which induces ATP release from the neuron (Huang and Neher 1996) and activates P2 receptors on SGCs surrounding the neuron, and on the neuron itself, causing an increase in $[Ca^{2+}]i$ in these cells. Because of the enhanced sensitivity of SGCs to ATP (Kushnir et al. 2011), this action will augmented, and the higher level of $[Ca^{2+}]i$ will lead to a greater release of ATP from the cells (Suadicani et al. 2010). ATP released from the SGCs and neuron, in combination with the greater communication by gap junctions (GJs) following injury, will cause an augmented spread of Ca^{2+} waves to a neighboring neuron (N2, which was not affected directly by the injury), and its surrounding SGCs (see Suadicani et al. 2010). The Ca^{2+} waves activate SGCs, and the overall effect will be augmented neuronal excitation. This model can explain how peripheral injury can increase excitability of a large number of sensory neurons, which may contribute to chronic pain

N-methyl D-aspartate (NMDA) receptors, and concluded that dysfunctional SGCs might contribute to migraine. NMDA receptors were also found in SGCs in rat DRG (Castillo et al. 2013). It should be added that work on mice did not show response to glutamate in SGCs in mouse TG (Kushnir et al. 2011; Weick et al. 2003), indicating possible species differences. Thus, more work on this topic is needed, but if indeed glutamate mediates SGC-neuron communications and contributes to abnormal neuronal activity, glutamate receptors could be considered as therapeutic targets. It should be kept in mind that glutamate receptors are extremely abundant in the CNS, and therefore ganglion selective drugs will be required.

20.4.5 GABA Receptors

γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the central nervous system, and GABA receptors have been identified in sensory neurons (White 1990). Vit et al. (2009) transfected glutamic acid decarboxylase (GAD, which synthesizes GABA) gene into SGCs of rat TG, which led to GABA expression in SGCs. They

found that in the infected animals behavioral pain responses were reduced; thus, GABA release from SGCs may have therapeutic value. The release mechanism of GABA was not studied. It was reported that GABA hyperpolarizes rat sensory neurons (Takeda et al. 2004), but another group showed that it depolarizes the neurons in rat DRG (Zhu et al. 2013), as also observed by us in mouse DRG (unpublished) DRG. Such an excitatory effect of GABA is observed frequently in central neurons of young animals due to intracellular chloride levels. However, in a later study (Takeda et al. 2013) it was shown that indeed the inhibitory action of GABA on TG. neurons may be indirect—activation of $GABA_B$ receptors in the neurons inhibits the excitatory effect of ATP that is mediated by P2X3 receptors in the neurons. The authors suggest that $GABA_B$ agonists (e.g., baclofen) have a potential for treating trigeminal nociception. Although there is no evidence that SGCs release GABA, the significance of the work of Vit et al. (2008) is in demonstrating the feasibility of inducing the expression in SGCs of molecules that can influence neuronal activity.

20.4.6 Pannexin1

As discussed above, there is evidence that SGCs can release ATP and other substances that can affect both neurons and SGCs. The release mechanism of ATP is not clear yet, but release via large channels (pannexin1 and P2X7 receptors) is a valid possibility. Pannexin1 (Panx1) can be activated by P2X7 receptor stimulation, allowing ATP release (Pelegrin and Surprenant 2006; Iglesias et al. 2009). It has been proposed that opening Panx1 channels following P2X7 receptor activation mediates ATP release from SGCs (Huang et al. 2013). We have observed Panx1 immunoreactivity in SGCs in mouse TG and found reduced orofacial hypersensitivity in Panx1 null mice subjected to submandibular CFA injection (Hanstein et al. 2013).

20.4.7 Anti-inflammatory Drugs

In most pain models the injury is directed at the periphery (e.g., axotomy or local inflammation of DRG axons). However, it was found that in these models inflammatory changes take place in the sensory ganglia, as evidenced by the infiltration of macrophages and lymphocytes into the ganglia and even into the space between SGCs and neurons (Hu and McLachlan 2002; Schmid et al. 2013). This is consistent with the elevation of the levels of various inflammatory mediators in the ganglia after peripheral injury (e.g., Takeda et al. 2009). These findings lead to several interesting questions: (1) What is the mechanism underlying this inflammatory response? (2) How are SGCs involved in this effect? (3) Does this response contribute to chronic pain and if so, can the inflammation and pain be reduced by anti-inflammatory drugs? The last question has been addressed by Li et al. (2011c), who induced pain in rat by spinal nerve ligation and examined pain behavior and changes in DRG. They found that a single injection of a corticosteroid near the

affected DRG, attenuated mechanical sensitivity and reduced SGC activation (as assayed by GFAP upregulation). The authors concluded that inflammation at the DRG level may play an important role in the mechanical pain induced by spinal nerve ligation. It is still unknown how the corticosteroid acted, but it can be speculated that at least partly, its effect is mediated by SGCs. Clearly, further investigation of the immune responses of SGCs can be highly rewarding for both understanding and treating chronic pain.

Conclusions

The account above shows that changes in SGCs are ubiquitous in pain models and that these cells are very likely to be involved in chronic pain. In analogy with the CNS, neuron-SGC interactions in sensory ganglia may play a key role in the function of the ganglia under normal and pathological conditions. As sensory ganglia are a major source of abnormal neuronal activity in chronic pain, understanding these interactions is of great importance. The absence of nerve-ganglion barrier makes sensory ganglia a target of choice for pain therapy. Further progress in the pharmacology and physiology of SGCs is required, and this field, which has attracted increasing attention in recent years, looks highly promising.

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Chapter 21
Enteric Glial Cells: Implications in Gut Pathology

Brian D. Gulbransen and Isola A. M. Brown

Abstract Gastrointestinal (GI) diseases are common, debilitating and all too often, humiliating disorders. Many of the most devastating, and permanent symptoms of GI disease are caused by alterations to the enteric nervous system (ENS); the local neural network that lines the walls of the intestines and controls moment-to-moment gut functions. The ENS is by far the largest, and most complex, component of the peripheral nervous system and the intrinsic neural circuitry within the ganglionated enteric neural networks can control most gut functions in the absence of input from the central nervous system (CNS). This "little brain" in the gut is more similar in size, complexity and autonomy to the CNS than other components of the autonomic nervous system. Like the CNS, this "gut brain" has its own distinct population of glia, called enteric glia, that are analogous to the astrocytes of the CNS. Recent evidence implicates enteric glia in almost every aspect of gastrointestinal physiology and pathophysiology but elucidating the exact mechanisms by which enteric glia influence gastrointestinal physiology and identifying how those roles are altered during gastrointestinal pathophysiology remains elusive. This chapter focuses on our current understanding of enteric glial physiology and how enteric glia contribute to, or are altered by, pathological events in the gut.

Keywords Gastrointestinal diseases · ENS · Enteric gliocyte · Enteric glia · Pro-inflammatory cytokines · Inflammatory mediators · IBD · Crohn's · *Necrotizing Enterocolitis* · Parkinson's · Diabetes

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21.1 Introduction

Gastrointestinal (GI) diseases are common, debilitating and all too often, humiliating disorders that encompass a spectrum of conditions affecting the alimentary canal, hepatobiliary system and pancreas. Treatment of GI diseases is difficult because GI disorders arise from multiple causes ranging from congenital and genetic abnormalities to acute and chronic infections, exposure to environmental factors such as drugs and toxins, and altered homeostasis with the gut microbiome. Often, GI disorders arise from idiopathic, or unknown origins. Some GI diseases, such as the inflammatory bowel diseases, Crohn's and ulcerative colitis, are primarily localized to the GI tract. Yet GI impairments are common complications of many disorders and can be more debilitating than the primary disease. For example, in type 2 diabetes, blood sugars can be monitored and effectively managed but GI complications associated with the disease can produce nausea, vomiting, abdominal pain, constipation and diarrhea that are difficult to resolve. Likewise, quality of life may not be significantly diminished by resting tremor in a hand or foot in an individual with Parkinson's but inability to control bowel movements is devastating. By interfering with digestion, these diseases interfere with the most fundamental physiological process essential for life. Current drugs used to combat gastrointestinal diseases are often not effective in all patients, carry serious side effects and are costly. Thus, novel drug targets to combat GI disease are desperately needed.

Many of the most devastating, and permanent symptoms of GI disease are caused by alterations to the enteric nervous system (ENS); the local neural network that lines the walls of the intestines and controls moment-to-moment gut functions. The ENS is by far the largest, and most complex, component of the peripheral nervous system and the intrinsic neural circuitry within the ganglionated enteric neural networks can control most gut functions in the absence of input from the central nervous system (CNS) (Furness 2012). In fact, this "little brain" in the gut is more similar in size, complexity and autonomy to the CNS than other components of the autonomic nervous system so it should not be surprising that this "gut brain" has its own distinct population of glia, called enteric glia, that are analogous to the astrocytes of the CNS.

The term enteric glia actually encompasses several morphologically, and likely functionally distinct subtypes of peripheral glia in various microenvironments throughout the thickness of the gut wall and along the length of the gastrointestinal tract (Gulbransen and Sharkey 2012). Recent evidence implicates the various populations of enteric glia in almost every aspect of gastrointestinal physiology and pathophysiology but elucidating the exact mechanisms by which enteric glia influence gastrointestinal physiology and identifying how those roles are altered during gastrointestinal pathophysiology remains elusive. This chapter focuses on our current understanding of enteric glial physiology and how enteric glia contribute to, or are altered by, pathological events in the gut.

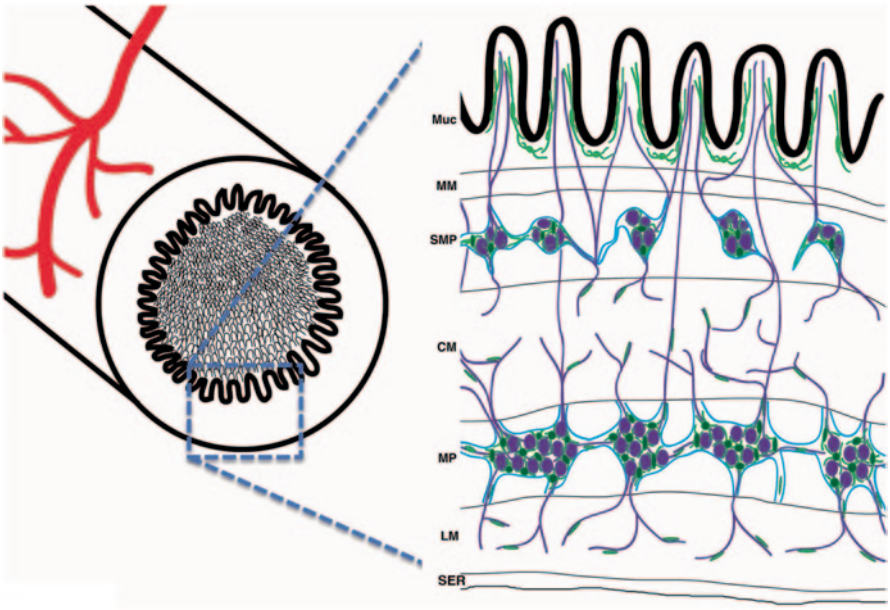

Fig. 21.1 Schematic representation of the unique environments within the gut wall. The innermost, toward the center of the gut tube, is the mucosa (*Muc*) with a thin muscle layer, the muscularis mucosa (*MM*) just below. Enteric ganglia are located within the submucosal (*SMP*) and myenteric (*MP*) plexuses which are separated by a thick layer of circular muscle (*CM*). Longitudinal muscle (*LM*) coats lie between the MP and the outer serosal (*SER*) surface of the gut

21.2 The Enteric Nervous System: A Brain in the Gut to Regulate Gastrointestinal Function

While this chapter is not meant to constitute a comprehensive description of ENS regulation of gut function, a brief overview of the cellular organization of the gut wall and functional ENS circuitry would be beneficial to appreciate the following sections discussing of the roles of enteric glia in gut physiology and pathophysiology.

In the most basic sense, the thickness of the gut wall can be separated into several distinct cellular signaling environments composed of mucosa, smooth muscle or nerve plexuses (Fig. 21.1) The mucosal compartment primarily functions to form a selective barrier to luminal substances and coordinate secretory and absorptive functions. A large component of the immune system resides here and the importance and complexity of the dynamic interactions between the gut immune system and microbes in the gut lumen are only recently becoming apparent (Markle et al. 2013; Garrett et al. 2010). Sensory transduction begins in the mucosal compartment and chemical and mechanical cues originating in the gut lumen drive intrinsic enteric neurocircuitry and programmed gut functions.

The cell bodies of enteric neurons lie within the submucosal and myenteric plexuses and primarily function to coordinate gut secretion/absorption and motor activity, respectively. The neurochemical coding and functions of enteric neurons are diverse and integration within the enteric nervous system by enteric sensory, inter- and motorneurons provides all the neurocricuitry necessary to carry out essential gut functions (Furness 2000). Unlike motor circuits in the spinal cord, enteric motorneurons and secretomotorneurons are sandwiched between their effector end organs and provide local, moment-to-moment coordination of gut musculature, glands and blood flow.

Nerve fibers of enteric motorneurons course through the smooth muscle coats and diffusely innervate regions of smooth muscle. In the gut, the benefits of coordination outweigh the need for precise control of individual muscle fibers and neuromuscular junctions are traded in favor of mechanisms to promote coordination. Neurotransmitters released from enteric varicosities act upon a network of gap junction coupled interstitial cells of Cajal (ICCs) that lie between nerve fibers and smooth muscle cells (Sanders et al. 2012). The ICCs thus distribute the neuron signal to a region of smooth muscle and function to coordinate gut activity.

We are now becoming increasingly aware that an additional cell type, called enteric glia, is ideally positioned to influence cellular interactions in each signaling compartments throughout the gut wall. Enteric glial cells intercalate between neurons within enteric ganglia and between neurons and cells within the mucosa and smooth muscle coats. Accumulating evidence suggests that enteric glia play essential roles in processes that control gut function. Importantly, alterations to glial functions have the potential to drive disease processes and gut pathology. Thus, we now recognize not only the need to incorporate enteric glial cells as additional players in physiological processes, but also that enteric glia are important drug targets to improve, or prevent gut pathophysiology.

21.3 Enteric Glia: The Astrocytes of the Gut

What exactly is an enteric gliocyte? The answer to this question may not be as simple as we once thought because discrete differences between populations of glial cells in the gut wall are becoming increasingly apparent. Dogiel published the first description of glial cells associated with enteric neurons in 1899 but enteric glia were practically omitted from further study for the next 70 years or only briefly noted and incorrectly described as Schwann cells. Dogiel described what he saw as “nucleated stellate cells” associated with enteric neurons and assumed that these nucleated cells were some sort of connective tissue (Dogiel 1899). Unfortunately, this conclusion was broadly accepted and enteric glia were not fully appreciated as a unique class of peripheral glia with distinct characteristics until the detailed anatomical analyses of Giorgio Gabella in the early 1970s (Gabella 1972).

The classical definition of an enteric gliocyte refers to glial cells associated with enteric neurons and nerve fiber bundles within the enteric plexuses. These cells are

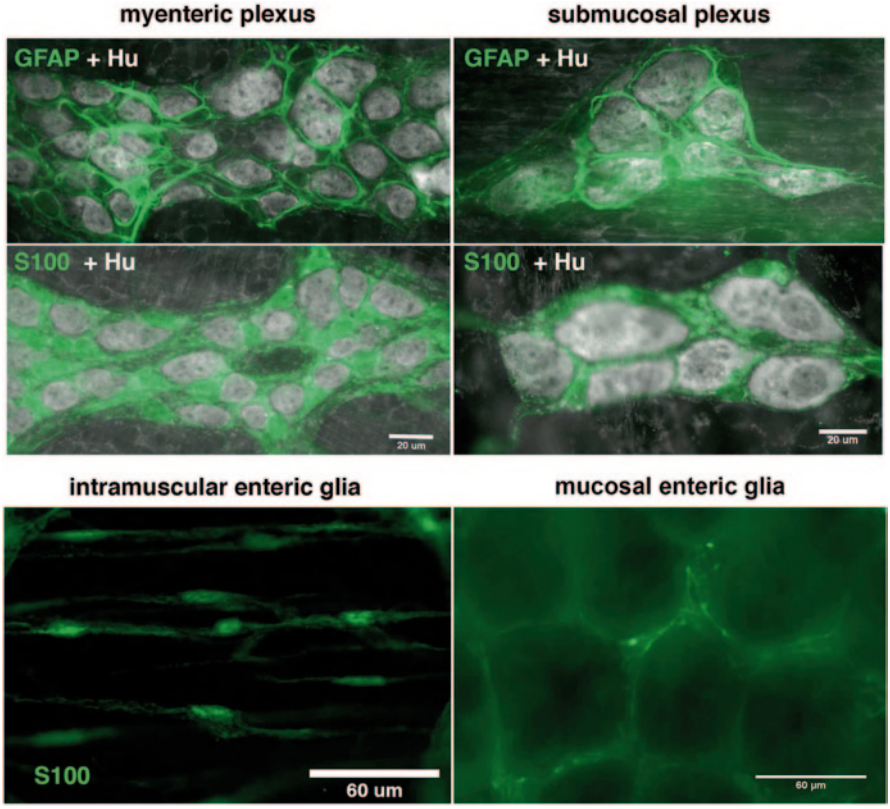

Fig. 21.2 Morphology of the main populations of enteric glia in the mouse colon. GFAP or S100 immunoreactive enteric glia (*green*, GFAP or S100 labeling where indicated) surround enteric neurons (Hu immunoreactive, grayscale) in the myenteric and submucosal plexuses of the mouse colon. Examples of S100 immunoreactive enteric glia (*green*) are shown within the smooth muscle coats (intramuscular enteric glia) and the mucosa (mucosal enteric glia)

easily identified by their stellate-shaped morphology with many highly irregularly branching processes (Gershon and Rothman 1991), similar to astrocytes in the CNS (Fig. 21.2) As in the brain, glial cells embrace enteric neuron cell bodies within enteric ganglia and the dense synaptic neuropil within enteric ganglia and interganglionic nerve bundles is solely comprised of enteric neurons and glia. The majority of the glial cell body is filled by a 2–3 µm nucleus (Gabella 1981) and based on nuclear diameter, enteric glia are estimated to be approximately 10 times smaller in size than enteric neurons. However, this estimate does not incorporate the multitude of processes extending from the glial cell body. Enteric glia are "molded" to the surface of enteric neurons but unlike the satellite cells of sympathetic ganglia, enteric glia do not form capsules around enteric neurons and large areas of enteric neurons have no glial coverage (Gabella 1981). Likewise, glia within interganglionic fiber tracts rarely ensheath an individual neurite and most often only act to separate

nerve processes into groups. Thus, most nerve processes are in direct membrane-to-membrane contact.

Although small in size, the number of enteric glia within ganglia is often touted as being far greater than neurons. However, the exact ratio of enteric glia to neurons varies widely between species and increases with animal size. In mice, enteric neurons and glia are present at approximately a 1:1 ratio within myenteric ganglia and neurons actually outnumber glia in the submucosal plexus by almost a 2:1 ratio (Gabella and Trigg 1984). In guinea pigs and rabbits enteric glia outnumber myenteric neurons by approximately 2:1 but are nearly equal to neuron numbers in the submucosal plexus. Sheep have over 4 times as many myenteric glia as neurons and 1.5 times as many submucosal glia than neurons (Gabella 1984). Humans have the highest ratio of enteric glia to neurons in any species examined to date and enteric glia outnumber human enteric neurons by a ratio of up to 7:1 in myenteric ganglia and 2:1 in submucosal ganglia (Hoff et al. 2008). Thus, enteric glia are by far the most abundant cell type within the human enteric nervous system.

The molecular makeup of enteric glia appears largely similar to astrocytes and enteric glia are commonly identified by expression of the astrocytic markers glial fibrillary acidic protein (GFAP) and S-100beta (Ferri et al. 1982; Jessen and Mirsky 1983) (Fig. 21.2). All glia within enteric ganglia are thought to express GFAP and S-100beta but expression of these glial markers may vary between enteric glial subtype, species, gut region and glial state in disease (discussed later). The transcription factors Sox8/9/10 are reportedly expressed by all enteric glia and immunoreactivity for Sox8/9/10 has been successfully used to quantify glial numbers in myenteric and submucosal ganglia (Hoff et al. 2008). Electrophysiological recordings from enteric glia indicate astrocytic-like membrane properties including low input resistance, large passive currents in response to voltage steps and inwardly-rectifying potassium and sodium currents (Hanani et al. 2000), indicating that a similar composition of cell membrane channels are expressed in both astrocytes and enteric glia.

21.4 Functions of Enteric Glia

As stated earlier, the term “enteric glia” encompasses multiple unique cell types along the length of the gut and through its thickness. All populations of enteric glia derive from a common pool of neural-crest-derived progenitors (Laranjeira and Pachnis 2009) and many maintain expression of common markers such as S100 (Ferri et al. 1982), GFAP (Jessen and Mirsky 1983) and the transcription factors SOX8, SOX9 or SOX10 (Hoff et al. 2008) into adult life. However, the phenotype of enteric glia is heavily influenced by external cues (Dulac and Le Douarin 1991; von Boyen et al. 2004) and the unique microenvironments throughout the gut wall promote the development of several morphologically and functionally distinct populations of enteric glia.

21.4.1 Glial Regulation of Barrier Function

In the mucosal compartment, a population of enteric glia lies just below the epithelial lining of the gut wall in contact with epithelial cells, nerve fibers and blood vessels (Neunlist et al. 2013). Increasing evidence suggests that enteric glia at the level of the mucosa regulate gut barrier function by releasing factors that modulate the transcriptome of gut epithelial cells. Maintenance of gut barrier function is essential to limit invasion by the multitude of bacteria within the gut lumen as well as viruses or other foreign substances. Indeed, an increase in epithelial permeability is associated with many gastrointestinal diseases and is regarded as a contributing event to the onset of disease pathology in the gut. Glia influence the differentiation, adhesion, migration and proliferation of epithelial cell monolayers in culture by releasing factors that include S-nitrosoglutathione (GSNO) (Savidge et al. 2007), 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) (Bach-Ngohou et al. 2010), transforming growth factor (TGF)-β1 (Neunlist et al. 2007) and proepidermal growth factor (proEGF) (Van Landeghem et al. 2011). Given the propensity of glia to change under culture conditions, it is unclear if mucosal glia maintain similar roles *in vivo* but conditional ablation of enteric glia does increase epithelial permeability (Savidge et al. 2007). Thus, it seems that a loss of glial support of the epithelial barrier has the potential to promote, or exacerbate disease processes in the gut.

21.4.2 Enteric Glia and Neurotransmission

Enteric glia within the enteric nerve plexuses are morphologically (Jessen and Mirsky 1983), and likely functionally similar to astrocytes in the central nervous system. Enteric glia are associated with neurons in submucosal ganglia, myenteric ganglia and the nerve bundles connecting ganglia. Glia within enteric ganglia display a star-shaped morphology that resembles protoplasmic astrocytes with many irregularly branched processes surrounding enteric neurons (Hanani and Reichenbach 1994). The elongated, fibrous glia within interconnecting nerve fiber bundles are more similar morphologically to the fibrous astrocytes of CNS white matter tracts and these glia populations likely share similar functions.

Glia within enteric ganglia are actively involved in homeostatic mechanisms that maintain enteric neurotransmission (Gulbransen and Sharkey 2012). Intraganglionic glia supply enteric neurons with essential precursors for the synthesis of neurotransmitters including nitric oxide (NO) (Aoki et al. 1991; Nagahama et al. 2001), glutamate and γ-aminobutyric acid (GABA) (Jessen and Mirsky 1983). NO has well characterized functions in both the physiology and pathophysiology of the enteric nervous system but the physiological significance of glutamate and GABA remain unclear. Interestingly, dysregulation of either glutamate or NO participates in disease by promote the death of enteric neurons (Kirchgessner et al. 1997). In health, enteric glia supply neurons with antioxidants that limit death caused by oxidative

stress (Abdo et al. 2010; Abdo et al. 2012) and mounting evidence suggests that enteric glia normally function to sustain enteric neurotransmission by regulating the bioavailability of neuroactive substances in the extracellular environment. Enteric glial cell surface enzymes such as the ectonucleotidase, nucleoside triphosphate diphosphohydrolase 2 (Braun et al. 2004; Lavoie et al. 2011) and transporters including those for peptides (Rühl et al. 2005) and GABA (Fletcher et al. 2002) play a key role in the removal of neuroactive compounds surrounding enteric neurons. Likewise, glial channels maintain neurotransmission and prevent excitotoxic neuron death by regulating, and buffering extracellular potassium (Hanani et al. 2000; Costagliola et al. 2009). Taken together, this evidence suggests that glia are essential points of homeostatic control in enteric ganglia and that disruption of glial functions could alter gastrointestinal physiology by altering enteric neurotransmission or permitting enteric neuron death.

In addition to the passive, homeostatic mechanisms discussed above, recent evidence suggests that enteric glia actively participate in enteric neurotransmission and are capable of detecting and in turn, modulating the activity of enteric neurons. Enteric glia express a diverse assortment of receptors that permits glia to "listen" to neuronal conversations and initiate intracellular signaling mechanisms in response to neurotransmitters released from enteric neurons (Gomes et al. 2009; Gulbransen and Sharkey 2009; Gulbransen et al. 2010, 2012; Broadhead et al. 2012). ATP and related purines are the most ubiquitous signaling molecules involved in enteric neuron-to-glia transmission *in vitro* (Kimball and Mulholland 1996; Gomes et al. 2009) and *in situ* (Gulbransen and Sharkey 2009; Gulbransen et al. 2010; Broadhead et al. 2012) but enteric glia have the potential to detect a number of neuroactive substances including norepinephrine (NE) (Nasser et al. 2006), glutamate (Nasser et al. 2007), thrombin (Garrido et al. 2002), lipid signaling molecules (Segura et al. 2004a; Segura et al. 2004b), serotonin (Kimball and Mulholland 1996; Boesmans et al. 2013), bradykinin (Kimball and Mulholland 1996), histamine (Kimball and Mulholland 1996) and endothelins (Zhang et al. 1997). Precisely how glial signaling mechanisms affect neurotransmission is still debated but recent evidence demonstrates that glial responses are associated with neuron activity during patterns of activity that underlie physiological gut function (Broadhead et al. 2012).

The tight association of glial responses with neuron activity raises the possibility that enteric glia function as a feedback loop to modulate enteric neurotransmission. Conceivably, enteric glia could modulate enteric neurotransmission by either utilizing the mechanisms described above to regulate the availability of neurotransmitters or by releasing neuroactive substances upon stimulation in a process called gliotransmission. Although few studies have addressed the potential of enteric glia to release neuroactive substances, the available evidence does support the notion of enteric gliotransmission. Indeed, calcium responses in enteric glia can initiate the release of ATP *in vitro* (Zhang et al. 2003) and ATP and its purine metabolites are well known neurotransmitters/neuromodulators in the enteric nervous system (Galligan and Bertrand 1994). *In vitro*, propagation of calcium responses between enteric glia depends on their release of ATP through hemichannels (Zhang et al.

2003). We recently found that this process is maintained *in situ* and that substances released through connexin-43 hemichannels mediate intercellular communication in enteric glia (McClain et al. 2014). Importantly, we found that gastrointestinal transit was delayed when connexin-43 was genetically ablated from glia. These results provide strong evidence that enteric glia have the potential to release gliotransmitters and that gliotransmitters influence gut function through actions on enteric neurons.

How other populations of enteric glia outside enteric ganglia interact with enteric neurons is currently unknown but enteric glia are associated with nerve fibers within the smooth muscle coats of the gut wall. Unfortunately, very little is known about this class of cells but they are morphologically similar to nonmyelinating Schwann cells. Intramuscular enteric glia do express receptors for neuroactive substances (Vanderwinden et al. 2003) but the interactions between nerve fibers and enteric glia in the muscle coats are still unexplored.

In summary, a growing body of evidence points toward enteric glia as important regulators of physiological gut functions at the level of the mucosa and within the enteric nerve plexuses. Importantly, these are two sites often associated with pathological processes in the gastrointestinal tract. In the following sections, we will discuss the evidence implicating enteric glia in gastrointestinal disease and how enteric glial alterations could contribute to disease pathology.

21.5 Enteric Glia in Gut Pathology

An emerging concept in gastroenterology is that inflammation within the enteric nerve plexuses is a key-contributing factor to organ dysfunction in many gastrointestinal diseases. A growing body of evidence supports the notion that enteric glia are altered by, and contribute to, pathological conditions in the gastrointestinal tract. Our current understanding of how enteric glia participate in gut pathophysiology stems mainly from histological studies but accumulating evidence suggests that specific glial changes can promote gastrointestinal disease and dysfunction.

21.5.1 Gastrointestinal Diseases Associated with Alterations to Enteric Glia

Glial alterations are associated with a wide array of diseases that affect the gastrointestinal system. These diseases can be broadly grouped as those primarily affecting the gastrointestinal tract (Primary) or diseases where gastrointestinal complications arise with other disease processes (Secondary). We begin this section by introducing the evidence for glial involvement in specific gastrointestinal diseases and then discuss the possible mechanisms by which glia could participate in pathology.

Table 21.1 Differences between Crohn's disease and ulcerative colitis

	Crohn's disease	Ulcerative colitis
Location	Entire GI tract	Colon only
Characterization of inflammation	Patchy inflammation-Healthy regions adjacent to inflamed regions, skip lesions	Continuous area of inflammation
Depth of inflammation	Transmural—changes to all layers of GI tract	Changes restricted to innermost layer primarily
Clinical symptoms	Ulcers, Fever, Weight loss	Bloody diarrhea
Cytokine response	Mixed Th1-Th17 response: IL-17, IL-23, IL-1β, TNF-α, IL-12, IFN-γ	Th2-like response: IL-13, IL-5, IL-4

21.5.1.1 Primary Intestinal Diseases

Inflammatory bowel disease Approximately 1.4 million people in the United States are affected by the Inflammatory bowel diseases (IBDs), Crohn's and ulcerative colitis (Loftus 2004). Although both diseases are characterized by chronic or recurring gastrointestinal inflammation, the pathology and clinical manifestations of these diseases differ significantly (Table 21.1). Inflammation in ulcerative colitis is restricted to the colon, while inflammation in Crohn's disease may occur anywhere along the GI tract from the mouth to the anus. These differences in inflammatory patterns result in slightly different clinical symptoms of the two diseases. The major symptoms of the IBDs are those typical of diseases of GI distress and include diarrhea, abdominal cramps, ulcers and constipation. Other less common symptoms include fever, weight loss and loss of appetite. Ulcerative colitis patients usually suffer from rectal bleeding, a symptom rarely seen in Crohn's disease patients.

The cytokine profile driving inflammation in Crohn's disease and ulcerative colitis differs considerably. Ulcerative colitis is characterized by a Th2-like immune response with increases in interleukins 3, 4, 5 and 13 (IL-3, IL-4, IL-5 and IL-13) while Crohn's disease features an increase in the Th1 response associated cytokines IL-12 and Interferon-γ (IFN-γ), which results in increases in interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα) (Sartor 1995; Bamias et al. 2011). Alterations to enteric glial survival, morphology and molecular makeup are now recognized as consistent features of IBD. However, the nature of glial alterations seems to differ between Crohn's and ulcerative colitis. Assessment of GFAP content by Western Blot, enzyme-linked immunosorbent assay (ELISA), or immunohistochemistry all demonstrate a significant reduction in GFAP protein levels in non-inflamed regions of colon from individuals with Crohn's disease but no change in non-inflamed regions during ulcerative colitis (Cornet et al. 2001; von Boyen et al. 2011). In contrast, GFAP levels were significantly elevated in areas of active inflammation in Crohn's disease and ulcerative colitis, although to a lesser extent in Crohn's (Cornet et al. 2001; von Boyen et al. 2011). Quantitative assessment S-100 immunoreactivity also showed a significant reduction in Crohn's disease samples as compared to controls (Cornet et al. 2001). These differences may reflect the influence of the

differing cytokine profiles in the diseases and possibly indicate differing glial roles in the two distinct diseases.

In animal models of IBD, glial alterations are recognized as coinciding with intestinal disease. Cornet et al. (2001) used an autoimmune targeting strategy to ablate enteric glia and found that glial ablation resulted in fulminant jejuno-ileo-colitis and premature death. Other animal models of IBD, including dextran sodium sulfate and trinitrobenzene sulfonic acid, demonstrate that colonic inflammation alters glial receptor expression (Nasser et al. 2007), enzyme expression (Green et al. 2004), and increases glial proliferation (Joseph et al. 2011). Whether glial changes reflect inflammatory state or actively promote inflammation is still debated and evidence for both possibilities is discussed below.

Functional Gastrointestinal Disorders In addition to the IBDs, evidence for glial alternations is becoming apparent in a number of other disorders causing gut dysfunction. Termed functional gastrointestinal disorders, these syndromes are characterized by alterations in gastrointestinal function with few associated pathological changes. Irritable bowel syndrome (IBS) is described a disease of unknown pathophysiology that is characterized by symptoms of GI dysfunction including diarrhea and constipation. Individuals with IBS experience altered gut function but structural changes to the gut wall are not readily apparent (Törnblom et al. 2002). However, closer examination of tissue from IBS patients using immunohistochemical analysis studies demonstrates evidence of inflammation and enteric neuropathy within enteric ganglia (Törnblom et al. 2002). Enteric glia are thought to play a key role in enteric neuroprotection (Cornet et al. 2001; Abdo et al. 2010; De Giorgio et al. 2012) and are altered by inflammation (see above). Although detailed assessment of enteric glia in IBS has yet to be performed, the presence of enteric ganglionitis with IBS strongly suggests that enteric glia are involved in the mechanisms of IBS pathology.

Intractable slow-transit constipation (STC) refers to a particularly severe form of constipation. Patients tend to be resistant to typical therapies (Schiller 2001) and often require surgery for relief from symptoms (Bassotti et al. 2003). The most prominent hypothesis explaining altered propulsion and peristalsis in STC is that the underlying mechanism involves alterations to the ENS, including enteric neuron dysfunction or death. In support, immunohistochemical analysis demonstrates an increase in the number of apoptotic neurons in tissue from STC patients (Bassotti et al. 2006). Further, the density of both enteric neurons and glia is significantly reduced (Bassotti et al. 2006). These alterations suggest that enteric glial alterations are associated with STC and raise the possibility that glial changes participate in mechanisms leading to altered motility.

Chronic idiopathic intestinal pseudo-obstruction (CIIP) is a syndrome that presents with symptoms of GI tract obstruction, although no physical blockage is present. Symptoms of CIIP are similar to those that would be observed with GI obstruction and include constipation, abdominal pain, nausea and vomiting. While the etiology of the disease is unknown, secondary forms of the disease have been observed with the presence of infectious viruses such as the JC virus. Current evidence

indicates that glial density is significantly reduced in patients with CIIP (Selgrad et al. 2009). Interestingly, the remaining glial cells contained the JC Virus. These results raise the intriguing possibility that the JC virus acts preferentially on enteric glial to cause pathology in CIIP.

Diverticular disease (DD) or diverticulosis is defined as the formation of protruding sacs or pockets within the colon wall. These diverticula are formed when there is an area of weakness in the colon wall, through which the mucosal layer can bulge. Diverticular disease is typically asymptomatic however, damage or inflammation of the diverticula can result in serious complications such as rectal bleeding, GI tract infection, GI tract blockage and colon or diverticula rupture. As with a number of other functional GI disorders, the etiology and pathology of diverticular disease are unknown but morphometric analysis of ENS plexuses indicates a significant decrease in enteric neuron and glial cell density in DD patients (Wedel et al. 2010).

Necrotizing Enterocolitis Necrotizing enterocolitis (NEC) is the most common and serious gastrointestinal disorder encountered among hospitalized preterm infants. As the name suggests, NEC is characterized by infection and inflammation that leads to the destruction of the terminal ileum and proximal colon. The causal nature of NEC is unknown but an ischemic event in the intestine is hypothesized to play a key role in disease pathology (Watkins and Besner 2013). Histological evidence suggests that myenteric and submucosal neurons and glia decline in NEC (Wedel et al. 1998). Interestingly, the loss of neurons and glia was most predominant at sites where ischemic lesions appear first. In animal models, intestinal ischemia reperfusion causes alterations in enteric glial morphology and disrupted glial morphology is associated with a decline in enteric neuron numbers (Thacker et al. 2011). These intriguing results raise the possibility that alterations to enteric glia initiated by ischemic events in the neonatal gut contribute to the pathology of NEC.

Infectious Diseases Infection is a well-recognized cause of inflammation in the gastrointestinal tract. Evidence supporting a role for enteric glia in gastrointestinal infections is mainly based on studies of Chagas disease: an infection by the protozoan *Trypanosoma cruzi* that commonly occurs in rural Latin America. Like the IBDs, Chagas disease causes systematic inflammation that can affect multiple organ systems. In the acute phase of the disease, which typically occurs within weeks of infection, symptoms are usually mild and may include fever, inflammation at the site of infection and gastrointestinal disturbances including diarrhea, vomiting and loss of appetite. The chronic phase develops a few months after infection and, in about 30% of individuals, is characterized by chagasic megacolon; an abnormal dilation of the GI tract. In Chagas disease, it is hypothesized that inflammatory destruction of the plexuses of the enteric nervous system contributes to the pathogenesis of chagastic megacolon.

Glial alterations are associated with the pathology of Chagas disease and chagastic megacolon. Immunohistochemical analysis of tissue from Chagas' patients reveals a significant decrease S-100 immunoreactivity (da Silveira et al. 2009). This decrease in glial S-100 is evident in both dilated and non-dilated colon segments

and is associated with a Chagas-mediated enteric neuropathy. However, GFAP expression was significantly higher in non-dilated segments than that in dilated colon segments. Thus, while glial alterations are clearly involved in the pathology of Chagas, it is unclear whether these changes indicate an alteration in glial numbers or a change in expression of glial markers.

JC virus is a polyomavirus which can infect both the central and enteric nervous system. JC virus infection in the brain causes progressive multifocal leuokoencephalopathy which is characterized by multiple inflammatory lesions throughout the brain. As indicated above, JC virus infection in the ENS is recognized as a secondary cause of CIIP. In CIIP, JC virus infects enteric glial cells (Selgrad et al. 2009). Consequently, glial cell density in JC virus-associated CIIP was found to be decreased compared to control samples.

21.5.1.2 Secondary Intestinal Diseases

Type II diabetes The incidence of type II diabetes has risen to the extent where diabetes is now the first metabolic disease to be classified as an epidemic by the world health organization. Gastrointestinal dysfunction is common in persons with diabetes and gastrointestinal complications can affect one or multiple regions of the gastrointestinal tract. Nausea, vomiting, abdominal pain, gastroparesis, constipation and diarrhea are commonly seen in type II diabetes patients (Enck and Frieling 1997) and changes to the ENS are hypothesized to contribute to these symptoms. Mice ingesting a high-fat diet (an established model for type II diabetes) experienced enteric neuron loss in the duodenum, along with an increase in apoptotic neurons (Stenkamp-Strahm et al. 2013b). Immunohistochemical classification identified that inhibitory motor neurons were preferentially lost in diabetes associated enteric neuropathy. Neuropathy was accompanied by a decrease in the glial cell network during high-fat mediated diabetes in mice (Stenkamp-Strahm et al. 2013a).

Parkinson's Disease Parkinson's disease (PD) is typically thought of as a disease that primarily affects neurons in the basal ganglia but a current hypothesis proposed by Braak and co-workers postulates that PD originates in the gastrointestinal tract (Braak et al. 2006). The formation of Lewy bodies is considered an important pathological feature of PD (Fujiwara et al. 2002). Braak et al. (2003) noted that the first appearance of Lewy pathology, during the initial stages of PD, is within the ENS and dorsal motor nucleus of the vagus nerve. Further support comes from findings showing that alpha-synuclein immunoreactive Lewy bodies are present within the ENS of Parkinson's patients (Pouclet et al. 2012; Shannon et al. 2012). Similar to the effects of PD on skeletal muscle motor control, one of the most common symptoms of PD is gastrointestinal dysfunction including constipation, impaired gastric emptying and dysphagia (Edwards et al. 1991; Pfeiffer 2011). Further, disease severity in PD correlates with increased expression of pro-inflammatory cytokines in the colon, indicating that inflammation within

the gut predicts disease activity and progression (Devos et al. 2013). Altered expression of enteric glial markers is also associated with Parkinson's disease severity. Devos et al. (2013) found that the expression of GFAP and Sox-10 both increased in colonic biopsies from individuals with PD. However, S-100 levels remained unchanged and GFAP and Sox-10 expression were not correlated with enteric Lewy pathology. A central tenant of Braak's hypothesis is that altered barrier function contributes to disease initiation. Enteric glia are known to potently regulate barrier function so the finding that glial alterations are associated with disease progression raises the intriguing possibility that enteric glia are involved in the initiation of PD.

21.5.2 Enteric Glial Response to Pro-inflammatory Cytokines and Inflammatory Mediators

A common thread associating the pathology of intestinal diseases is inflammation. Inflammation is a key process driving pathology in the gastrointestinal tract and mounting evidence suggests that enteric glia are both a site of action of, and producers of, inflammatory mediators within the ENS. This reactive gliosis is characterized by glial cell hypertrophy, increases in the synthesis of GFAP and other proteins and secretion of cytokines (Rühl et al. 2004).

An altered cytokine profile and the presence of other inflammatory mediators is intimately linked to disease pathology in IBD. Specifically, Crohn's disease and ulcerative colitis are characterized by increases in pro-inflammatory cytokines (Nikolaus et al. 1998). These diseases can be differentiated between based on the pro-inflammatory cytokines they upregulate, supporting the idea of different pathologies for the diseases. Ulcerative colitis is generally characterized by a Th2-like response with increases in IL-4 and IL-13 while Crohn's disease is associated with a Th1 response predominated by IL-1β andTNFα (Sartor 1995). Similar to Crohn's, IL-1β and TNFα are upregulated in NEC, a common condition in preterm infants that destroys of segments of bowel. The effect of the proinflammatory cytokines IL-β and TNF-α on enteric glia provides some evidence of a role for enteric glia in the pathology of IBDs.

In vitro treatment of enteric glia with the pro-inflammatory cytokines IL-1β and TNF-α or lipopolysaccharide (LPS) induces an increase in GFAP expression (von Boyen et al. 2004). *In vivo*, LPS initiates an immune response within the GI tract mediated by the proinflammatory cytokines TNF-α and IL-1β, raising the possibility that the effect of LPS could be secondary to cytokine release. However, because these studies were performed *in vitro*, they suggest that LPS has a direct effect of glial GFAP content. The increase in GFAP expression was accompanied by both an initiation of expression of GFAP in GFAP-negative glial cells and further upregulation of expression in already GFAP-positive cells (von Boyen et al. 2004). An increase in GFAP-positive glia was also observed in tissue from patients with ulcerative colitis and Crohn's disease, supporting the *in vitro* data above (Cornet

et al. 2001). However, the role of increased GFAP expression in the inflammatory process of the IBDs is not fully understood. Indeed, mice lacking GFAP develop and reproduce normally (Pekny et al. 1995). Increased GFAP expression in CNS astrocytes is proposed to act to isolate inflammatory regions (Rühl et al. 2001a). Thus, upregulation of enteric glial GFAP expression may play a similar role in the ENS and act as a defense mechanism of the ENS to protect against inflammation.

Pro-inflammatory cytokines and inflammatory molecules directly activate enteric glial cells. *In vivo* treatment with the pro-inflammatory cytokine IL-1β upregulates expression of the immediate early gene, *c-Fos*, in enteric glia in both the myenteric and submucosal plexuses (Tjwa et al. 2003). Glial activation is also induced by TNF-α and *in vitro* treatment of cultured rat enteric ganglia with TNF-α caused translocation of the signal transducer and activator of transcription 5 (STAT5) protein in glia (Rehn et al. 2004). Translocation of STAT5 is considered a measure of cellular activation in response to TNF-α signaling. Thus, enteric glial cells are activated by pro-inflammatory cytokines which are upregulated during IBD-associated inflammation.

Inflammation in the ENS can cause upregulation of other pro-inflammatory molecule besides cytokines such as thrombin and purine molecules. Thrombin is a serine protease that is involved in activating the blood coagulation pathway. A novel role for thrombin has been identified in linking the blood coagulation pathway and inflammation (Dugina et al. 2002). Thrombin and other proteases activate a class of receptors known as the protease-activated receptors (PARs). PARs are expressed by glial cells (Garrido et al. 2002) and PAR activation results in the expression and secretion of molecules including transcription factors and proinflammatory cytokines in astrocytes (Shpacovitch et al. 2002; Zeng et al. 2013). Although PARs have not been definitively localized to enteric glia *in situ*, PAR agonists initiate calcium responses in enteric glia in culture (Garrido et al. 2002) and *in situ* (Mueller et al. 2011). These results suggest that thrombin and other proteases may either directly, or indirectly activate enteric glia during inflammatory events.

Purines play an important role in inflammation and altered purinergic signaling is increasingly recognized as a key-contributing factor to the pathophysiology of IBD in both humans and experimental models. Increased release (Wynn et al. 2004; Lomax et al. 2005) and decreased hydrolysis (Wynn et al. 2004; Friedman et al. 2009) elevate extracellular purine levels during inflammation and purine receptor genes and proteins are dysregulated in affected humans (Yiangou et al. 2001; Rybaczyk et al. 2009) and animals models (Guzman et al. 2006). Experimentally increasing extracellular purine levels exacerbates colitis (Atarashi et al. 2008) and impaired purine hydrolysis (and hence increased purine levels) is associated with increased susceptibility to colitis in humans (Friedman et al. 2009). Increasing evidence demonstrates that enteric glia are intimately tied to purinergic signaling in the enteric nervous system. As noted earlier, enteric glia express purine receptors and are highly responsive to extracellular purines (Kimball and Mulholland 1996; Gomes et al. 2009; Gulbransen and Sharkey 2009; Gulbransen et al. 2010, 2012; Broadhead et al. 2012). Activation of enteric glial purinergic receptors is a potent inducer of glial calcium responses but the significance of glial calcium responses

in physiology and pathophysiology is currently debated. Additionally, enteric glia express ectonucleotidases that regulate extracellular purine availability within the enteric nervous system (Braun et al. 2004; Lavoie et al. 2011) and enteric glia are capable of releasing ATP (Zhang et al. 2003).

21.5.3 The Potential of Enteric Glia to Promote Pathological Processes in the Gastrointestinal Tract

To this point, we have discussed available evidence supporting the notion that inflammatory mediators act on, and cause alterations to, enteric glia in gastrointestinal pathology. However, mounting evidence suggests that enteric glia play an active role in disease progression. In this section, we discuss possible mechanisms whereby enteric glia could promote gastrointestinal disease.

21.5.3.1 Glial Production of Inflammatory Molecules

Activation of glial cells alters their function and behavior within the ENS (Rühl et al. 2004). Activated enteric glial cells have the potential to synthesize and secrete a number of compounds including cytokines (Rühl et al. 2001b), growth factors (von Boyen et al. 2006a) and inflammatory mediators such as ATP (Zhang et al. 2003). As discussed above, proinflammatory cytokines and purines activate glial cells and may initiate the production and release of proinflammatory mediators. For example, cultured enteric glia release IL-6 upon incubation with the proinflammatory cytokine IL-1β (Rühl et al. 2001b). Treatment with IL-6 inhibited IL-6 release, suggesting that a feedback inhibition loop regulates IL-6 release. IL-6 has the ability to act as both a pro- and an anti-inflammatory cytokine and thus its release by enteric glia plays an important role in the regulation of inflammation in the gut. In addition, enteric glial calcium responses can initiate the release of ATP (Zhang et al. 2003), a molecule with well known pro-inflammatory effects in the ENS (Gulbransen et al. 2012). Further, enteric glia up-regulate expression of inducible nitric oxide synthase during inflammation (Green et al. 2004), suggesting that enteric glia may promote oxidative stress in disease. Oxidative stress is associated with increased susceptibility to IBD in humans and plays a key-role in disease progression (Sido et al. 1998; Esworthy et al. 2001; Rezaie et al. 2007; Zhu and Li 2012). Enteric glia also release a number of factors involved in regulation of gut barrier function including 15dPGJ2 (Bach-Ngohou et al. 2010), GSNO (Savidge et al. 2007) and TGF-β1 (Neunlist et al. 2007). A loss of glia, and hence a loss of glial factors regulating barrier function, results in profound inflammation in the gut (Bush et al. 1998; Cornet et al. 2001). It is thus becoming increasingly clear that enteric glia are an important source of both pro- and anti-inflammatory mediators in the gastrointestinal tract. Alteration of these glial properties has a high potential to act as a driving source of disease in the gut.

21.5.3.2 Recruitment of Inflammatory Cells by Enteric Glia—Glia as Antigen Presenting Cells

An important step in the inflammation process is the presentation of antigens to T-cells, causing their activation and differentiation. The appropriately named antigen presenting cells (APCs) express one of two classes of major histocompatibility complex (MHC) proteins, coupled to the antigen in question. This complex is recognized by the T-cell receptors and results in activation of T-cells and the appropriate immunological response. Following antigen presentation, T-cells are further activated by the presentation of non-specific co-stimulatory compounds. For example, the T-cell surface molecule CD28 is activated by CD80 and CD86 molecules that are present on antigen-presenting cells (Ellis et al. 1996). The expression of both co-stimulatory receptors and ligands are upregulated during inflammatory events and ensures sufficient T-cell differentiation and activation to sustain the inflammatory event. In order for T-cells to be properly activated, both antigen presentation and co-stimulation are necessary. Both of these steps involve APCs, which thus serve a very important role in the initiation of a GI inflammatory incident.

The typical APCs in the GI tract are dendritic cells and macrophages (Rescigno 2010). However, glial cells have the ability to become antigen presenting cells in incidences of inflammation. This phenomenon is observed in both Chagas disease (da Silveira et al. 2011) and Crohn's disease (Koretz et al. 1987; Geboes et al. 1992). In Chagas patients with chagasic megacolon, both the human leukocyte antigen (HLA)-DR class II peptide and the costimulatory proteins CD80 and CD86 are expressed on the surface of enteric glia (da Silveira et al. 2011). These molecules were not detected on enteric glia of uninfected patients or on neurons in either infected or uninfected patients. Similarly, immunostaining of tissue from Crohn's disease patients showed an upregulation of HLA-DR expression on glial cells when compared to normal tissue (Koretz et al. 1987). Interestingly, glial HLA-DR expression was found to correlate with the density of lymphocyte infiltrate present. In addition, mast cells are recruited to enteric ganglia during inflammatory conditions and appear to contact enteric glia (Bassotti et al. 2012). These findings support the hypothesis that enteric glia act as APCs during inflammatory events and recruit inflammatory cells to the enteric plexuses.

Astrocytes, the CNS equivalents of enteric glia, also possess the ability to express MHC class II molecules and can transform into APCs during inflammation. This antigen presenting role of astrocytes is important in the pathogenesis of cerebral autoimmune diseases such as multiple sclerosis (MS). MHC class II expression is induced in astrocytes by the pro-inflammatory cytokine IFN-γ (Wong et al. 1984) but astrocytes also transform into APCs in non-autoimmune diseases such as Parkinson's disease (Harms et al. 2013), initiating or potentiating inflammatory episodes in these diseases. MHC II expression on astrocytes, and thus their antigen-presenting ability, is tightly regulated by neurotransmitters in the brain. NE and NO both inhibit astrocyte MHC II expression (Frohman et al. 1988; Heuschling 1995). Thus, neuronal production of these compounds is important in regulating activation of an immune response and inflammation within the CNS. More importantly, the

balance MHC stimulators (such as IFN-γ) and inhibitors (NE and NO) is important in regulating inflammation with disease. Whether enteric glial MHC II expression is regulated by similar mechanisms is currently unknown but the similarities between astrocytes and enteric glia suggest this is highly likely.

21.5.3.3 Epithelial Barrier Regulation by Enteric Glia in Disease

The gastrointestinal epithelium performs an essential role in maintaining gut homeostasis by forming a selective barrier between the potentially hazardous environment in the gut lumen and the rest of the body. The epithelial barrier allows the passage of water, ions and other nutrients to, and from the gut lumen while blocking the movement of bacteria, viruses and other harmful organisms beyond the gut lumen. Maintenance of the epithelial barrier function is achieved through strict regulation of epithelial cell differentiation, proliferation and migration, ensuring that tight junctions between epithelial cells are maintained (Tsukita et al. 2001; Van Itallie and Anderson 2006). This regulation prevents the infiltration of harmful compounds and organisms further into the GI tract. Dysfunction of this barrier role is associated with, and predictive of gastrointestinal disease (Clayburgh et al. 2004; Turner 2009).

Epithelial barrier dysfunction in Crohn's disease and ulcerative colitis promotes inflammation by allowing foreign, and potentially harmful substances access into the gut wall where they come in contact with, and activate inflammatory cells (Cario 2008). Numerous lines of evidence now support the hypothesis that enteric glia maintain epithelial barrier function in health and that altered glial regulation can promote disease. As we noted earlier, enteric glia release several mediators that regulate barrier function through their potent influence on epithelial cell function can activate or change epithelial cells, thus regulating barrier function. One such factor is 15dPGJ2, an endogenous ligand for the peroxisome proligerator-activated receptor γ (PPARγ) receptor (Bach-Ngohou et al. 2010). Activation of PPARγ on epithelial cells promotes cell differentiation (Kitamura et al. 1999) and thus control of 15dPGJ2 levels and PPARγ activation is important in maintaining barrier function. Likewise, enteric glial-derived GSNO interacts directly with epithelial cells and upregulates the expression of tight junction proteins such as F-actin that are essential to maintain barrier integrity. Enteric glia may also secrete glial-derived neurotrophic factor (GDNF) during IBD-associated gut inflammation (von Boyen et al. 2011). GDNF has anti-apoptotic on epithelial cells and thus has the potential to improve barrier function (Steinkamp et al. 2003). Genetic models of glial cell ablation clearly demonstrate that these glial-derived factors are necessary to maintain barrier function (Bush et al. 1998; Cornet et al. 2001; Savidge et al. 2007). Glial ablation induces an abrupt increase in epithelial permeability followed by an inflammatory response (Cornet et al. 2001; Savidge et al. 2007). Supplementing glial factors such as GSNO restores mucosal epithelial barrier function and prevents inflammation *in vivo* (Savidge et al. 2007) and *in vitro* (Cheadle et al. 2013). Likewise, glial-derived factors protect against barrier dysfunction in cultured epithelial cells induced by a

mixture of proinflammatory cytokines (Cheadle et al. 2013). These findings indicate that enteric glia are normally protective by maintaining barrier function and that changes to glial survival or function may promote disease.

21.5.3.4 Role of Enteric Glia in Enteric Neuron Death

The most prominent hypothesis explaining abnormal gut function in disease is that inflammation alters the function and/or structure of the ENS (De Giorgio and Camilleri 2004). Although enteric neuron death is associated with, and predictive of, many gastrointestinal diseases, the mechanisms that promote neuron death are only beginning to be characterized (Gulbransen et al. 2012). What is clear is that enteric glia play an essential role in neuroprotection in the ENS. This was made particularly evident by the fact that glial ablation results in a substantial decrease in enteric neuron density (Bush et al. 1998). Although the exact mechanism promoting neuron death in these models are unknown, neurotrophic factor secretion, regulation of oxidative stress and regulation of extracellular purines by enteric glia are all proposed to play a role. Cultured enteric glia secrete the neurotrophic factor, GDNF (von Boyen et al. 2006b) and GDNF is known to promote enteric neuron survival (Anitha et al. 2006; Rodrigues et al. 2011). Stimulation of glial cells with proinflammatory cytokines also increases production and secretion of nerve growth factor (von Boyen et al. 2006a), which both modulates inflammation in the ENS and has direct neuroprotective effects (Reinshagen et al. 2000). In addition to neurotrophic factors, cultured enteric glia secrete potent antioxidants including glutathione (Abdo et al. 2010) and 15d-PGJ2 (Bach-Ngohou et al. 2010). Both are integral in regulating oxidative stress in enteric neurons and are capable of protecting neurons from oxidative stress-mediated death (Abdo et al. 2010, 2012). Further, enteric glia are essential sites of purine regulation within the ENS and are responsible for both degrading (Braun et al. 2004; Lavoie et al. 2011), and producing ATP (Zhang et al. 2003) and its metabolites. Disruption of either of these functions has the potential to promote neuron death during disease because the accumulation of extracellular purines during inflammation drives enteric neuron death by activation of P2×7 purine receptors (Gulbransen et al. 2012). Thus, current evidence supports the hypothesis that enteric glia protect neuron survival in health but that glial alterations in disease promote neuron death.

Conclusion

As we begin to appreciate the diverse functions of enteric glia, we are increasingly aware that their classical characterization as passive, supportive cells couldn't be further from the truth. The rapidly expanding body of enteric glial literature supports numerous roles for enteric glia in both physiological and pathophysiological gut processes. Immunohistochemical and morphological analysis have identified

changes in glial cells and their proteins in a number of diseases. Yet, the exact mechanisms by which these changes occur and the consequences of these glial cell alterations are still unclear. Enteric glia are increasingly implicated as key players in the pathology of inflammatory-mediated GI disorders. From reactive gliosis in response to inflammatory mediators to the production and secretion of inflammatory mediators, glial cells are intimately involved in pathology of inflammation in the gut. By better understanding the role of glial cells in gut pathology, we can potentially develop improved clinical interventions for those suffering with GI disorders. GI complication are not limited to primary GI disorders but are seen in a number of secondary disorders such as diabetes. Thus, the effects of these improved mediations will widespread and highly relevant.

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