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Neuroglia in Neurodegenerative Diseases

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Preface

Over the last century the mankind suddenly became old, as the average life span increased from ~45 years in 1900 to approximately 75 years in the year 2000 and to almost 79 years in 2019; in Japan, Spain, Singapore and Switzerland the life expectancy in 2019 reached in excess of 85 years. This abrupt ageing of the population changed the epidemiological landscape and brought forward something entirely unknown to the humanity over the history—the growing epidemic of neurodegenerative diseases, which end up with senile dementia. The numbers are almost impossible to accurately define; rough estimates account for ~ 50–70 million of senile people worldwide, with each year adding extra 10 million cases. The neurodegenerative diseases are several, of which the most known are Alzheimer's disease, Parkinson disease, Huntington disease, frontotemporal dementia and tauopathies; the common denominator is senility as the outcome and absence of therapeutic hopes; all available pharmacological options remain merely symptomatic.

The ultimate result of neurodegenerative process is the atrophy of the brain and loss of brain function. The direct link between a decrease in the size (i.e. atrophy) of brain tissue and a decrease in cognitive capabilities (i.e. dementia) was suggested by Thomas de Willis at the end of the seventeenth century. Despite a remarkable progress in understanding the biochemistry and genetics of neurodegenerative processes, the genesis and pathobiology of the majority of sporadic cases remain obscure. Neurodegeneration, as well as the majority of other neurological diseases, are commonly believed to reflect pathological evolution that is conceived and developed primarily, if not solely, in neurones. This neurone-centric view emerged and expanded with the triumphal spread of the neurone doctrine born at the end of the nineteenth century. This development was, however, at odds with neuropathological thoughts of earlier neuroanatomists who recognized the remarkable pathological potential of neuroglia that represent the homeostatic arm of the nervous tissue. Rudolf Virchow, who invented the concept of neuroglia was convinced that 'This very interstitial tissue of the brain and spinal marrow is one of the most frequent seats of morbid change' (R. Virchow, (1858). *Die Cellularpathologie in ihrer Begründung auf physiologische and pathologische Gewebelehre*. August

Hirschwald, Berlin; p. 317 of the 1st English Edition, 1860). The fundamental contribution of neuroglia to neuropathology was also considered by such towering figures as Santiago Ramón y Cajal, Alois Alzheimer, Franz Nissl, Pió del Rió-Hortega and William Lloyd Andriezen, to name only a few.

The term Neuroglia defines the class of neural cells of ectodermal (neuroepithelial) and mesodermal (myeloid) origin, which are responsible for support, maintenance and defence of the nervous tissue. In the brain and in the spinal cord (which together form the central nervous system, CNS) neuroglia are represented by astrocytes (main homeostatic cells of the CNS), oligodendrocytes (cells myelinating and supporting axons) and microglia (cell of mesodermal origin, which, in the guise of foetal macrophages, migrate into the neural tube in the early embryonic development and disseminate throughout the CNS); microglial cells represent innate immune and defensive system of the nervous tissue. The neurone-centric neuropathological ideas have been challenged in the recent decade, when contribution of neuroglia to neurological disorders begun to be widely considered. It emerged that complex neuroglial reactions and metamorphoses contribute to most if not all neurological diseases, either as primary factors, or as factors instigated by exogenous pathology and neuronal abnormalities or (most frequently) as combination of both. Neurogliopathology is manifested in many forms from degeneration, loss of function, glial asthenia and paralysis to glial reactivity in which neuroglial cells undergo context-specific biochemical, morphological and functional remodelling producing neuroprotective or neurotoxic phenotypes. Understanding neuropathology ultimately requires in-depth analysis of glial pathological changes, which in turn may stimulate the development of novel, glia-specific therapies. In this volume we collected essays, written by leading gliologists. These essays highlight glial contribution to neurodegenerative diseases and we hope that this book (being first of its kind on the market) may be appreciated by academics, professionals and students of medicine, neuroscience, biology, biochemistry and pharmacology.

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Chapter 1
The Concept of Neuroglia

Alexei Verkhratsky, Margaret S. Ho, Robert Zorec and Vladimir Parpura

Abstract Neuroglia represent a diverse population of non-neuronal cells in the nervous systems, be that peripheral, central, enteric or autonomic nervous system. Arguably, these cells represent about half of the volume of the human brain. This volumetric ratio, and by extension glia to neurone ratio, not only widely differ depending on the size of the animal species brain and its positioning on the phylogenetic tree, but also vary between the regions of an individual brain. Neuroglia derived from a dual origin (ectoderm and mesodermal) and in an assorted morphology, yet their functional traits can be mainly classified into being keepers of homeostasis (water, ions, neurotransmitters, metabolites, fuels, etc.) and defenders (e.g., against microbial organisms, etc.) of the nervous system. As these capabilities go awry, neuroglia ultimately define their fundamental role in most, if not, all neuropathologies. This concept presented in this chapter serves as a general introduction into the world of neuroglia and subsequent topics covered by this book.

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1.1 The Birth of the Concept of Homoeostatic Neuroglia

The complexity of the human brain is remarkable: a population of more than 200 billion (i.e. 2×10^{11}) neural cells (neurones and neuroglia) is packed within a limited volume (average human brain occupies 1200–1400 cm^3). These neural cells form complex networks, connected through 15–20 trillions of chemical and electrical synapses that provide for computing power of this organ. The logistical support underlying this highly complex organ is provided by a specific class of cells known as neuroglia.

The concept of connective tissue of the nervous system emerged in the nineteenth century [16, 47]; this concept was initially formalised by Rudolf Virchow who introduced the term neuroglia in the 1850s [100, 101]. According to Virchow the neuroglia was '…connective substance that forms in the brain, in the spinal cord and in the higher sensory nerves a type of putty (neuroglia), in which the nervous elements are embedded…' [100]. Prominent neuroanatomists of the second half of the nineteenth century characterised the cellular nature of glia in great detail, and described many types of glial cells [16]. At the same time numerous theories have considered the functional role of neuroglia in the brain homeostasis, nutritional support, regulation of blood flow, sleep and conscience, as well as in neuropathology [6, 29, 30, 72, 79]. The first major type of glia, the astroglia, has been defined in 1895, when Michal von Lenhossék suggested to name a sub-population of parenchymal glia astrocytes, star-like cells (from Greek αστρον κυτος). At the same time the parenchymal glia was also sub-classified into protoplasmic (grey matter) and fibrous (white matter) cells [6]. The myelinating cells of the central nervous system (CNS) were first drawn by the Scottish pathologist William Ford Robertson [74, 75], and subsequently Pío del Río Hortega named them oligodendrocytes and recognised their myelinating function [24]. It was also Pío Del Río Hortega who identified and named microglia as the defensive cellular elements of the CNS, by demonstrating that these cells undergo remarkable metamorphosis in pathology and suggesting their role as 'garbage collectors' [21–23]. Finally, in the 1980s the fourth type of neuroglia, the NG2 glia (also known as oligodendrocyte progenitor cells or polydendrocytes), was discovered by William Stallcup and colleagues, after they developed an antibody to a chondroitin sulphate proteoglycan, dubbed NG2 [88]. Based on their developmental origin (neuroepithelial or mesodermal), neuroglia of the CNS have been classified as macroglia (astrocytes, oligodendrocytes, NG2) and microglia, respectively.

1.2 The Definition of Neuroglia

The definition of neuroglia is based on the unifying fundamental function of these cells, which, regardless of their origin, is homeostasis of the nervous system. This function is fundamental for both physiological context, when glial cells perform their routine housekeeping duties, as well as for pathological context, when glial cells can undergo reactive remodelling in order to preserve, repair and restore brain homeostasis. Failure in this function results in the development of the neurological disease and damage to the nervous tissue. Therefore, neuroglia can be defined as **homeostatic and defensive cells of the nervous system**, represented by highly heterogeneous cellular populations of different origin, structure and function [94].

In this sense neuroglia are the **ultimate supportive cells** of the nervous system, keeping it in a functional state. This reflects upon evolution of the nervous system, which resulted in the division of labour: the information processing and electrical excitability became confined to the neuronal networks, whereas homeostatic support and defence became the sole prerogative of the neuroglia [95]. This homeostatic support occurs at all levels of organisation of the nervous system: at molecular level (control over homoeostasis of ions, neurotransmitters, protons, reactive oxygen species, metabolites, etc.), at cellular level (astrocytes involvement in neurogenesis), at network level (both astroglia and microglia regulate synaptogenesis, synaptic maturation and extinction), connectome level (which is maintained by myelinating oligodendroglia and Schwann cells), organ level (astrocytes control blood-brain barrier and glymphatic flow and regulate functional hyperaemia) and systemic level (glial cells emerge as central chemoceptors and contribute to systemic control over ventilation, ion homeostasis and energy metabolism); for comprehensive coverage of neuroglial homeostatic capabilities see [1, 2, 7, 19, 31, 34, 37, 41, 45, 46, 49, 50, 59, 68, 70, 93, 96, 97, 103, 105].

This ultimate homeostatic capability of neuroglia underlies their fundamental role in neuropathology, the latter being broadly defined as a homeostatic failure of the nervous system. Environmental stress and/or pathological insults trigger glial homeostatic response (when glial cells attempt to keep homeostatic equilibrium or steady state) and glial reactivity, which represents an evolutionary conserved programme of glial cells remodelling aimed at mounting defence of the nervous tissue. Neuroglial reactivity is manifested in reactive astrogliosis, microglial activation and Wallerian degeneration (for oligodendrocytes). At the same time glial asthenia or atrophy, which is observed in numerous neurological conditions, facilitates evolution of the disease because of compromised homeostatic and defensive capabilities. Although the fundamental role of neuroglia in neuropathology has been predicted by prominent neurologists of the nineteenth and the beginning of the twentieth centuries (such as Rudolf Virchow, Carl Frommann, Alois Alzheimer, Nicolas Achucarro and Franz Nissl), the pathophysiological role of glia begun to be universally recognised only in the recent decade; for references and concepts see [11, 12, 53, 66, 67, 76, 81, 84–86, 99]. The concept of astrotauopathology, recently introduced by Kovacz [51],

supports the notion that the neuroglia emerges in the limelight when considering the evolution of neurological diseases.

1.3 Glial Numbers

The numerical preponderance of glial cells in the brains and spinal cords of different species and glial to neurone ratio (GNR) have been a matter of the most common fallacy over recent decades. The notion of glial cells outnumbering neurones in the human brain by a factor of 10 or even 50 [10, 18, 44] represented an undisputed general knowledge that has been repeatedly proclaimed in glial literature (for critical analysis see for example [39, 98, 102]). The story of exceedingly high number of glial cells in the human brain goes back to Franz Nissl [58]; this idea became rather popular and reached the climax in writings of Robert Galambos who considered that neuroglia represent the primary seat of intelligence, consciousness, emotions and are overall responsible for our 'humanity'. 'Glia is … conceived as genetically charged to organize and program neuron activity so that the best interests of the organism will be served; the essential product of glia action is visualized to be what we call innate and acquired behavioural responses. In this scheme, neurons in large part merely execute the instructions glia give them' [28]. The notion was further promoted by the finding that the Einstein's brain had a rather higher glia to neurone ratio in his associated cortex than that found in the control human population [25], leading to speculations that this could be the reason for his remarkable intellectual abilities (https://www.theguardian.com/science/2007/feb/21/neuroscience.highereducation) (https://www.npr.org/templates/story/story.php? storyId=126229305). The public myth of glia has extended into that of an untapped part of the brain that we may not use, perhaps gloriously captured in Starbuck's The Way I see it? (http://www.stevekmccoy.com/blog/2005/08/starbucks_the_w) #236 quote 'Scientists tell us we only use 5% of our brains. But if they only used 5% of their brains to reach that conclusion, then why should we believe them?' Of course, based on any functional imaging, this myth has been debunked and the authors would like to assure the readers that we had used the vast majority if not all of our brains to write this chapter.

None of these concepts had experimental confirmation. Exceptionally high glia to neurone ratio of the human brain was not related to actual cell counts; to the contrary most of stereological investigations produced the GNR values in the neocortex somewhere around 1.5 (see Table 1 in [42, 102]), with the number of neuronal counts in the range of 20–30 billion and glial cells in the range of 27–38 billion. In the cerebellum, which contains the largest number of brain neurones (around 70 billion) the number of glial cells is much smaller, with GNR not exceeding 0.1 [3, 4]. These stereological data obviously made the total GNR estimate of 10:1 unrealistic. Further advances in defining the glial numbers are associated with the application of isotopic fractionation technique, which counts nuclei of neurones and non-neuronal cells in the homogenates of the nervous tissue [8, 40, 54]. This technique demonstrated that the total numbers of neuronal and non-neuronal cells in the human brain are more or

less on par, both being in the range of ~80–100 billion. After subtracting the population of endothelial cells which may account for about 20% of all non-neuronal cells, the true number of glial can be estimated at ~60 billion and total GNR for the whole brain is therefore less than 1:1. The density of glia is quite different in various brain areas. For example, the GNR varies between 1.2 in the grey matter of the occipital cortex and 3.6 in the grey matter of frontal cortical regions [73, 82], it is technically an infinity in the white matter that does not contain neuronal cell bodies, and hence inclusion of white matter counts increases the total GNR in the cortex to ~3. As already alluded above the GNR in cerebellum is very low probably not exceeding 0.1. Much higher GNR values were reported for the striatum (3.7:1), for the superior colliculus (10:1), for the ventral pallidum (12.2:1), for the lateral vestibular nucleus (30–50:1), while for the globus pallidus a very high GNR of 160:1 has been calculated from stereological counts [5, 8, 64, 71, 80, 91, 102]. Similarly, the GNR for the spinal cord was determined at 5:1 in cynomolgus monkey and almost 7:1 in humans [9].

Evolution of the nervous system paralleled with an increase in GNR, which however was not entirely linear and was not directly related to the intelligence. The nervous system of invertebrates has, as a rule, relatively smaller numbers of glial cells, with a GNR between 0.01:1 and 0.2:1 (50 glial cells derived from neuronal/epithelial progenitors and six glial cells that are mesodermally derived per 302 neurones in *Caenorhabditis elegans* [63, 89]; 10 glial cells per 400–700 neurones in every ganglion of the leech [20]; ~9000 glial cells per 90,000 neurones in the CNS of Drosophila [26, 52]). At the same time, the buccal ganglion of the great ramshorn snail *Planorbis corneus* contains 298 neurones and 391 glial cells [69], thus having a GNR of 1.3, very similar to that of humans. Similarly, in vertebrates there is no hard-and-fast increase in the GNR with an increasing brain size; for example, in the cortex, the GNR is about 0.3–0.4 in rodents, ~1.1 in cat, ~1.2 in horse, 0.5–1.0 in rhesus monkey, 2.2 in Göttingen minipig, ~1.5 in humans and as high as 4–8 in elephants and the fin whale [15, 27, 38, 43, 55, 65, 92]. The largest absolute number of glial cells has been counted in the neocortex of whales [27, 56]; stereological cell counts in the neocortex of the long-finned pilot whale (*Globicephala melas*) brain determined there are approximately 37.2 billion neurones and 127 billion glial cells and this gives a GNR of 3.4 [56]. The largest GNR was found in the neocortex of the common Minke whale (*Balaenoptera acutorostrata*), which contained ~12.8 billion neurones and 98 billion of glia giving therefore a GNR of ~7.6 [27].

1.4 Classification and Main Functions

Neuroglia (Fig. 1.1, see also [94]) are classified into glia of the peripheral nervous system (PNS) and of the CNS. The glial cells of the PNS originate (similarly to peripheral neurones) from the neural crest and are classified into:

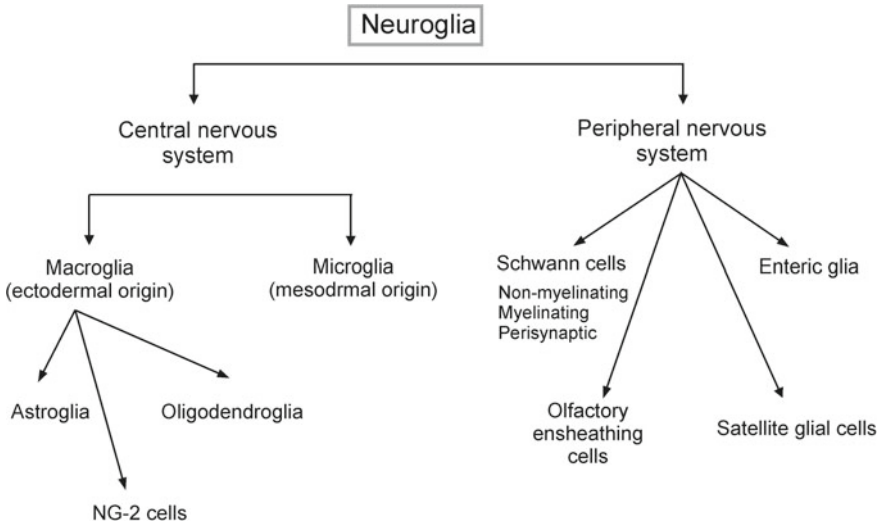

Fig. 1.1 Classification of neuroglia

(1) Schwann cells [48] associated with sensory, motor, sympathetic and parasympathetic axons; Schwann cells are further subdivided into (i) myelinating Schwann cells that myelinate peripheral axons; (ii) non-myelinating Schwann cells that surround multiple non-myelinating axons and (iii) perisynaptic Schwann cells, which enwrap peripheral synapses and maintain homoeostasis in the perisynaptic milieu.
(2) Satellite glial cells [35, 36], which are surrounding neurones in sensory, sympathetic and parasympathetic ganglia. These satellite glial cells control local homeostasis and are capable of reactive remodelling in pathology.
(3) Olfactory ensheathing cells [77], which are a part of the olfactory system. These cells extend very fine processes that enclose large numbers of unmyelinated olfactory axons
(4) Enteric glia [32, 33], represented by homeostatic glial cells of the enteric nervous system.

Neuroglia of the CNS are subdivided into macroglia (cells of ectodermal, neuroepithelial origin) and microglia (cells of mesodermal, myeloid origin). The macroglia is further classified into:

(1) Astroglia or astrocytes. Astrocytes are heterogeneous population of primary homeostatic glia residing throughout the brain and the spinal cord, in both grey and white matter. Astroglia include [94, 96]:

    (i) protoplasmic astrocytes of grey matter;
    (ii) fibrous astrocytes of white matter
    (iii) surface-associated astrocytes associated with the cortical surface in the posterior prefrontal and amygdaloid cortex;

(iv) Velate astrocytes, which are localised in the parts of the brain that are densely packed with small neurones, for example in the olfactory bulb or in the granular layer of the cerebellar cortex;
(v) Radial glia, which are the pluripotent neural cells precursors that generally disappear at birth in mammals
(vi) Radial astrocytes, which include Bergmann glia in the cerebellum, Müller glia of the retina, radial glia-like neural stem cells of the neurogenic niches and tanycytes of the hypothalamus, hypophysis and the raphe part of the spinal cord;
(vii) Pituicytes, which are the glial cells of the neurohypophysis;
(viii) Gomori astrocytes rich in iron and positive for Gomori's chrome alum hematoxylin staining identified in the hypothalamus and in the hippocampus;
(ix) Perivascular and marginal astrocytes, which are placed near the pia mater, where they form endfeet with blood vessels. These astrocytes do not establish contacts with neurones and their main function is in establishing the pial and perivascular glia limitans barriers.
(x) Ependymocytes, choroid plexus cells and retinal pigment epithelial cells. These cells line up the ventricles and the subretinal space; the choroid plexus cells produce the cerebrospinal fluid. Ependymocytes possess small movable processes (microvilli and kinocilia), which by rhythmic movements produce a stream of cerebrospinal fluid.

In addition, the brain of higher primates (including humans) contains several types of specialised astrocytes [17, 98], which include:

(xi) Interlaminar astrocytes;
(xii) Polarised astrocytes;
(xiii) Varicose projection astrocytes.

Function of these hominoid astroglia remain unknown.

Parenchymal astrocytes of the human brain are substantially larger and more complex compared with astroglial cells of rodents, and have distinct gene expression pattern [60–62, 87, 104]. Human protoplasmic astrocytes have about 10 times more primary processes and a more complex secondary process arborisation, with an average volume about 16.5 times larger than that of the corresponding astrocytes in a rat brain [61]. The larger human protoplasmic astrocytes also have extended outreach onto neuronal structures, on average contacting and encompassing up to 2 million synapses residing within astrocytic territorial domains, significantly more than the integrating capacity of rodent protoplasmic astrocytes, which covers ~20,000–120,000 synaptic contacts [13, 61]. Similarly, human fibrous astrocytes have a 2.14-fold larger domain compared to that in rodents [61].

(2) Oligodendroglia or oligodendrocytes, the myelinating cells of the CNS are subdivided into 4 classes [94]:

(i) *Type I oligodendrocytes* are most numerous in the cortex and grey matter; they have small rounded somata and fine branching processes that myelinate 30 or more small diameter axons;
(ii) *Type II oligodendrocytes* are similar to type I, but have parallel arrays of intermediate length internodes (100–250 μm), and are most common in white matter, such as the corpus callosum, optic nerve, cerebellum and spinal cord;
(iii) *Type III oligodendrocytes* have larger (than type I and II) irregular cell bodies, with one or more thick primary processes that myelinate a small number of large diameter axons with long internodes (250–500 μm). These cells are localised in the cerebral and cerebellar peduncles, the medulla oblongata, and the spinal cord funiculi;
(iv) *Type IV oligodendrocytes*, are somewhat similar to Schwann cells, being directly associated with a large diameter axon to form a single long internodal myelin sheath (as long as 1000 μm), and are restricted to tracts containing the largest diameter axons near the entrance/exit of nerve roots into the CNS.

(3) NG-2 glia also known as oligodendrocyte progenitor cells or OPCs, or synantocytes, or polydendrocytes [14, 57]. The NG2 glia can have homeostatic role and contribute to adulthood myelination, albeit their functions are yet to be better characterised.

Microglia originate from the foetal macrophages that migrate into the neural tube very early in the embryonic development; arguably, microglia represent the first parenchymal glia to populate neural tissue in development. Microglial cells carry numerous physiological functions, including shaping neuronal synaptic connectivity, removing of redundant or apoptotic neurones in the development and regulating synaptic transmission [45, 46, 90]. Microglia form the main defence system of the CNS through evolutionary conserved programme of activation (microgliosis) which can produce numerous neuroprotective and neurotoxic phenotypes [78, 83].

In terms of numbers, the most numerous glia are oligodendrocytes and NG2 cells combined (40–60%), with astrocytes accounting for 20–40% and microglia for ~10% of neuroglia population, although there is, of course, a considerable variability between the brain regions, developmental stage and species.

1.5 Envoi and Outlook

One of the two goals of this chapter is to serve as a general introduction into the world of Neuroglia. The other goal is to pique an interest of the reader into subsequent chapters in this book. As we tersely reviewed Neuroglia we establish the origin of these cells, their classification and their general functions in homeostasis and defence of the brain. In the following chapters, we explore the role of these cells in the progression of neuropathologies, especially neurodegenerative disorders. For

a long time, the neurone-centric view dominated neuropathological thinking, and only recently the role of glia has been reassessed and the perception is mounting of specific importance of neuroglia that to a very large extent defines the progression and outcome of most (if not all) neurological diseases.

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Chapter 2
Evolution of Neuroglia

Alexei Verkhratsky, Margaret S. Ho and Vladimir Parpura

Is evolution a theory, a system or a hypothesis? It is much more - it is a general postulate to which all theories, all hypotheses, all systems must henceforward bow and which they must satisfy in order to be thinkable and true. Evolution is a light which illuminates all facts, a trajectory which all lines of thought must follow-this is what evolution is.
Pierre Teilhard de Chardin.
quoted from Theodosius Dobzhansky, *Nothing in Biology Makes Sense Except in the Light of Evolution*, The American Biology Teacher, Vol. 35, No. 3 (Mar., 1973), pp. 125–129.

Abstract As the nervous system evolved from the diffused to centralised form, the neurones were joined by the appearance of the supportive cells, the neuroglia. Arguably, these non-neuronal cells evolve into a more diversified cell family than the neurones are. The first ancestral neuroglia appeared in flatworms being mesenchymal in origin. In the nematode *C. elegans* proto-astrocytes/supportive glia of ectodermal origin emerged, albeit the ensheathment of axons by glial cells occurred later in prawns. The multilayered myelin occurred by convergent evolution of oligodendro-

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cytes and Schwann cells in vertebrates above the jawless fishes. Nutritive partitioning of the brain from the rest of the body appeared in insects when the hemolymph-brain barrier, a predecessor of the blood-brain barrier was formed. The defensive cellular mechanism required specialisation of bona fide immune cells, microglia, a process that occurred in the nervous system of leeches, bivalves, snails, insects and above. In ascending phylogeny, new type of glial cells, such as scaffolding radial glia, appeared and as the bran sizes enlarged, the glia to neurone ratio increased. Humans possess some unique glial cells not seen in other animals.

Keywords Astrocytes · Blood/haemolymph-brain barrier · Brain size · Complexity of glia · Glia to neuron ratio · Microglia · Myelination · Oligodendrocytes · Radial glia

2.1 Evolution of the Nervous System

Several taxonomy charts are currently in use. Here we use the system that classifies all living forms into Superkingdoms or Empires of Prokaryota and Eukaryota (Fig. 2.1). The Empire of Prokaryota comprises a single Kingdom of Bacteria, whereas the Empire of Eukaryota includes the Kingdoms of Protozoa, Animalia,

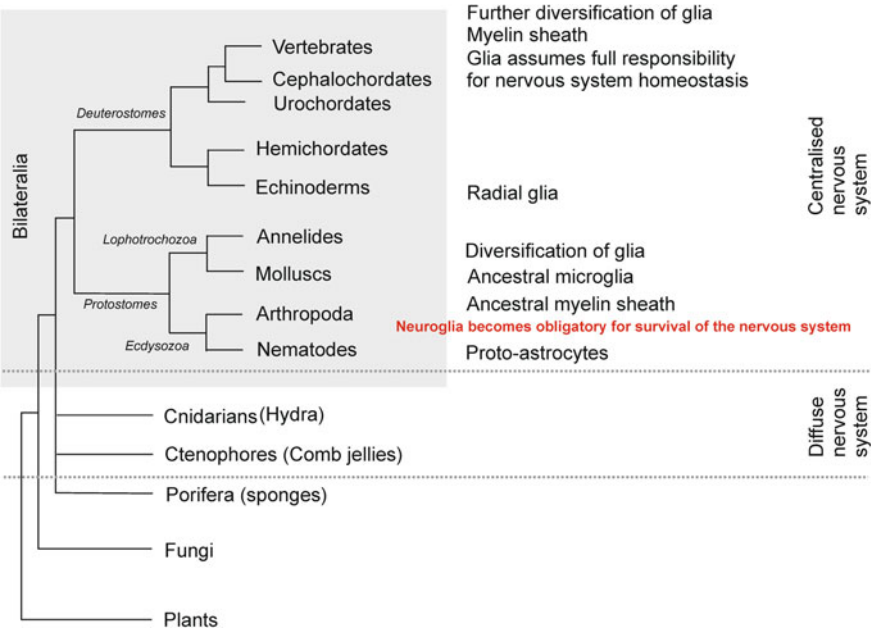

Fig. 2.1 Tree of life and evolution of the nervous system and of neuroglia. Adapted from Verkhratsky and Butt [143]

Fungi, Plantae and Chromista [17, 18]. The nervous system is the sole prerogative of the Kingdom of Animalia, which is represented by radially symmetrical Cnidaria and Ctenophora, and Bilateralia. The bilateralia are further subclassified into Protostomia and Deuterostomia, the latter including Echinodermata, Hemichordata and, finally, Chordata to which vertebrates belong.

Arguably, the most ancient nervous systems appeared in Ctenophora (comb jellies) and Cnidarians (hydras and sea jellies). This was represented by the so-called diffuse nervous system, formed by homogeneously distributed network of neurones connected with their processes [45]. The principal cells of the diffuse nervous system are multipolar and unipolar neurones, organised in several semi-independent networks connected through chemical synapses that mainly used peptides as neurotransmitters. The neurones remain the sole elements in the diffuse nervous system with no evidence of (and obviously no need for) any kind of specialised supportive cells. Of note, the primordial neurones evolved from epithelial cells [58], which expressed ion channels and transporters (that formed basis for electrical excitability) and the secretory vesicular system that was the morphological substrate for chemical neurotransmission [123].

Further evolution of the nervous system saw the change from diffuse nervous system to the centralised nervous system. At that stage the first conglomerates of neuronal somatas forming ganglia had emerged. The tendency to concentrate nervous elements was observed already in some Cnidarian polyps where neural networks appear denser around the oral opening. The first true centralised nervous systems, however, appear in early Bilateralia, in flatworms, earthworms and roundworms. For example, in roundworms the centralised nervous system is composed of several ganglia surrounding the oral orifice. In more advanced protostomes (e.g., insects and crustacea), the centralisation advanced further, with the polyganglionic brain. In vertebrates the new brain organisations in layers have appeared and progressed. The centralisation of the nervous system also signalled the appearance of supportive neural cells, the neuroglia.

Characterisation and morphological analysis of glial cells in early invertebrates and in phylogenetically lower taxa have been very much hampered by the absence of specific markers similar to those found for higher species. The main criteria for identification of glia is their close association with and coverage of neuronal elements, these being fundamental features of supportive cells. It is most probable that neuroglia has evolved several times in different species. Despite very similar functions, the appearance of glial cells is rather different between non-vertebrates and vertebrates, for example. Even genes responsible for glial differentiation can be distinct. In the insects, for example, development of glia is controlled by the gene glial cells missing (*gsm*—[60, 63]). In contrast, the *gsm* homologue gene is not even expressed in the CNS of mammals [69].

The very first glial cells provided for support of neuronal cell bodies within the ganglia (primeval astrocytes) as well as support of axons (primeval oligodendrocytes/Schwann cells). Another important function of primordial glia was formation of peripheral sensory organs, or sensilla. The glia-like cells are found in Acoelomorpha, the primitive flatworms, which are generally considered to be the earliest (or

one of the earliest) Bilateralia. Electron microscopy of the brains of *Symsagittifera roscoffensis*, *Convoluta psammophila*, *Amphiscolops sp*. and *Otocelis rubropunctata* (free-living Acoela worms) characterised non-neuronal cells with electron-dense cell bodies in which nuclei occupy most of the cytosol, and lamellar processes extend into neuropil and surround groups of neurites [10, 11]. More advanced neuroglia is present in the nematode *Caenorhabditis elegans*. For example, the sheath glia of the cephalic sensilla in *C. elegans* possess some anatomical and functional characteristics that parallel those of astrocyte and oligodendrocyte lineages in the mammalian nervous system [133]. In Platyzoa (another member of early Bilateralia), glial cells have been found in polyclad flatworms and in some (but not in all) triclad planaria, whereas neuroglia seem to be absent in Rotifera (wheel animals) and in many platyhelminthes (for example, in tubellarian flatworms, Catenulida or Macrostomida). Neuroglia are generally present throughout Ecdysozoa and Lophotrochozoa, being well developed in molluscs, in Annelida, and even more developed and rather diverse in Arthropoda. In Deuterostomes the organisation of the brain as well as newly emerged spinal cord has changed form polyganglia to the layers, as a result of the appearance of the radial glia, a new type of neuroglia, which provided both for neurogenesis and migration of neuronal precursors to their appropriate layers. In early Chordata, the radial glial cells predominate and are present throughout life, while the parenchyma is quite underdeveloped. An increase in brain thickness instigated the emergence of parenchymal glia that diversified and became responsible for major homeostatic tasks in the CNS of mammals. The radial glial, however, remain active only in the prenatal period and largely disappear after birth. Below, we shall provide an account of the evolution of the main types of neuroglia.

2.2 Neuroglia in Invertebrates

2.2.1 Primitive Glia of Flatworm

The flatworms are the most primitive bilateralia with clearly formed centralised nervous system with the ‘brain’ represented by cerebral ganglia. The cerebral ganglia of at least two flatworms, *Fasciola hepatica* and *Notoplana acticola*, apparently contain some type of supportive cells that might be considered the ancestral neuroglia [135, 136]. These primitive glia, defined as mesenchymal cells, have long processes emanating from the cell body; some of these processes encircle the cerebral ganglion, some invaginate into giant nerve cells processes, whereas some other send processes into the ganglion and enclose clusters of neuronal processes. The ‘glia-like’ mesenchymal cells most likely originate from parenchymal cells of the worm and undergo morphological specialisation after contacting the nervous elements. Some of these cells contain glycogen and may act as a source of energy substrates [139].

2.2.2 Complex Neuroglia of the Earthworm

In the earthworm *Eisenia fetida* several types of glia have been characterised according to the morphology and localisation of the supportive cells. These neuroglia types were classified as neurilemmal-, subneurilemmal-, supporting-nutrifying- and periaxonal sheath-forming glial cells [26]. The neurilemmal glia are elongated with long processes. Subneurilemmal glia are small spindle-like cells with few processes. Supporting-nutrifying glial cells are positive for glial fibrillary acidic protein (GFAP, a well-accepted marker for mammalian astrocytes) and appear as brushes on the surface of the neuronal perikarya. Sheath-forming glial cells are found around giant axons [26]. The membranes of glial cells form close contacts with neuronal membranes, these possibly being involved in glia- to-neurone transport. Glial cells in the earthworm contain multiple intracellular vesicular structures with diameters between 200 and 400 nm, some with dense cores, perhaps indicating glial secretion.

2.2.3 Proto-astrocytes in Caenorhabditis Elegans

The nervous system of C. *elegans* has been precisely mapped with a wealth of details and meticulously categorised with structural information allowing identification of each neural cell [147]. The neuroglia of *C. elegans* were described and characterised based on light and electron microscopy (none of these glial cells express markers of mammalian glia), with these cells being defined as neural in origin lacking morphological characteristics of neurones, for example pre-synaptic structures [138, 147]. The nervous system of *C. elegans* contains 302 neurones, 50 supportive (glial) cells derived from the ectoderm and six supportive cells of the mesodermal origin [101, 134, 147]. The central nervous system of *C. elegans* comprises the nerve ring located in the frontal part of the body. The nerve ring receives processes of peripheral sensory neurones and also contains cephalic and motor neurones, axons of which convey efferent signals through the ventral and dorsal nerve cords.

Most of the neuroglial cells (that is 46 cells out of 56) of *C. elegans* are associated with the sensory system (Fig. 2.2). These cells are classified into 26 socket cells and 20 sheath cells that (together with neuronal processes) form sensory organs known as sensilla [112]. Four glial cells of the ectodermal origin known as cephalic sheath (CEPsh) cells are localised in the nerve ring (Figs. 2.2 and 2.3). The anterior processes of CEPsh cells cover cephalic neuronal dendrites and form sensilla in the lips of the animal. Posterior processes of CEPsh cell with lamellar morphology ensheath the nerve ring and send processes to the neuropil, where they contact and possibly enwrap synapses [101, 134]. The CEPsh cells control ion homeostasis in perisynaptic regions and are involved in neuronal development and morphogenesis. Complete ablation of glia in of *C. elegans* results in complex morphological, developmental, sensory and behavioural deficits, although it does not affect survival of the worm [4].

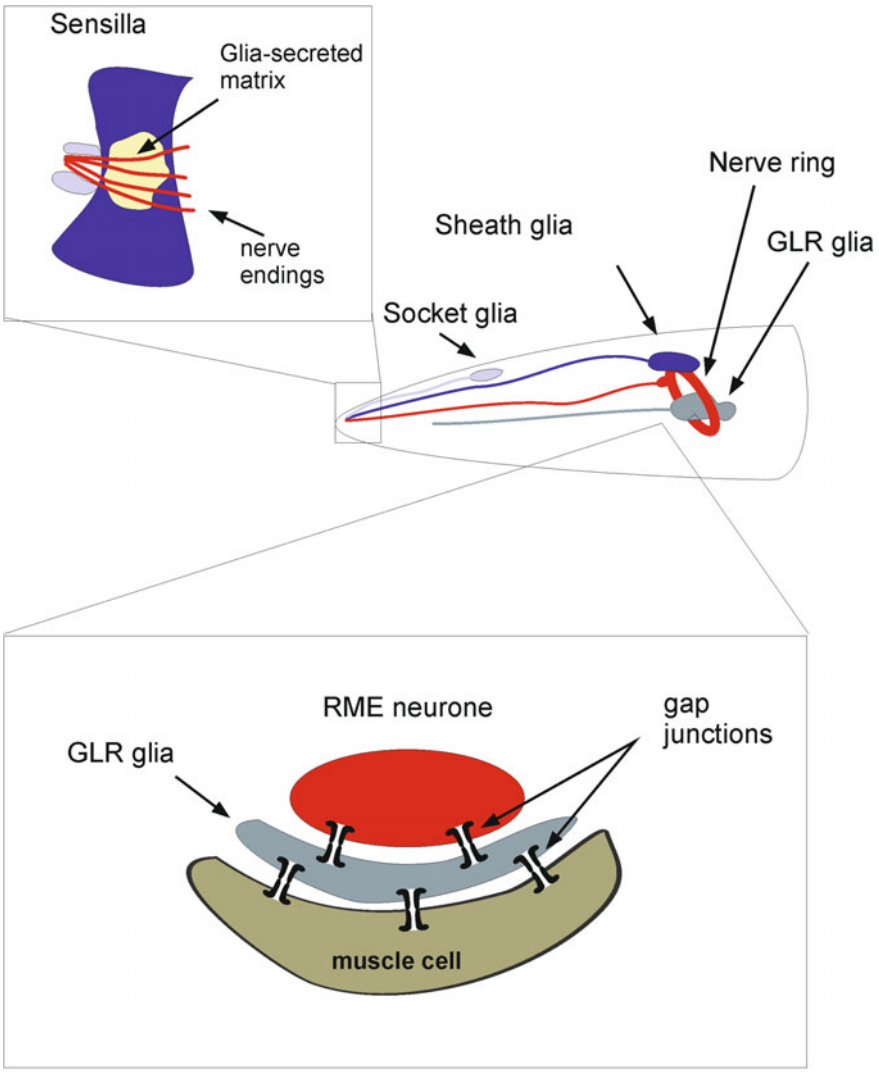

Fig. 2.2 Glial cells in *Caenorhabditis elegans*. The "brain" of *C. elegance* is represented by the nerve ring. Most of the glial cells are part of sensory organs known as sensilla. Each sensilla has two glial cells: the sheath cell and socket cell. In the anterior part there are 4 CEP (cephalic) glial cells that ensheath nerve ring. The nerve ring also has 6 GLR glial cells which establish gap junctional contacts between ring motor neurones (RME) and muscle cells

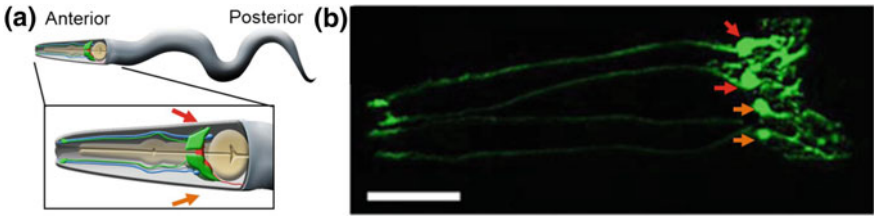

Fig. 2.3 The CEPsh glia. **a** A cartoon of an adult worm showing the four CEPsh glial cells (green) positioned in the anterior of the worm (inset). The CEPsh cell bodies with their velate processes are positioned around the central nerve ring (red) which they enwrap along with the proximal section of the ventral nerve cord. Additionally, each CEPsh glial cell possesses a long anterior process, emanating to the anterior sensory tip, which closely interacts with the dendritic extension of a nearby CEP neuron (blue). Arrows indicate the dorsal (red arrow) and ventral (orange arrow) side of the worm. **b** A confocal image showing green fluorescent protein expression driven by the *hlh-17* promoter to visualize the four CEPsh glial cells (worm strain VPR839). The anterior (head) of a juvenile (larval stage 4) worm is shown; the worm is turned ~45° from "upright" such that all four CEP sheath cells are visible. The sheath portion of the cells that form a tube around the dendritic endings of the CEP neurones are seen at the left of the image. The dorsal (red arrow) and ventral (orange arrow) CEPsh cell bodies are seen. The thin sheet-like extensions that surround and invade the nerve ring are seen in the rightmost part of the image. Scale bar, 20 μm. Image adapted from [134]

The glia of *C. elegans* have numerous differences from neuroglial cells of higher animals. Porto-astrocytes of the worm do not express GFAP; their physiology has both neuronal and non-neuronal properties—for example, Ca^{2+} signals in *C. elegans* glial are generated by Ca^{2+} entry through voltage-gated channels, while functional intracellular Ca^{2+} stores appear to be rudimentary or absent (Fig. 2.4, [133]). Glial cells of the worms, of course, do not form the glia limitans barrier because of the absence of the circulatory system. Main homeostatic functions of *C. elegans* glial cells similarly remain unknown; arguably, they may include K^+ buffering and neurotransmitter catabolism [134]. At the same time several genetic pathways controlling development and differentiation of glia are shared between the worm and mammals. For example, the transcription factor LIN-26 contributes to glial cell development and ablation of the *lin-26* gene may turn glial cells into neurones [75]. Another gene expressed in worms, the *hlh-17* gene (the promotor of which was used to generate markers for CEPsh glia [86]) has homology to the mammalian regulator of glial development *Olig2* [101, 154].

Neuroglia in *C. elegans* is responsible for the following functions: (i) establishment of the location of neuronal structures; (ii) regulation of size and morphology of sensory endings; (iii) creation of a barrier that bundles and separates neuronal elements from other cells; and (iv) modulation of neuronal activity. Conceptually, these functions are similar to the functions of glia in higher invertebrates and in vertebrates. Glial cells in *C. elegans* support development of the nervous system. In particular, glial cells modulate sensory activity by controlling the development of cellular compartments surrounding sensory cilia [114]. The sensory organs of the worm are singular aspects of the nematode nervous system, and their correct

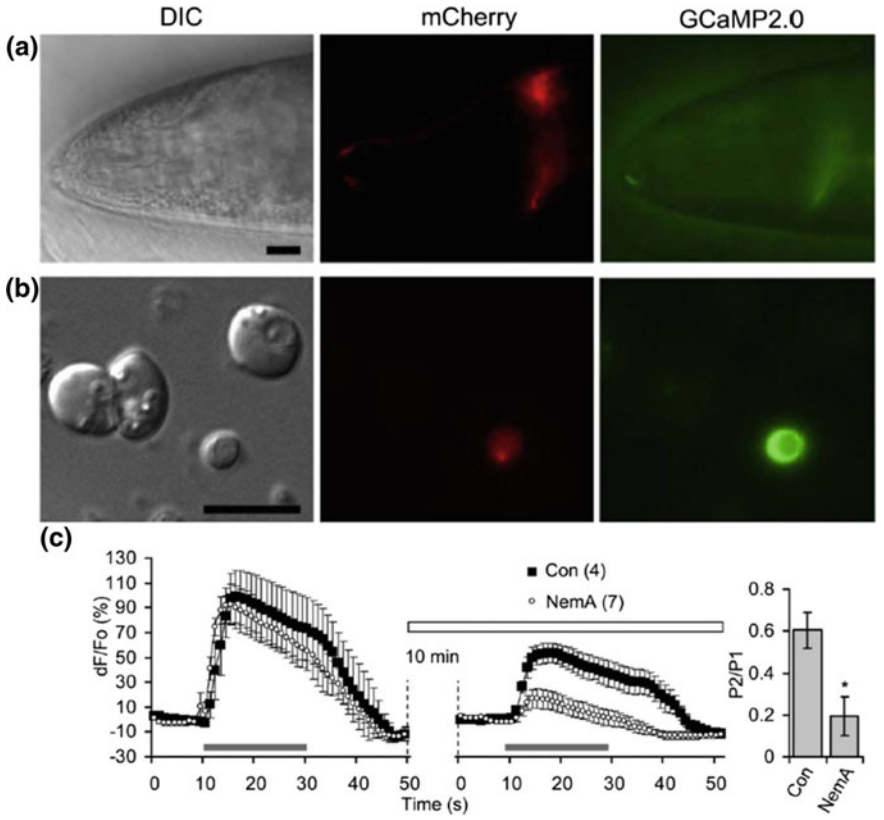

Fig. 2.4 L-type voltage-gated Ca^{2+}channels (VGCCs) play a role in depolarization-induced intracellular Ca^{2+} elevations in CEPsh glial cells. **a** The hlh-17 promoter can be used to drive expression of a red fluorescent protein marker (red, mCherry) in the CEPsh glia along with a fluorescent-protein based Ca^{2+} sensor (green, GCaMP2.0). DIC, differential interference contrast. An anterior portion of an L4 stage worm (VPR108 strain) is shown. **b** CEPsh glial cells in mixed culture prepared from embryos can be identified based on their mCherry/GCaMP2.0 expression. **c** Time-lapse of GCaMP2.0 fluorescence emission from CEPsh glial cells. Paired-pulse application of a depolarization stimulus, high extracellular potassium (HiK^+, 100 mM; horizontal bar), to CEPsh glial cells results in an elevation of intracellular Ca^{2+} levels (black squares). Nemadipine-A (NemA), a pharmacological L-type VGCC blocker, can be used to test the channels present in glial cells in culture; Con, sham stimulated control. (right, bar graph). Ratio of the peak Ca^{2+} level in response to the second HiK^+ application (P2) over the first application (P1). *Indicates a significant difference. Adapted from [133]

development is impaired in the absence of glia; moreover, development of sensory structures requires the expression of gene sets both in neurones and in glia [102]. Factors released by glia control sensory dendrites attachment during migration of neurones in development [53].

The four CEPsh glial cells (see above and Figs. 2.2, 2.3) differentially express netrin (UNC-6). Two ventral CEPsh cells express netrin, which regulates axon guidance, while the dorsal pair of CEPsh glia lacks the expression of this protein [52, 146, 154, 52, 146, 154].

There are some hints indicating that the CEPsh cells modulate dopamine-dependent behaviours in the worm, including feeding pattern and certain forms of learning [41]. Disruption of the *hlh-17* gene, expressed in CEPsh cells affected the egg-laying behaviour, instigated deficits in feeding behaviour and plasticity, and disrupted gustatory associative learning. The CEPsh glia are closely associated with four CEP neurones, which mediate the aforementioned behaviours through release and up-take of dopamine, and hence CEPsh glia seem to modulate neuronal function [41, 134].

The *C. elegans* is also in possession of a rather unique class of glial cells, which connect neurones with myocytes. These so-called GLR cells are of the mesodermal origin [1]. The GLR cells are integral part of the nervous system of the *C.elegans*; these cells are involved into the development of the nerve ring and the muscle-based feeding organ of the worm known as pharynx. The most idiosyncratic feature of GLR cells is that the cells are being sandwiched between and connected to both neurones and muscle cells in the head by gap junctions. This arrangement arguably represents a signalling circuit for producing specialised fine motor movements of the frontal part of the worm during foraging [120, 148]. The GLR-neuronal circuit contains both coincidence detection and shunting activity that is based on gap junction intercellular communication [115]. Gap junctional connectivity between glial cells and neurones are not limited to invertebrates only; there are some indications of this form of neuronal-glial communications in higher animals [113] as well as in developing nervous system of the mammals [95, 105]. The special set of cells connecting neurones and muscle cells is present in vertebrates including mammals; these are the telocytes initially discovered by Santiago Ramón y Cajal in the gut as interstitial cells of Cajal at the beginning of the twentieth century; the telocytes are found now in various tissues [113].

2.3 Homeostatic Proto-astrocytes in Annelida

The nervous system of the medicinal leech *Hirudo medicinalis* includes the anterior and posterior brains and the chain of 21 ganglia that are positioned in between (Fig. 2.5a). The anterior brain is made of six ganglia fused into two neuronal masses, while seven fused ganglia in the tail form the posterior brain. Somatic ganglia innervate corresponding segments of the leech body [22, 30]. Every ganglion contains ~400 neurones (with exception of the 5^{th} and 6^{th} ganglia innervating the reproduc-

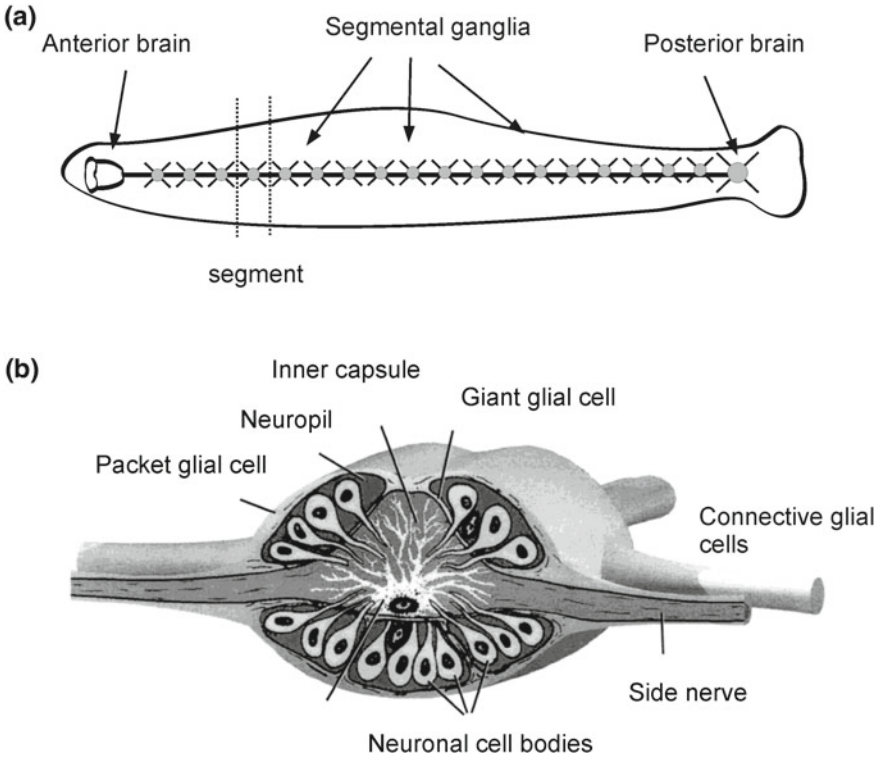

Fig. 2.5 Neuroglia in medicinal leech *Hirudo medicinalis.* **a** General structure of the nervous system. **b** Structure of a segmental ganglion, which contains three types of glial cells: the giant glial cell; packet glial cells and connective glial cells. Adapted from Verkhratsky and Butt [143]

tive system, which have about 700 neurones) and ten glial cells. These glial cells are (Fig. 2.5b): (i) two connective glial cells, which ensheath axons; (ii) six packet cells covering neuronal cell bodies and (iii) two giant glial cells [30]. Glial cells of the leech nervous system are interconnected by gap junctions assembled from innexins [37, 65, 79] thus creating a panglial syncytium. The nervous system of the leech additionally contains some amount of microglial cells that can be activated in response to lesions [76]. Within the ganglia the homeostatic support is maintained by packet glia and giant glial cells which in this function resemble mammalian astrocytes. The packet glial cells buffer extracellular K^+, especially at high extracellular K^+ concentrations [97, 124]. The giant glial cells have somata of 80–100 μm in diameter; these somata are localised in the centre of the ganglion. The processes of giant glial cells are 300–350 μm long; they extend through the entire neuropil and contact neuronal dendrites [93]. The neuropil is partitioned by these processes into several functional domains. In addition, glial processes sometimes invaginate into neuronal somata creating a structure described as 'trophospongium' [59]. The membrane of giant glial cells is highly permeable to K^+, which underlies hyperpolarised resting

potential of about−75 mV. Giant glial cells express multiple types of neurotransmitter receptors including ionotropic glutamate, acetylcholine and serotonin receptors as well as metabotropic receptors to glutamate, serotonin, myomodulin and possibly P2Y-like purinoceptors and A_1-like adenosine receptors [30, 92]. Giant glial cells regulate many homeostatic responses, including regulation of pH involving plasmalemmal Na^+–HCO_3^- co-transporter, Na^+–H^+ and Cl^-–HCO_3^- exchangers [28, 31, 32]. They also regulate neurotransmitters uptake and catabolism through plasmalemmal Na^+-dependent glutamate and Na^+-dependent choline transporters [33, 56, 149]. Giant glial cells respond to neuronal activity and to evoked behaviours by changes in membrane potential [29] and by generation of cytosolic Ca^{2+} signals that occur in both somata and processes [34]. In contrast to mammalian glia, and rather similar to *C. elegans* CEPsh glial cells, the main source of Ca^{2+} signal generation in leech glia is associated with the opening of plasmalemmal Ca^{2+} channels. Termination of Ca^{2+} signal is mediated by plasmalemmal Ca^{2+} pump and Na^+/Ca^{2+} exchanger. The intracellular Ca^{2+} stores seem to play a minor role in Ca^{2+} dynamics of leech glia [34, 80].

2.4 Proto-astrocytes in Insects

The brain of *Drosophila* contains ~90,000 cells of which 10% belong to neuroglia; some glial cells also exist in the peripheral nervous system. Neuroglia of *Drosophila* are classified (Fig. 2.6) into the following classes [39, 43, 49, 108]: (i) Wrapping glia of the peripheral nervous system; (ii) Surface glia, which make the brain-hemolymph barrier, is further subclassified into perineural glia (small cells lying on the ganglionic

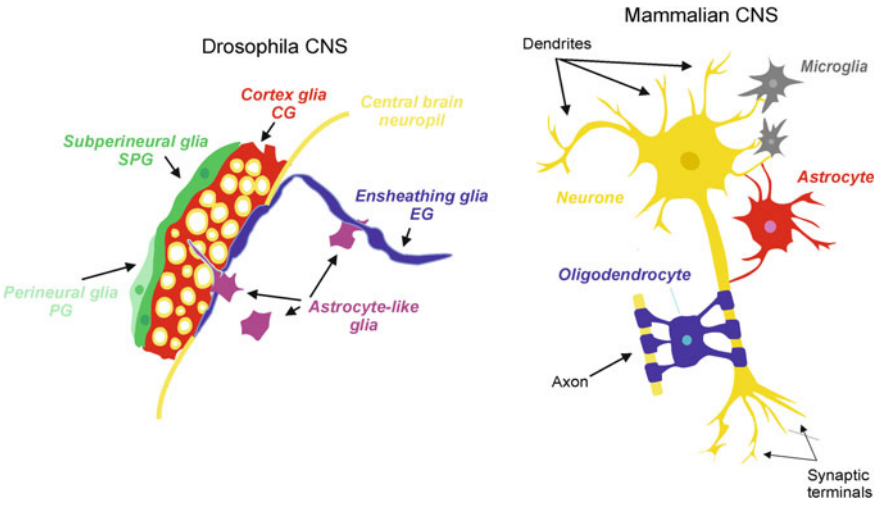

Fig. 2.6 Neuroglia in Drosophila and mammals

surface) and subperineural or basal glia (which are large sheet-like cells connected with septate junctions that form the barrier); (iii) Cortex glia that contact neuronal cells somata in the CNS, with each glial cell establishing contacts with many neurones; (iv) Neuropil glia, which are located in the neuropil and cover axons and synapses. The neuropil glia are further subdivided into ensheathing or fibrous and astrocyte-like glia, which forms a perisynaptic glial cover. Finally, (v) Tract glial cells cover axonal tracts connecting different neuropils. The cell mapping using the glia-specific GAL4-UAS system derived numerical calculation of various glial cell types in the Drosophila CNS. The perineural glia accounted for ~17%, subperineural—2%, cortex glia—20%, astrocyte-like glia—34% and ensheathing glia for 27% of total glial cells [70].

The cells of the above classes ii, iii and iv (i.e. surface glia, cortex glia and neuropil glia) are homeostatic cells being thus similar to astrocytes in mammals; the degree of morphological specialisation is, however, greater with specialised cell types responsible for distinct set of functions. A layer of perineural glia covers and delineates the brain as an organ. This coverage is provided by ~100 perineural glial cells, which create a physical barrier of the CNS. Perineural glia and primary surface glia of the ventral nerve cord are derived from embryonic neuro-glioblasts. Both types of primary glial cells increase in size, but not in number, during the larval stage. These cells are retained throughout metamorphosis and become the functional adult glia [35]. Primary perineural glia are mitotically active in the larva, undergo a late phase of proliferation during late larval stage, and differentiate into the optic lobe wrapping glia and then into optic lobe distal satellite glia [104].

Immediately beneath the perineural glial layer, the subperineural glia forms the hemolymph-brain barrier that controls exchange of substances between the CNS and the rest of the body [3, 131]. The subperineural glial cells have a flat morphology and are connected with innexin-based septate junctions, which seal the barrier. Molecular components of septate junctions include Neurexin IV, Gliotactin, Neuroglian and both of α and β subunits of Na^+/K^+ ATPase [8, 44, 132]. Loss of function of subperineural glia results in aberrant physiological properties such as the alternation in the permeability of extracellular dextrans [132] and reversed polarity of the electroretinogram [150, 151]. The G-protein coupled receptors moody expressed in the surface glia is essential for proper barrier formation. Glial cells in *moody* locus mutant flies exhibit disruptive actin cytoskeleton, which leads to reduced number of septate junctions and a leaky and malfunctional brain-hemolymph barrier [6, 50, 126]. Mutations in the ATP-binding cassette (ABC) transporter gene *mdr65* expressed in surface glia alters the passage of substrates and increases the sensitivity of the haemolymph-brain barrier to toxic pharmaceuticals, thus playing a role in neuroprotection [85]. The glial barrier in the insects is functionally analogous to the endothelial blood-brain barrier in vertebrates. This glial barrier in Drosophila is particularly important in guarding the CNS against substantial fluctuations in systemic K^+ that occur after feeding [39].

The specialised type of parenchymal glia, known as cortex glia covers neuronal somata; these cells are very much different from mammalian CNS glia in that a single cell can provide coverage for many neuronal cell bodies. The cortex glial cells also make contact with the hemolymph-brain barrier and provide trophic support

to neurones. The cortical glial cells generate activity-dependent intracellular Ca^{2+} oscillations and regulate seizure susceptibility [88]. Similarly to the perineural glia, primary cortex glial cells enter a phase of proliferation during the late larval period, likely forming the secondary population of adult cortical glia. Glial cells in the insect CNS also plaster tracheoles, which deliver oxygen to the nerve tissue [111].

The neuropil glia in *Drosophila* are represented by the ensheathing glia and the astrocyte-like glia, both of which are critical elements for the neuropil homeostasis, synaptogenesis and synaptic transmission. Physical contacts between neuropil glia and axons as well as with axon fascicles allow these glial cells to function in a variety of synaptic contexts to regulate neuronal activity and survival. The flat ensheathing glial cells line the borders of the neuropil thus segregating it from the cortex. In addition, ensheathing glia enwrap glomeruli in the antennae lobe. The astroglia-like cells are morphologically similar to parenchymal astrocytes in vertebrates with elaborated arborisation; these cells extend processes into the neuropil and provide for synaptic coverage [36]. Astrocyte-like glia express plasmalemmal amino acid transporters to regulate uptake and release of neurotransmitters to modulate synaptic activity. The ensheathing glia express the engulfment receptor Draper and dCed-6, which control clearing axonal debris due to injury in adult brains. The astrocyte-like glia, also express Draper, which regulates pruning of axons during the development [137] Both types of neuropil glia, the ensheathing glia and astrocyte-like glia share the same origin being the progenies of the embryonic lateral glioblast. The lateral glioblast-generated primary glia undergo several rounds of mitotic divisions to produce a cluster of cells that differentiate into the ensheathing glia and astrocyte-like glia. The primary glial cells are subject to programmed cell death and are not retained into adulthood [9]. Instead, a secondary wave of proliferation from larval neuro-glioblasts is responsible for generating adult ensheathing and astrocyte-like glia [104].

Neuroglial cells in *Drosophila* exhibit classical intracellular ionic excitability, which contributes to neuronal-glial interactions. Different types of insect glia demonstrate spontaneous and evoked Ca^{2+} signalling mediated by both intracellular Ca^{2+} release (mostly in soma) and plasmalemmal Ca^{2+} entry [88]. Mutation in glial Na^+/Ca^{2+}-K^+ exchanger (NCKX), which arguably mediates plasmalemmal Ca^{2+} flux in cortical glia, results in seizures [88]. The astrocyte-like glial cells in *Drosophila* demonstrate prominent Ca^{2+} oscillations, seen as fast fluctuations of intracellular Ca^{2+} in processes. Acute induction of Ca^{2+} influx into these astroglia-like cells triggered rapid behavioural paralysis and suppressed neuronal activity [156].

Homeostatic functions of glial cells in *Drosophila* CNS include regulation of ionic balance and control over clearance, recycling and metabolism of neurotransmitters. In the retina, for example, glial cells are providing for recycling of the principal neurotransmitter histamine. Histamine, released from photoreceptors, is accumulated by glial cells, processes of which enwrap relevant synapses [87]. After entering the glial cytoplasm histamine is converted into β-alanyl-histamine (also known as carcinine) by Ebony (N-β-alanyl-biogenic amine synthetase)-catalysed reaction [13]. This carcinine is then shuttled back to photoreceptors, which is mediated by a plasmalemmal carT (in humans—organic anion transporter family *Slc22a*) transporter [130]. In

the photoreceptor cell, carcinine is hydrolysed to histamine by Tan (acyltransferase) protein [140]. Mutations in the components of this histamine/carcinine shuttle impair vision of the fly *Drosophila* [19, 157]. Genetic alterations of glial cells in *Drosophila* glia which interfere with vesicle trafficking (by specific expression of temperature-sensitive dynamin) and ionic transport (by glia-specific expression of bacterial Na^+ channel) alter circadian rhythm [96].

Another important function of homeostatic neuroglia lies in regulation of neurotransmitters turnover and catabolism. For example, *Drosophila* neuropil glia express plasmalemmal transporters for excitatory amino acid transporters dEAAT1and dEAAT2 [66, 127], as well as glutamine synthetase [27], all these being key components of glutamate-glutamine shuttle. The plasmalemmal glutamate transporters are preferentially localised at glial perisynaptic processes [121]; decreased expression of these transporters triggers neurotoxicity, degeneration of the neuropil and premature death [78]. In addition, *Drosophila* glia regulate the homeostasis of inhibitory neurotransmitter γ-aminobutyric acid (GABA) through activity of relevant transporters [103]. The glia-specific cascades regulating glutamate transport are involved in control of sexual behaviour and courtship of the flies. These cascades are represented by glial cystine-glutamate transporter, which controls ambient glutamate concentration and therefore affects the strength of glutamatergic transmission. Loss of function mutation of these transporters (observed in a mutant known as a genderblind, *gb*) results in 'homosexual' courting [47].

The neuroglia in insects is fundamental for metabolic support of neurones and for neuroprotection. In the retina of the honeybee, glial cells supply photoreceptors with alanine, which subsequently is converted to pyruvate for use in the Krebs cycle and production of energy [141]. Targeted ablation of glial cells in the fly instigates extensive neuronal death [12]. Similarly, neuronal loss and progressive neurodegeneration are observed in mutants with aberrant or non-functional glia (mutants designated as *drop dead*, *swiss cheese* and *repo* [14, 71, 150]). Insect neuroglia provides for nervous tissue defence through reactive gliosis and phagocytosis activated in response to lesions [72, 81].

Neuroglia in *Drosophila* form the neurogenic niche, a specialised anatomical location where stem cells reside, proliferate and differentiate [20, 90]. The cortex glial cells in particular regulate neuroblast proliferation; the neuroblasts establish a specific adhesive contact with cortex glia, this process involving phosphoinositide 3-kinase (PI3-kinase) and *D*E-cadherin. The cortical glia also regulate neuroblast proliferation through secretion of nutritional signal molecules, such as insulin-like peptides (ILPs) or the glycoprotein encoded by *anachronism* locus [38, 128, 129]. Cortex glia also produce and secrete ILPs in response to nutrition; these peptides activate the PI3 K/AKT signalling in neuroblasts, thus stimulating neuroblast growth and proliferation. The insulin/insulin-like growth factor (IGF) receptor pathway is necessary for neuroblasts to exit quiescence [20]. Likewise, a fat-body-derived signal required for neuroblast activation is linked to rapamycin (TOR) signalling cascade [128]. Another type of glia, the optic lobe-associated cortex glia, promote neuroepithelial proliferation and neuroblast formation by activating epidermal growth factor receptor. *Drosophila* microRNA mir-8 (the homolog of vertebrate miR-200 family)

is expressed in a subpopulation of optic-lobe-associated cortex glia processes, which ensheath the neuroepithelium. In the absence of glial mir-8, excess proliferation and ectopic neuroblast transition were detected, suggesting that optic-lobe-associated cortex glia use signalling via mir-8 to communicate with the neuroepithelium. The optic-lobe-associated miR-8-positive cortex glia thus acts as a niche component that contributes signals for the growth and morphogenesis of the neuroepithelium [89]. Taken together, these findings suggest that glia are indispensable components of the neurogenic niche in insects; glial cells regulate formation, proliferation and differentiation of neuroblasts.

Drosophila glia also positively regulates and promotes synaptogenesis as well as synaptic maturation through activation of a homologue of the Gabapentin receptor α2δ1 [73].

The glia-regulated signalling cascade involving the peripheral glia expressing Eiger, the first invertebrate tumour necrosis factor (TNF) superfamily ligand [61], and the motorneurone-specific *Drosophila* TNF-α receptor (TNFR) Wengen [64], regulates neuromuscular junction synaptogenesis. This glia-initiated TNF signalling depends on caspase and mitochondria to regulate neuromuscular junction degeneration, further demonstrating the importance of glia in regulating neuromuscular junction synaptogenesis [67].

2.5 Astrocytes in Chordata and Low Vertebrates

In the CNS of Chordata, Hemichordata and Echinodermata, the main (and often the only) type of neuroglia is represented by radial glial cells. The radial glia, although occurring at some developmental stages in the insects and being identified in some protostomes (for example, in Annelida and Scalidophora [54]), is mainly associated with vertebrates [116]. In the Echinodermata (sea urchin, star fishes or sea cucumber), radial glial cells are the only glia in the CNS. These radial glial cells produce and secrete the Reissner's substance [84, 145], which mainly contains the glycoprotein known as SCO-spondin, that acts as cell adhesion modulator [46]. This Reissner's substance has been identified in radial glia throughout Chordata from cephalochordates to *Homo sapiens*. Glia of Echinodermata have a characteristic radial morphology with elongated shape; these cells have long processes penetrating the whole thickness of the neural parenchyma, and orienting perpendicularly to the surface of the neuroepithelium and high level of expression of intermediate filaments in the cytoplasm [83, 84].

Radial glia represent the main type of neuroglia in the CNS of many early vertebrates, which are almost completely devoid of other types of parenchymal glia. This in particular is characteristic for the brains with thin parenchyma. In Elasmobranchii (chondrichthyan/cartilaginous fish, such as sharks and rays), the brains are sub-classified into the type I, or 'laminar' brain (with thin brain wall and large ventricles) and the type II or 'elaborate' brain (with thicker parenchyma and smaller ventricles) [2, 16]. In type I brains radial glia predominate, whereas type II brains

contain numerous well-developed parenchymal glia resembling astrocytes [2]. Emergence of parenchymal astrocytes in elaborate brains probably reflects an increased homeostatic challenge of the enlarged nervous tissue that cannot be met by radial glia. This constrains homeostatic capabilities of the radial glia and hence prompts an increase in numbers and complexity of parenchymal astrocytes [118].

Similarly radial glia are well developed in bony fish, with teleosts (e.g., zebra fish) being a particular example. The radial glia of the zebrafish, extend their processes through the entire thickness of the brain from the ependymal cells of the ventricles to the pial surface. In these radial glial cells high expression of GFAP has been detected; in addition, these cells possess glutamine synthetase contributing to glutamate homeostasis, and express aquaporin-4 contributing to water homeostasis, [48]). Zebrafish does not possess parenchymal glia (i.e., astrocyte-like cells) and hence radial glial cells are specially important for the responses of zebrafish nervous tissue to injury. Brain lesions in the teleost do not instigate astrogliosis. The stab wounds are closed in several days without formation of the scar; because of rapid increase in the neurogenesis, that generates new cells to fill up the wound [7]. Of note the blood-brain barrier in teleosts is formed by endothelial cells lining brain capillaries, which is similar to all higher vertebrates.

2.6 Astrocytes in Higher Vertebrates and Hominids

An increased complexity of the brain, which developed in parallel with increased intellectual power and increased homeostatic and energy demands stipulated high diversification of neuroglia in mammals [99, 100, 117, 119]. Glial cells became heterogeneous in their form and function. This evolutionary advance in astroglial complexity is specifically prominent in the brains of higher primates and humans [99, 144].

The number of glial cells varies substantially between different species and the GNR does not simply increase with increasing brain size (Fig. 2.7). Albeit already discussed in Chap. 1, we here revisit the glial-to-neurone ratio (GNR) in the nervous system. In invertebrates, it varies between 0.001 and 0.1 (56 glia per 302 neurones in *C. elegans* [101]; 10 glial cells per 400–700 neurones in every ganglion of the leech [30]; ~9000 glial cells per 90,000 neurones in the central nervous system (CNS) of Drosophila [39, 70]). There are exceptions though: the buccal ganglia of the great ramshorn snail *Planorbis corneus* contains 298 neurones and 391 glial cells with GNR of 1.3 [110]. In vertebrates the GNR is about 0.3–0.4 in rodents, ~1.1 in cat, ~1.2 in horse, 0.5–1.0 in rhesus monkey, 2.2 in Göttingen minipig, ~1.5 in humans, and as high as 4–8 in elephants and the fin whale [21, 40, 51, 62, 77, 107]. The largest absolute number of glial cells has been identified in the neocortex of whales [40, 31, 35]; for example the neocortex of the long-finned pilot whale (*Globicephala melas*) contains ~ 37.2×10^9 neurones and 127×10^9 glial cells with GNR of 3.4 [91]. In the human brain, the total number of glia is more or less the same as number of neurones (about ~80 billion of neurones and ~60 billion of glia) with remarkable

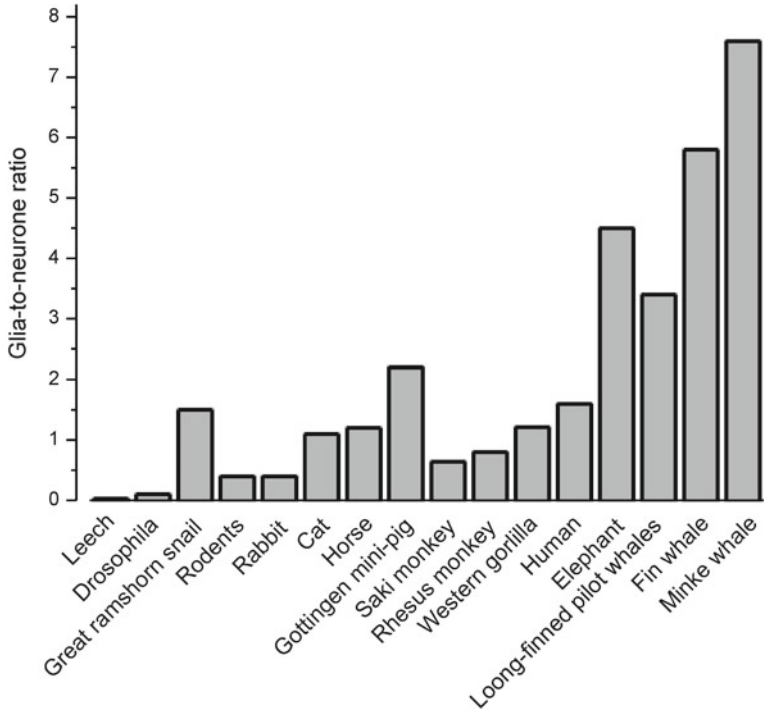

Fig. 2.7 Phylogenetical advance of neuroglia. Glia-to-neurone ratio in the nervous system of invertebrates and in the cortex of vertebrates. Glia-to-neurone ratio is generally increased in phylogeny; more or less this ratio linearly follows an increase in the size of the brain

regional differences. Of note, the more primitive parts of the human brain have a higher GNR of 7–10 in the brainstem, or even more according to some studies [106]. The GNR of ~5 was determined for the spinal cord of cynomolgus monkey and GNR of almost 7 for spinal cord in humans [5]. These trends argue against the concept that a high GNR reflects evolutionary advance and increased intelligence. Nonetheless, it is important to be aware that evolution brought with it substantial changes in the morphology and complexity of astroglia in the human cortex, which also contains several highly specialised types of glial cell which are absent in the brains of lesser vertebrates.

Human astrocytes are much larger and far more complex than astroglial cells in, for example, rodent brain (Fig. 2.8). In the human brain the average diameter of the domain belonging to a human protoplasmic astrocyte is ~2.5 times larger than the domain formed by a rat astrocyte (142 vs. 56 μm). The volume of the human protoplasmic astrocyte domain was ~16.5 times larger than that of the corresponding domain in a rat brain. Human protoplasmic astrocytes have ~10 times more primary processes, and correspondingly much more complex processes arborisation than rodent astroglia. Similarly, fibrous astrocytes, localised in the white matter are ~2.2

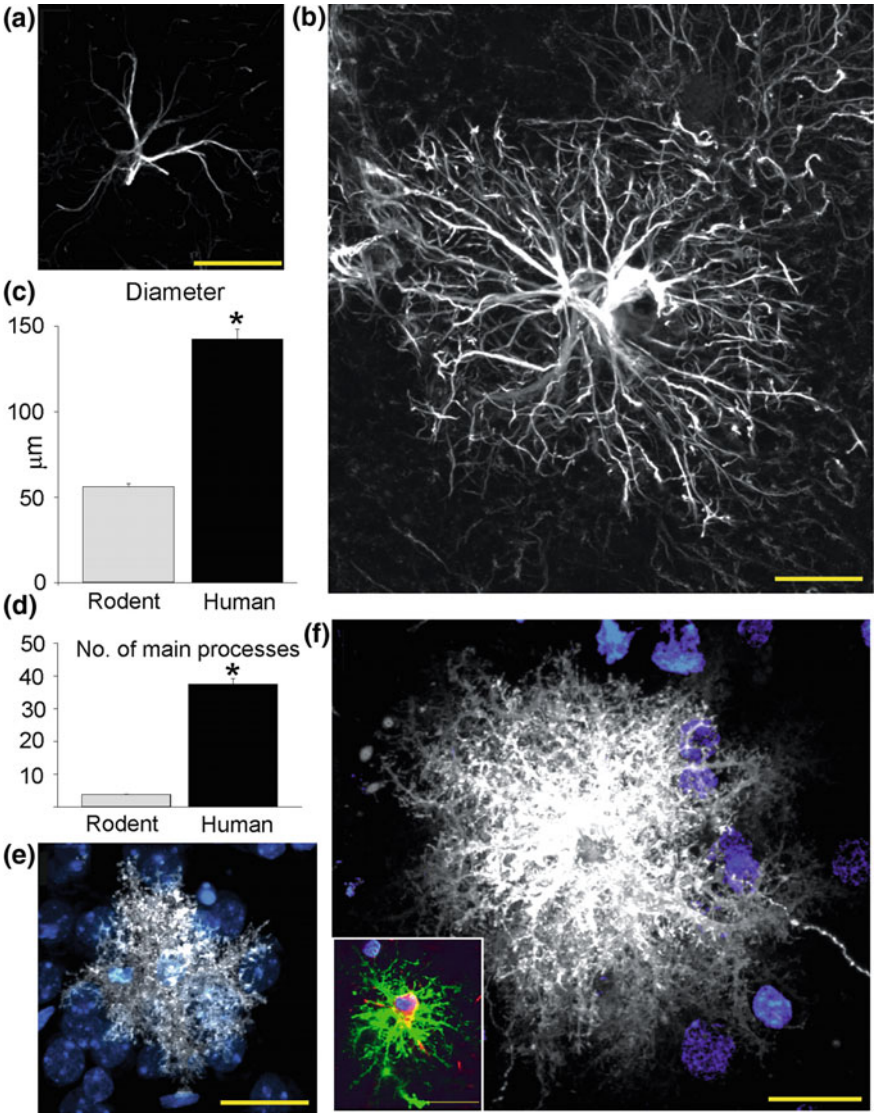

Fig. 2.8 Comparison of rodent and human protoplasmic astrocytes. **a** Typical mouse protoplasmic astrocyte. GFAP, White. Scale bar, 20 μm. **b** Typical human protoplasmic astrocyte at the same scale. Scale bar, 20 μm. **c**, **d** Human protoplasmic astrocytes are 2.55-fold larger and have 10-fold more main GFAP processes than mouse astrocytes (human, n = 50 cells from 7 patients; mouse, n = 65 cells from 6 mice; mean ± SEM; *p < 0.005, t test). **e** Mouse protoplasmic astrocyte diolistically labelled with DiI (white) and sytox (blue) revealing the full structure of the astrocyte including its numerous fine processes. Scale bar, 20 μm. **f** Human astrocyte demonstrates the highly complicated network of fine process that defines the human protoplasmic astrocyte. Scale bar, 20 μm. Inset, Human protoplasmic astrocyte diolistically labelled as well as immunolabelled for GFAP (green) demonstrating colocalisation. Scale bar, 20 μm. Reproduced, with permission from [99, 144]

times larger in humans when compared to rodents. Due to this increased complexity human protoplasmic astrocytes enwrap ~2 millions of synapses localised in their territorial domains, whereas in rodents single astrocyte covers only 20,000–120,000 synaptic contacts [15, 99].

Special subpopulations of astrocytes are found in the brains of higher primates and humans (Fig. 2.9). The first type of these specialised astroglial cells is known as interlaminar astrocytes [23–25]. The somata of these interlaminar astrocytes are localised in the layer I of the cortex, which has low density of neuronal cell bodies and high density of synaptic connections. Somata of interlaminar astrocytes are rather small not exceeding 10 μm, from these somata emanate several short and one or two very long processes. These long processes, which can be as long as 1 mm pen-

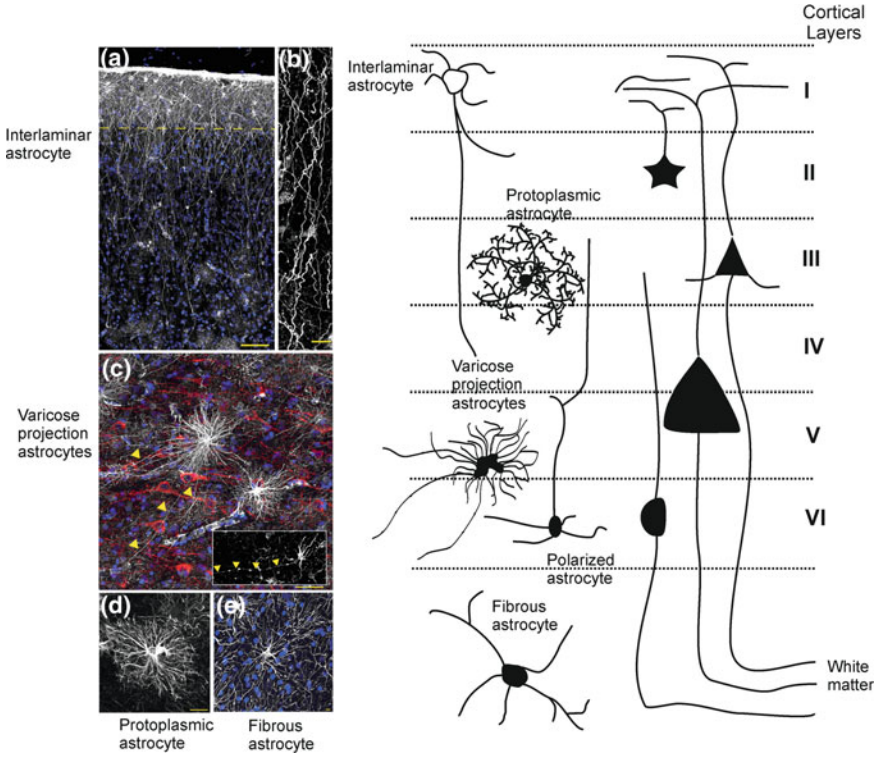

Fig. 2.9 Morphological heterogeneity and subtypes of astrocytes in the cortex of higher primates. **a** Pial surface and layers 1–2 of human cortex. GFAP staining in white; DAPI, in blue. Scale bar, 100 μm. Yellow line indicates border between layer I and II. **b** Interlaminar astrocyte processes. Scale bar, 10 μm. **c** Varicose projection astrocytes reside in layers V and VI and extend long processes characterized by evenly spaced varicosities. Inset: Varicose projection astrocyte from chimpanzee cortex. Yellow arrowheads indicate varicose projections. Scale bar, 50 μm. **d** Typical human protoplasmic astrocyte. Scale bar, 20 μm. **e** Human fibrous astrocytes in white matter. Scale bar, 10 μm. (modified with permission from [98]. Left panel schematically shows different astrocytes and their relatuons to cortical layers. Adapted from [143]

etrate through the cortex, and terminate in layers III and IV. At the tips of these long processes special structures known as the 'terminal mass' or 'end bulb' have been detected. These terminal masses often contain mitochondria. Physiological properties of interlaminar astrocytes remain completely unknown. It has been suggested that they may form astroglial component of neuronal columns that are regarded as cortical functional units. It has been further speculated that interlaminar astrocytes may contribute to long distance signalling and integration within cortical columns.

The second type of specialised human astrocytes is represented by polarised astrocytes, represented by uni- or bipolar cells, somata of which are in cortical layers V and VI close to the white matter. These cells possess one or two very long (up to 1 mm) and very thin (2–3 μm in diameter) processes that end in the neuropil. In addition these processes have numerous varicosities with yet unknown functions [99, 144].

In contrast to neuroglia neurones did not increase their size that much. Similarly, the density of synaptic contacts in rodents and primates is similar (the mean density of synaptic contacts in the rodent brain is ~1397 millions/mm^3, whereas in humans synaptic density in the cortex is around 1100 millions/mm^3). Likewise, the number of synapses per neurone is not much different between primates and rodents. The average size of human neurones is ~1.5 times larger than in rodents. Thus, at least morphologically, evolution resulted in much more prominent changes in glia than in neurones, which most likely has important, although yet undetermined, significance.

2.7 Evolution of Myelination and Oligodendroglia

The emergence and evolution of myelination is linked to an increase in animal size that requires faster nerve conductance; increase in action potential propagation velocity can be achieved either through an increase in axon diameter, or through introduction of saltatory nerve impulse propagation. Increase in axon diameter reduces resistance of the axon proportionally to the square of diameter, with the conductance velocity being directly proportional to the square root of the axon diameter [57]. In the *Loligo* squid, for instance, thick axons of 0.5 mm in diameter sustain action potential propagation velocity of about 30 m/s. Large axons, however, present several problems to the complex nervous system. Firstly, the conduction through large axons is energetically costly, because Na^+/K^+ pump responsible for ion gradients maintenance consumes substantial amounts of ATP [82]. Secondly, large diameter axons are associated with prominent space constrains incompatible with a rather compact design of advanced CNS.

The saltatory propagation of action potential, which allows high speed nerve impulse conductance, solves both problems due to restriction of ion fluxes to small portions of axonal membrane. Namely, axons are covered with multiple layers of lipid membranes, interrupted by gaps known as nodes of Ranvier. The nodal membranes have high densities of voltage-gated Na^+ and K^+ channels responsible for action potential generation [94]. The lipid-rich membranous lamellae insulate parts of the

axons in between the nodes, thus increasing axonal transverse resistance and reducing transverse capacitance.

The very first structures unsheathing the axons and allowing saltatory propagation of action potentials emerged early in evolution. Arguably, the first organisms in possession of this mechanism are prawns, which appeared in the Cambrian period (500–540 million years ago). At that time, the giant prawn, *Anomalocaridids*, was the sole and the most dangerous predator of the sea [142]. These predatory prawns had about 1 m in length, and they were endowed with large compound eyes. These eyes were exceptionally big (according to the fossil studies the visual surface was 22 mm long and 12 mm wide) being composed of tens of thousands of hexagonal ommatidial lenses ~70–110 μm in diameter [109]. Feeding thousands of axons into the CNS of these animals most likely required insulating ensheathing of axons.

Modern Crustacean (e.g., prawns, shrimps and crabs) retain this arrangements having elaborated axonal ensheathment. In the prawns of the genus *Penaeus* (such as Japanese tiger shrimp, or Chinese white shrimp), axons are surrounded by glial membranes and by a large submyelin space positioned between the axonal membrane and the first layer of glial membrane. The ion currents thus are trapped in this space as if the normal axon is surrounded by a giant axon (the submyelin space acts in essence as a low-resistance pathway), this topography allows for an unprecedented speed of action potential propagation of up to 210 m/s [74, 152, 153]. The submyelin spaces are tightly sealed at the nodes thus allowing the saltatory conductance. The node (which in invertebrates is called the 'fenestration node') diameter and internodal distance is proportional to the axon diameter and, in prawns, vary between 5 and 50 μm and 3 and 12 μm, respectively. The thickness of the glial membranous sheath is ~10 μm; it is comprised of 10–60 stacked membrane layers separated by 8–9 nm. Like in vertebrates, voltage-gated sodium channels in prawns are concentrated at the nodes where their density can reach thousands of channels/μm^2. There is a fundamental difference between vertebrates and prawn axonal coverage. In vertebrates the single Schwann cell (peripheral nerves) or a process of an oligodendrocyte (CNS) spirals around the axon forming multiple membranous lamellae. In the prawns a single myelinating glial cell sends multiple processes forming multiple layers, with each process encircling the axon once, meeting itself on the opposite side in a seam [55]. Another difference is location of the nuclei of myelinating cell. In vertebrates it is located as a rule at the outer edge of myelin sheath, whereas in prawns the nuclei are randomly dispersed between membrane laminae [153].

Axons covered with multilayered glial membranes (although these glial cells do not produce myelin) are operative in some other invertebrates. In the earthworm *Lumbricus terrestrils* the central axons of 50–100 μm in diameter are enwrapped with 60–200 layers of cell membranes produced by many glial cells, nuclei of which are scattered along the axon [122]. In this structure the nodes are not clearly seen; nonetheless, the conductance velocity reaches ~20–45 m/s, which is higher compared to thicker giant axons of *Loligo* squid. The glial axonal coverage was also found in marine Annelida phoronids; in these animals axons are covered with 9–20 membranous layers [42]. Similar number of layers of glial membranes covers the axon of the aquatic sludge worm *Branchiura sowerbyi* [158].

Emergence of myelin is associated with relatively developed vertebrates. Compacted myelin sheaths are absent in lower vertebrates, such as hagfish and lampreys, and begun to develop in sharks and bony fish. There are some arguments indicating that the most ancient forms of myelin sheath emerged in placoderms (now extinct jawed armoured fish from the early Silurian period ~420 million years ago). These fish form the base for chondrichtyan and bony fishes. The fossil records indicate that the diameters of the foramina for oculomotor nerves in the jawed fish and in jawless Osteostraci fish (which do not have myelin) are the same (about 0.1 mm), whereas the length of nerve in placoderms was 10 times larger, that highlights the necessity for myelin to maintain the same speed of action potential-mediated signal transduction [155]. Further reasoning suggests the connection between appearance of the jaw in early Gnathostomata (jawed vertebrates, which embrace all higher vertebrates living today, including mammals) and myelination. By acquiring myelinated nerves, the jawed fishes arguably acquired better ability to hunt the prey, while keeping the axonal diameter the same or even smaller compared to their jawless predecessors [155].

Coverage of axons with glial membranes emerged in early evolutionary forms. In the beginning this coverage arguably supported axonal mechanical stability and provided energy support. At the same time glial membranous lamellae increased action potential propagation velocity, and once emerged, myelination provided obvious evolutionary advantages. One of the advantages was an increase in compactness of the nervous system and decrease in energy expenditure for restoring ion balances.

2.8 Evolution of Microglia

The evolutionary origins of microglia remain largely unexplored. It is possible to suggest, however, that appearance of innate immune and phagocytic cells in the nerve tissue coincided with the emergence of barriers separating early brains from the circulation. Such barriers restricted pathways for entry of immune/defence cells into the brain parenchyma, thus calling for a specialised intra-brain defence system. This problem was solved after immune cells found the way to migrate and retain in the nerve tissue; exposure to the specific neurochemical environment as well as epigenetic trends most likely stimulated acquisition of specific microglial phenotype. Evidence for phylogenetically early microglial cells is available for Annelida (leech), Mollusca (Bivalvia and snails) and some Arthropoda (insects) (see [68] for detailed review).

The nervous system of medicinal leech contains substantial numbers of microglial cells. The microglia of the leech is represented by small spindle-like shape cells. Insults to the leech nervous system trigger microglial activation; these cells migrate to the site of the insult and become phagocytes. The weak silver carbonate staining (a classical microglial staining technique developed by Pío del Río Hortega) is generally used to visualise activated microglia in leech. In response to infectious attack the leech microglial cells were found to produce and secrete antimicrobial peptides [125].

Well developed microglia were also found in nerve ganglia of molluscs. In the marine bivalve *Mytilus edulis* microglial cells can be activated and their migration can

be instigated in response to various molecular signals including nitric oxide, opioids, cannabinoids and cytokines. Similarly, migrating microglia were observed in the snail *Planorbarius corneus* and in the insect *Leucophaea maderae*. The microglial cells (morphologically distinguished by phagocytic inclusions) of snail *Planorbis corneus*, were mainly concentrated in the neuropil of nerve ganglia while mechanical lesion increased the number of these phagocytic cells [110].

2.9 Conclusions

Most ancient glial cells developed as a supportive element of the sensory organs. The centralisation and increase in complexity of the nervous system created high demand for homeostatic support which was met by diversification of glial cells. Such a diversification resulted in multiple phenotypes in invertebrates, which are in possession of very specialised glial cells such as giant glial cells in the leech or cortex glia in *Drosophila*. These glial cells of invertebrates have performed many supportive functions from regulation of ion and neurotransmitter homeostasis to metabolic support and regulation of neuronal development. In the invertebrates glial cells formed brain to body barriers which stipulated the emergence of specialised immune and defence cells known as microglia. Increase in complexity of brain connectome and increase in axonal density were factors defining evolutionary benefits of the myelin sheath and development of myelinating cells. The evolution of myelination formed the basis for increased complexity of the nervous system that relies on interneuronal connections. In early Chordata radial glia become the main sub-type which was instrumental in formation of the layered brain. Subsequent increase in brain thickness promoted evolution of homeostatic astroglia. Finally, in the brain of primates and especially in the brain of humans, astrocytes become exceedingly complex and new types of astroglial cells involved in interlayer communication/integration have evolved.

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Chapter 3
Physiology of Astroglia

Alexei Verkhratsky, Vladimir Parpura, Nina Vardjan and Robert Zorec

Abstract Astrocytes are principal cells responsible for maintaining the brain homeostasis. Additionally, these glial cells are also involved in homocellular (astrocyte-astrocyte) and heterocellular (astrocyte-other cell types) signalling and metabolism. These astroglial functions require an expression of the assortment of molecules, be that transporters or pumps, to maintain ion concentration gradients across the plasmalemma and the membrane of the endoplasmic reticulum. Astrocytes sense and balance their neurochemical environment via variety of transmitter receptors and transporters. As they are electrically non-excitable, astrocytes display intracellular calcium and sodium fluctuations, which are not only used for operative signalling but can also affect metabolism. In this chapter we discuss the molecules that achieve ionic gradients and underlie astrocyte signalling.

Keywords Astrocytes · Brain homoeostasis · Neurotransmitter receptors · Ion channels · SLC transporters · Ca^{2+} signalling · Na^{+} signalling

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3.1 Definition of Astroglia

Astroglia (also known as astrocytes) are a class of neural cells of ectodermal, neuroepithelial origin that sustain homeostasis and provide for defence of the central nervous system (CNS) (Fig. 3.1; [410]). The term astrocyte (αστρον κψτοσ; *astron*, *star and kytos*, *a hollow vessel*, later *cell*; that is a star-like cell) was introduced by Michael von Lenhossék in 1895 [213]; of note, he proposed to name all parenchymal glia spongiocytes, with only a subtype of these cells having characteristic morphology in Golgi's stained preparations being identified as astrocyte. On this matter Lenhossék 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'. The terms of protoplasmic (white matter) and fibrous (grey matter) glia were introduced by Albert von Kölliker and William Lloyd Andriezen [13, 195]).

Astrocytes demonstrate quite heterogeneous morphology across different brain structures (see Chap. 1). Nevertheless, the main physiological features of astroglial cells are somewhat similar, being specifically tailored for their homeostatic function. Astrocytes maintain homeostasis of the CNS at all levels of organisation [408–410] from molecular (ion and transmitter homeostasis, regulation of pH, metabolic energy support, Fig. 3.1), cellular (neurogenesis), network (synaptogenesis and synaptic maturation, maintenance and extinction), organ (regulation of the blood-brain barrier, operation of the glymphatic system) and systemic (chemosensing of oxygen, CO_2 and systemic Na^+ concentration).

In the CNS, astrocytes are integrated into cellular networks (known as syncytia), by gap junctions, which are specialised areas of apposing membranes of adjacent cells pierced by many hundreds of intercellular channels or connexons that form the conduit for intercellular transport of ions, second messengers and other biologically active molecules with a molecular weight lesser than 1000 Da. In the mammalian

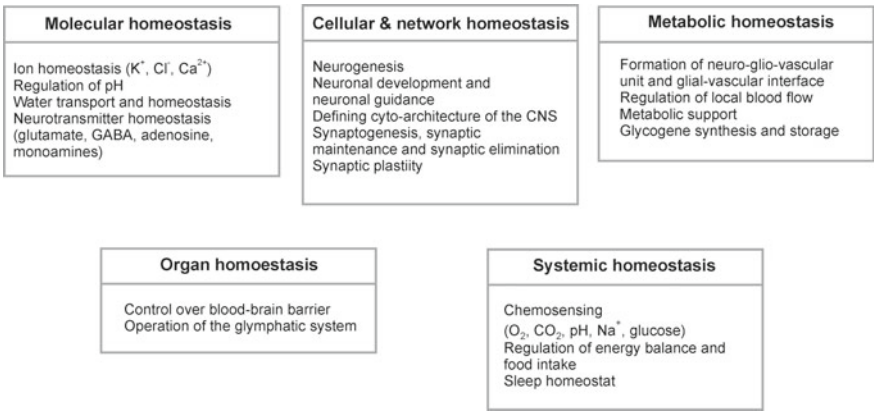

Fig. 3.1 Homoeostatic functions of astroglia

CNS, astroglial syncytia are anatomically segregated within different anatomical structures. In the sensory cortex, astroglial syncytia are confined to individual barrels and in the olfactory bulb to individual glomeruli [118, 148, 332]. Panglial syncytia that connect astrocytes and oligodendrocytes have been identified in the thalamus, neocortex and hippocampus [59, 123, 181, 296]. Whether astroglia form syncytia with neurones, remains an open issue.

3.2 Membrane Physiology and Ion Distribution

Astrocytes are electrically non-excitable cells, with a rather negative resting membrane potential (V_m) of about −80 mV. Disparity between cytosolic and extracellular ion concentrations (Fig. 3.2), together with the specific membrane ion permeability, define this negative V_m of astrocytes. At rest intra-astrocytic concentration of K^+ is between 120 and 140 mM and extracellular K^+ concentration is about 3 mM, which sets the equilibrium potential for K^+ (E_K) at −98 mV (at 37 °C). Concentration of cytosolic Na^+ in astrocytes (15–20 mM) is generally higher than in the majority of neurones (8–10 mM). With Na^+ concentration in the cerebrospinal fluid (CSF) around 145–155 mM, the corresponding E_{Na} ranges between +55 and +60 mV [192, 331]. Concentration of ionised Ca^{2+} in the cytosol of astrocytes ranges between 50 and 150 nM, which for extracellular $[Ca^{2+}]$ of 1.4 mM sets the $E_{Ca}{}^{2+}$ at +120 to +

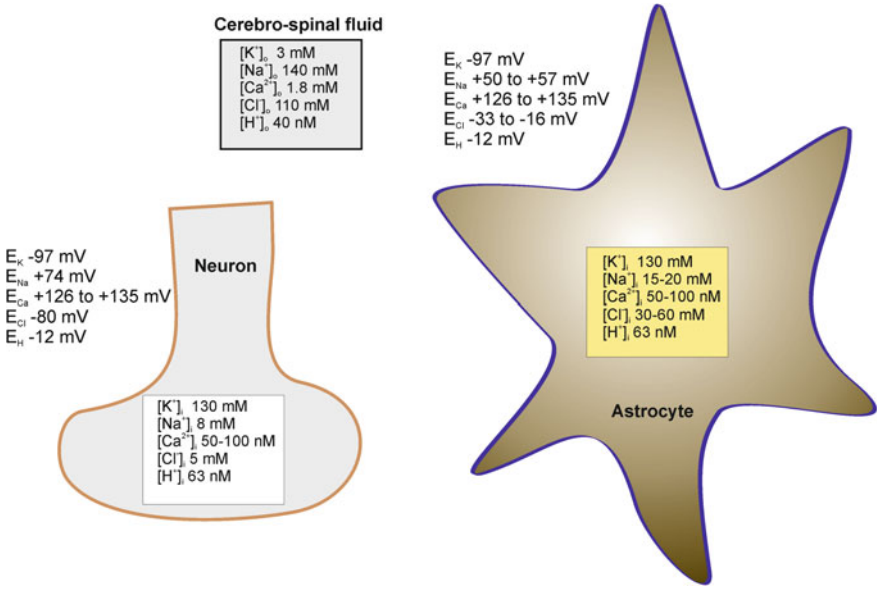

Fig. 3.2 Ion distribution (and corresponding values of equilibrium potentials for different ions) between the cerebrospinal fluid and cytosol of astrocytes and neurones. Modified from [413]

140 mV. Cytosolic concentration of free Mg^{2+} in cultured astrocytes measured with a fluorescent probe Mag-Fura-2 is around 125 μM [20]; the CSF Mg^{2+} has been determined at ~0.9 mM [384] giving $E_{Mg}{}^{2+}$ ~ +25 mV.

High cytosolic Cl^- concentration (30–50 mM) has been measured in cultured astrocytes and Bergmann glial cells in cerebellar slices [403]; this sets the $E_{Cl}{}^-$ around −35 mV (the $[Cl^-]_o$ is ~120 mM). The concentration of protons in astroglial cytosol is ~63 nM (pH 7.2), which, assuming the extracellular H^+ concentration to be ~40 nM (pH 7.4) sets the $E_H{}^+$ at ~−12 mV. The cytosol of astrocytes is rich in CO_2 (~1.2 mM) and $HCO_3{}^-$ (~17 mM).

The most characteristic electrophysiological signature of mature astrocytes is hyperpolarised resting potential (~−80 mV) and low input resistance (5–20 MΩ) indicative of high resting membrane permeability for K^+ [242, 243]; the current to voltage relationship of astroglial cells is nearly linear [2, 73, 161, 168]. Fluctuations of astroglial V_m generally reflect changes in extracellular K^+ concentration [11, 81].

3.3 Ion Channels

3.3.1 Potassium Channels

Glial membrane permeability is dominated by K^+ channels, several types of which are expressed in astrocytes (Fig. 3.3). These channels have distinct voltage-dependence, which covers the whole range of physiological membrane potentials, thus ensuring

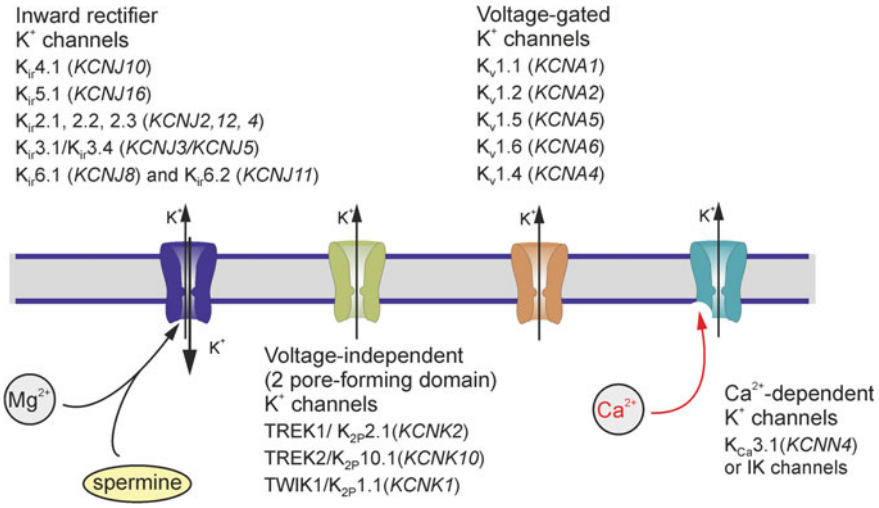

Fig. 3.3 Potassium channels in astroglia. Gene names for a given channels are shown in parentheses. Modified from [413]

the passive properties of astroglial plasma membrane. This prominent K^+ conductance of astrocytic plasmalemma defines the homeostatic capabilities of astrocytes [269], supporting movement of ions and providing electrical driving force for membrane transporters.

3.3.1.1 Inward Rectifier Potassium Channels, K_{ir}

Inward rectifying K^+ channels are so-called because they pass K^+ ions more easily into the cell (the inward direction) than out of the cell (the outward direction). The inward rectification occurs due to a voltage-dependent intracellular block by Mg^{2+} and polyamines [225]. These two transmembrane domain channels belong to a gene super-family represented by 16 subtypes (*KCNJ1–KCNJ18*), which are further divided into 7 families from $K_{ir}1.x$ to $K_{ir}7.x$ [139].

The main astroglial inward rectifying K^+ channels are represented by the $K_{ir}4.1$ subtype (product of *KCNJ10 gene*). These channels are detected in many types of astroglia including protoplasmic and fibrous astrocytes, from all brain areas including hippocampus, neocortex, optic nerve, cerebellum, spinal cord and retina [58, 140, 169], with some regional variations; astroglia such as Müller glia and Bergmann glia express them as well. High $K_{ir}4.1$ immunoreactivity was detected in hippocampal astrocytes, in astroglial cells in the cerebral cortex, in the deep cerebellar nuclei, in Bergmann glia and in Müller cells but not in astrocytes in white matter [309]. In the spinal cord, the expression of $K_{ir}4.1$ channels is the highest in astrocytes from the ventral horn and the lowest in astrocytes from the apex of the dorsal horn [273]. The $K_{ir}4.1$ channels are major contributors to the resting membrane potential of astroglial cells: functional inhibition or genetic deletion of K_{ir} currents markedly increases input resistance (up to 20-fold) and depolarises astrocytes (by ~20 mV) [273, 350].

The $K_{ir}4.1$ may co-assemble with $K_{ir}5.1$ channels forming heteromers, which were found in parenchymal and radial astrocytes, in the olfactory bulb, neocortex, cerebellum and retina [50, 138, 159, 251]. The $K_{ir}4.1/K_{ir}5.1$ heteromeric channels are concentrated in perisynaptic and perivascular processes of astrocytes and their processes close to the pia mater [138]. Some astrocytes in the hippocampus and cerebellum (including Bergmann glia) along with retinal Müller glia were found to express $K_{ir}2.1$, $K_{ir}2.2$ and $K_{ir}2.4$ channels [194, 214, 317, 380]. Astrocytes also express ATP-sensitive inward rectifying K^+ channels assembled from $K_{ir}6.x$ subunit and SUR1/2; these channels open upon intracellular ATP depletion [96, 174, 365, 395, 431].

3.3.1.2 Voltage-Independent K^+ Channels

Another type of K^+ channels, which contributes to the resting membrane permeability of astrocytes is represented by members of the two-pore-domain potassium channels (K_{2P}) family encoded by 15 KCNK genes [100]. Hippocampal astrocytes were

found to express functional TREK1/K_{2P}2.1(*KCNK2*), TREK2/K_{2P}10.1(*KCNK10*) and TWIK1/K_{2P}1.1(*KCNK1*) channels [350, 432]. In cultured cortical astrocytes and astrocytes in hippocampal slices expression of TWIK-1/TREK-1 heterodimer (formed by disulphide bridge between cysteine-cysteine residuals of both subunits) has been demonstrated as the predominant channel type [156].

3.3.1.3 Voltage-Gated K^+ Channels, K_v

Astrocytes express delayed rectifying (K_D) and transient (K_A) voltage-gated K^+ channels. The delayed rectifying K^+ currents were identified in astrocytes throughout the CNS including the cortex, hippocampus, cerebellum and spinal cord [48]. At the molecular level, K_v1.5 (*KCNA5*), K_v1.4 (*KCNA4*) and K_v11.1/ERG1 (KCNH2) have been identified in astrocytes from the hippocampus and spinal cord [97, 98, 334]. Astrocytes are also in possession of fast (rapidly activating and inactivating) A-type K^+ currents mediated by K_v1, K_v3 and K_v4 channels [32].

3.3.1.4 Ca^{2+}-Dependent K^+ Channels, K_{Ca}

Several types of Ca^{2+}-dependent K^+ channels (K_{Ca}) were found in astrocytes in vitro and in situ. At the mRNA level SK (small conductance K_{Ca} 2.3/KCNN3) and IK (intermediate conductance K_{Ca}3.1/KCNN4) channels were detected in mouse cortical astrocytes in acutely isolated slices [222]. The K_{Ca}2.3 immunoreactivity was found in astroglial processes in the rat supraoptic nucleus [17]. BK (big conductance) channels (K_{Ca}1.1/KCNMA1) were identified in the perivascular astroglial endfeet in the hippocampus and cerebellum [315], whereas patch-clamp recordings obtained from endfeet revealed large-conductance (225 pS) BK-single channel currents [107].

3.3.2 Sodium Channels

3.3.2.1 Voltage-Gated Sodium Channels

Although being non-excitable cells, astrocytes express voltage-gated Na^+ channels (Na_v), which were detected in vitro and in situ, albeit at low density [26, 28, 42, 371–374]. Cultured astrocytes from the optic nerve, hippocampus and spinal cord were found to express fast tetrodotoxin (TTX)-sensitive and slow TTX-resistant Na^+ currents [373, 374]. At the molecular level astrocytes mainly express Na_v1.5 subunit which was identified both in vitro and in situ at mRNA and protein levels [40, 41, 286]; relatively low expression of Na_v1.2, Na_v1.3 [44] and Na_v1.6 [321, 345] were also detected; incidentally expression of Na_v1.6 channels was found to increase in reactive astroglia [434].

Distribution of Na^+ channels in astrocytes from different brain regions as well as their physiological role remain poorly understood; hitherto Na^+ currents have not been recorded from astroglial cells in vivo. Possibly voltage-gated Na^+ channels contribute to Na^+ signalling; it was also suggested that Na^+ influx mediated by these channels is needed for sustained activity of Na^+/K^+ pump (see [43, 285, 287] for further details).

3.3.2.2 $[Na^+]_O$—Regulated Na^+ Channels, Na_x

Specific type of Na^+ channels regulated by extracellular Na^+ concentration ($[Na^+]_o$) are named Na_x channels. They are expressed in astrocytes of the subfornical organ and organum vasculosum of the lamina terminalis (that are parts of circumventricular organs surrounding ventricles, structures where the blood-brain barrier is not as tight as in other parts of the brain) [267, 420]. These channels in vitro are opened following an increase in $[Na^+]_o$ to 150 mM. In vivo, in the presence of endothelin-3, which activates ET_B receptors expressed in astroglia, the threshold for Na_x channel activation is lowered to 140 mM of $[Na^+]_o$ [141]. The Na_x channels appear to operate as molecular sensors for $[Na^+]$ in the circulation (Fig. 3.4).

3.3.2.3 Epithelial Sodium Channel, ENaC

The epithelial Na^+ channels are non-voltage gated, amiloride-sensitive Na^+ channels widely expressed in the CNS [10, 238, 416]. Immunohistochemistry found strong expression of ENaCs in astrocytes in circumventricular organs, white matter and pia mater [237]. These channels, together with Na_x channels, may be involved in the regulation of systemic Na^+ homeostasis.

3.3.3 Calcium Channels

3.3.3.1 Voltage-Gated Ca^{2+} Channels

Early patch clamp recordings from astrocytes in culture revealed Ba^{2+} and Ca^{2+} currents sensitive to classic voltage-gated Ca^{2+} channel antagonists and enhanced by norepinephrine or by an increase in cytosolic 3′,5′-cyclic adenosine monophosphate, i.e. cAMP [27, 72, 228], the signalling by this second messenger complementing Ca^{2+} signalling in astrocytes [146]. Subsequently $Ca_v1.2$ and $Ca_v1.3$ channels were detected in the transcriptome of rodent cortical astrocytes [60, 430]. Several types of Ca^{2+} channel subunits (α1B or N-type, α1C/D or L-type, α1E or R-type and α1G or T-type) were detected at mRNA and protein levels in astrocytes in culture [209]. Pituicytes analysed immunohistochemically in situ were found to possess $Ca_v2.2$ (N-type) and $Ca_v2.3$ (R-type) channels [418]. Evidence for functional activity of

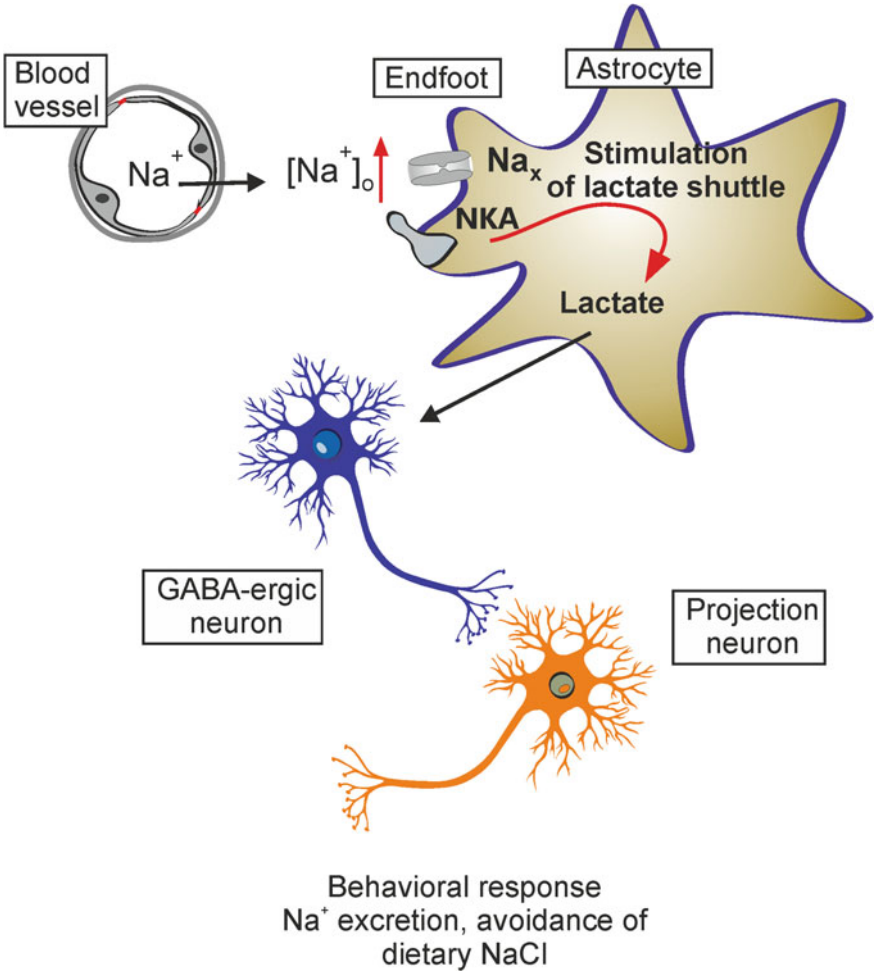

Fig. 3.4 Astroglial Na_x channels in systemic Na^+ regulation. Increases in blood Na^+ concentration activate Na_x sodium channels localised in astrocytes residing in the subfornical organ. This leads to an increase in cytosolic Na^+ concentration, which in turn increases astroglial production of lactate. Lactate released by astrocytes is accumulated by neighbouring neurones (release and uptake carried by MCT1 in astrocytes and MCT4 in neurones, respectively), thus increasing ATP production in neurones. Increased ATP in turn closes neuronal ATP-sensitive K^+ channels, which results in depolarisation and subsequent activation of neuronal networks responsible for systemic Na^+ homeostasis. NKA, sodium-potassium ATPase. Modified from [413]

voltage-gated Ca^{2+} channels in situ is rather thin and indirect (see for example [215, 294]) and no evidence from the in vivo experiments exist at all. There are some indications for increased expression of voltage-gated Ca^{2+} channels in pathological or reactive astrocytes [419, 421].

3.3.3.2 Orai or Ca^{2+}-Release Activated Ca^{2+} Channels

The Ca^{2+}-release activated Ca^{2+} channels of Orai family (Orai1,2,3) represent one of the main molecular pathways of the store-operated Ca^{2+} entry (SOCE) in non-excitable cells [289]. Activation of these plasmalemmal channels is controlled by the stromal interacting molecules, STIM1 and STIM2, which act as Ca^{2+} sensors of the endoplasmic reticulum (ER) [368]. In cultured astrocytes Orai1 and STIM1 were detected at a protein level; over-expression of Orai1 increased the amplitude of SOCE, whereas siRNA knock out decreased SOCE [248]. Electrophysiological recordings of I_{CRAC} performed on acutely dissociated Müller cells demonstrated sensitivity of the current to ORAI inhibitor Synta 66 [246].

3.3.3.3 Ca^{2+} Release Channels

Release of Ca^{2+} from the ER store in astrocytes is mainly mediated by Inositol 1,4,5-trisphosphate receptors ($InsP_3R$), of which type 2 predominates [133, 144, 354, 356, 414]. Genetic deletion of $InsP_3R$ type 2 ($InsP_3R2$) has been shown to significantly reduce or even completely abolish Ca^{2+} signalling in astroglial cells from the hippocampus and cortex [171, 302]. Other studies, however, reported $[Ca^{2+}]_i$ transients in $InsP_3R2^{-/-}$ mice astrocytes [130]. There is evidence for functional expression of $InsP_3Rs$ type 1 and 2 in astroglia [125, 327, 357].

Ryanodine receptors also have been identified in astrocytes [364, 414], although their functional role remains unclear. They supply Ca^{2+} necessary for Ca^{2+}-dependent glutamate release from cortical astrocytes in culture. Ca^{2+}-dependent glutamate release involves two classes of ER Ca^{2+} stores in astrocytes [150]. There is some evidence for astroglial expression of the two-pore channels (TPC) that release Ca^{2+}, and are activated by nicotinic acid adenine dinucleotide phosphate, i.e. NAADP [25, 301].

3.3.4 Transient Receptor Potential (TRP) Channels

Astrocytes express several types of cationic channels of TRP (transient receptor potential) family (Fig. 3.5). The ‘ankyrin’ channel TRPA1 was found in somata and processes of astrocytes in the brain stem in the rat trigeminal caudal nucleus using immunogold electron microscopy [211]. Functional expression of TRPA1 was also demonstrated in a sub-population of hippocampal astrocytes [360, 362]. The TRPC

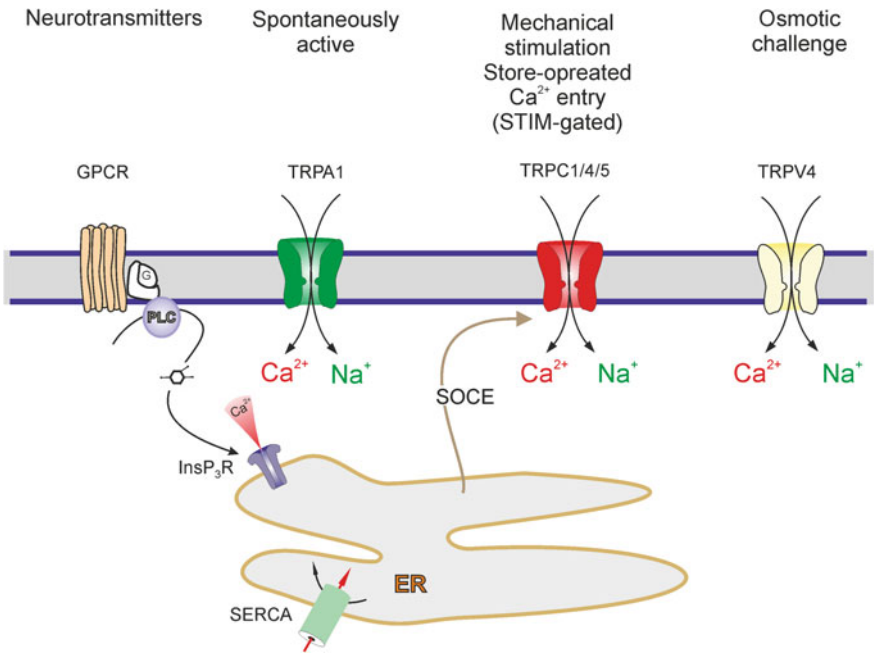

Fig. 3.5 Astroglial TRP channels. Activation of G-protein coupled receptors (GPCR), i.e. metabotropic stimulation, can lead to production of $InsP_3$ and release of Ca^{2+} from the ER store. The Ca^{2+} content of the ER store is refilled by Sarco(Endo)Plasmic Reticulum Ca^{2+} ATPase, i.e. SERCA. Depletion of the ER Ca^{2+} store activates (via STIM) TRPC channels in astrocytes which are therefore acting as a store-operated channel, contributing to capacitative Ca^{2+} entry. Activation of all TRP channels mediates Ca^{2+} and Na^{+} influx. Modified from [413]

('canonical') channels were detected in freshly isolated and in primary cultured astrocytes, which were reported to express all subtypes of these channels from TRPC1 to TRPC6 [124, 244, 308]. These TRPC channels contribute to astroglial Ca^{2+} signalling induced by purinergic, glutamatergic and mechanical stimulation [231, 323, 324]. Astroglial TRPC channels represent a substantial pathway for store-operated Ca^{2+} entry in astroglia [412].

Rodent astrocytes from the brain and the spinal cord have been also reported to express TRPV1 channels [91, 151, 398]. Similarly TRPV4 channels have been identified in cortical and hippocampal astrocytes [22, 33, 57, 220]; these channels can be activated by hypo-osmotic stress and by cell swelling [33, 57] in the absence of addition of membrane by exocytosis [283].

3.3.5 Hyperpolarisation-Activated Cyclic Nucleotide-Gated (HCN) Channels

The cationic (Na^+/K^+) HCN channels are found in both healthy and reactive astrocytes in situ [145, 337].

3.3.6 Acid-Sensitive Ion Channels

The acid-sensitive ion channels (ASIC1-3) were described in reactive astrocytes in the context of chronic epilepsy, and their activation was claimed to contribute to seizure generation [423].

3.3.7 Anion Channels

Astrocytes express the following anion channels: (i) cystic fibrosis transmembrane conductance regulator or CFTR channels, (ii) voltage-dependent anion-selective channels or VDAC, (iii) Ca^{2+}-dependent Cl^- channels (iv) volume-regulated anion channels or VRAC and (v) ClC-1, -2 and -3 channels [186, 291, 410, 429]. The ClC-2 channels are concentrated in astroglial processes enwrapping GABAergic synapses [363], which may indicate their role for regulation of intra-cleft Cl^- concentration and hence of GABAergic transmission. Astrocytes also express an anion channel of Bestrophin (*Best*) family; these channels were suggested to contribute to the Ca^{2+}-dependent secretion of glutamate and GABA [290, 422].

3.3.8 Aquaporins

Three types of aquaporins, the AQP1, AQP4 and AQP9 were identified in astroglia although AQP4 is the most abundant [21, 256, 342]; the plasma membrane contains mainly the AQP4e isoform [219, 313]. Astroglial AQP4 channels are concentrated in the perivascular and subpial endfeet [256]. Genetic deletion of AQP4 affects olfaction [224] and hearing; it results in a decrease in astroglial water permeability [370], deficient K^+ buffering, compromised volume regulation [39, 219], and deficits in synaptic plasticity [346, 367], and memory [367, 427].

3.3.9 Connexons

Connexons form gap junctional channels that integrate astrocytes into functional syncytia, subject to regulation by G-protein coupled receptors [392]. Astrocyte-astrocyte homocellular gap junctions are composed of Cx26, Cx30 and Cx43 [117, 198, 258], of which Cx43 is the most abundant [257]. The Cx43 is expressed in astroglial cells in all CNS regions, whereas Cx30 is mostly expressed in the thalamus and leptomeninges [257, 369]. The Cx26 subtype has been detected in astrocytes in the hypothalamus, reticular thalamic and subthalamic nuclei [259]. Astrocyte-oligodendrocyte heterocellular gap junctions are composed of heterotypic channels represented, in vitro, by four complexes: Cx47/Cx43, Cx47/Cx30, Cx32/Cx30 or Cx32/Cx26 [230], although in situ Cx32/Cx30 and Cx47/Cx43 complexes appear to predominate [8, 277]. There are some sporadic reports of astrocyte-neuronal heterocellular contacts [9, 255, 279], and these contacts are arguably limited to developing brain.

Unpaired connexons, or hemichannels, have been identified in astrocytes in vitro and in vivo; all three major connexons (Cx26, Cx30 and Cx43) expressed in astrocytes can act as hemichannels [119]. The hemichannels are non-operational in healthy astrocytes, but can be activated by low external calcium concentration, by substantial depolarisation, by some specific intracellular Ca^{2+} signals, or by exposure to pro-inflammatory agents [274, 275]. The hemichannels can contribute to secretion of neurotransmitters and neuromodulators [293] and are subject to regulation via transmitters and G-protein coupled receptors.

3.3.10 Pannexons

Transcripts for pannexin 1 (Panx1) were identified in astroglia in vitro and in situ [153, 320], with Panx1 currents characterised in cultured cortical astrocytes [157]. Astroglial pannexons are activated by voltage and by activation of $P2X_7$ receptors; they are inhibited by broad-spectrum gap junction antagonists carbenoxolone and mefloquine, and they are permeable to fluorescent tracer YoPro [157]. Panx1 containing pannexons have been considered as a transmembrane conduit for ATP [78].

3.4 Receptors

Conceptually, astrocytes have been shown to express virtually any type of receptor found in the CNS. At the same time the pattern of receptors expressed by astrocytes in situ and in vivo is restrictive and is regulated by the local neurochemical environment [183, 410, 411].

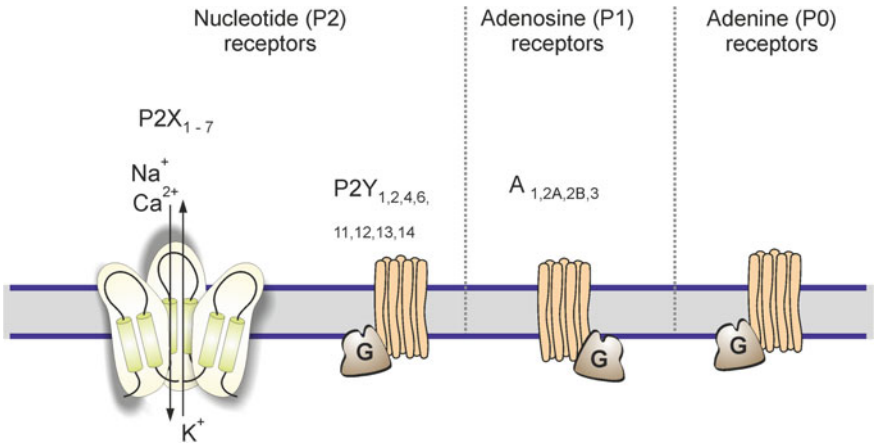

Fig. 3.6 Classes of purinoreceptors. ATP after being released from neurones and glia is rapidly degrading by ectonucleotidases into ADP, AMP and adenosine, which act on P1 metabotropic adenosine receptors, P2X ionotropic and P2Y metabotropic nucleotide receptors. Adenine stimulates A0 adenine metabotorpic receptors, which hitherto have not been detected in astrocytes. Modified from [406]

3.4.1 Purinoceptors

3.4.1.1 Adenosine Receptors

All four types of adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) were found in astrocytes in the in vitro and in situ preparations [84]. These receptors are linked to intracellular second messenger systems including $InsP_3$, Ca^{2+} and cAMP cascades (Fig. 3.6). Activation of A_1 receptors triggered intracellular Ca^{2+} release as well as Ca^{2+} entry and potentiated histamine-induced Ca^{2+} mobilisation [297, 310]. Similarly, A_{2A} receptors were responsible for Ca^{2+} signalling in astrocytes from the olfactory bulb [90], whereas A_{2B} receptors triggered Ca^{2+} signals in cortical astroglia [306]. The A_3 receptors mediated adenosine- and guanosine-evoked $[Ca^{2+}]_i$ transients in cultured mouse astrocytes [68].

3.4.1.2 P2X Purinoceptors

Transcripts for all seven types of ionotropic purinoceptors ($P2X_1$–$P2X_7$, Fig. 3.6) have been detected in astrocytes in vitro and in tissue extracts [89, 109, 113, 162]. At the protein level, $P2X_{2,3,4}$ receptors were found in astrocytes from the nucleus accumbens [109]. $P2X_1$ and $P2X_2$ receptors were found in astroglial cells in the cerebellum [173, 221]; $P2X_4$ receptors were identified in astrocytes from the brainstem [18] and in Müller glia [142], whereas hippocampal astrocytes were immunoreactive for $P2X_{1\text{-}4}$, $P2X_6$ and $P2X_7$ receptors [197].

Ion currents mediated by heteromeric $P2X_{1/5}$ receptors were characterised in cortical mouse astrocytes [204]). These receptors contribute to 'glial synaptic currents' monitored in astrocytes in response to stimulation of neuronal afferents [200, 203]; in addition $P2X_{1/5}$ receptors produced spontaneous 'miniature' post-synaptic currents in astrocytes in cortical slices [203]. Astroglial $P2X_{1/5}$ receptors have intermediate Ca^{2+} permeability ($P_{Ca}{}^{2+}/P_{monovalent} \sim 2.2$), and their activation by endogenous agonists, or by synaptically released ATP, triggers transient cytoplasmic Ca^{2+} signals [281].

Astrocytes have been reported to express $P2X_7$ receptors both in healthy and pathological contexts [111, 158]. In astrocytes in vitro $P2X_7$ receptors were detected at mRNA and protein levels [89, 93, 113, 154, 164, 260, 282, 417]. Astroglial expression of $P2X_7$ receptors, as a rule, increases after brain injury of various aetiology [109, 110, 260]. In astrocytes in culture both $P2X_7$-mediated Ca^{2+} signals and membrane currents have been detected [93, 113, 266, 268, 335]. Astroglial $P2X_7$ currents were also characterised in rat and mouse cortical slices. Activation of $P2X_7$ receptors in cultured astrocytes may be associated with release of glutamate, GABA, ATP [24, 93, 94, 382]. The $P2X_7$-mediated release of glutamate was also identified in astrocytes in hippocampal slices [106].

3.4.1.3 P2Y Receptors

Astrocytes in cortex express transcripts for $P2Y_{1,2,4,6,12,13}$ and UDP-glucose $P2Y_{14}$ receptor [1, 36, 89, 113], whereas spinal cord astrocytes predominantly express mRNA for $P2Y_{1,2}$ receptors [104]. Stimulation of astroglial P2Y receptors triggers Ca^{2+} signals originating from $InsP_3$-induced ER Ca^{2+} release [38, 56, 160, 163, 175, 298, 299, 407].

3.4.2 Glutamate Receptors

3.4.2.1 Ionotropic Glutamate Receptors

Astrocytes from different regions of the brain express α-amino-3-hydroxy-5-methyl-isoxazole propionate (AMPA) receptors (Fig. 3.7), which have been characterised at expression and functional levels. All main subunits of AMPA receptors (GluA1–GluA4) have been detected in astroglial cells. In the hippocampus, AMPA receptors are assembled predominantly from GluA2 and GluA4 subunits, which are reflected by a linear I-V relation and low Ca^{2+} permeability [351]. In cortical astrocytes these receptors are composed of GluA1 and GluA4 subunits [75]. In Bergmann glial cells AMPA receptors do not contain GluA2 subunit, and accordingly they have a double-rectifying I–V relationship and low ($P_{Ca}{}^{2+}/P_{monovalent} \sim 1$) Ca^{2+} permeability [115, 253]. Conditional deletion of AMPA receptors, composed of GluA1 and GluA4

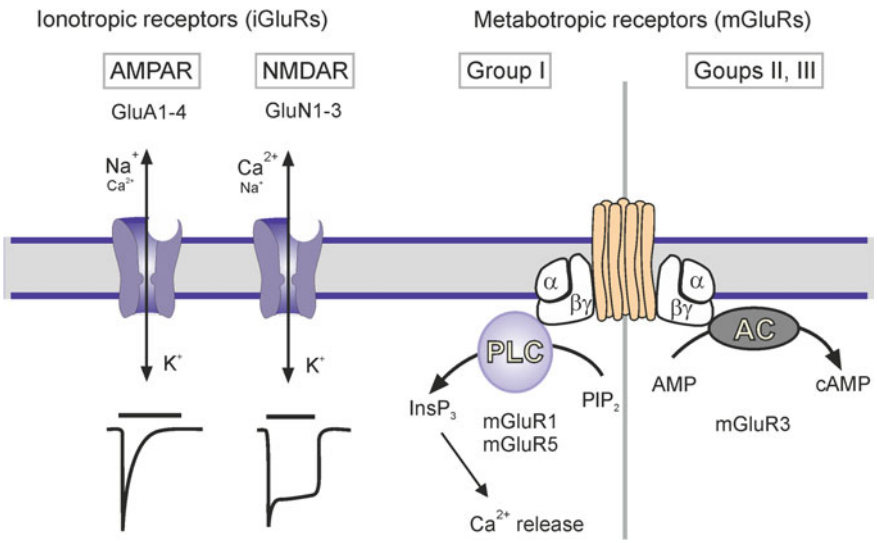

Fig. 3.7 Classes of glutamate receptors expressed in astrocytes. Current traces show a faster time course for AMPAR than NMDAR. AC, adenylyl cyclase; AMP, adenosine monophosphate; cAMP, cyclic AMP; $InsP_3$, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C. Modified from [406]

subunits, from Bergmann glia led to a retraction of glial perisynaptic processes and deficient fine motor coordination [338].

Astrocytes also possess functional N-methyl D-aspartate (NMDA) receptors. The transcriptome of human astroglia contains all 7 NMDA receptors subunits (GluN1, GluN2/A-D and GluN3A,B—[210]). In acute slices the NMDA-mediated currents and Ca^{2+} signals were found in astrocytes in the neocortex [202, 272, 348], in the spinal cord [437], and in some cells in the hippocampus [311, 376]. Astroglial NMDA receptors heterotetramers assembled from obligatory two GluN1 and additional subunits of each GluN2 C or D and GluN3; this composition underlies a weak Mg^{2+} block (which develops at ~−120 mV) and relatively low Ca^{2+} permeability ($P_{Ca}/P_{monovalent}$ ~ 3), as well as sensitivity to memantine and GluN2C/D subunit-selective antagonist UBP141 [95, 203, 280, 281].

3.4.2.2 Metabotropic Glutamate Receptors

The most abundant type of astroglial metabotropic receptors in mature CNS is represented by mGluR3, which inhibits adenylyl cyclase [385]. In younger animals astrocytes express mGluR1/5 receptors linked to Ca^{2+} signals and $[Ca^{2+}]_i$ oscillations [76, 191, 208, 312].

3.4.3 GABA Receptors

Astrocytes express both ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. The $GABA_A$ receptors mediated Cl^- currents have been characterised in astroglial cells in culture and in situ in the hippocampus, cerebellum, retina, hypothalamus, supraoptic nucleus and spinal cord [74, 179, 180, 182, 229, 252]. The subunit composition of astroglial $GABA_A$ receptors is not certain; α1 and β1 subunits were detected in hippocampal astrocytes [112] and α2 and γ1 in Bergmann glia [326]. Metabotropic $GABA_B$ receptors evoke astroglial Ca^{2+} signalling by triggering Ca^{2+} release from the ER [264].

3.4.4 Glycine Receptors

Glycine receptors mediate Cl^- currents in astrocytes in the spinal cord slices [295]. Single-cell RT-PCR performed on these astrocytes revealed expression of α1 and (in ~50% of cells) β-subunits of the receptor [188].

3.5 Acetylcholine Receptors

The ionotropic nicotinic acetylcholine receptors (nAChRs) have been characterised in astrocytes in culture and in acute slices. Activation of these receptors mediates Ca^{2+} influx and triggers Ca^{2+}-induced Ca^{2+} release [271, 353, 389]. Analysis of mRNA expression in rodent cortical astrocytes found α4, α7 and β2 subunits, whereas in human astroglial cells from the hippocampus and entorhinal cortex α3, α7 and β4 subunits were identified by immunocytochemistry [121, 390].

The metabotropic M_1 and M_2 AChRs mediate Ca^{2+} signalling in astrocytes from hippocampal slices [15].

3.6 Receptors for Monoamines

Astroglial cells express receptors for major monoamines including adrenoceptors, and receptors for serotonin, dopamine and histamine. Both α- and β-adrenoceptors have been identified and characterised in astrocytes in culture, in slices, and in vivo at transcript, protein and functional levels [14, 135]. The α_1-adrenoceptors are coupled to phospholipase-C (PLC) and $InsP_3$ signalling and hence to Ca^{2+} release from the ER [55, 193, 352]. Immunoreactivity for α_2-adrenoceptors was found in astrocytic processes in the brain tissue [14, 239]; activation of α_2-adrenoceptor also triggers Ca^{2+} signalling [265, 339]. β_1-, β_2- and β_3-adrenoceptors were characterised in astro-

cytes in vitro and in vivo [62, 232, 352]. β_1-adrenoceptors contribute to regulation of glycogen synthesis, while β_2-adrenoceptors coupled to adenylyl cyclase through G_s proteins together with possibly β_3-adrenoceptors regulate glucose uptake by modulating GLUT1 plasmalemmal glucose transporter [92, 155].

Astrocytes express 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{5A} metabotropic serotonin receptors, [19, 61, 341]. 5-HT_2 receptors activate PLC/$InsP_3$/Ca^{2+} signalling cascade [341]. 5-HT_{2B} receptor seems rather abundant in astrocytes [196] with its expression as twice as large as in neurones [428]. Serotonin-specific reuptake inhibitors (major anti-depressant agents such as fluoxetine or sertraline) directly activate astroglial 5-HT_{2B} receptors [136, 428] with subsequent Ca^{2+} signalling [347] or phosphorylation of extracellular regulated kinases 1/2 (ERK1/2) or up-regulation of Ca^{2+}-dependent phospholipase A2 (cPLA2) [136, 428].

Dopamine D_1, D_2, D_4 and D_5 receptors have been detected in astroglia at transcript and protein levels [245] with higher expression of D_1 [425] or D_2 receptors [23]; astrocytes from the striatum were claimed to express D_5 receptors [52]. Strong presence of D_2 receptors was found in astroglial processes enwrapping cortical interneurones [184]. Activation of D_1 and D_2 receptors in astrocytes triggers Ca^{2+} signalling originating from the $InsP_3$-induced ER Ca^{2+} release [184, 322].

Astroglia express H_1, H_2 and H_3 histamine receptors [166]. Astrocytic H_1 receptors are coupled to PLC/$InsP_3$/Ca^{2+} signalling cascade [193, 355], regulation of glucose metabolism [16] and up-regulation of EAAT2/GLT-1 plasmalemmal glutamate transporter [105]; the latter also modulated by cytosolic Ca^{2+} [377].

3.7 Bradykinin Receptors

The B_2 bradykinin receptors were identified in cultured astrocytes [71]; stimulation of these receptors induces $InsP_3$ production, Ca^{2+} signalling and glutamate release [292, 378].

3.8 Cannabinoid Receptors

Astrocytes express cannabinoid CB_1 receptors, which are involved in regulation of cellular metabolism [46, 340]. The CB_1-mediated astroglial Ca^{2+} signalling was detected in response to neuronal release of endocannabinoids [261]. Activation of CB_1 receptors in astroglia was also claimed to regulate neuronal synaptic plasticity [262].

3.9 Neuropeptide Receptors

V_1 vasopressin receptors induced Ca^{2+} signalling was found in cultured astrocytes and in pituicytes [129, 167]. Oxytocin receptors linked to PLC/$InsP_3$/ER Ca^{2+} release were identified in rat embryonic cultured hippocampal astrocytes [87] and in hypothalamic astrocytes [199].

ET_A and ET_B endothelin receptors were initially described in cultured astrocytes [147, 405]. Stimulation of $ET_{A/B}$ receptors resulted in astroglial Ca^{2+} signalling [47, 233]. In mouse cerebellar Bergmann glial cells, endothelin evoked $[Ca^{2+}]_i$ transients sensitive to the selective ET_B receptor antagonist BQ-788 [399]. Activation of $ET_{A/B}$ receptors also suppresses astroglial gap junctions due to dephosphorylation of Cx43 [47, 116].

Receptors to atrial natriuretic peptide (NPR) were first identified in cultured mouse astrocytes [393]. Subsequently NPR-A [383], NPR-B [383, 438] and NPR-C [366, 383] receptors have been characterised. NPR-A and NPR-B increase intracellular cyclic guanosine monophosphate (cGMP), while NPR-C acts as a 'clearance receptor' that removes peptides from the extracellular space [314].

Astrocytes have been found to express δ and κ-opioid receptors [101, 102]; κ-opioid receptors induced Ca^{2+} signals sensitive to nifedipine [103], while δ-receptors, are linked to ER Ca^{2+} release [396]. Opioid receptors were claimed to regulate expression of plasmalemmal glutamate transporters [218], and astroglial growth [379].

3.10 Receptors for Leptin and Insulin

Expression of leptin receptors was detected in astrocytes in the subcommissural organ [79], in the nucleus tractus solitarius [80] and in the hypothalamus [149].

Insulin receptors have been characterised in astrocytes in vitro [131, 435]. Genetic deletion of insulin receptors from astrocytes affected brain glucose sensing, and reduced astroglial coverage of hypothalamic neurones [114]. Insulin may also act via the insulin-like growth factor 1 (IGF-1) receptor. Activation of insulin and IGF-1 receptors upregulates levels of glycogen in cultured rodent astrocytes [250].

3.11 Platelet-Activating Factor Receptor

Receptors for platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) were detected in astrocytes in vitro [53]. Activation of platelet-activating factor receptors stimulated production of $InsP_3$ [254, 304], induced secretion of nerve growth factor [54] and prostaglandin E_2 [391].

3.12 Protease-Activated Receptors (PAR)

Thrombin-activated PAR-1 and trypsin-activated PAR-2 receptors were found in cultured rat newborn astrocytes; activation of these receptors induced ER Ca^{2+} release [400, 401]. Astroglial PAR receptors are coupled to several signalling pathways, including stabilisation of hypoxia inducible factor-1α through ERK, JNK and PI3K/Akt cascades [436]. Activation of PAR-1 receptors (for example, by the selective peptide agonist TFLLR) is often used for selective stimulation of astroglia in situ [201, 359], although there are reports of neuronal Ca^{2+} signals triggered by the TFLLR-sensitive receptors [127].

3.13 Astroglial Membrane Transporters

3.13.1 ATP-Dependent Transporters

The most prominent and functionally important astroglial membrane P-type ATPase is represented by the Na^+-K^+ ATPase (NKA), that counter-transports Na^+ and K^+ with a stoichiometry of 3 Na^+ (expelled from the cell): 2 K^+ (imported into the cell). This defines electrogeneity of NKA. Astrocytes exclusively express α2 catalytic subunit [137, 165], which defines its peculiar properties. In contrast to neurones (which express α1 subunit) astroglial NKA is activated by physiological rises in $[K^+]_o$, while neuronal NKA is activated by an increase in $[Na^+]_i$ [134, 207, 329]. This stipulates the leading role of astroglial NKA in K^+ buffering. Astrocytes also express plasmalemmal Ca^{2+}-ATPases (PMCAs) and Sarco(Endo)Plasmic Reticulum Ca^{2+} ATP-ase (SERCA) both being responsible for Ca^{2+} homeostasis [410]. Astroglial plasma membrane and astroglial secretory vesicles possess the vacuolar V-type H^+ ATPase [288, 305].

3.13.2 Secondary Plasmalemmal Transporters of Solute Carrier (SLC) Family

3.13.2.1 Glutamate Transporters

Astrocytes represent the main sink for glutamate in the CNS [82, 410, 433]. Astrocytes express two types of plasmalemmal glutamate transporters: the excitatory amino acid transporters 1 and 2 (EAAT1/SLC1A6 and EAAT2/SLC1A2), which in rodent experiments are also referred to as GLAST1 (glutamate-aspartate transporter 1 [381]) and GLT-1 (glutamate transporter 1 [307]). The EAAT1 is predominantly expressed in the cerebellum [212], in the retina [319] and in circumventricular organs [37]; in all other parts of the brain the EAAT2 is the major type. The average den-

sity of EAAT1 is ~4700/μm^2 in Bergmann glia, and ~2300/μm^2 in the CA1 region of the hippocampus; the density of EAAT2 is ~8500/μm^2 in the hippocampus and ~740/μm^2 in the cerebellum [212]. Both transporters are concentrated in perisynaptic astroglial processes [66].

The stoichiometry of EAAT1 and EAAT2 is 3 Na^+, 1 H^+, 1 glutamate$^-$ in (glutamate is an anion at physiological conditions): 1 K^+ out [278, 426]. The equilibrium potential E_{EAAT} is the function of ions and glutamate concentration; the extracellular glutamate concentration varies between 25 nM at rest and 1 mM during synaptic transmission. The intracellular concentration of glutamate in astrocytes is ~0.3 mM due to high activity of glutamine synthetase [49, 132]. At the rest the E_{EAAT} is about +9 mV, whereas at 1 mM of glutamate in the cleft the transporter reverses at +145 mV [410]. The transporter is electrogenic and generates transmembrane current carried mainly by Na^+ ions [190, 404]. This Na^+ influx may elevate $[Na^+]_i$ by 10–30 mM [331].

Astrocytes also express the cystine/glutamate antiporter Sxc^-, which localises extrasynaptically [7, 70, 176]. This transporter is important for accumulation of cystine needed for production of glutathione.

3.13.2.2 Glutamine Transporters

The obligatory glutamate (and in proxy GABA) precursor glutamine is exported from astrocytes by SNAT3/SLC38A3 and SNAT5/SLC38A5 plasmalemmal glutamine transports, which are coupled with co-transport of 1 Na^+ and counter-transport of 1 H^+ [343]. Astroglial Na^+ signals stimulate glutamine efflux [397].

3.13.2.3 GABA Transporters

Astrocytes predominantly express GAT3 GABA plasmalemmal transporter with much lower expression of GAT1. The GAT3 are concentrated in astroglial processes [241, 325]. In the cerebellum GAT3 is localised in the perisynaptic processes of Bergmann glia, enwrapping inhibitory synapses [241]. In thalamic astrocytes GAT1 is concentrated in perisynaptic membranes, whereas GAT3 is localised more distantly being thus responsible for extrasynaptic GABA transport [31]. The stoichiometry of both transporters is 1 GABA: 2 Na^+: 1 Cl^-, being thus electrogenic [177, 223, 318]. The reversal potential E_{GAT} lies around −50 mV; hence, relatively small depolarisation and/or an increase in $[Na^+]_i$ favour the reverse mode operation of these transporters [402].

3.13.2.4 Glycine Transporters

Astroglial cells express GlyT1/SLC6A9 glycine plasmalemmal transporters [424]. Their stoichiometry is 1 glycine: 2 Na^+: 1 Cl^- [333]. The transporter can reverse at

physiological membrane potentials [358]. This transporter-mediated glycine release was shown to be stimulated by dopamine [152].

3.13.2.5 Adenosine Transporters

Astrocytes express both equilibrative (i.e. controlled by adenosine transmembrane gradient, [187]) plasmalemmal transporters ENT-1/SLC29A1, ENT-2/SLC29A2, ENT-3/SLC29A3 and ENT-4/SLC29A4, and Na^+-dependent concentrative nucleoside plasmalemmal transporters CNT2/SLC28A2 and CNT3/SLC28A3, which co-transport adenosine together with 1 Na^+ [217, 300].

3.13.2.6 Transporters for Monoamines

It seems that the main astroglial monoamine plasmalemmal transporter is represented by norepinephrine transporter NET/SLC6A2 that couples monoamine transport with 2 Na^+ and 1 Cl^-, this transporter has higher affinity for dopamine than norepinephrine [349, 387].

3.13.2.7 D-Serine Transporters

Transmembrane transport of D-serine in astrocytes is mediated by a neutral amino acid transporter subtype ASCT2 (SLC1A5), which is an alanine-, serine-, cysteine-preferring neutral amino acid plasmalemmal transporter [234]. The ASCT2 is a Na^+-dependent with Na^+ to amino acid stoichiometry of 1:1 [235].

3.13.2.8 Sodium-Calcium Exchangers

All three known plasmalemmal sodium-calcium exchangers, NCX1/SLC8A1, NCX2/SLC8A2 and NCX3/SLC8A3, are expressed in astroglia [287]. The NCXs are mainly concentrated in astroglial perisynaptic processes, and co-localise with glutamate transporters and possibly with glutamate ionotropic receptors [45, 240]. The stoichiometry of astroglial NCX is 3 Na^+: 1 Ca^{2+}, and hence the equilibrium E_{NCX} lies at ~−85 to −90 mV at rest making it prone for fluctuating between forward and reverse modes [415]. Membrane depolarisation and increase in intracellular Na^+ concentration favour NCX to operate in the reverse mode, whereas increase in $[Ca^{2+}]_i$ promotes the forward mode. Operation of astroglial NCX was shown in vitro [120, 388] and in situ [189].

3.13.2.9 Sodium-Proton Exchanger, or NHE

Astrocytes express NHE1/SLC9A1 Na^+–H^+ exchanger [69, 85] with electroneutral stoichiometry 1 Na^+ (in): 1 H^+ (out) [276]. The NHE1 is primarily responsible for efflux of protons generated by cytoplasmic metabolism and accumulated by astrocytes through glutamate uptake (each glutamate brings a single H^+ ion) and Ca^{2+} extrusion (PMCA exchanges 2 H^+ for each Ca^{2+} ion expelled).

3.13.2.10 Sodium-Bicarbonate Co-transporter, NBC

The sodium-bicarbonate transporter NBCe1/SLC4A4 has been identified in astrocytes in culture [270] and in hippocampal slices [122]. The NBC stoichiometry is 1 Na^+: 2 HCO_3^- or 1 Na^+: 3 HCO_3^- [263], and this transporter can operate in both forward and reverse modes [394].

3.13.2.11 Sodium-Potassium-Chloride Co-transporter, NKCC1

The Na^+–K^+–Cl– co-transporter NKCC1/SLC12A2 has been detected in Bergmann glia [170], in astrocytes from the optic nerve [227] and spinal cord [316]. It has an electroneutral stoichiometry of 1 Na^+: 1 K^+: 2 Cl^- [226]. Experiments in situ in hippocampal slices questioned the functional role of NKCC1 in protoplasmic astrocytes in the healthy brain [207].

3.13.2.12 Glucose Transporters

Astrocytes express the glucose transporter GLUT1/SLC2A1 [6], which is predominantly localised in endfeet and perisynaptic processes. Immunostaining revealed the presence of this transporter in grey matter astroglia [249]. It also contains GLUT4, a transporter sensitive to insulin in skeletal muscle, however the flux of glucose in astrocytes is not upregulated by insulin [250].

3.13.2.13 Monocarboxylate Transporters, MCT

Monocarboxylate transporters 1 and 4 (MCT1/SLC16A1, MCT4/SLC16A3) provide for export of lactate from astroglial cells [126]. They may, however, mediate both export or import of lactate depending on concentration gradients for monocarboxylate and H^+ [126].

3.14 Ionic Signalling in Astroglia

3.14.1 Calcium Signalling

Discovery of astroglial Ca^{2+} signals and propagating Ca^{2+} waves [63, 76, 83, 99, 108, 185, 236] led to the formulation of the concept of astrocytic ionic signalling as a basis for their excitability [411]. Astroglial Ca^{2+} signalling depends on both intracellular and extracellular sources (Fig. 3.8) [414]. Somatic Ca^{2+} signals almost entirely depend on Ca^{2+} release from the ER mediated by $InsP_3$ receptor type 2; deletion of this channel often substantially reduced or even eliminated somatic $[Ca^{2+}]_i$ transients [4, 171, 302, 303]. At the same time, Ca^{2+} signals in astroglial processes remain even in the $InsP_3R2^{-/-}$ mice [130, 172, 375]. These signals were mediated by plasmalemmal Ca^{2+} influx [336]. This Ca^{2+} influx may reflect upon Ca^{2+} entry through ionotropic receptors and plasmalemmal channels, or Ca^{2+} influx mediated by the reverse mode of NCX [29, 361, 414].

Mechanisms underlying Ca^{2+} signalling differ between astrocytes from different brain regions. Local Ca^{2+} microdomains in Bergmann glia and in the main processes of hippocampal astrocytes were mediated solely by $InsP_3Rs$ [86, 191]. Local $[Ca^{2+}]_i$ transients in hippocampal astrocytes in contrast are mediated by TRPA1 channels [360]. In neocortical astrocytes Ca^{2+} signals involve ryanodine receptor-mediated $[Ca^{2+}]_i$-induced Ca^{2+} release [284], which is not operative in hippocampal astroglia [30]. In astrocytes in vivo sensory stimulation triggers global synchronised Ca^{2+} signals in astrocytes in somato-sensory cortex, which depend entirely on $InsP_3R2$ [171]. In cortical astrocytes spontaneous local Ca^{2+} signals in fine processes originate from Ca^{2+} release from mitochondria [3].

Global Ca^{2+} signals in the mature astrocytes in vivo are mediated by α_1-adrenoceptors [88]. Similar global astroglial signalling is observed in attention and vigilance state, when widespread astrocytic responses are evoked by acetylcholine release from projection of the nucleus basalis of Meynert and are mediated through metabotropic cholinergic receptor-$InsP_3$ pathway [67, 386]. Global astroglial Ca^{2+} signals spreading through the entire cortex were observed in response to transcranial direct current stimulation; these signals were mediated through α_1-adrenoceptors [247].

Astroglial propagating Ca^{2+} wave is mediated either by intercellular diffusion of $InsP_3$ through gap junctions [5, 143, 216] or through regenerative paracrine ATP-mediated signalling [12, 77, 128] or through the combination of both [344]. Whether propagating Ca^{2+} waves develop in the in vivo brain in awake and behaving animals remains an open question.

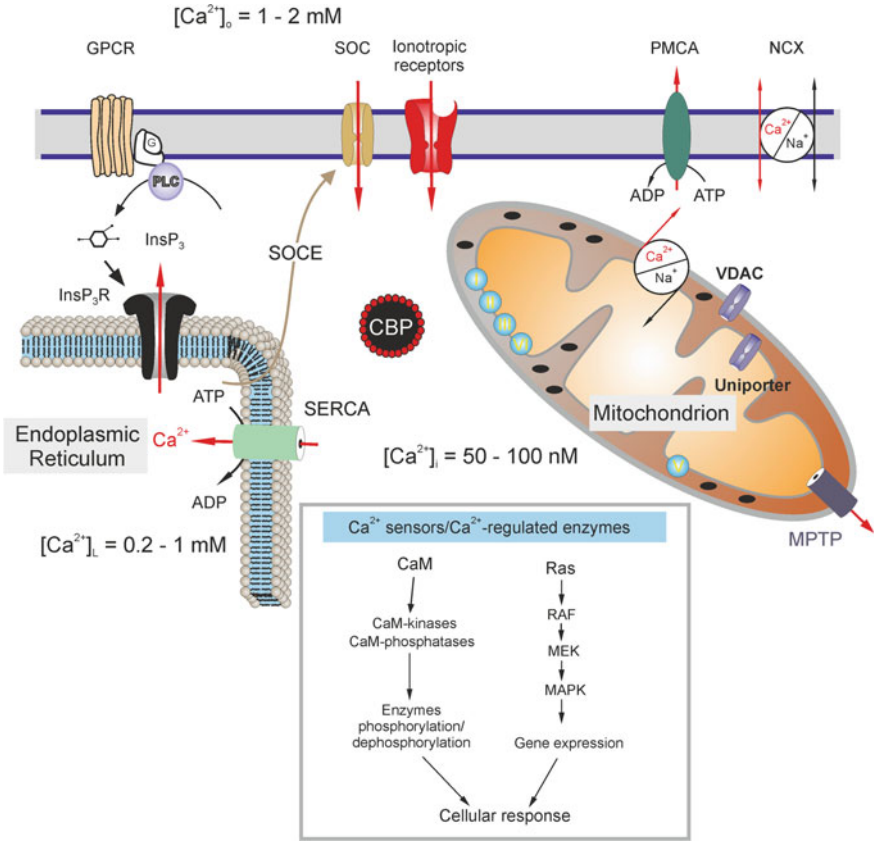

Fig. 3.8 Calcium distribution and calcium signalling cascades in intracellular compartments. Stimuli-induced increases in $[Ca^{2+}]_i$ could be caused by the entry of Ca^{2+} from the extracellular space through ionotropic receptors or store-operated channels (SOC). Plasmalemmal Ca^{2+} pumps/ATPases (PMCA) can extrude cytosolic Ca^{2+}, while the plasmalemmal sodium-calcium exchanger (NCX) can operate in both directions depending on intercellular Na^+ concentration and membrane potential. An additional source of Ca^{2+} is available from the ER internal store that possesses inositol 1,4,5 trisphosphate ($InsP_3$) receptors, which can be activated by the activity of metabotropic G-protein coupled receptors (GPCRs) and phospholipase C (PLC). The ER store is (re)filled by the activity of the store-specific Ca^{2+}-ATPase (SERCA). Cytosolic Ca^{2+} levels can be affected by a variety of cytosolic Ca^{2+}-binding proteins (CBPs) and by the action of mitochondria. A negative membrane potential exists across the inner mitochondrial membrane. Mitochondrial Ca^{2+} uptake occurs through voltage-dependent anion channels (VDACs) present in the outer membrane and by the uniporter in the inner membrane as the electrochemical gradient drives Ca^{2+} into the matrix, while free Ca^{2+} exits the mitochondrial matrix through the mitochondrial Na^+/Ca^{2+} exchanger and transient opening of the mitochondrial permeability transition pore (MPTP). Concentrations of free Ca^{2+} in different compartments are indicated on the scheme. Inset shows various Ca^{2+} effector molecules, sensors and enzymes. CaM, calmodulin; RAF, Rapidly Accelerated Fibrosarcoma, MAPK, mitogen-activated protein kinase (MAPK), MEK, MAPK kinase. Modified from [413]

3.15 Sodium Signalling

The concept of astroglial Na^+ signalling has been developed rather recently [192, 331]. Physiological stimulation triggers $[Na^+]_i$ transients in astrocytes in vitro [178, 329, 330] and in situ [189, 190, 205, 328]. Generation of Na^+ signals is accomplished through plasmalemmal Na^+ entry either through plasmalemmal channels or Na^+ coupled SLC transporters, whereas extrusion of Na^+ is primarily mediated by NKA [192, 331]. Resting $[Na^+]_i$ in astrocytes is higher than in neurones, being in the range of 15–20 mM.

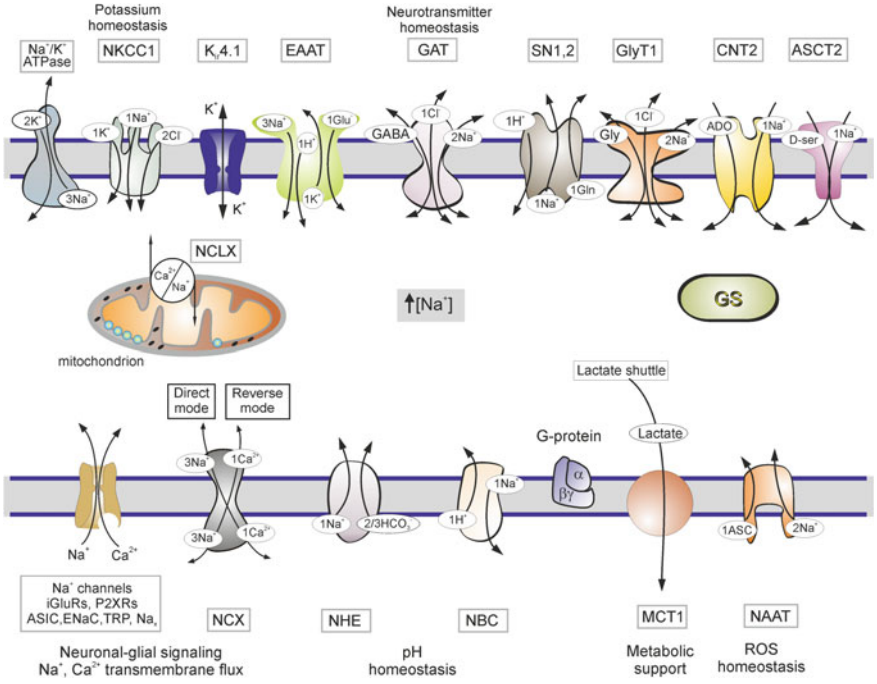

Fig. 3.9 Molecules of Na^+ homeostasis and targets of Na^+ signalling in astroglia. Schematic diagram showing receptors and transporters involved in and sensitive to changes in $[Na^+]_i$ and their relations to main homeostatic functions of astroglia. Abbreviations ASCT2, alanine-serine-cysteine transporter 2; ASIC—acid sensing ion channels; CNT2, concentrative nucleoside transporters; EAAT—excitatory amino acid transporters; ENaC—epithelial sodium channels; GAT—GABA transporters; GS—glutamine synthetase, GlyT1—glycine transporter. iGluRs—ionotropic glutamate receptors; Na_x—Na^+ channels activated by extracellular Na^+; NAAT—Na^+-dependent ascorbic acid transporter; NBC—Na^+/HCO_3^- (sodium-bicarbonate) co-transporter; NCX—Na^+/Ca^{2+} exchanger; NCLX—mitochondrial Na^+/Ca^{2+} exchanger; NHE—Na^+/H^+ exchanger; NKCC1—$Na^+/K^+/Cl^-$ cotransporter, MCT1—monocarboxylase transporter 1; P2XRs—ionotropic purinoceptors; SN1,2—sodium-coupled neutral amino acid transporters which underlie exit of glutamine; TRP—transient receptor potential channels. Reactive oxygen species (ROS). Modified from [413]

One of the main sources for Na^+ influx activated in response to neuronal activity is associated with operation of EAATs that co-transport 3 Na^+ with 1 glutamate; increase in extracellular glutamate may increase $[Na^+]_i$ by 10–30 mM [35, 65, 190]. Sodium influx may also be mediated by GABA transporters, ionotropic receptors, by TRP channels or by NCX operating in the forward mode [189, 205, 323]. Sodium entry may produce long-lasting $[Na^+]_i$ microdomains which, as per computational modelling, may be facilitated by fairly negative resting potential of astroglial plasmalemma [51]. Propagating Na^+ waves have been also detected in astrocytes in culture and in situ in hippocampal slices; these waves are propagating through gap junctions [206].

Astroglial Na^+ signals regulate multiple SLC transporters sensitive to transmembrane Na^+ gradients (Fig. 3.9); $[Na^+]_i$ also regulates glutamine-glutamate (GABA) shuttle through direct action on glutamine synthetase [34] and regulation of glutamine transporters [397]. Changes in $[Na^+]_i$ regulate K^+ buffering through the NKA transport and pH homeostasis by regulating NBC and NHE. By controlling reversal potential of NCX, astroglial Na^+ signals may contribute to Ca^{2+} signalling by initiating local Ca^{2+} influx in distal processes. Finally, fluctuations of $[Na^+]_i$ are coupled to astroglial metabolism, through controlling glycolysis and lactate production and possibly regulating ATP synthesis [64]. The sodium signalling system thus provides for fast coordination of neuronal activity with 'homeostatic' response of astroglia mediated through Na^+-dependent transporters, concentrated in perisynaptic processes.

3.16 Summary

Astroglial physiology is defined by a complement of ion channels, receptors for neurotransmitters, and neurohormones and membrane transporter systems. High expression of K^+ channels stabilises the membrane potential at negative level, thus ensuring electro-driving forces for operation of membrane transporters. Multiple receptors for neuroactive agents on astrocytes provide for input signals reflecting upon neuronal activity. Astrocytic ionic signalling regulates operation of transporters responsible for astroglial homeostatic response, central for astrocytic support of neuronal networks.

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Chapter 4
Gliocrine System: Astroglia as Secretory Cells of the CNS

Nina Vardjan, Vladimir Parpura, Alexei Verkhratsky and Robert Zorec

Abstract Astrocytes are secretory cells, actively participating in cell-to-cell communication in the central nervous system (CNS). They sense signaling molecules in the extracellular space, around the nearby synapses and also those released at much farther locations in the CNS, by their cell surface receptors, get excited to then release their own signaling molecules. This contributes to the brain information processing, based on diffusion within the extracellular space around the synapses and on convection when locales relatively far away from the release sites are involved. These functions resemble secretion from endocrine cells, therefore astrocytes were termed to be a part of the gliocrine system in 2015. An important mechanism, by which astrocytes release signaling molecules is the merger of the vesicle membrane with the plasmalemma, i.e., exocytosis. Signaling molecules stored in astroglial secretory vesicles can be discharged into the extracellular space after the vesicle membrane fuses with the plasma membrane. This leads to a fusion pore formation, a channel that must widen to allow the exit of the Vesiclal cargo. Upon complete vesicle membrane fusion, this process also integrates other proteins, such as receptors, transporters

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and channels into the plasma membrane, determining astroglial surface signaling landscape. Vesiclal cargo, together with the whole vesicle can also exit astrocytes by the fusion of multivesicular bodies with the plasma membrane (exosomes) or by budding of vesicles (ectosomes) from the plasma membrane into the extracellular space. These astroglia-derived extracellular vesicles can later interact with various target cells. Here, the characteristics of four types of astroglial secretory vesicles: synaptic-like microvesicles, dense-core vesicles, secretory lysosomes, and extracellular vesicles, are discussed. Then machinery for vesicle-based exocytosis, second messenger regulation and the kinetics of exocytotic vesicle content discharge or release of extracellular vesicles are considered. In comparison to rapidly responsive, electrically excitable neurons, the receptor-mediated cytosolic excitability-mediated astroglial exocytotic vesicle-based transmitter release is a relatively slow process.

Keywords Exocytosis · Astrocytes · Fusion pore · Gliocrine system · Secretory vesicles

4.1 Vesicular Network and Astroglial Secretion

Similarly to all eukaryotic cells, astrocytes (homoeostatic glial cells of the central nervous system, CNS), contain a complex cytoplasmic network of vesicles. Lysosome, a vesicular organelle discovered in 1955 [29], is present in astrocytes, and plays a prominent intermediate role in endo- and exocytotic vesicle pathways (Fig. 4.1) [152]. It has been hypothesized almost a century ago, that astrocytes act as secretory cells, when in 1910 Jean Nageotte, based on the microscopic observations, considered that astrocytes act as secretory cells [90]. In the last two decades, using a variety of experimental approaches (e.g., by optical and membrane capacitance measurements, electrochemical amperometry, and selective interference with proteins of the exocytotic machinery), it has been determined that astrocytes can release signaling molecules via a vesicle-based mechanism (i.e., exocytosis) and are thus actively involved in information processing in the brain [136, 139]. Although being electrically non-excitable, astrocytes, similarly to neurons, possess (i) exocytotic vesicles, (ii) express proteins for regulated SNARE (Soluble NSF Attachment protein REceptor)-dependent vesicular exocytosis and (iii) can respond to various extracellular stimuli with an increase in cytosolic second messengers triggering Vesiclal exocytosis. The SNARE components of exocytotic machinery in astrocytes are not identical to neurons, nor are the vesicle types, their fusion sites and regulation of exocytosis [77, 86, 136, 139, 152, 153].

4.2 Astroglial Secretory Vesicles

Astrocytes contain various different types of secretory vesicles loaded with different types of molecules (such as ATP, D-serine, glutamate, atrial natriuretic peptide

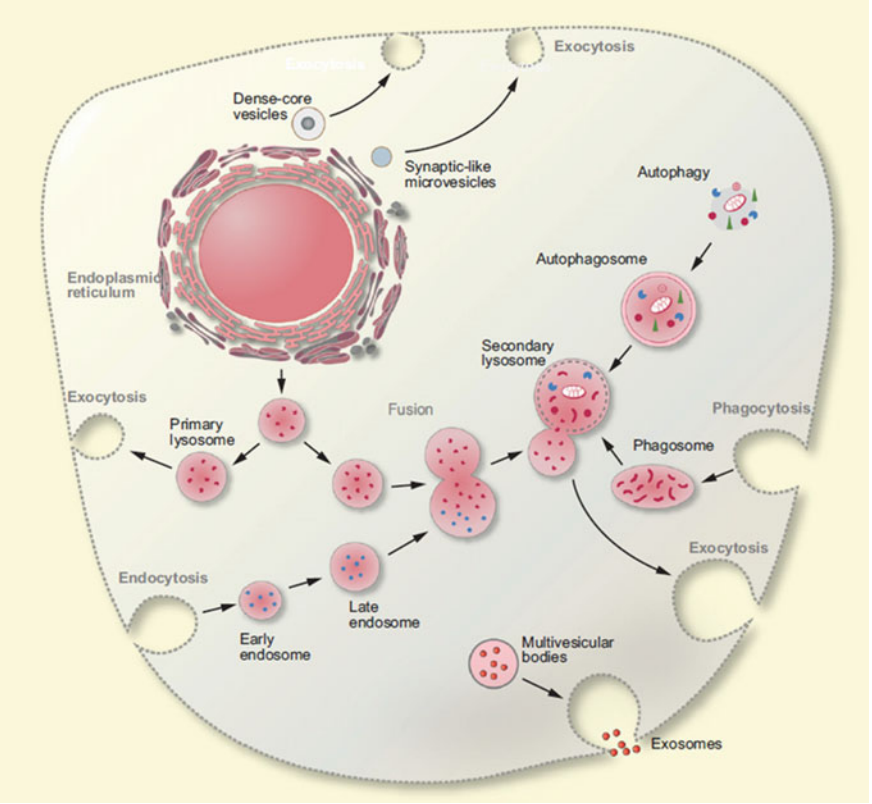

Fig. 4.1 The vesicle network in astrocytes. Lysosomes, first described in 1955, represent a central, prominent intermediate of endo- and exocytotic pathways in all eukaryotic cells, including astroglia. Intracellular secretory organelles (synaptic-like vesicles, dense-core vesicles and primary lysosomes) originate from the endoplasmic reticulum and Golgi complex. Primary lysosomes fuse with endosomes, phagosomes and autophagosomes and convert to secondary lysosomes that undergo exocytosis, thus expelling products of degradation. The multivesicular bodies contain exosomes that may carry various signaling factors. Modified with permission [152]

(ANP), brain-derived neurotrophic factor (BDNF), etc., Fig. 4.2) [39, 40, 101, 139]. These secretory vesicles are classified into synaptic-like microvesicles (SLMVs), [8, 12, 27, 58], dense-core vesicles (DCVs) [17], secretory lysosomes (SL) [71], and extracellular vesicles (EVs) [38].

4.2.1 Synaptic-Like Microvesicles

Astroglial SLMVs are clear electron-lucent vesicles, which are similar to neuronal synaptic vesicles [28, 60], their diameters range between 30 and 100 nm and these

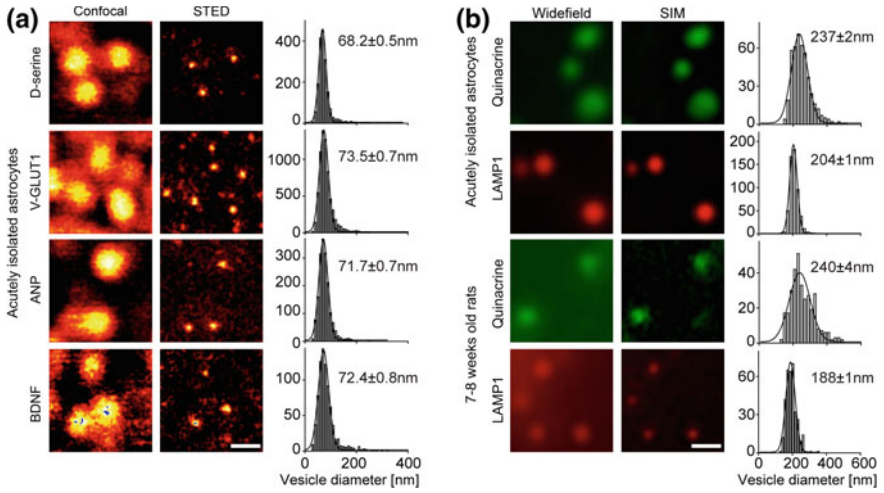

Fig. 4.2 Secretory vesicles studied by STED and SIM microscopies in acutely isolated rat astrocytes. **a** Confocal and STED microscopy images of immunostained vesicles D-serine-, V-GLUT1-, ANP- and BDNF-positive vesicles in acutely isolated astrocytes. Histograms display STED-acquired vesicle diameter distributions for 1788 (D-serine), 6787 (V-GLUT1), 1747 (ANP) and 798 (BDNF) vesicles (2 cells per staining). The black curves show Gaussian fits of the diameter distributions; the numbers next to the distribution peaks indicate the average vesicle diameter (expectation value ± SEM). Recalculated values taking into account the microscope's optical resolution (45 nm) are 80.8 nm for D-serine, 88.4 nm for V-GLUT1, 85.9 nm for ANP and 86.8 nm for BDNF. Scale bar, 500 nm. **b** Wide-field microscopy and SIM were used to determine the vesicle diameter of immunostained LAMP1 endolysosomes and ATP-loaded vesicles (quinacrine dihydrochloride). Histograms show SIM-acquired vesicle diameter distributions for 557 (LAMP1, 2 cells) and 445 (quinacrine, 2 cells) vesicles in acutely isolated astrocytes (upper two panels) and 338 (LAMP1, 3 cells) and 333 (quinacrine, 6 cells) vesicles in astrocytes isolated from 7- to 8-week-old rats (lower two panels). The black curves show Gaussian fits of the diameter distributions; the average vesicle diameter (expectation value ± SEM) is labeled next to the distribution peaks. Scale bar, 500 nm. Modified with permission [39]

SLMVs store low molecular weight signaling molecule glutamate (~147 Da) and in some astrocytes also D-serine (~105 Da). In hippocampal slices, larger SLMVs have been identified in astrocytes (1–3 μm in diameter), which may be generated by intracellular fusion of smaller vesicles or other organelles or both upon sustained Ca^{2+} or mechanical stimulation [59], perhaps a manifestation of a pathological status. D-serine has been recently proposed to reside preferentially in neurons, since biosynthetic enzyme of D-serine serine racemase is expressed almost entirely by neurons [97, 145], with astrocytes arguably being the source of L-serine, which cannot be synthesized in neurons. As revealed with electron microscopy, astrocytes with SLMVs lack the structurally organized active zones with clearly defined synaptic vesicle pools with hundreds to thousands of synaptic vesicles (SVs) per synapse (the readily releasable and the reserve vesicle pools), which are typically found in presynaptic neurons [12, 58]. However, SLMVs in astrocytes do organize in small spaced clusters (2–15 vesicles) located near the astrocytic plasma membrane of the perisy-

naptic astrocytic process. Endoplasmic reticulum appears located in close proximity to these clusters, suggesting that astrocytes contain functional nanodomains, where a local Ca^{2+} increase can trigger release of glutamate and/or D-serine [9, 12, 58, 82]. However, astrocytic perisynaptic processes are mainly devoid of subcellular organelles [105, 124].

Whether signaling molecules glutamate and D-serine are stored inside the same astroglial SLMVs is still a matter of debate. In cultured astrocytes vesicular SNARE protein vesicle-associated membrane protein 2 (VAMP2) and cellubrevin (VAMP3) were found colocalized with both vesicular glutamate transporters (VGluTs; [12, 14, 87]) and D-serine [80, 88], while studies on tissue astrocytes showed that glutamate and D-serine can be stored in distinct SLMVs within the same astrocyte [9]. Examination of immunopurified astroglial SLMVs showed that SLMVs can co-store both glutamate and D-serine [82]. The observation that isolated astroglial SLMVs and isolated neuronal SVs contain different signaling molecules (isolated SLMVs contain D-serine and glutamate [27, 82] and isolated neuronal SVs contain glutamate, glycine GABA and are devoid of D-serine [82, 125]) (although D-serine has been recently proposed to reside preferentially in neurons [97]) might indicate distinct physiological roles of SLMVs and SVs in the CNS.

SLMVs use VGluTs to move glutamate from the cell cytosol into vesicular lumen using a H^+ gradient, created by vacuolar-type H^+-ATPase (V-ATPase), with associated chloride flux. VGluTs 1, 2, and 3 were identified in the membrane of SLMVs in astrocytes in culture, and VGluTs 1 and 2 were shown to associate with SLMVs in tissue astrocytes of several brain areas of hippocampus (CA1), cerebral cortex, striatum, dentate-molecular layers [12, 82, 87, 92, 148], although VGluTs 1-3 were not identified in tissue astrocytes from mice grey matter, thalamic ventrobasal nucleus primary somatosensory cortex, hippocampus and cerebellum [70], suggesting that astrocytes from different brain regions may carry different vesicle types consistent with the regional and functional heterogeneity of astrocytes [91]. Vesicular D-serine transporters (VSerT) were identified in immunopurified astrocytic vesicles. They are likely D-serine/chloride co-transporters and use the H^+ gradient created by V-ATPase to refill the vesicles with D-serine [81, 82].

4.2.2 Dense-Core Vesicles

Astroglial dense-core vesicles (DCVs) are ultrastructurally similar to the large-dense core vesicles (LDCVs) that release neuropeptides and hormones from neuroendocrine cells [16] and neurons [60]. Although DCVs are not very abundant in astrocytes [27], both DCVs and SLMVs can coexist within the same astrocyte [94, 113]. Moreover, DCVs appear larger (100–600 nm; [17, 54, 109]) than SLMVs. The DCVs in cultured astrocytes may contain secretogranins II [17, 94, 109] and III [95], chromogranins [54], ANP [61, 94], neuropeptide Y [109, 113], and ATP [24, 96]. Secretogranins containing DCVs were identified also in astrocytes in human brain tissue [54], indicating the presence of DCVs in astrocytes in situ. Inositol-

1,4,5-triphosphate (IP_3) receptors (IP_3Rs), acting as IP_3-gated Ca^{2+} channels were detected on DCV membranes in astrocytes in brain tissue suggesting that DCVs also serve as IP_3-sensitive intracellular Ca^{2+} stores [54].

Using a super-resolution microscopy approach it was shown that the peptidergic ANP- and BDNF-containing vesicles have diameters less than 100 nm [39] and that the ANP-antibody retrieving vesicles do not exhibit a dense core [107]. Also, astrocytes contain fewer smaller and less dense secretory granules containing secretogranin II [17]. Thus, it appears that peptidergic granules in astrocytes are not uniform in morphological appearance.

Tissue-type plasminogen activator (tPA) is considered to be released by neurons but taken up by astrocytes, possibly into recycling vesicles as these vesicles can uptake ANP-antibodies [107]. Interestingly, tPA is constitutively endocytosed by astrocytes via the low-density lipoprotein-related protein receptor, and is then exocytosed in a regulated manner. Extracellular glutamate inhibits the exocytotic recycling of tPA by astrocytes and on the other hand, capturing extracellular tPA into astrocytes reduces the NMDA-mediated responses potentiated by tPA [20].

4.2.3 Secretory Lysosomes

Secretory lysosomes with diameters between 300 and 500 nm [23, 150] that store signaling molecule ATP, have been identified in cultured astrocytes [56, 71, 96, 150]. Secretory lysosomes in astrocytes as in other cell types are likely involved in membrane repair [3]. Astroglial secretory lysosomes express lysosomal-specific markers, including cathepsin D and lysosomal-associated membrane protein 1 (LAMP1 [150]), monomeric GTP-protein Rab 7, SNARE protein tetanus neurotoxin (TeNT)-insensitive VAMP (TI-VAMP/VAMP7), which contributes to TeNT-independent exocytotic release of ATP [138], and vesicular nucleotide transporter VNuT [120], which is involved in ATP storage [93] within secretory lysosomes in astrocytes and hence warranting ATP release [63] from these astrocytic secretory organelles. Secretory lysosomes in astrocytes can be specifically labeled with dextrans [55, 134], FM dyes, and by a fluorescent ATP analogue MANT-ATP [150]. They may coexist with SLMVs in the same astrocyte [72]. Fusion of secretory lysosomes is regulated and induced with slow, locally restricted Ca^{2+} elevations [71], which are distinct from Ca^{2+} spikes inducing SLMV fusion [138].

4.2.4 Extracellular Vesicles: Exosomes and Ectosomes

Exosomes and ectosomes are extracellular vesicles (EVs) released from cells to deliver signals to target cells (Fig. 4.3). EVs control different biological processes by transferring membrane proteins, lipids, signaling molecules, mRNAs, microRNAs (miRNAs), and activating receptors of recipient cells, possibly playing a role

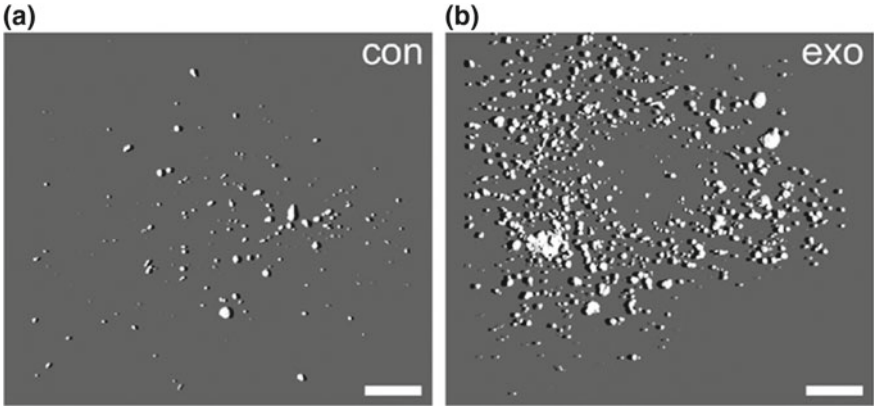

Fig. 4.3 Internalization of exosomes into astrocytes. Internalization of PKH26 nanoparticles and PKH26-positive particles of the exosome-containing samples into subcellular compartments of cultured astrocytes. **a**, **b** Representative three-dimensional shaded display of individual live cultured astrocytes that internalized PKH26 nanoparticles (**a**, con) and PKH26-positive particles present in the PKH26-labeled exosome-containing samples (**b**, exo) into intracellular compartments, observed as numerous bright fluorescent puncta. Scale bars, 10 μm. Modified with permission [112]

in autocrine regulation. Exosomes are released by exocytosis of multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) that are called exosomes when released from cells into the extracellular space. Ectosomes (also called microvesicles) are assembled by outward budding of the plasma membrane and released (shed) from the plasma membrane into the extracellular space. Exosomes are vesicles of 50–100 nm in diameter, while ectosomes are larger vesicles from 100 to >1,000 nm in diameter [25]. Astrocytes release both types of EVs [38].

Ectosomes carrying interleukin-1β (IL-1β) may shed from cultured astrocyte upon ATP stimulation through activation of ionotropic purinoreceptor $P2X_7$. This is associated with rapid activation of acid sphingomyelinase, which moves from luminal lysosomal compartment to the plasma membrane outer leaflet altering membrane structure/fluidity leading to vesicle blebbing and shedding 1–2 min after ATP stimulation [13]. Diameters of ectosomes shed by cultured astrocytes vary between 100 and 1,000 nm [13, 110]. Moreover, upon repetitive ATP stimulation cultured astrocytes release vesicles from the cell surface that can be from 1 up to 8 μm in diameter and express on their surface β1-integrin proteins and contain mitochondria and lipid droplets together with ATP [34]. Although it has not been directly demonstrated [34], these vesicles likely represent ectosomes due to their large size. In addition to interleukin-1β (IL-1β) [13], mitochondria, lipid droplets, and ATP [34], culture astrocyte-derived ectosomes may also carry fibroblast growth factor 2 and vascular endothelial growth factor [110], ectoenzyme nucleoside triphosphate diphosphohydrolases that hydrolyze extracellular nucleotides [21], and matrix metalloproteinases and their inhibitors [121]. Ectosomes shed from astrocytes in response to lipopolysaccharide-induced stress contain miRNA miR-34a that enhances the vulner-

ability of dopaminergic neurons to neurotoxins by downregulating the anti-apoptotic protein Bcl2 [78]. Recently, it has been shown that cytokines tumor necrosis factor α and IL-1β can modify the miRNA cargo of EVs shed from astrocytes to regulate neurotrophic signaling in neurons [22]. Astrocyte also shed EVs that promote transmigration of leukocytes into the brain through regulation of the peripheral acute cytokine response to IL-1β–induced inflammatory brain lesion [31].

Exosomes containing heat-shock protein 70 are released from cultured astrocytes in response to oxidative and heat stress, suggesting a mechanism by which astrocytes provide antioxidant protection to neurones [132]. Retinal astrocytes release exosomes that contain anti-angiogenic components that inhibit laser-induced choroidal neovascularization [43]. Exosomes secreted from astrocytes carrying synapsin I promote neurite outgrowth and neuronal survival [142]. Astrocyte-derived exosomes have been reported to contain mitochondrial DNA [41] and may carry also disease-specific cargo and promote neurological disorders by spreading pathology. Indeed, cultured astrocytes expressing mutant copper-zinc superoxide dismutase 1 (SOD1) secrete exosomes, which carry mutant SOD1. Astroglial derived mutant SOD1-positive exosomes can transfer mutant SOD1 to cultured neurons and induce motor neuron death. This suggests a role of EVs in the pathogenesis of amyotrophic lateral sclerosis [6]. Moreover, cultured astrocytes exposed to amyloid peptide release exosomes enriched with pro-apoptotic ceramide and prostate apoptosis response 4 (PAR4). These exosomes are taken up by astrocytes and promote their apoptosis suggesting that exosome-mediated astrocyte death may contribute to neurodegeneration in Alzheimer's disease. Exosome-mediated miRNA transfer from astrocytes to neurones has been suggested to participate in HIV-associated neurological disorders. Treatment of cultured astrocytes with pathogenic HIV trans-activator of transcription (Tat) protein and morphine triggers shuttling of miRNA miR29b via exosomes to neuronal cells, which results in decreased trophic factor platelet-derived growth factor (PDGF)-B expression and neuronal viability [52]. Nef (Negative Regulatory Factor), a protein encoded by primate lentiviruses such as HIV-1, has been shown to be released in EVs derived from astrocytes and human microglia and may accumulate in neighboring cells (Fig. 4.3) contributing to Nef-mediated neurotoxicity [118, 129]. Interestingly, this release appeared inhibited by elevated cytosolic calcium in human microglia [129]. Recently, it has been shown that reactive astrocytes release vimentin, an intermediate filament of the cytoskeleton, via exosomes. This promotes binding of exoenzyme *Clostridium botulinum* C3 transferase (that enzymatically inhibits small GTPases of the Rho family) to neuronal surface, which can be than internalized and promotes neuronal plasticity and growth [1].

As the field of studying EVs is still developing, it is important to note that when studying the internalization of EVs into cells, the methods and approaches have to be evaluated carefully. For example, when monitoring the internalization of EVs into astrocytes, EVs were labeled by a fluorescent dye PKH26, and it has been reported that a significant false-positive signal due to internalization of PKH26-nanoparticles was observed (Fig. 4.3), which can compromise the interpretation of EV internalization [112]. Thus, for EV uptake and functional studies it is critical

to consider potential artifacts, since EVs are very small, often below the optical microscopy resolution.

4.3 SNARE and SNARE-Associated Proteins in Astrocytes

Astrocytes express vesicular R-SNARE and plasma membrane Q-SNARE proteins (Fig. 4.4). R-SNARE proteins synaptobrevin 2 (VAMP2), VAMP3 [27, 74, 80, 88, 99, 144], and TI-VAMP/VAMP7 [138] and Q-SNARE proteins SNAP23 and syntaxins 1, 2, 3, and 4 [48, 94, 148] have been identified in astrocytes as well as SNARE-associated proteins Munc18 [94] and synaptotagmin 4 [147]. In mammals, synaptotagmin 4 is not a Ca^{2+}-sensor for regulated exocytosis like synaptotagmin 1 in neurones is [143], but is important in modulating Ca^{2+}-evoked exocytosis [131]. SNARE proteins VAMP2 [144], VAMP3 [8, 12, 58, 123, 148], TI-VAMP/VAMP7 [138], SNAP23 [123], and syntaxin 1 [123] were confirmed also in brain tissue astrocytes using immunogold cytochemistry and confocal microscopy. Expression of synaptotagmins and other SNARE-associated proteins, such as Sec1/Munc18-like proteins, in brain tissue astrocytes still needs to be determined, although studies examining mRNA of astrocytes in brain tissue suggest expression of several synaptotagmin isoforms [84, 149] and SNARE-associated proteins [149].

The ternary SNARE fusion complex between vesicular and plasma membrane SNAREs [35, 36] in astrocytes is likely made of vesicular SNARE proteins VAMP2/3 (SLMVs) or TI-VAMP/VAMP7 (secretory lysosomes) and the plasma membrane SNAP23 and syntaxins [45, 85]. The formation of up to five SNARE complexes containing VAMP2 is believed to be sufficient to carry a single vesicle fusion in astrocytes [127].

Astroglial VAMP2 and VAMP3 colocalize with ATP [74] or D-serine [80]-storing vesicles. VAMP3 in astrocytes colocalizes also with the VGLUT1 and 2, vesicular glutamate transporters present on SLMVs that store glutamate [8, 12, 58, 148], and likely D-serine [82]. TI-VAMP/VAMP7 is present in the membrane of the astroglial late endocytic/lysosomal compartments [138] storing ATP [4, 24, 96, 150].

The functionality and physiological role of exocytotic apparatus in astrocytes consisting of aforementioned SNARE proteins has been addressed and confirmed in multiple studies. It has been shown that cleavage of SNARE proteins with tetanus (TeNT) and botulinum neurotoxins (BoNT) in cultured astrocytes attenuates exocytotic release of glutamate [4, 10, 11, 12, 53, 87, 104] as well as a reduction in membrane capacitance (C_m) increases [39, 61] and in amperometric spikes [23], implying the role of SNARE proteins in the release of glutamate from cultured astrocytes. The inactivation of VAMP2/VAMP3 in astrocytes by TeNT abolishes the release of glutamate or D-serine from astrocytes in brain tissue slices [47, 58, 106]. Additionally, in a mouse model in which a dominant negative SNARE transgene is expressed in astrocytes to interfere specifically with astroglial VAMP2/3 [44, 103] the synaptic transmission and plasticity in these animals were altered [49, 63, 89, 103, 133]. Furthermore, in mice with targeted expression of BoNT/B in Müller cells,

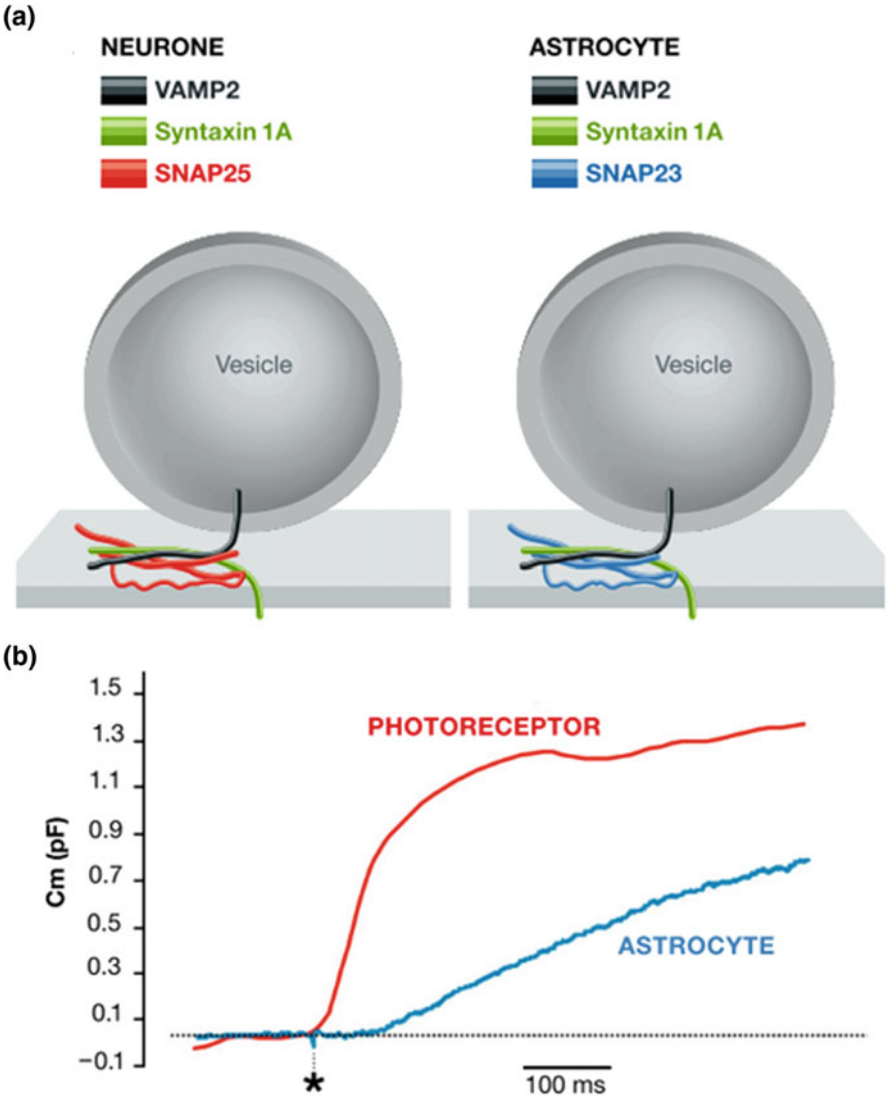

Fig. 4.4 Slowness of astroglial exocytosis. **a** Neuronal versus astrocytic SNAREs. Neurones and astrocytes alike express SNAREs VAMP2 and syntaxin 1; many astrocytes can also express VAMP3 in lieu of or in addition to VAMP2. Astrocytes express SNAP23, a homologue of neuronal SNAP25. At the plasma membrane, syntaxin 1A can form a binary cis complex with SNAP25B or SNAP23A, which then interacts with vesicular VAMP2 to form a ternary complex. A single ternary complex can tether the vesicle at the plasma membrane for a longer period of time, when it contains SNAP25B rather than SNAP23A, respectively. Of note, truncated syntaxin 1, lacking the N-terminal Habc domain and the linker region to the SNARE domain, is shown for simplicity. Drawings are not to scale. **b** Comparison of kinetics of neuronal and astroglial exocytosis. Time-dependent changes in membrane capacitance (Cm) recorded in a neuronal cell (trace in red, photoreceptor) and an astrocyte (trace in blue), elicited by a flash photolysis-induced increase in cytosolic Ca^{2+}. Note that the blue trace recorded in an astrocyte displays a significant delay between the stimulus (asterisk) and the response (trace components above the dotted line). Modified with permission [139]

a subtype of astroglia that expresses BoNT/B-sensitive VAMP2/3, the disruption of the Ca^{2+}-dependent vesicular glutamate release from Müller cells was observed [128]. Moreover, it has been shown in mice with inducible expression of TeNT in astrocytes that TeNT-sensitive vesicular release from astrocytes is necessary for sustaining gamma oscillations associated with recognition memory in mice [68]. Interestingly, the use of various botulinum toxins and dominant negative SNARE peptides has demonstrated that SNARE proteins determine the fusion frequency of individual vesicles monitored by the high-resolution membrane capacitance technique [39]. All these data clearly imply that SNARE-mediated exocytosis is present in astrocytes and essential for normal brain function.

4.4 Regulation and Kinetics of Secretory Vesicle Release in Astrocytes

4.4.1 GPCR-Mediated Regulation of Secretory Vesicle Release: Ca^{2+} and cAMP Signals

Neurones are electrically excitable and release neurotransmitters from synaptic vesicles in synaptic terminals in response to depolarization. In contrast to neurones, astrocytes are electrically silent and display only receptor-mediated cytosolic excitability. Astrocytes sense extracellular signalling molecules via plasma membrane receptors. They express a large number of various types of receptors and many of these receptors are metabotropic high affinity G-protein-coupled receptors (GPCRs) [2, 100, 140, 151]. Binding of signalling molecules to these receptors may increase cytosolic levels of free Ca^{2+} as well as other astrocytic cytosolic secondary messengers, including the cyclic adenosine monophosphate (cAMP). Such cytosolic excitability may lead to secretory vesicle release of signaling molecules from astrocytes (see Vesicular Network). These gliosignalling molecules can then interact with the receptors on neurons affecting neuronal excitability [17, 100] or affect receptors on other neighbouring cells.

Stimulation of astroglial GPCRs coupled to G_q protein leads to increases in intracellular levels of cytosolic Ca^{2+}. Activation of G_q GPCRs triggers IP^3 signaling cascade that releases Ca^{2+} into the cytosol from the IP^3-sensitive intracellular organelles acting as Ca^{2+} stores, such as endoplasmic reticulum (ER) [53, 69] and secretory vesicles [54]. Mitochondria can modulate those cytosolic calcium dynamics in astrocytes by taking up Ca^{2+} from the cytosol or releasing this ion into the cytosol at time of high or low Ca^{2+} cytosolic levels, respectively [115, 126]. Ca^{2+} can also partially enter astrocytes from the extracellular space through voltage-gated Ca^{2+} channels [67, 73, 102], ionotropic receptors [64], sodium-calcium exchanger [116] and through the transient receptor potential canonical type 1-containing channel [75]. G_q-induced cytosolic Ca^{2+} increases in astrocytes occur as oscillations or sustained elevations [100, 141, 151], spontaneously or in response to signaling molecules [26]. Astrocytes

can intercellularly communicate through gap junction channels. They can propagate cytosolic Ca^{2+} excitability by diffusion of IP_3 or Ca^{2+} through gap junctions to neighboring unstimulated astrocytes in the form of intercellular Ca^{2+} waves [122]. They can also release glutamate or ATP in response to Ca^{2+} excitability [15, 26, 42].

Stimulation of astroglial GPCRs coupled to G_s proteins activates adenylyl cyclase (AC), an enzyme catalyzing the conversion of ATP to cAMP [114, 135]. cAMP activates a number of effectors in the cell, primarily cAMP-dependent protein kinase A, but signalling via cAMP-activated GTP-exchange protein [30], cAMP-gated ion channels, and Popeye domain-containing proteins [37] may also be triggered [7]. G_s protein activation induces persistent cAMP elevations [135, 137], which are at least in the case of adrenergic receptor activation 10-fold slower compared to G_q protein-triggered Ca^{2+} elevations [50, 51]. Whether G_s-induced cAMP excitability can be propagated via gap junctions needs to be evaluated [33]. It has been suggested that G_q- and G_s-mediated pathways in astrocytes can interact, since G_s-signaling pathway may enhance G_q-mediated Ca^{2+} responses and vice versa [5, 50, 51, 57].

GPCR G_q- and G_s-protein signalling pathways were shown to be involved in the regulation of secretory vesicle release of chemical messengers from astrocytes. Ca^{2+} elevations in astrocytes trigger the release of glutamate [10, 12, 98, 104, 148], ATP [4, 24], secretogranin II [17], ANP [62], and D-serine [88] from secretory vesicles. cAMP elevations can trigger the release of secretogranin II from astroglial peptidergic vesicles [17]. In astrocytes pretreated with the membrane-permeable cAMP analogue dibutyryl-cAMP the Ca^{2+}-triggered release of ANP from secretory vesicles was enhanced [94]. cAMP might trigger the fusion of secretory vesicles de novo or it may modulate the fusion pore dynamics of already pre-fused secretory vesicles by increasing the diameter and open time of a fusion pore between the vesicle and plasma membranes, which needs to be still determined. The latter mechanism has been observed in neuroendocrine cells [18].

4.4.2 Kinetics of Secretory Vesicle Content Release in Astrocytes

Temporal dynamics of secretory vesicle release from cultured astrocytes has been monitored using (i) electrophysiological techniques (amperometry [23] and membrane capacitance (C_m) measurements [61, 117] in combination with UV-flash photolysis-induced increases in cytosolic Ca^{2+} levels [61]), and (ii) optical techniques (real-time confocal microscopy and total internal reflector fluorescence microscopy, TIRFM) in combination with fluorescent markers of vesicular cycling/fusion, such as FM dyes [71, 72, 150], acridine orange [12, 32], quinacrine [96, 111], fluorescent dextrans [56], MANT-ATP [150], and genetically encoded chimeric proteins of specific membrane/luminal vesicle markers and green fluorescence proteins (GFP) or mCherry-derived proteins [77, 79].

4.4.2.1 Secretory Vesicle Fusion in Astrocytes Occurs with a Delay upon Stimulation

Compared to neurones it has been shown for all 4 secretory vesicles types described in astrocytes (Sect. 4.1) to fuse with the plasma membrane with a delay upon stimulation. As determined with C_m measurements the kinetics of secretory vesicle fusion in astrocytes is at least two orders of magnitude slower than that in neurons (Fig. 4.4b) [61], where secretory vesicle fusion occurs within <0.5 ms upon intracellular Ca^{2+} increase [131].

In respect to astroglial SLMVs the rise of cytosolic Ca^{2+} evoked by activation of metabotropic glutamatergic receptors [12, 19, 79] or purinergic receptors [119] triggers fusion events of SLMVs within hundreds of milliseconds after stimulation as determined in studies using fluorescently tagged VGluT1/2-containing vesicles (i.e., VGluT-pHluorin and VGluT-EGFP, which are chimeric proteins of VGluT and a pH-sensitive GFP protein ecliptic synapto-pHluorin (SpH; [83]) or EGFP. Ionomycin, a Ca^{2+} ionophore, triggers exocytotic fusion of SpH-labeled SLMVs within seconds [72]. In another study exocytotic bursts of SpH-labeled SLMVs occur within 6 s after mechanical stimulation of astrocytes, but other stimuli such as ATP, bradykinin, the Ca^{2+} ionophore 4-Br-A23187, α-latrotoxin, or hypertonicity cause fusion of SpH-labeled SLMVs following a delay of >1 min [77]. Secretion of astroglial peptidergic vesicles also occurs with a delay. Exocytosis of neuropeptide Y-positive peptidergic vesicles upon glutamate [113] or ionomycin stimulation [109] occurs with a delay of >1 min and exocytosis of emerald green-tagged AMP from peptidergic vesicles in 8-Br-cAMP-differentiated astrocytes occurred over a time scale of minutes upon ionomycin stimulation [94]. A similar time-course was observed, when exocytosis of FM-dye-labeled lysosomes was studied. FM-dye labeled lysosomes began to fuse with the plasma membrane with a delay of >1 min upon stimulation of astrocytes with Ca^{2+} ionophores A-23187 [71] and ionomycin, or upon ATP stimulation [150]. The exocytotic fusion of the majority of TI-VAMP positive quinacrine-labeled secretory vesicles [138], that likely represent secretory lysosomes, occurred with a delay of >2 min upon addition of different stimuli, including glutamate, ATP, ionomycin or upon stimulation with UV-induced Ca^{2+} uncaging [96, 111]. EGFP-LAMP1 (lysosomal-associated membrane protein 1)-labeled lysosomes and FITC-dextran-labeled lysosomes also undergo fusion with a delay of >40 s upon application of ionomycin [72], ATP and, a group I metabotropic glutamate receptor agonist (R/S)-3,5-dihydroxyphenylglycine [56]. Moreover, ectosomes carrying IL-1β start to bleb and shed from astroglial plasma membrane with a 1–2 min delay upon ATP stimulation [13].

The reason for such a loose excitation-secretion coupling in astrocytes [136] may be that (i) the major source of Ca^{2+} in astrocytes is not the extracellular space as in neurones, but intracellular IP_3-sensitive Ca^{2+}-storage organelles, which release Ca^{2+} only upon activation of receptor-mediated intracellular signalling cascades and production of IP_3, (ii) the lack of active zones in astrocytes and slower delivery of secretory vesicles to the plasma membrane fusion sites upon stimulus application compared to neurons, where there are active zones [108], or (iii) differences

in exocytotic vesicle fusion machinery between astrocytes and neurons (Fig. 4.4) with astroglial machinery exhibiting a slower vesicle fusion dynamics compared to neuronal exocytotic machinery [85].

4.4.2.2 Modes of Astroglial Secretory Vesicles Fusion

Exocytotic fusion of secretory vesicles in astrocytes exists in two major forms [23], as observed in neurones and neuroendocrine cells [46]. Namely, amperometric studies revealed that dopamine-loaded astrocytic vesicles fuse with the plasma membrane either by transient (kiss-and-run) exocytosis, with vesicle content only partially released, or by full-fusion exocytosis [23]. In optical studies in which the exocytosis of SLMVs expressing spH [14, 77] or SLMVs co-expressing VGluT1-mCherry/VGluT1-pHluorin [79] was studied, both modes, the transient and the full-fusion, of SLMV exocytosis were shown to occur in the same astrocyte simultaneously under spontaneous or stimulated conditions. 50–60% of all spontaneous exocytotic events were the full-fusion events, while 40–50% were the transient fusion events. Depending on the type of a stimulus, the percentage of either type of event shifted toward transient or full-fusion modes of exocytosis upon stimulation. This indicates stimulus-dependent regulation of fusion pore opening [14, 23, 77]. Secretory lysosomes can also exhibit both transient and full-fusion modes of exocytosis. A rapid, total release of an FM dye was observed, followed by a slower and complete loss of EGFP-sialin (a lysosomal sialic acid transporter), from the same lysosomes upon mechanical stimulation, suggesting that secretory lysosomal fusion in astrocytes completes upon mechanical stimulation within seconds, without evidence for transient fusion [71]. Upon glutamate and ATP stimulation the release of FM dyes and MANT-ATP from LAMP1-positive lysosomes is only partial, implying the transient mode of secretory lysosomal exocytosis [150]. Vesicular nucleotide transporter mCherry has been shown to remain associated with the lysosomal membrane during the release of cathepsin D-Venus from the same lysosomes upon ATP, L-glutamate, and calcium ionophore A23187 stimulation, further suggesting that secretory lysosomes in astrocytes may not fully fuse with the plasma membrane [93]. Discrete increases in membrane capacitance, indicating single-vesicle fusion, revealed that astrocyte stimulation increases the frequency of predominantly transient fusion events in smaller vesicles (likely SLMVs and peptidergic vesicles), whereas larger vesicles (likely secretory lysosomes) transitioned to full fusion suggesting that vesicles with different diameters in astrocytes exhibit different capacities to discharge their cargo, due to distinct fusion pore properties [39].

The underlying molecular mechanisms controlling the fusion pore state are not clearly known, but may among others involve SNARE proteins and dynamin. Dynamin, a multidomain GTPase involved in vesicle scission from the plasmalemma during endocytosis, has been shown to be involved in the regulation of a fusion pore during spontaneous exocytosis in astrocytes, since activators of dynamin Ryngo™-1-23 promoted fusion pore closure by prolonging closed and by shortening open fusion pore dwell times [66]. DnSNARE (dominant-negative domain of synapto-

brevin 2 protein) peptide, which interferes with endogenous VAMP2 expression and thus prevents VAMP2-mediated membrane fusion, has been shown to stabilize the fusion-pore diameter to narrow, release-unproductive diameters regardless of vesicle diameter, implying the regulatory role of SNAREs in governing vesicle fusion in astrocytes [39]. The fusion pore can alone be a subject of regulation by ketamine, an anesthetic that exhibits analgesic, psychotomimetic, and rapid antidepressant effects. It has been shown recently, using high-resolution cell-attached membrane capacitance measurements, that ketamine evokes long-lasting flickering of a narrow fusion pore that is incapable of transiting to full fission [65]. Furthermore, ketamine treatment also suppressed ATP-triggered vesicle fusion and BDNF secretion by increasing the probability of a narrow fusion pore open state and/or by reducing astrocytic Ca^{2+} excitability [130].

4.5 Non-vesicular Astroglial Secretion

Astrocytes can also release signalling molecules by a non-vesicle-based mechanisms (i) through plasmalemmal channels (e.g., volume-regulated anion channels, connexons/pannexons (hemichannels), ionotropic pore-forming $P2X_7$ purinergic receptors, the two-pore-domain potassium channel Trek-1, or Bestrophin-1 channels [146], and (ii) through plasmalemmal transporters (e.g., reversal uptake by plasma membrane excitatory amino acid (glutamate) transporters, (hetero)exchange via the cystine–glutamate antiporter or organic anion transporters) [76]. With the exception of Bestrophin-1, these non-vesicular release mechanisms are Ca^{2+}-independent and might be activated only under pathological conditions [2, 45].

4.6 Concluding Remarks

Astrocytes are involved in many processes in the CNS through sensing extracellular signaling molecules by surface GPCRs, responding to this with cytosolic excitation, which then stimulates the release of their own astroglial chemical messengers, gliosignaling molecules. Many studies support the existence of vesicule-based secretion of transmitters from astrocytes, in response to GPCR-mediated stimulation. These studies have shown that astrocytes possess various types of secretory vesicles. The exocytotic fusion of these vesicles is regulated at the level of a single fusion pore and it occurs in two modes, as transient and full-fusion exocytosis. Moreover, astrocytes, which are electrical silent, but exhibit GPCR-mediated cytosolic excitability, respond to stimulation with a delay in exocytosis compared to fast responsive electrically excitable neurones. Such slow release kinetics of vesicle signaling apparatus suggests that astrocytes are acting as integrators of information, modulating neuronal activity in a slow-time domain. Although the physiological relevance of astroglial

exocytosis in vivo is still not clear, it is predicted that astrocytes participate in information processing in the brain by exocytotic release of signaling molecules.

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Chapter 5
Physiology of Oligodendroglia

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Abstract Oligodendrocytes are the myelinating cells of the CNS, producing the insulating myelin sheath that facilitates rapid electrical conduction of axonal action potentials. Oligodendrocytes arise from oligodendrocyte progenitor cells (OPCs) under the control of multiple factors, including neurotransmitters and other neuron-derived factors. A significant population of OPCs persists in the adult CNS, where they are often referred to as NG2-glia, because they are identified by their expression of the NG2 chondroitin sulphate proteoglycan (CSPG4). In the adult brain, the primary function of NG2-glia is the life-long generation of oligodendrocytes to replace myelin lost through natural 'wear and tear' and pathology, as well as to provide new oligodendrocytes to myelinate new connections formed in response to new life experiences. NG2-glia contact synapses and respond to neurotransmitters and potassium released during neuronal transmission; to this end, NG2-glia (OPCs) express multiple neurotransmitter receptors and ion channels, with prominent roles being identified for glutamatergic signalling and potassium channels in oligodendrocyte differentiation. Myelinating oligodendrocytes also express a wide range of neurotransmitter receptors and ion channels, together with transporters and gap junctions; together, these have critical functions in cellular ion and water homeostasis, as well as metabolism, which is essential for maintaining myelin and axon integrity. An overriding theme is that oligodendrocyte function and myelination is not only essential for rapid axonal conduction, but is essential for learning and the long-term integrity of axons and neurones. Hence, myelination underpins cognitive function and the massive computing power of the human brain and myelin loss has devastating effects on CNS function. This chapter focuses on normal oligodendrocyte physiology.

Keywords Oligodendrocyte · Oligodendrocyte precursor cell · OPC · NG2-glia · Myelin · Axon

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5.1 Introduction

Individual oligodendrocytes can myelinate 30 or more axons and each myelin sheath extends along the axon for over 50–100 μm to form internodes that are interrupted by the nodes of Ranvier [12, 55]. In this way, the axonal membrane is divided into alternating nonconductive portions underneath the myelin sheath and the conductive nodes of Ranvier, where the sodium and potassium channels that mediate the action potentials are localised [76]. The myelin sheath is formed by concentric lamellae of the oligodendroglial plasmalemma wrapped around the axon; compacted myelin is formed by fused phospholipid bilayers, which gives the myelin its insulating properties [65]. The compacted myelin is surrounded by a cytoplasmic ridge that forms a conduit through which proteins and other chemicals are transported from the oligodendrocyte cell body [47]. At the node of Ranvier, the cytoplasmic ridges form the paranodal loops that establish complex adhesion junctions with the axon, which is essential for the separation of axonal potassium and sodium channels that generate the action potential [25]. The loss or disruption of myelin, such as occurs in demyelinating diseases and other pathologies, results in disruption of action potential propagation or conduction block, and ultimately loss of axonal integrity and neuronal death [58].

5.2 Myelin

The main constituents of myelin are lipids (70% of its dry weight) and proteins (30% of the dry weight), many of which are specific to myelin and are used to identify oligodendrocytes [47]. Cholesterol is a major component of myelin (27%) and is essential for myelination [60]; the blood–brain barrier prevents dietary cholesterol from entering the brain and astrocytes are proposed to be the main source of cholesterol in the brain, although oligodendrocytes are capable of de novo synthesis [35]. In addition, myelin contains phospholipids that are rich in glycosphingolipids, in particular, galactocerebroside (GalC), and sulphatides [34]. The major myelin proteins are myelin basic protein (MBP) and proteolipid protein (PLP), which constitute about 80% of CNS protein, together with numerous proteins that make up a small but significant fractions of myelin, including 2′, 3′-cyclic nucleotide-3′-phosphodiesterase (CNP), myelin-associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG); notably, the absence of PLP or CNP result in axonal degeneration [48]. In addition, myelin contains gap junctions, predominantly formed by connexins Cx32 and Cx47, the latter being specific to oligodendrocytes in the CNS, together with Cx29, which are crucial for ion homeostasis, myelination and axonal metabolism and integrity [70]. A consistent theme is that ablation of individual oligodendrocyte genes, ranging from myelin genes to connexins, results in axonal demise, demonstrating that oligodendrocytes and axons are interdependent functional units.

5.3 Oligodendrocyte Differentiation

Oligodendrocytes are generated from OPCs that arise from multipotent neural stem cells (NSCs) in the subventricular zone (SVZ) [5]. From these focal sources, OPCs migrate to populate the entire CNS, where they undergo local proliferation and pass through a number of intermediate stages to differentiate into myelinating oligodendrocytes [68]. A significant population of OPCs persists in the adult CNS, where they are often referred to as NG2-glia, which have the stem cell-like property of self-maintaining and the capacity to generate oligodendrocytes throughout life [2]. OPCs are identified by their expression of PDGFRα and NG2 (cspg4) [56] and, as they differentiate, they exit cell cycle and lose Pdgra and NG2, and transiently express GPR17, before expressing the transcription factor myelin gene regulatory factor (MRF) and myelin-related proteins, such as MBP and PLP [19, 23]; GPR17 and MRF appear in oligodendrocytes shortly before the onset of myelination and, respectively, negatively and positively regulate terminal differentiation into myelinating oligodendrocytes. The specification, migration, proliferation and differentiation of OPCs are regulated by a highly complex interplay between intrinsic and extrinsic factors that both negatively and positively influence oligodendrocyte generation and myelination [18]. These include key growth factors, such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), which act, respectively, via PDGFRα and FGF receptors-1, -2 and -3 (FGFR1-3), and insulin-like growth factor (IGF-1) [54]. In addition, Wnt signalling drives OPC expansion throughout life [5], and oestrogen positively regulates oligodendrocyte differentiation and myelination [72]. Cytokines and chemokines also act on oligodendrocytes and OPCs through a wide range of receptors, such as interleukins (e.g., IL-1β and IL-6) and the CXCL12/CXCR4/CXCR7 axis, which regulate OPC proliferation, migration, and differentiation [35, 54]. Furthermore, OPC expresses a range of neurotransmitter receptors and ion channels that regulate their migration, proliferation and differentiation [27]. The disruption or loss of OPCs, or their capacity for generating myelinating oligodendrocytes, has devastating effects on CNS function and ultimately leads to death. Hence, the key feature of OPC is that their self-maintenance and differentiation into oligodendrocytes are regulated by multifarious factors, helping to ensure that disruption of any single factor does not result in their loss of function. Most of these factors are also involved in oligodendrocyte pathologies and are important in regeneration and remyelination in diseases such as multiple sclerosis (MS).

5.4 Neurotransmitter Receptors

Oligodendrocytes and OPCs express ligand-gated ion channels and G protein-coupled receptors (GPCR) for a wide range of neurotransmitters (Fig. 5.1) [43, 71]. In oligodendrocytes, neurotransmitter receptors mediate intercellular communication with the neurons/axons they myelinate [37]; in OPCs, they are generally con-

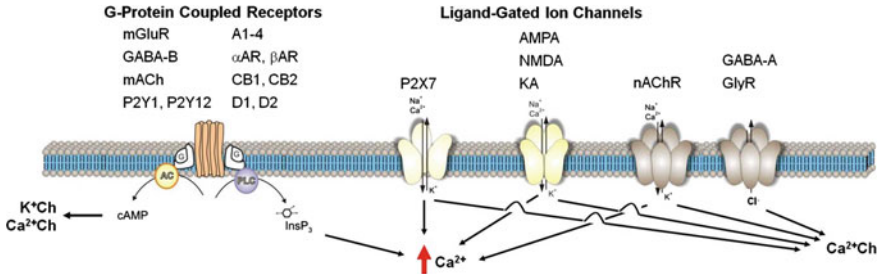

Fig. 5.1 Oligodendrocyte lineage cells express ligand-gated ion channels and G-Protein-coupled receptors (GPCR) for multiple neurotransmitters. A prominent feature of oligodendrocytes at all stages of differentiation is the expression of glutamate receptors, which play key roles in OPC proliferation and differentiation, and in homeostasis and metabolism in oligodendrocytes. A general characteristic is that most receptors can mediate increased intracellular Ca^{2+}, which has diverse effects on OPCs and oligodenrocytes (see text for further details)

sidered to regulate differentiation and many mediate their effects through changes in intracellular Ca^{2+} [10, 11].

Glutamate receptors are highly expressed by oligodendrocytes and OPCs [37]. Ionotropic glutamate receptors (iGluRs) of the AMPA, kainate and NMDA types are abundantly expressed throughout the oligodendrocyte lineage, which allows the flow of K^+, Na^+ and Ca^{2+}. In OPCs, AMPAR is permeable to Ca^{2+} [7, 26, 28], and Ca^{2+} permeability may be downregulated during oligodendrocyte development [33]. AMPAR activation in oligodendrocyte lineage cells mediates signalling from axons that regulate OPC differentiation and myelination [15]. Oligodendrocytes also express functional NMDARs, although their physiological importance is unclear [36]. All three groups of mGluR have been demonstrated in OPCs, but maybe downregulated as they differentiate into mature oligodendrocytes [13].

GABA receptors have been demonstrated to be functional in OPCs and are proposed to regulate their differentiation into oligodendrocytes [27]. Intracellular $[Cl^-]$ is maintained high in glia and activation of $GABA_AR$ leads to Cl^- efflux and cell depolarization, which activates voltage-operated calcium channels (VOCC) and increased intracellular Ca^{2+} in OPC and oligodendrocytes [4]. Metabotropic $GABA_BR$ is also expressed by OPCs and stimulate proliferation and migration [41].

Purine receptors are widely expressed by OPCs and oligodendrocytes, where they are important in development, myelin maintenance and pathology [11, 57]. Adenosine receptors (AR) are GPCR are of four subtypes (A1-4), all of which have been identified in OPCs, where they regulate migration, proliferation, and differentiation, and appear to be downregulated in oligodendrocytes [17]; A2A-R activation decreases cAMP and stimulates outward rectifying potassium channels (Kv) and OPC differentiation (see below). P2XR are expressed by OPCs and oligodendrocytes, with most robust evidence for the $P2X_7$ subtype. P2X7R mediate a rise in intracellular Ca^{2+}, as well as activating multiple intracellular pathways, including MAPK, PKC, and PI3 K, all of which regulate OPC proliferation, differentiation and myelination. In addition, $P2X_7R$ is implicated in the loss of oligodendrocytes

and myelin in ischemia and demyelination. P2YR are GPCR and a key feature in OPCs and OLs are the prominent expression of $P2Y_1R$ that mediate raised intracellular Ca^{2+}, which regulates migration, proliferation and differentiation in OPCs. In addition, $P2Y_{12}R$ are enriched in oligodendrocytes and are implicated in demyelination in MS [3].

Numerous other neurotransmitter receptors are reported in OPCs and oligodendrocytes that are implicated in the regulation of OPC differentiation and myelination [43]; these include acetylcholine receptors (AChR), both nicotinic and muscarinic [21], as well as cannabinoid receptors CB_1 and CB_2 [32].

5.5 Ion Channels and Transporters

Oligodendrocyte lineage cells express diverse ion channels (Fig. 5.2) [39, 64]. Kv (Kv1.3, Kv1.4, Kv1.5, Kv1.6) are prominent in OPCs and regulate their proliferation and differentiation, and are generally downregulated during differentiation [69]. OPCs and oligodendrocytes also express inward rectifying potassium channels (Kir), with a prominent role for Kir4.1, as homomers and as heteromers with Kir5.1 [9]; Kir4.1 facilitate clearance of K^+ released during axonal firing and selective deletion of Kir4.1 from OPCs or mature oligodendrocytes results in profound functional

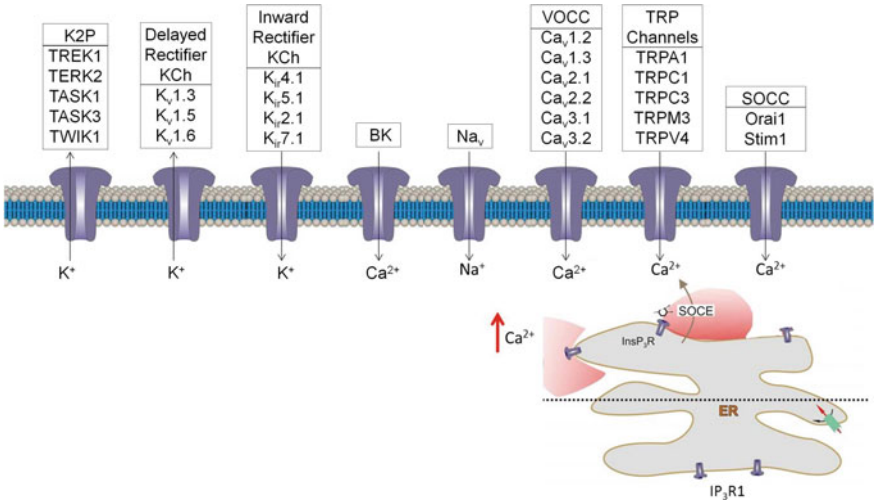

Fig. 5.2 Oligodendrocyte lineage cells express diverse ion channels. A key feature is the expression of potassium channels: delayed rectifier Kv are prominent in OPCs, where they regulate proliferation and differentiation, and are generally downregulated in myelinating oligodendrocytes; inward rectifier channels are expressed throughout the oligodendrocyte lineage and play an important role in ion and water homeostasis. In addition, oligodendrocyte lineage cells express multiple ion channels that mediate Ca^{2+} flux, which is important for myelination and are implicated in pathology

impairments and axon degeneration [38, 62]. Other potassium channels may also be important in maintaining oligodendroglial function and integrity, including Kir2.1, Kir7.1 and TASK1 channels [8, 31, 52].

A key feature of oligodendrocyte lineage cells is the important role for calcium in regulating OPCs and myelination, by influx through ion channels or by activation of receptors [71]. OPCs express voltage-operated calcium channels (VOCC) that regulate OPC maturation and myelination [51, 61]. In addition, store-operated calcium channels (SOCC) and TRP channels are another important mechanism of calcium influx [52]; TRPA1, TRPM3 and ASIC are expressed by oligodendrocytes [20, 30, 52], whilst TRPV4 are expressed by OPC [49]. OPC also express BK channels that mediate Ca^{2+} influx [14]. Oligodendrocytes express the major cation and anion transporters, including Ca-ATPase and Na–Ca exchangers (NCX), which are important in regulating intracellular calcium, together with Na-K-pumps and a variety of anion transporters proteins [53]; Na–K–Cl and K–Cl co-transporters in oligodendrocytes can promote pathological Na^+ entry into oligodendrocytes, which then triggers reverse Na–Ca exchange resulting in Ca^{2+} entry and injury. The physiological role of these diverse mechanisms appears to be regulation of proliferation and differentiation in OPCs and homeostasis and maintenance of cellular and myelin integrity in oligodendrocytes.

5.6 NG2-Glia

As noted above, a significant population of OPCs persists in the adult CNS, where they are often referred to as NG2-glia, since they are identified using antibodies against NG2. Genetic fate mapping has demonstrated that NG2-glia generate oligodendrocytes in the adult brain, which is essential for functional mtyelin repair [50] and for myelination of new connections formed in response to new life experiences [44, 75]. It is evident that neuronal activity enhances myelin formation, termed adaptive myelination, and this is important for nervous system plasticity and repair [16, 22, 50, 74]. This supports the concept that neurotransmission may drive differentiation of NG2-glia (Fig. 5.3), consistent with abundant evidence that NG2-glia express a wide range of neurotransmitter receptors and ion channels [39] and respond to synaptic transmission [7], as well as contacting axons at nodes of Ranvier and responding to axonal electrical activity [28]. In this context, regulation of NG2-glial cell proliferation and differentiation have been indicated for glutamatergic, GABAergic, purinergic and potassium signalling [27, 37, 57, 69]; these may act via changes in intracellular Ca^{2+} and calcium-dependent intracellular signalling pathways, including ERKs and CREB [63]. Adenosine and ATP have been shown to mediate axonal control of differentiation and myelination via raised $[Ca^{2+}]_i$ in NG2-glia [66]. Similarly, glutamate released from electrically active axons acting on AMPAR promotes proliferation and differentiation of NG2-glia [15, 73], whereas GABA acting on GABAAR finely tunes OPC self-maintenance capacity and negatively regulate the generation of oligodendrocytes [6, 29]. NG2-glia also sense changes in extracellular K^+ dur-

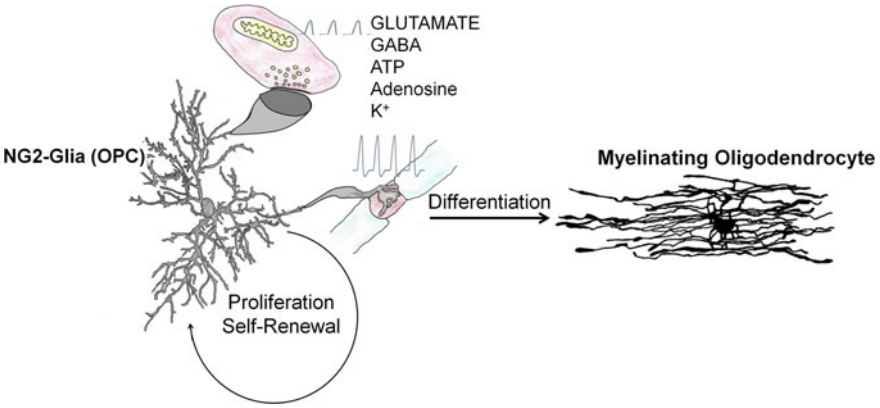

Fig. 5.3 Relationships of NG2-glia with neurons. NG2-glial cells function as adult OPCs. They are multiprocessed cells that extend processes to contact neurons at their sites of electrical and chemical activity at synapses, the sites of chemical transmission between neurons, and nodes of Ranvier, the sites of action potential propagation along axons. Neurotransmitters and K^+ released from synapses and axons act on ion channels and neurotransmitter receptors on NG2-glia to regulate their self-renewal and differentiation into myelinating oligodendrocytes. In this way, neuronal activity can drive myelination during development and in the adult

ing neuronal activity [42], and Kv regulate their proliferation and differentiation [69]; in contrast, selective deletion of Kir4.1 in OPCs did not appear to impair their development [38]. The balance of evidence is that neuronal activity drives adaptive myelination and that NG2-glia are the source of newly generated oligodendrocytes. However, blocking or stimulating synaptic signalling directly has only subtle effects on NG2-glia, indicating neurotransmitters alone do not drive oligodendrogenesis.

5.7 Oligodendrocyte–Axon Interactions and Metabolism

In addition to enabling rapid electrical conduction, myelin is required for axonal integrity, and an important mechanism is metabolic support (Fig. 5.4) [1]. Oligodendrocytes may provide support in the form of glucose [45], but, in general, it appears oligodendrocytes deliver lactate to axons, which they release through MCT1 into the periaxonal space, from where it is taken up by axons via MCT2 [24, 40]. A recent study provides a mechanism by which metabolic support is coupled to axonal activity: action potentials trigger axonal release of glutamate, which activates oligodendroglial NMDAR to evoke a rise in intracellular Ca^{2+} [46]; this stimulates oligodendroglial expression of the glucose transporter GLUT1 and glucose uptake, which is metabolised to lactate and released to axons [59]. The physiological importance of oligodendrocyte–axon metabolic support is unclear and may be more critical under conditions of glucose deprivation and high-frequency activity [67].

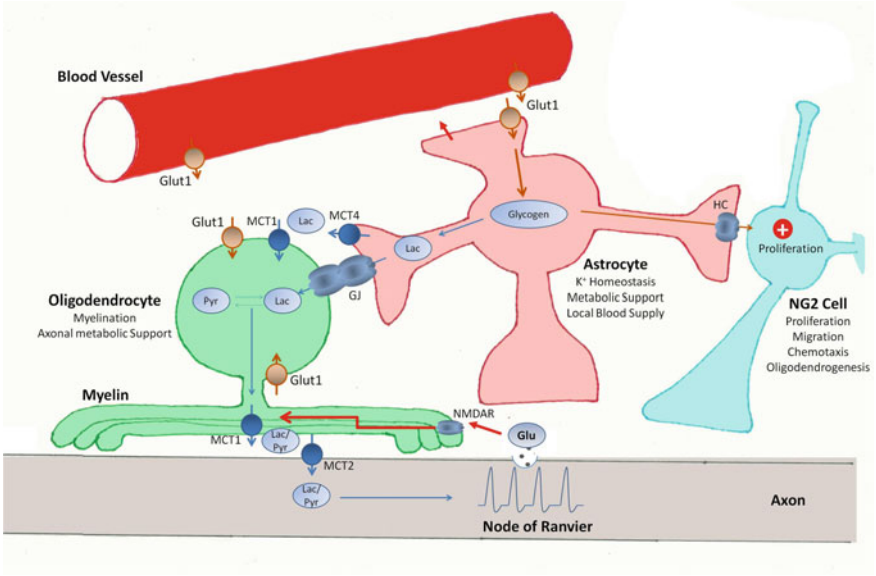

Fig. 5.4 Oligodendrocytes provide metabolic support for axons. Glutamate released during action potential propagation can act directly on oligodendrocyte NMDA-type receptors to stimulate their metabolic support of axons. NMDAR raise intracellular Ca^{2+} which increases activity of glucose transporters (Glut1), which is converted to lactate and released into the periaxonal space via monocarboxylic transporters (MCT1), which are highly expressed by oligodendrocytes, and taken up by the axons via the neuronal MCT2. Astrocytes are another source of lactate for oligodendrocytes, via gap junctions (GJ) and by release through monocarboxylate transporters (MCT4) and subsequent uptake by oligodendrocytes (MCT1). Astrocytes also release glucose via hemichannels to provide metabolic support for NG2-glia, which is necessary for their proliferation and regeneration of oligodendrocytes

5.8 Concluding Remarks

Oligodendrocytes are defined by their myelinating function in the CNS. Myelination provides rapid nervous transmission, without which the brain could not achieve its massive computing power. Myelination is also essential for axonal integrity: oligodendrocytes and the axons they myelinate are completely interdependent functional units and dysfunction in one results in loss of function of the other. The underlying mechanisms are not fully resolved, but recent findings indicate oligodendrocytes are an important source of metabolic support for axons. In addition, new studies demonstrate the importance of adaptive myelination for neural circuit plasticity and learning. The wide range of neurotransmitter receptors and ion channels expressed by oligodendrocytes and OPCs play key roles in these functions and are increasingly recognised as being important in both oligodendrocyte and axonal integrity.

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Chapter 6
Physiology of Microglia

Tuan Leng Tay, Micaël Carrier and Marie-Ève Tremblay

Abstract Microglia constitute the major immune cells that permanently reside in the central nervous system (CNS) alongside neurons and other glial cells. These resident immune cells are critical for proper brain development, actively maintain brain health throughout the lifespan and rapidly adapt their function to the physiological or pathophysiological needs of the organism. Cutting-edge fate mapping and imaging techniques applied to animal models enabled a revolution in our understanding of their roles during normal physiological conditions. Here, we highlight studies that demonstrate the embryonic yolk sac origin of microglia and describe factors, including crosstalk with the periphery and external environment, that regulate their differentiation, homeostasis and function in the context of healthy CNS. The diversity of microglial phenotypes observed across the lifespan, between brain compartments and between sexes is also discussed. Understanding what defines specific microglial phenotypes is critical for the development of innovative therapies to modulate their effector functions and improve clinical outcomes.

Keywords Microglia · Origin · Development · Homeostasis · Physiological roles · Periphery · Environment

6.1 Introduction

Microglia are a prominent type of glia in the central nervous system (CNS) originating from a single mesodermal source in contrast to all other brain parenchymal cells

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that have multiple neuroectodermal lineages. In the past, studies on microglia centred on their function as resident macrophages of the brain and mediators of injury, inflammation and neurodegeneration [22, 113, 114]. Advances in mouse genetics enabled fate mapping of mammalian microglia across conditions of health and disease [51, 52, 110, 143, 160, 166]. Importantly, in vivo imaging techniques revealed real-time microglial activities in the brain milieu [32, 86, 104, 147, 155]. Adult microglia appear morphologically uniform at steady state, but they are functionally heterogeneous in their physiological responses, which may be attributed to their local environment including neuronal activity [4, 26, 33, 58, 86, 124].

Numerous studies have shifted the field's attention to the physiological functions of microglia in brain development, activity and plasticity. Microglia are crucial regulators of CNS development and homeostasis via neuronal–microglial interactions, scavenging of cellular debris, secretion of trophic factors and synaptic modelling [145]. Broadly speaking, physiological microglial functions are required for learning, memory and cognition through the modulation of neuronal numbers and neural connectivity [158]. Microglia regulate neuronal density through coordinated control of neurogenesis, oligodendrogenesis, as well as neuronal survival and turnover. These processes mainly take place during perinatal development [3, 62, 132, 149, 162], but also persist during adolescence and adulthood [18, 128, 133]. Microglia provide neurotrophic support to neurons, notably through the secretion of insulin-like growth factor 1 for cortical layer V neuronal survival [149], the maintenance of embryonic forebrain basal progenitors [3] and the facilitation of neuroblast survival and migration to the adult olfactory bulb [116]. As scavenging phagocytes, microglia accumulate in regions containing high densities of neural precursors or apoptotic neurons, to facilitate neuronal turnover during developmental cell death [6, 29, 90, 111, 141].

In the prenatal mouse brain, microglia additionally regulate the wiring of forebrain dopaminergic circuits [136]. Multiple electrophysiological and high resolution microscopy studies in zebrafish [86] and mouse [7, 10, 27, 72, 74, 89, 118, 120, 121, 164] demonstrated microglial modulation of activity-dependent synaptic maturation, activity and plasticity [145]. Once neuronal circuits are established, microglia contribute to the refinement of synaptic connections across adolescence and adulthood [11, 147, 155]. Microglia–synapse interactions were characterised in the thalamus, cerebral cortex, amygdala and hippocampus, in postnatal development, adolescence, adulthood and normal ageing, [2, 95, 109, 124, 147, 148]. For instance, correlative light and electron microscopy of postnatal day (P) 15 mouse hippocampus confirmed that microglia mediate both the elimination and formation of synaptic elements [159]. The evidence that microglial processes selectively and partially phagocytose presynaptic structures and induce postsynaptic spine head filopodia supports the hypothesis of a microglia-dependent mechanism for the remodelling and maturation of synaptic circuits [159].

The plurality of microglial functions suggests that environmental conditions impairing microglia during brain development could compromise essential processes including neural connectivity. During adulthood, impaired microglial remodelling of neuronal circuits could severely impair learning and memory functions [158]. Dys-

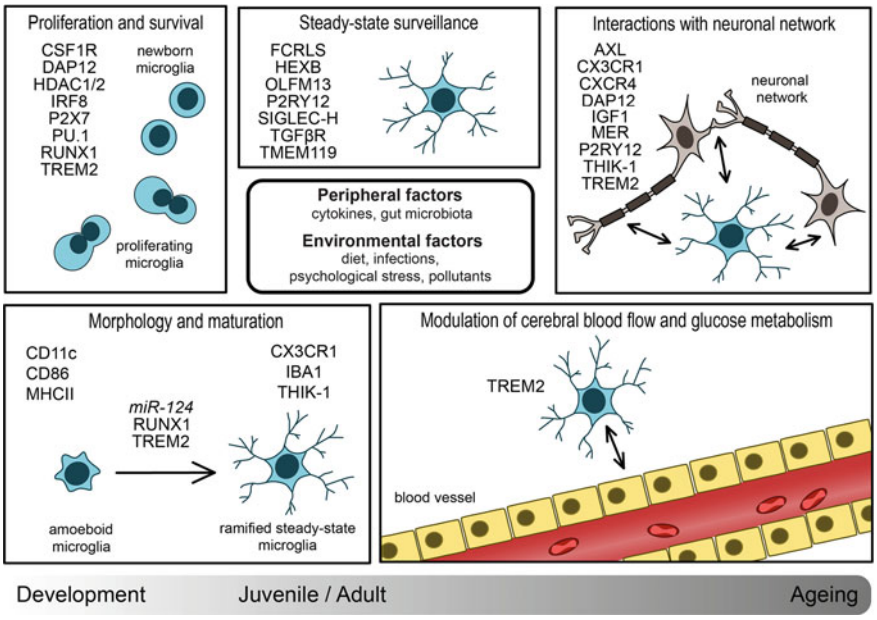

Fig. 6.1 Factors that impact physiological microglial development, homeostasis and function across the lifespan

functional or perturbed microglial homeostasis could have direct consequences on the onset of severe neurodegenerative or neuropsychiatric disorders at an early age or later in adulthood [142]. Here, we examine the innate conditions and external circumstances that support the proper maturation, homeostasis and function of physiological microglia (Fig. 6.1).

6.2 Origin and Maintenance of Microglia

Microglial colonisation of the CNS occurs before neuron and glial cells such as astrocytes and oligodendrocytes develop, and is conserved across vertebrate species [125, 141, 153]. Unlike neuroectodermal cells, microglia have a myeloid origin. Several fate mapping studies have established that microglia arise solely from yolk sac (YS) primitive macrophages [51, 54, 70, 71, 77, 126]. The microglial fate of YS precursors is specified between embryonic day (E) 7.0 and E7.5 [51]. Multi-lineage c-kit^{+} erythromyeloid YS precursor cells reside in blood islands of the proximal YS where they mature from A1 (CD45^{+} c-kit^{lo} CX_3CR1^{-} F4/80^{-}) into A2 (CD45^{+} c-kit^{-} CX_3CR1^{+} F4/80^{hi}) amoeboid macrophages [77]. They thereafter adopt a phenotype of mature macrophages in the neuroepithelium at E10.5 [97, 121]. Microglia seed the rudimentary brain via the leptomeninges and lateral ventricles before E9.5

and distribute themselves throughout the cortical wall from both directions at different speeds with varying rates of proliferation and maturation, depending on the brain region and developmental stage [4, 51, 141]. Normal blood circulation is necessary for YS macrophage recruitment [51]. Human amoeboid microglia penetrate the developing cerebral cortex at 4.5 gestational weeks (gw) via the pial surface, ventricles and choroid plexus [98, 153]. Microglia migrate radially and tangentially from the periphery towards the putative white matter, subplate and cortical plate layers, while pial cells populate the prospective cortical layer I. At 12–13 gw, the second wave of microglia invade only the white matter via the vasculature [98, 153]. Microglial cell numbers rise steadily in the first two postnatal weeks of mouse development, followed by a gradual decline by 50% from week three to six, after which their density stabilises [103]. The decline in the rate of proliferation is concomitant with an increase in apoptosis, contributing to the overall reduction in microglial cell numbers [103]. Mature microglia maintain their numbers in the brain throughout life via a process of self-renewal [5, 51, 66, 115, 145].

The contribution of definitive haematopoiesis as an origin to brain microglia under steady-state conditions has so far been excluded by specific targeting of *Kit*-positive cells, foetal liver- or aorta-gonad-mesonephros-derived haematopoietic stem cells in myeloid-specific genetic mouse models for *Csf-1r* (colony-stimulating factor-1 receptor), *Flt3* (Fms-like tyrosine kinase 3), *Myb* (myeloblastosis), *Runx1* (runt-related transcription factor 1) and *Tie2* (angiopoietin receptor) [51, 54, 70, 126, 131]. In contrast, pulse labelling of zebrafish macrophage precursors demonstrated that adult microglia derived from the ventral walls of the dorsal aorta in zebrafish [165]. Moreover, the existence of a significant number of *Hoxb8* lineage of haematopoietic mononuclear cells in the newborn and adult mouse brain under homeostasis [24] remains unexplained and challenges the single YS source of microglia. Mice lacking *Hoxb8* expression in microglia display obsessive–compulsive-like over-grooming behaviour. A plausible point of entry for these cells is via the brain ventricular choroid plexus [130], since *Hoxb8*-expressing cells distribute in a gradient from the pial surface and ventricular lining to the parenchyma during the first two postnatal weeks [24]. Even though genetic tracing studies predominantly support the single YS origin view, physiological microglia exhibit heterogeneous gene expression profiles, self-maintenance rates and morphological phenotypes across CNS compartments, as will be discussed later. We next examine the intrinsic and external factors that impact microglial development, identity and homeostasis.

6.3 Factors Required for Microglial Development and Homeostasis

Multiple studies have focused on elucidating the origin and renewal of microglia, as well as identifying factors which may affect their maturation, proliferation and apoptosis under normal physiological conditions (Fig. 6.1). Understanding these attributes is important to expand the available possibilities to target and control the effector functions of microglia in CNS diseases.

6.3.1 Cell Signalling Pathways Required for Microglial Proliferation and Maturation

The transcription factors Pu.1, a member of the Ets family [119], and interferon regulatory factor Irf8 both function as heterodimers in the determination of brain macrophage phenotype during the development of YS microglial precursors [8, 77, 96]. Another transcription factor that regulates the differentiation of myeloid cells is Runx1, which is expressed in a subpopulation of amoeboid microglia restricted to the ventricles during early postnatal forebrain development [169]. Runx1 has been described to mediate microglial proliferation. However, by P10, colocalisation of the proliferation marker Ki67 with Runx1 was no longer observed, with microglia shifting towards a ramified morphology, suggesting its role as a maturation factor [169]. It is still unknown how Runx1 regulates these events in a spatiotemporal dependent manner. The microRNA miR-124, which binds the mRNA of transcription factor C/EBPalpha and which in turn downregulates Pu.1, was specifically identified on microglia among myeloid cell populations [112]. miR-124 is functionally conserved in zebrafish and mouse. It controls the motility and phagocytic activities of microglia by promoting ramified surveillant phenotypes over reactive amoeboid ones [112, 140]. In the E13.5 mouse spinal cord, the purinergic ionotropic receptor P2X7 was also shown to mediate the proliferation of embryonic microglia, consequently regulating microglial density [117].

An essential pathway that defines microglial cell number involves Csf-1R (CD115). Csf-1R knockout mouse embryos are depleted of microglia and display impaired brain architecture [42]. Neuron-derived interleukin (IL)-34, the second ligand for Csf-1R, is more critical for regulating microglial cell density than Csf-1 in the adult brain; however its requirement during perinatal development is still controversial [59, 156]. The upregulation of brain Csf-1 levels did not mitigate the physiological decline in microglial numbers after the third postnatal week in mouse [103], suggesting that alternative microglial responses, to still unknown developmental signals, could favour microglial cell death during CNS maturation. Evidence for the requirement of Csf-1R signalling in microglial survival was provided by the near complete loss of mature microglia observed in adult mice treated with Csf-1R inhibitors [41]. Similarly, mice deficient for the Csf-1R adaptor protein DAP12 (DNAX activation protein of 12 kDa) reportedly have reduced microglial cell numbers at adulthood [107], without overt impact on their density during development [77]. While young DAP12-deficient mice do not show neurological deficits up to four weeks of age, synaptic function and plasticity are impaired, suggesting that DAP12 contributes to microglial physiology and communication with neurons [120]. Loss of signalling through the triggering receptor expressed on myeloid cells 2 (TREM2), another interaction partner of DAP12, has been implicated in CNS pathology [151]. Dissecting the impact of TREM2 knockout or mutation on microglial biology revealed that it plays key roles in regulating microglial proliferation, survival, clustering, autophagy, metabolism, as well as phagocytosis [150, 168], with consequences on CNS development and health maintenance. For instance, TREM2 knockout mice dis-

play enhanced excitatory neurotransmission, reduced functional brain connectivity, as well as repetitive behaviour and altered sociability [46]. In addition, TREM2 loss-of-function mutation in mouse reduced cerebral blood flow and glucose metabolism, thus uncovering new roles for microglia in regulating brain metabolism [79]. Moreover, the expression of *histone deacetylases* (*Hdac*) *1* and *2* and their target genes was shown to be developmentally regulated in prenatal murine microglia [31]. CX_3CR1-targeted ablation of *Hdac 1* and *2* during microglial precursor stages led to cellular malformation, impaired cell proliferation and the induction of apoptosis. Altered microglial cell density was observed up to 6 weeks of age while the morphological deficits persisted. Both enzymes are, however, redundant for the maintenance of adult microglia [31].

6.3.2 Cell Signalling Pathways Required for Microglial Homeostasis

Microglia utilise a vast number of surface receptors for cytokines, chemokines, purines, hormones and neurotransmitters in order to quickly react to changes in brain homeostasis. Similar to other tissue-resident macrophages, steady-state microglia express high levels of common markers including the fractalkine receptor CX_3CR1, Csf-1R, the integrin CD11b, surface glycoproteins F4/80 and CD68, ionised calcium-binding adapter molecule 1 (IBA1) and intermediate levels of pan-haematopoietic CD45 [60]. While earlier bulk transcriptomic studies have identified several genes to distinguish microglia from other cell types in the CNS or myeloid cells located in the periphery [20, 21, 25, 50, 129], groundbreaking single-cell transcriptomic analyses of microglia are unveiling the heterogeneity of their total pool in healthy and disease conditions [63, 75, 76, 85, 91, 92, 144]. Microglia 'signature' factors are now known to comprise *P2ry12*, *Fcrls*, *Tmem119*, *Olfml3*, *Hexb*, *Tgfbr1* and *Siglec-H*. It remains unknown, however, whether these factors contribute to specifying microglial motility, morphology and functions [20, 25, 50, 69]. Among these factors, the transforming growth factor β (TGFβ) appears critical for mediating microglial survival and phenotypic differentiation, since microglial density is drastically reduced in TGFβ receptor-deficient mice [20]. TGFβ signalling induces microglia to adopt a ramified morphology concomitant with reduced levels of CD86, MHC class II and CD11c and upregulation of CX_3CR1 and IBA1 in vitro [1]. The transmembrane protein TMEM119 was also described to distinguish resident $IBA1^+$ microglia from CNS-associated macrophages of the choroid plexus, meninges and $CD163^+$ perivascular cells and IBA^+ $CD68^+$ infiltrating macrophages in the postnatal mammalian brain, but its function remains unknown [9, 122]. The surveillant state of microglia is widely believed to be maintained by signalling between the neuron-secreted fractalkine (CX_3CL1) and its microglial CX_3CR1 receptor [12]. Several studies have shown the requirement of fractalkine signalling for the recruitment of microglial cells into the early postnatal hippocampus and cerebral cortex [72, 108, 149]. A transient

reduction of microglial numbers was detected in CX_3CR1 knockout mice during the early postnatal period. Concomitant deficits in microglia-mediated synaptic pruning, weakened synaptic transmission and decreased functional brain connectivity were reported [109, 167], with consequences on cognition both early in life and into adulthood [158].

6.3.3 Modulation of Microglial Process Remodelling and Surveillance

Microglia constantly remodel the structure of their processes as required for surveilling the CNS [32, 86, 104, 147, 155], even following the death of the organism [36]. Studies that focused on elucidating the mechanisms regulating microglial cell migration and process motility revealed that purinergic signalling through microglial P2RY12 drives process response to laser injury in the cerebral cortex in vivo [32, 67], process remodelling in retinal explants [47] and filopodia extension in mouse models of status epilepticus [45] and neuropathic pain [61]. In addition, ATP release triggered by dendritic neuronal NMDA receptor activation induces the outgrowth of microglial processes in acute mouse hippocampal slices, suggesting a purine-mediated form of neuron–microglia communication [38]. Extracellular calcium reduction induced microglial process convergence onto neuronal dendrites independently from action potential firing in mouse brain slices and in vivo. This process is mediated by microglial P2RY12, suggesting that microglial interactions with neurons are guided by dendritic calcium reduction in the healthy brain [44]. Furthermore, the inhibition of the two-pore domain K^+ channel THIK-1 revealed its necessity for microglial membrane potential, process ramification, dynamic surveillance and release of pro-inflammatory IL-1β, using pharmacological or gene knockout interventions, in mouse brain slices. However, blocking P2RY12 did not affect microglial membrane potential, ramification or surveillance ex vivo [88]. Lastly, genetic deletion of the TAM receptor tyrosine kinases Mer and Axl revealed their involvement in the regulation of microglial physiology, including their process motility and response to laser injury in vivo, as well as phagocytic elimination of apoptotic newborn neurons generated during adult neurogenesis [48]. Whether and how these different pathways intersect still remains elusive.

6.3.4 Impact of the Periphery

Despite being residents of the so-called immune-privileged CNS, numerous studies point to an interrelationship between microglia and the periphery in the absence of pathology. Microglia are constantly modulated by blood cytokine diffusion and transport into the brain during infection, immune-related molecules secreted from the

endothelial cells of the blood–brain barrier, and peripheral immune signals from the autonomic nervous system [37]. In particular, the gut microbiota was implicated in the normal development and maintenance of microglial cell homeostasis. Rats exposed to prenatal helminth infection have aberrant microglial tiling and reduced microglial response to early-life immune challenges [161]. Mice bred in germ-free conditions have increased microglial cell density and delayed maturation, with the effects regulated by short-chain fatty acids derived from bacterial fermentation by-products of microbiota [43]. Rodents raised without gut microbiota also display increased blood–brain barrier permeability [17]. Introduction of short-chain fatty acids into germ-free mice could potentially allow signalling to peripheral splenic macrophages which subsequently traffic into the CNS to promote maturation of microglia [99]. The microglial response to gut microbiota is subject to sex-specific effects, namely, germ-free male embryonic and female adult microglia were more severely perturbed than other groups [146]. Altered microglial transcriptome, chromatin accessibility and colonisation of the developing neocortex were further observed in the absence of the microbiota [146]. Altogether, maintaining the physiological CNS immune system depends on complex yet little known interactions between the peripheral and central nervous systems, apart from microglial responses to their immediate surroundings.

6.3.5 Impact of the External Environment

Microglial phenotype is constantly shaped by the exposure to various environmental factors, acting on both the brain and periphery, across the lifespan. Inflammation associated with psychological stress, infection, dietary imbalance and environmental pollutants alters physiological microglial phenotype and function [13, 23, 142, 158]. Brief exposure to these external insults (particularly during CNS development and maturation) can exert long-lasting impacts on microglia through 'priming', a process which increases their sensitivity to later challenges. This increased sensitivity accompanied by exacerbated phagocytosis and cytokines release impacts cognitive processes such as learning and memory. The environmental influence on microglia and its implications in brain health and disease are discussed in greater detail elsewhere [64, 142, 158, 163].

One example of an environmental impact on microglial brain colonisation, maturation and/or function is prenatal maternal immune activation. It can be induced, for instance, using the viral mimic polyriboinosinic-polyribocytidylic acid (poly I:C), which exerts different results dependent on the timing. Exposure to poly I:C at E14.5 accelerated the transcriptomic maturation profile of early postnatal mouse microglia towards an adult signature [91]. Complex changes in hippocampal microglial gene signature and decreased phagocytic activity were further induced in mice treated with poly I:C at E15 and correlated with schizophrenic-like behavioural abnormalities at P60 [93]. Furthermore, poly I:C treatment at E9.5 led to more profound changes in microglial inflammatory states and cell density in male than female mice, as well as produced schizophrenia-associated behavioural deficits at P80-90 [73].

Hippocampal microglial molecular profile, density, morphology and process motility were all affected in juvenile mice exposed to early-life stressors such as separation anxiety [35] and dietary deficiency in the essential n-3 polyunsaturated fatty acid [87]. High-fat diet in adult rodents also alters microglial reactivity and activity in selected brain milieus with adverse implications in the CNS inflammatory status and function that were, however, reversible with a low-fat diet [15, 65, 152]. To intervene in neuropsychiatric disorders and progressive neurodegeneration, the variable susceptibility of normal microglia in young and adult brains to their environments needs to be better understood.

6.4 Heterogeneity of Microglia

The increasing accessibility of single-cell transcriptomic analysis has revealed the highest diversity of microglial cellular states during normal physiological development and ageing [63, 85]. Significant distinctions in bulk microglia gene expression, morphology, ultrastructure, distribution, bioenergetics, immunophenotype and cellular properties compared across regions have been reported in healthy rodent brains [33, 34, 39, 58, 82, 123]. Some of these differences correlate with functional requirements for steady-state CNS development or surveillance. The immediate surroundings of a microglial cell influence its identity through a selection pressure for exclusive gene enhancers [55, 56, 81]. For example, reciprocal influence on cell densities exerted by microglia and neuronal progenitors in the ventricular and subventricular zone (SVZ) of the developing cortex was shown to depend on signalling between microglial receptor CXCR4 and CXCL12 secreted by basal progenitors [3]. Microglia in the adult SVZ and rostral migratory stream also comprise a morphologically and antigenically distinct phenotype, distinguishable by lower expression of purinergic receptor P2RY12 and its lack of ATP-driven chemotaxis [116]. Under healthy conditions, specific upregulation of the ubiquitin-specific protease 18 within white matter microglia, but not grey matter, mitigates tissue destruction via tonic interferon signalling [53]. Elevated neuronal death in the adult mouse cerebellum correlates with an enriched cell clearance phenotype which can be epigenetically suppressed in cortical or striatal microglia by the polycomb repressive complex 2 [6]. Evidence of varying microglial turnover rates across brain regions in mouse and human was provided by lineage tracing studies using genetic, thymidine analog or carbon dating [5, 83, 115, 143]. Taken together, the specific interactions between microglial cells and neuronal progenitors or neurons in each microenvironment may be prerequisites for the proper recruitment of particular microglial phenotypes that are required for the maturation, maintenance and plasticity of local neural circuits.

6.5 Sexual Dimorphism of Microglia

To elucidate the control of microglial phenotype and homeostasis, it has become increasingly important to examine the influence of sex. After all, sexual dimorphism in immune responses has been well documented in the peripheral innate and adaptive immune systems across the animal kingdom [78]. Physiological microglial density differs between males and females across stages of the lifespan and brain regions comprising the preoptic area (POA), the hippocampus, parietal cortex and amygdala [84, 101, 102, 127]. Sex-specific microglial response to neuropathic pain [135], chronic stress [16] and gut microbiota [146] were also reported in rodent and human foetal microglia. Isolated microglia from postnatal or adult mice of both sexes express glucocorticoid receptor, mineralocorticoid receptor and oestrogen receptor alpha together with 17β-hydroxysteroid dehydrogenase type 1 and 5α-reductase type 1 involved in steroid hormones metabolism [28, 57, 134]. It is thus conceivable that microglia contribute to sex-dependent brain regulation via their interaction with the endocrine system during CNS development. Steroid hormones act on their receptors, which belong to the superfamily of nuclear receptor transcription factors, to recruit enzymes and other protein components for histone modifications that exert epigenetic effects on the developing brain, thus determining sex differences in brain and behaviour [94]. At adulthood, female microglia grafted in male mice keep their sex-specific gene expressions and remain more immunoprotective than male microglia, in a context of ischemia, suggesting differences that may emerge during development [154]. Reduced DNA methylation in rodent POA, which is associated with male-specific behaviours, enables the expression of masculinising genes during a small perinatal time window [105]. While the POA in male rats reportedly displays more reactive microglial phenotype and a higher dendritic spine density compared to females [84], a direct relationship between microglia and brain sexualisation has not been established due to cellular heterogeneity in the POA. Considering that sexual dimorphic microglial cell density, (phagocytic) function and response may significantly modulate synaptogenesis and shape neuronal circuitry differently, existing data based on a single sex or mixed sex cohorts should be interpreted with care.

6.6 Microglia in Normal Ageing

Several phenotypes of non-steady state microglia become more common in physiologically aged brains absent of overt pathology compared with younger ones. Aged microglia are prone to upregulate pro-inflammatory genes and antigen-presenting markers, while anti-inflammatory cytokines and microglial activation inhibitory factors are down-regulated [100]. Genome-wide analysis of bulk isolated microglia from discrete brain areas revealed regional variability in ageing [58]. Dystrophic or senescent microglial cells with altered morphology, diminished reactivity and motility and reduced phagocytic capacity have been described in normally aged human

and rodent brains [30, 68, 80, 137–139, 148, 157]. In addition, the prevalence of 'dark' microglia characterised by augmented signs of oxidative stress at the ultrastructural level is associated with ageing and several disease models in mice [14]. The reciprocal effects of age-associated microglial phenotypes to their environment are under active investigation [19, 40, 49, 106].

6.7 Conclusion

Microglia are active contributors to normal brain development and physiology. Of note, microglial dysfunctional or loss has been implicated in normal ageing and diseases, as discussed in other chapters. Within this context, it is necessary to understand the intrinsic factors and external conditions that shape the maturation, homeostasis and functions of these long-residing YS-derived tissue-resident macrophages of the CNS. Intrinsic cellular, molecular and epigenetic mechanisms in addition to external peripheral responses and environmental changes determine microglial phenotype, as well as dynamics, phagocytic behaviour, synaptic interactions and release of various mediators that modulate cognition. A better understanding of the mechanisms that govern microglial effector functions during normal physiological conditions will contribute significantly to the strategic development of better targeted treatments for CNS diseases across the lifespan.

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Chapter 7
General Pathophysiology of Astroglia

Alexei Verkhratsky, Margaret S. Ho, Nina Vardjan, Robert Zorec and Vladimir Parpura

Abstract Astroglial cells are involved in most if not in all pathologies of the brain. These cells can change the morpho-functional properties in response to pathology or innate changes of these cells can lead to pathologies. Overall pathological changes in astroglia are complex and diverse and often vary with different disease stages. We classify astrogliopathologies into reactive astrogliosis, astrodegeneration with astroglial atrophy and loss of function, and pathological remodelling of astrocytes. Such changes can occur in neurological, neurodevelopmental, metabolic and psychiatric disorders as well as in infection and toxic insults. Mutation in astrocyte-specific genes leads to specific pathologies, such as Alexander disease, which is a leukodystrophy. We discuss changes in astroglia in the pathological context and identify some molecular entities underlying pathology. These entities within astroglia may repent targets for novel therapeutic intervention in the management of brain pathologies.

Keywords Astrocyte · Neuropathology · Alexander disease · Stroke · Psychiatric diseases · Metabolic diseases · Neurotrauma · Infectious diseases · Systemic

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inflammation · Sepsis-associated encephalopathy · Toxic encephalopathy · Hepatic encephalopathy · Autistic spectrum disorders · Epilepsy

7.1 Prologue: Neuroglia in Neurological Diseases

The role of neuroglia in neurological disorders have been widely accepted by leading neuroanatomists and neurologists of the nineteenth century, from Rudolf Virchow (who indicated that the 'interstitial tissue of the brain and spinal marrow are one of the most frequent seats of morbid change' [222]) to Carl Fromann, Alois Alzheimer and Nicolas Achucarro [1, 6, 8, 77], to name but a few. The neuropathological philosophy of the twentieth century was dominated by neurono-centric views, while the last decade witnessed the resurgence of neurogliopathology. Recently, the pathological potential of neuroglia in general, and astrocytes in particular, has been extensively studied and the fundamental principles of astrogliopathology have been defined [33, 74, 79, 152, 156, 173, 191, 192, 216, 219, 220, 234].

Neuroglial cells are primary homeostatic and defensive cells of the nervous system; and naturally, all types of glia are contributing to neuropathological developments. Astrocytes are a part of neural networks; they interact with neurones, with other glia and with blood vessels, thus, maintaining the structural and functional integrity of the neural tissue. Astrocytes are indispensable for maintaining neuronal functional and neuronal survival both in physiology and in pathology [214]. Therefore, astroglial failure creates a disease-permissive landscape and underlies neuronal malfunction, neuronal death and neurological deficits.

7.2 Principles of Astrogliopathology

Pathological changes in astroglia in neurological diseases are complex and diverse (Fig. 7.1). These changes can be generic or disease-specific. They often vary at different disease stages. In the context of human pathology, changes are affected by age and comorbidity. Astrogliopathology is classified into (i) reactive astrogliosis; (ii) astrodegeneration with astroglial atrophy and loss of function; and (iii) pathological remodelling of astrocytes (Fig. 7.1, [74, 156, 220]); all these pathological reactions occur together or in isolation.

7.2.1 Reactive Astrogliosis

Reactive astrogliosis is observed in many neurological disorders. Until very recently, astroglial reactivity was considered the sole manifestation of astrogliopathology. From histopathological point of view, astroglial reactivity is characterised by mor-

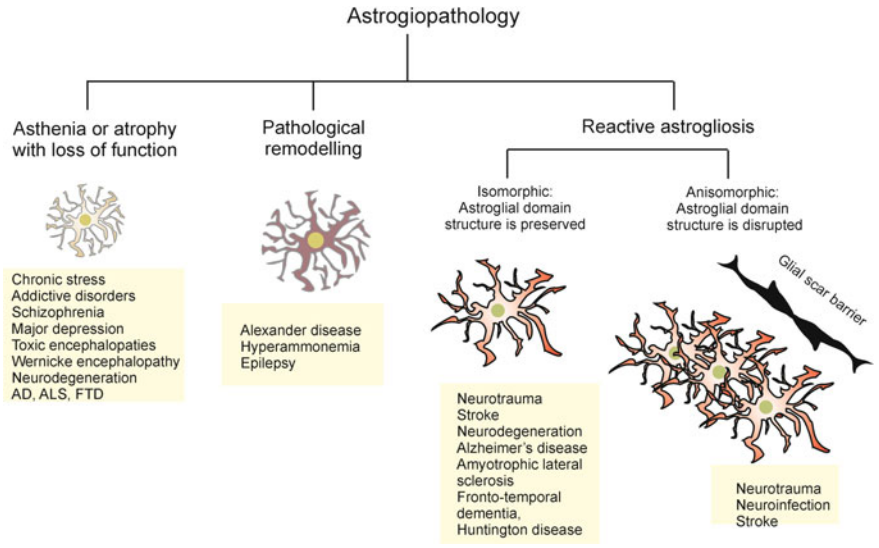

Fig. 7.1 Principles of astrogliopathology. Astrocytes undergo several types of morpho-functional changes in the brain pathology (see text for details). AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; FTD, fronto-temporal dementia

phological hypertrophy and up-regulation of two major cytoskeletal intermediate filaments/proteins, glial fibrillary acidic protein (GFAP) and vimentin [95, 155, 191]. Reactive astrocytes undergo a variety of substantial modifications, showing multiple phenotypes with both neuroprotective and neurotoxic features. These phenotypes arguably are disease-specific, although they all can share some common properties [120, 121, 156]. Transcriptomes of reactive astrocytes in ischemia and endotoxin activation, for example, show significant differences [233].

Conceptually, reactive astrogliosis represents an evolutionary conserved (the first manifestations of astroglial reactivity are observed in many invertebrates including annelids and insects) defensive reprogramming of astroglia aimed at: (i) increased neuroprotection and trophic support of the nervous tissue; (ii) isolation of the lesioned area; (iii) reconstruction of the compromised blood–brain barrier; and (iv) facilitating the post-lesion regeneration of the nervous tissue [7, 156, 191]. The astrogliotic programme, therefore, has a high degree of flexibility and tailors functional and biochemical reprogramming of astrocytes to the nature and strength of the insult. Even within the same lesioned regions, astrocytes demonstrate a degree of heterogeneity in expression of transcription factors, inflammatory agents and signalling molecules [78, 92].

The initiation of astrogliosis is regulated mainly by damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs). The DAMPs are endogenous molecules released from damaged or dying cells (ATP being the most prominent example), blood-borne factors that infiltrate brain parenchyma, etc. The PAMPs are exogenous molecules associated with infectious invaders such as bacte-

ria or viruses; they mostly act through Toll-like receptors (TLRs) widely expressed in astrocytes [99, 203]. Astroglial cells express a wide range of receptors to both DAMPs and PAMPs: $P2X_7$ purinoceptors, TLRs, nucleotide-binding oligomerisation domains (NOD)-like receptors (NLRs), double-stranded RNA-dependent protein kinase, scavenger receptors, mannose receptor and receptors for complement components and mediators, such as CXCL10, CCL2, interleukin-6 and B-cell-activating factor of the tumour necrosis factor (TNF) family [70]. Often, exposure of astrocytes to DAMPs and PAMPs evokes cytosolic Ca^{2+} increases due to its release from the endoplasmic reticulum (ER) intracellular store. These Ca^{2+} signals are critical for instigating the astrogliotic programme. For instance, genetic deletion of predominant astroglial Ca^{2+} release channel of the ER, inositol 1,4,5-triphospate receptor type 2, suppresses astrogliotic response [101]. Similarly, pharmacological inhibition of Ca^{2+} release from the ER restrains astroglial reactivity triggered by amyloid-β [2]. Stimulation of astrocytes with ATP (a classical DAMP) not only triggers Ca^{2+} signalling [34, 211] but also induces formation of inflammasomes comprised of the NLR protein-1 or -2 LR, the adaptor protein apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and caspase-1. Activation of these inflammasomes leads to the processing of inflammatory caspase-1 and interleukin-1β (IL-1β) [137].

Reactive astrogliosis is classified according to the morphological properties and severity (Fig. 7.1). From the morphological perspective, astrogliosis is divided into isomorphic and anisomorphic astrogliosis. The isomorphic astrogliosis preserves astroglial territorial domains and it is fully reversible, whereas anisomorphic astrogliosis proceeds with violation of territorial domains, cell migration and territorial overlap, formation of astroglial palisades and ultimately the scar formation [156]. According to the severity, astrogliosis is classified into (i) mild to moderate astrogliosis; (ii) severe diffuse astrogliosis; and (iii) severe astrogliosis with compact scar formation [190, 191]. Fundamentally, astrogliosis provides for defence of the nervous tissue; it increases neuroprotection and is ultimately important for post-lesion regeneration. Even scar formation carries clear definitive function isolating the damaged area of the CNS and saving the whole at the expense of its part [156, 210]. Suppression of reactive astrogliosis usually exacerbates the course of pathology [156]. Inhibition of astroglial reactivity enlarges the size of the traumatic lesions and aggravates neurological deficit [148]. Deletion of GFAP and vimentin, both being critical for the execution of astrogliotic programme, facilitates the development of ischaemic infarcts [116] and exacerbates post-traumatic synaptic loss [154]. Ablation of astroglial reactivity increased the accumulation of β-amyloid and reduced microglial association with senile plaques in the animal model of the Alzheimer's disease [157]. Nonetheless, in conditions of prolonged stress or severe damage, reactive astrocytes may acquire neurotoxic potential and astrogliosis as a process can become maladaptive [155].

7.2.2 Astroglial Atrophy

Astrodegeneration is a widespread class of astrocytopathy, which is represented by morphological atrophy, increased astroglial death and hence decrease in astroglial density and an impairment of homeostatic functions. Astrodegeneration has been observed in various types of neuropathologies [90, 212, 221]. Astrodegeneration is particularly prominent in major psychiatric diseases. For instance, schizophrenia, major depressive disorder, Wernicke–Korsakoff encephalopathy, and addictive disorders are all accompanied with a reduction in the packing density of astrocytes and a failure of their homeostatic cascades, the latter most notably associated with glutamate homeostasis and glutamate–glutamine shuttle, which are both impaired [50, 53, 54, 134, 163, 165, 178, 218]. Aberrant astroglial glutamate transport and catabolism are arguably responsible for abnormal neurotransmission as well as for excitotoxic neuronal death, both resulting in psychotic symptoms. In amyotrophic lateral sclerosis, insufficient astroglial glutamate clearance from the extracellular space instigates excitotoxic death of large motor neurones [177, 207], whereas in Alzheimer's disease, reduced astroglial synaptic coverage contributes to early synaptic extinction and cognitive deficiency [215].

7.2.3 Pathological Remodelling of Astrocytes

Pathological remodelling of astrocytes covers abnormalities associated with an acquisition of abnormal molecular cascades or functional properties, which drive pathology [74, 156]. Pathological remodelling of astroglia contributes to various leukodystrophies, most notably to Alexander disease, megalencephalic leukoencephalopathy with subcortical cysts or vanishing white matter syndrome, in all of which the astrocytopathy initiates lesions of the white matter [113]. In Alexander disease, astroglial expression of sporadically mutated GFAP gene results in early and severe leukomalacia [131]. Pathological remodelling in astroglia has been also described in mesial temporal lobe epilepsy, in which astrocytes acquire aberrant morphology, reduce gap junctional coupling and down-regulate expression of $K_{ir}4.1$ channels; all these changes compromise K^+ homeostasis thus contributing to the initiation of seizures [19].

7.3 Astrogliopathology in Neurological Diseases

7.3.1 Neurotrauma

Traumatic injury of the brain and of the spinal cord are classified according to their nature (penetrating wounds or concussions; the later when occurring in the cervical

spinal cord is known medically as cervical cord neurapraxia), their severity (mild, moderate or severe), volume (focal or diffuse), outcome (death, vegetative state, severe disability, moderate disability and good recovery) and anatomical localisation. According to its very nature, a traumatic injury to the CNS has a complex pathophysiology associated not only with direct damage to neural cells, but also with a damage to the whole organ with destruction of the blood–brain barrier and blood vessels, ischaemic insults, opening the way for secondary infection, etc. Neurotrauma predominantly triggers astrogliotic response; reactive phenotypes, however, very much depend on the pathological context [32, 33] with the severity of the damage and its anatomical localisation affecting astroglial activation.

In the healthy brain, astrocytes form numerous barriers with blood vessels and with cerebrospinal fluid; endfeet of astroglial cells together with the parenchymal basement membrane create *glia limitans*, which physically separates the brain parenchyma from blood vessels, perivascular spaces and the meninges. In response to a neurotrauma, an astrogliotic scar barrier is formed that delineates and isolates the areas of a focal damage from the healthy brain. Suppression of astrogliosis with consequent malformation of an astroglial scar markedly exacerbates tissue damage and neurological deficit [189]. The heterogeneity of reactive astroglial phenotypes very much depends on the distance to the lesion core. Close to the lesion astrocytes lose their domains, their processes overlap and the astroglial palisades are formed, reflecting anisomorphic astrogliosis. Astrocytes gather around the damaged sites and form the scar [32]. Astrocytes distant to the lesion core undergo isomorphic gliosis; they become hypertrophic and arguably neuroprotective. Contribution of astrocytes to tissue pathology in neurotrauma is multifaceted. Besides forming a protective scar astrocytes regulate inflammatory response, provide for homeostatic protection of the nervous tissue through the removal of extracellular glutamate, buffering K^+ or releasing scavengers of reactive oxygen species and regulate post-traumatic remodelling of synaptic networks. Reactive astrocytes are indispensable in remodelling as suppression of astrogliosis down-regulates post-traumatic regeneration of synaptic connectivity and neuronal networks [7].

7.4 Infectious Diseases

7.4.1 Infection of Nervous Tissue

Infections of the CNS caused by bacteria, viruses, fungi and parasites are classified into meningitis, encephalitis or brain abscess. Not every infectious agent can invade the CNS. Rather, only certain neurotropic viruses, bacteria, fungi and parasites can penetrate into the brain and the spinal cord with relative ease. Furthermore, most of the pathogens are effectively stopped by the brain barriers [110]. Infectious agents may cross the blood–brain barrier using the paracellular route, via transcytotic mechanism, inside entering monocytes (the Trojan horse hypothesis) as well as by other

mechanisms such as, for example, hijacking of β-adrenergic receptors as shown for *Neisseria meningitidis* (meningococcus) [47].

Neuroglial contribution to the infectious lesions of the CNS is of fundamental importance. Neuroprotective activation of astrocytes and microglia to a large extent defines the spread of infection through the nervous tissue and hence determines the outcome of the disease. The glial response, in turn, depends on the nature of an infectious agent. For example, contact of astrocytes with the Gram-positive bacteria such as *Pneumococcus* or *Staphylococcus* triggers rapid astrogliotic activation with marked cellular hypertrophy and up-regulation of GFAP expression [96] accompanied with synthesis and secretion of pro-inflammatory agents such as TNF-α, ILs and macrophage inflammatory protein 1α [122]. Activation of astrocytes by infectious agents (see for example [70, 197, 228]) is mediated mainly through pattern recognition receptors (PRRs), which are represented by TLR 2, 3, 4, 9 [65], NLRs, retinoic acid-inducible gene (RIG)-like receptors (RLRs) and cytokine receptors [108]. The NOD2 receptor, operational in astrocytes, recognises a minimal motif present in all bacterial peptidoglycans and it is required for astroglial reactive reprogramming in response to *N. meningitidis* and *Borrelia burgdorferi* [43].

Activation of astrocytes is also linked to TLR receptors [67]. Distinct TLR subtypes recognise and respond to different PAMPs. Lipopolysaccharide (LPS), for example, signals through TLR4; TLR3 is activated by double-stranded RNA; peptidoglycans interacts with TLR2, while TLR9 senses CpG DNA [40]. Activated TLRs interact with adaptor proteins myeloid differentiation factor 88 (MyD88) and/or a TIR-containing adaptor molecule, Toll/interferon-1 receptor domain-containing adaptor inducing interferon-γ (TRIF), which acts as a part of relevant signalling cascade [40]. Bacterial infection of the nervous tissue down-regulates expression of connexins hence decreasing gap junctional connectivity of astroglial syncytia [66]. Direct interaction of several bacteria such as *Streptococcus pneumonia*, *B. burgdorferi* and *N. meningitides* triggers astroglial reactivity as well as increases the production of pro-inflammatory cytokines and chemokines such as IL-6, TNF-α, IL-8, CXCL-1 and CXCL-10 [240]. Besides activation, astrocytes may undergo pathological remodelling and act as a reservoir for infection. Furthermore, astrocytes can promote apoptotic death of their uninfected neighbours through gap junction route [68, 240]. Astroglial reactivity, that includes overexpression of GFAP, keeps infectious process at bay. Indeed, genetic deletion of GFAP associated with suppressed astrogliosis, significantly exacerbated the neurological damage induced by intrabrain injection of *S. aureus* [197].

Astrocytes are fundamental players not only in bacterial but also in viral infections of the CNS. First and foremost, astrocytes can be directly infected by a virus. For example, astrocytes accumulate human immunodeficiency virus-1 (HIV-1) in a cluster of differentiation 81 (CD81)-lined vesicles. Inside these vesicles, the virus is protected from degradation [83]. The very same vesicles contributed to the secondary *trans*-infection of T-cells [83]. In the dementia caused by HIV brain infection, astrocytes undergo both reactive remodelling and astroglial degeneration and astroglial death. These processes may reduce homeostatic support and hence contribute to cognitive deficit [46]. The astroglial infection with HIV-1 (similarly to

bacterial infection) decreased expression of connexins and syncytial connectivity [150]. In a similar manner, astrogliotic response is mounted in response to infection with the herpes simplex virus 1 (HSV1). Here, activation of astrocytes is mediated by TLR3 and it is neuroprotective. Deletion of TLR3 suppressed astrogliosis and exacerbated HSV pathology in mice [168] as well as in humans [91]. Infection with cytomegalovirus (CMV) was associated with astroglial homeostatic failure. The CMV infected astrocytes showed decreased released of thrombospondins and deficient glutamate uptake, possibly linked to an increased excitotoxicity [235, 236]. Neurotropic viruses of the family of Flaviviridaem represented by Zika virus and tick-borne encephalitis virus (TBEV) invade astrocytes by endocytosis [159, 240]. Astroglial infection with TBEV does not visibly affect their survival or function, and it is generally believed that astroglial cells act as a reservoir for this type of virus [240]. Astrocytes also represent the cellular target for some protozoan parasite, most notably for *Toxoplasma gondii*. Astrocytes infected with *T. gondii* undergo biochemical remodelling associated with up-regulated synthesis of kynurenic acid that in turn may be linked to some forms of schizophrenia, which will be discussed in appropriate section below. In addition, infection of astrocytes with this protozoan results in the loss of gap junctions [37].

7.4.2 Systemic Infections and the Brain: Sepsis-Associated Encephalopathy

Systemic inflammation frequently accompanies various infectious and non-infectious diseases including degenerative and metabolic disorders. This systemic inflammation often is manifested in the form of sepsis. Sepsis (and in particular abdominal sepsis) is frequently accompanied with an acute brain dysfunction, generally defined as sepsis-associated encephalopathy or SAE [187]. From the clinical perspective, the SAE is regarded as a sign of the severity of a septic state, which potentially worsens the prognosis [158]. The SAE is defined as a clinical syndrome associated with the general brain dysfunction that develops in sepsis in the absence of primary infection of the nervous tissue. The histopathological signs of the SAE include infarctions, petechial and small focal haemorrhages, septic-embolic abscesses and septicopyemic microabscesses, disseminated intravascular coagulation (DIC) syndrome with fibrinous microthrombi, multifocal necrotising leukoencephalopathy, necrotic or apoptotic neuronal death, perivascular and cytotoxic oedema, damage of the blood–brain barrier and reactive neuroinflammation [89, 186, 187]. Sepsis is often associated with the formation of abscesses and microabscesses in the brain parenchyma, which can be regarded as directly associated with the SAE. The SAE, especially at the early stages is often associated with ‘sickness behaviour’, the syndrome accompanying system inflammation. The symptomatology of sickness behaviour syndrome includes anxiety, anorexia, anhedonia, depression, cognitive changes, including decreased concentration, learning and memory [56].

At the neurochemical level, the leading pathological changes in an SAE are represented by aberrant neurotransmission, which is responsible for cognitive and psychotic symptoms. Substantial changes in expression of main neurotransmitter receptors including receptors for γ-aminobutyric acid (GABA), serotonin, dopamine and noradrenaline have been observed in the brain in systemic infections [100, 208]. Changes in neurotransmitter homeostasis in sepsis are arguably related to alterations of amino acids levels in the blood characterised by a decrease in branched chain amino acids together with relative increase in aromatic amino acids [15]. In addition, compromised brain barriers allow a substantial influx of aromatic amino acids, such as tyrosine, phenylalanine and tryptophan, which may act as false neurotransmitters and alter biosynthesis of *true* neurotransmitters (e.g., dopamine, noradrenaline and serotonin—[188]).

Astrocytes, endfeet of which form *glia limitans* and hence can be considered as the parenchymal portion of brain barriers (the blood–brain and the blood–cerebrospinal fluid), define, to a very large extent, the resistance of the nervous tissue to the systemic inflammation. Intimate contacts of astrocytes with all other cellular elements of the nervous tissue allow them to regulate the relationship between the CNS and systemic physiology and pathology. In the context of SAE, astroglial reactivity is the principal mechanism that limits the propagation of pathological agents through the nervous tissue [41, 193]. Inhibition of astrogliotic response compromises astroglial barrier function and aggravates encephalopathy in the context of systemic inflammation or infectious lesion to the brain. For example, in transgenic mice with deleted gene for GFAP (this intervention suppresses astrogliosis), brain abscesses caused by *Staphylococcus aureus* or *Toxoplasma gondii* were much larger. Lesions become poorly demarcated, bacterial penetration significantly increased, neuronal death was much exacerbated and severe brain oedema developed [197]. Suppression of astroglial reactivity by activation of NF-κB signalling cascade in retinal ischemia or in spinal cord injury, is associated with an increased neuronal damage [27, 63]. Finally, inability of astrocytes to acquire reactive phenotype results in swelling, cytotoxic oedema and spread of damage in infectious abscesses [187].

Astrocytes contribute to the pathology of the blood–brain barrier, which is classified as disruptive and non-disruptive alterations, with both variants present in systemic inflammation [187]. The non-disruptive BBB pathology develops at the molecular and cellular levels when BBB permeability is affected following up- or down-regulation of receptors and transporters expressed in endothelial cells and astrocytes [209]. Disruptive BBB alterations develop through anatomical changes, which include degradation of glycocalyx, a loss of integrity of tight junctions, mitochondrial damage, appearance of fenestrae between endothelial cells, endothelial cells death, collapse of *glia limitans* and astrocytopathy. Disruption of BBB in systemic inflammation is mediated by blood-derived metalloproteinases, prostanoids, nitric oxide and reactive oxygen species [38, 136]. The switch between non-disruptive and disruptive BBB pathology depends on the severity of systemic inflammation. At the early stages of SAE the non-disruptive changes prevail, whereas in severe sepsis, both non-disruptive and disruptive changes occur [209].

7.5 Toxic Damage of the CNS

7.5.1 Heavy Metal Toxic Encephalopathies

Heavy metals, which cause severe brain damage with cognitive deficits, target primarily astrocytes. This is because heavy metals (such as manganese, lead, aluminium or mercury, in the form of methylmercury) mainly accumulate into astrocytes through different plasmalemmal transporters. In general, heavy metals down-regulate astroglial expression of glutamate transporters which decrease glutamate clearance and trigger excitotoxicity [198, 199, 217, 232].

Poisoning by methylmercury is known as Minamata disease named after the city of Minamata in Japan where the disease was first described [129]. The symptoms of Minamata disease include visual abnormalities, sensory lesions, cerebellar ataxia, hearing loss, weakness, tremor and cognitive decline. Methylmercury primarily accumulates in astroglia, where it inhibits glutamate and cystine uptake [5]. Suppression of glutamate uptake instigates exocytotic neuronal death, whereas inhibition of cystine transport limits astroglial synthesis of glutathione hence reducing astroglial capacity to counteract the accumulation of reactive oxygen species; both these processes contribute to neurotoxicity and neuronal death [57, 232].

Exposure to toxic concentrations of lead similarly causes neurodegeneration. Lead primarily accumulates in astroglia, where it down-regulates expression of EAAT-2 glutamate transporter, increases astroglial production of vascular endothelial growth factor, and impairs astroglia-associated water homeostasis by increasing the water permeability of aquaporin 4 [85]. Arguably, these mechanisms contribute to cytotoxic and vascular brain oedema observed in patients with lead poisoning.

Aluminium toxic encephalopathy is manifested by cognitive impairments, speech alterations, seizures and flapping wrist tremor (asterixis). Treatment of cultured astrocytes with aluminium led to swelling, destruction of the cytoskeleton, reduction in gap junctional connectivity, inhibition of glutamate uptake and increased astroglial apoptosis. Loss of astroglial glutamate uptake triggered neuronal death in neuronal–glial co-cultures [198, 199].

The main symptom of acute manganese neurotoxicity is an acute psychosis, whereas chronic manganese poisoning leads to parkinsonism. Astrocytes possess the high capacity manganese transport system; treatment of primary cultured astrocytes with manganese suppresses glutamate uptake and promotes apoptosis [57].

7.5.2 Hyperammonemia and Hepatic Encephalopathy

Increase in blood ammonium accompanies several diseases. The most frequent cause of hyperammonemia is, however, associated with an acute or chronic liver failure (the liver being the main organ for ammonia clearance). Hyperammonemia affects the brain and triggers a condition generally known as hepatic encephalopathy, manifested

by cognitive and behavioural impairment; symptoms include confusion, forgetfulness, irritability and alterations of consciousness, such as lethargy and somnolence. Severe hyperammonemia provokes brain oedema, coma and death [31, 35, 73]. In the CNS ammonia is detoxified by glutamine synthetase localised exclusively in astrocytes; this enzyme catabolises ammonium reaction with glutamate and produces glutamine [3, 143, 175]. This reaction is central for glutamate-glutamine shuttle; it also fixes ammonium, which is liberated during physiological neuronal activity [126]. Ammonium overload occludes this pathway and blocks glutamine synthetase hence causing major disturbances of glutamatergic and GABAergic (as glutamate is the precursor to GABA) neurotransmission, which underlie all the symptoms outlined above [31, 35].

Hyperammonemia also affects homeostatic astroglial functions. Exposure of astrocytes to ammonium results in a down-regulation of expression of inward rectifying $K_{ir}4.1$ channels, an event mediated through astrocytic NMDA receptors by a yet uncharacterised mechanism. Decrease in the density of $K_{ir}4.1$ channels, in turn, affects astroglial K^+ buffering which may impair neuronal excitability [144, 166]. Exposure of astrocytes to ammonium also produces aberrant Ca^{2+} signalling by increasing expression of Ca^{2+}-permeable TRPC1 channels, up-regulating expression of $Ca_v1.2$ voltage-gated Ca^{2+} channels and facilitating Ca^{2+} release from the intercellular stores [86, 119, 223]. Increased Ca^{2+} load of astroglial cytosol, in turn, triggers the exocytotic secretion of glutamate which further exacerbates excitotoxic damage of the nervous tissue [82, 139]. Finally, increased ammonium compromises astroglial transport of Na^+ and H^+ which contributes to aberrant pH regulation in the CNS [105, 106]. All these molecular changes result in impaired synaptic transmission, synaptic plasticity and cognitive capabilities [45].

7.6 Astrogliopathology in Stroke

A disruption of the blood flow results either from a blood vessel rupture (that causes a haemorrhage), or by a restriction of blood supply to the brain or parts of the brain, because of a vascular occlusion (thrombosis or embolism), or to a systemic decrease in blood supply (resulting, for example, from a heart failure). This status is generally referred to as brain ischaemia. As a consequence, brain ischaemia can be either global, or focal, the latter corresponding to a stroke.

Astrogliopathology in stroke is complex and multifaceted, with astrocytes being both neuroprotective and neurotoxic [81, 239]. Focal ischaemia results in the infarction of nervous tissue creating a zone of pan-necrosis or an infarction core. At this core, all cells, neurones, glia and other non-neuronal cells undergo rapid necrosis. The size of the core is determined by anatomical location and duration of the ischemic attack. Quite frequently the focal ischemia is transient, as the blood flow can be restored when the vessel blockage is removed. In this case, restored blood flow results in reperfusion of the damaged area, which itself is potentially damaging because of the production of reactive oxygen species and secondary ion imbalances.

The infarction core is surrounded by the ischemic penumbra, which contains viable cells, although with compromised metabolism and function. The infarction core is formed rapidly, within minutes to hours after initiation of the stroke. This is followed by a much slower process of expansion of the infarction zone through the penumbra, which develops over many hours and days. The final neurological deficit is often defined by the limits of the infarction expansion, which in turn depends on astroglial response.

Astrocytes support neurones in the ischaemic penumbra through several homeostatic pathways. First and foremost, astrocytes maintain homeostasis of glutamate in the ischaemic zones. They also feed neurones with metabolic substrates such as lactate. Energising astroglial mitochondria, for example, increase neuroprotection in the ischaemic context [180]. Taming glutamate excitotoxicity, which always follows stroke, almost solely falls onto astroglial cells. Down-regulation of expression of the astroglial glutamate transporter GLT-1/EAAT1 with siRNA increases the size of the infarct [167], whereas targeted overexpression of GLT-1 in astrocytes limits the infarction volume and alleviates neurological deficit [88]. Similarly, stimulation of glutamate uptake with pharmacological agents such as tamoxifen or riluzole decreased infarction volume in animal models [227, 238]. Of note, astroglial glutamate transporters are Na^+ dependent, and hence maintenance of Na^+ transmembrane gradients is critical for glutamate clearance [109]. Another important component of astroglia-dependent neuroprotection in the ischaemic penumbra is associated with antioxidant defence. Astrocytes are critical for both glutathione and ascorbic acid systems, which are the most powerful scavengers of reactive oxygen species [61, 62, 125]. Progression of cell death through the ischaemic penumbra is mediated by spreading depolarisation, which stresses astroglial ionostatic capacity. Furthermore, astrocytes may propagate death signal, triggering distant neuronal death [115, 142].

An important component of astroglial response to stroke is associated with reactive astrogliosis. Ischaemic damage to the brain tissue rapidly instigates astroglial activation through the release of DAMPs; the severity of astrogliotic remodelling and reactive phenotypes depend on the distance to the ischemic core [33]. Astrocytes close to the ischaemic core undergo anisomorphic gliosis, form astroglial palisades and produce astroglial scar that limits the damage to the nervous tissue. In parallel, distantly to the core astrocytes undergo isomorphic, neuroprotective astrogliotic remodelling, which is critically important for post-lesion regeneration The main outcome of astrogliosis in the immediate vicinity of the necrotic area is the formation of an astroglial scar, whereas more peripheral reactive astrocytes are important for post-lesion regeneration [81].

7.7 Metabolic Disorders

7.7.1 Congenital Glutamine Deficiency with Glutamine Synthetase Mutations

Congenital glutamine synthetase deficiency, a rather rare recessive inborn disease, results from mutations to the gene *GLUL* that encodes astroglia-specific glutamine synthetase, thus, this disorder can be considered as a specific astrogliopathy. This disease is characterised by pronounced malformation of the brain with severe white matter deficiency and abnormal gyri formation. Functionally, this deficiency is manifested as epileptic encephalopathy. The deficit in glutamine synthetase in the liver promotes chronic hyperammonemia. In addition, levels of glutamine are reduced in the brain as well as in other organism fluids. The disease results in prenatal malformation of various organs and is generally incompatible with life. Most of the infants die shortly after birth. The leading pathophysiological mechanism is associated with impaired ability of astrocytes to produce glutamine, which affects excitatory and inhibitory transmission; in addition, deficient glutamine synthetase cannot properly detoxify ammonium [195].

7.7.2 Pyruvate Carboxylase Deficiency

Pyruvate carboxylase is an enzyme of gluconeogenesis and it also contributes to anaplerotic metabolic pathways (i.e. producing intermediates for metabolic chains such as the Krebs cycle). In the CNS, pyruvate carboxylase is predominantly expressed in astrocytes. Pyruvate carboxylase deficiency is an autosomal recessive disease associated with impaired metabolism. The symptoms include retardation of mental development, recurrent seizures and metabolic acidosis [127]. There are three clinically distinct forms: type A, or the infantile form, in which children die in the early years; type B, or the severe neonatal form, with many neurological signs including pyramidal symptoms, in which babies die within 3 months after birth; type C or the benign form, which is characterised by mild neurological developmental deficits. The cellular pathogenesis remains largely unknown, but it is probably linked to reduced astroglial homeostatic function, such as glutamate buffering and regulation of angiogenesis [57]

7.7.3 Niemann–Pick Type C Disease

Niemann–Pick disease type C is a progressive neurodegenerative disease associated with hepatosplenomegaly. It is characterised as an autosomal recessive lysosomal storage disease, which results from loss-of-function mutations of genes encoding

NPC-1 or NPC-2 proteins [176]. These proteins are localised in astroglial perisynaptic processes and may be involved in the regulation of cholesterol transport and, hence, synaptogenesis or synaptic maintenance [153]. Astroglia-specific genetic deletion of *Npc1* from mice resulted in reduced neuronal cholesterol, which was associated with decreased neuronal and glial death and three times increase in the life span [237]. There is also evidence of a possible contribution for NPC-1 protein in calcium homeostasis and signalling.

7.7.4 Aceruloplasminemia

The enzyme ceruloplasmin (also known as ferroxidase) is a part of iron metabolism. In the CNS this enzyme is expressed almost exclusively in perivascular astrocytes. Ceruloplasmin is an important component of protection of the nervous tissue against iron-associated lipid peroxidation and formation of hydroxyl radicals. Mutation of the ceruloplasmin gene with loss-of-function causes the autosomal recessive disease known as aceruloplasminemia, which can be defined as an inherited neurodegenerative disorder with systemic iron-overload syndrome [138]. This disease is characterised by primary lesions to astrocytes, which affects their morphology and results in an appearance of foamy spheroid bodies at the vascular endfeet [147]. Aceruloplasminemia is also associated with neuronal death and the appearance of iron deposition.

7.8 Alexander Disease

Alexander disease (AxD), named after William Stewart Alexander, a neuropathologist who described it for the first time [4], is a rare, chronic and usually fatal neurodegenerative disorder. Clinically, AxD may be defined as a severe leukodystrophy; pathophysiologically, it is a primary genetic astrogliopathology [131]. The AxD results from a dominant gain-of-function mutation of the gene encoding GFAP. This leads to astroglial pathology that, in turn, results in a severe damage to the developing white matter. The histopathological hallmark of AxD is an accumulation of protein aggregates, known as Rosenthal fibres, around astroglial nuclei and endfeet [131]. AxD is subclassified into: (i) Type I, characterised with an early onset and severe mental and physical disabilities, megalencephaly, seizures, spasticity, difficulty speaking and swallowing, and (ii) Type II, with a later onset and somewhat different and milder clinical manifestations with normal development and head size, with rare occurrence of seizures, but with ataxia, visual and autonomic abnormalities, troubles in sleeping patterns, hyperreflexia, difficulty speaking and swallowing [160].

Astrocytes in AxD demonstrate reactive morphology. These glial cells also remodel their biochemistry and secretome. In particular, astrocytes start to release

pro-inflammatory factors TNF-α and IL-1β. In addition, astrocytes in AxD have reduced expression of glutamate plasmalemmal transporters, decreased activity of proteasomes, increased autophagy and increased activity of stress-activated protein kinase/c-Jun N-terminal kinase (JNK) pathway [131]. Multiple mechanisms by which pathological mutation of GFAP affects cellular functions have been considered. These include: (i) mutated GFAP through positive feedback loop inhibits proteasome function which activates JNK, and activated JNK directly further inhibits proteasome [204]; (ii) mutated GFAP inactivates one or more proteins by degradation of the Rosenthal fibres, where fragments of the small stress proteins, HSP27, αB crystalline, the 20S proteasome subunit, p-JNK, p62 and plectin, have been detected [131]. So far the AxD remains incurable, although several therapeutic strategies aimed at reducing GFAP expression are in development.

7.9 Neurodevelopmental Disorders

7.9.1 Autism Spectrum Disorders (ASD)

The class of autistics spectrum disorders (ASD) embraces numerous pathological conditions of heterogeneous clinical presentation and pathophysiology. They all, however, are manifested by deficits in social interactions and restrictive patterns of behaviours. Some of the autistic diseases are associated with intellectual deficits [162]. The underlying mechanism of ASDs is most likely associated with malformation of neuronal networks and aberrant neurotransmission in embryonic development caused by environmental and/or intrinsic factors [42, 80, 130, 174]. Formation of neuronal ensembles, synaptogenesis and synaptic elimination all critically depend on the performance of the astroglial cradle, which controls birth, life and death of synapses [213]. Astrocytes are responsible for neuroprotection and detoxification of harmful agents, including reactive oxygen species (through secreting antioxidants such as glutathione and ascorbic acid [30, 229]). Astrocytes tame excitotoxicity through glutamate uptake and they also control neurotransmitters catabolism and supply of neurotransmitter precursors [214]. In parallel, astrocytes are the main target for neurotoxic factors, such as heavy metals, which are linked to the aetiology of ASD [234]. Astrogliopathology in ASD has not been investigated in great details; there are some indications for astrogliosis [234], increased expression of connexin 43 and decreased expression of aquaporin 4 [71].

7.9.2 Down Syndrome

Down syndrome (DS), which is linked to the trisomy of chromosome 21, is characterised by mental retardation. In DS, the density of astrocytes is significantly reduced

in the cortex [102] with decreased ability to properly support synaptogenesis and neuronal maturation [44].

7.9.3 Fragile X Syndrome

Expression of Fragile X mental retardation protein (FXMRP) causes a specific form of a neurodevelopmental disease manifested in ASD symptoms and mental disability, Fragile X syndrome that is also known as Martin–Bell syndrome or Escalante's syndrome [107]. Expression of FXMRP in astrocytes weakens their homeostatic function and neuroprotection in the in vitro experiments. Co-culturing healthy neurones with astrocytes harbouring FXMRP leads to abnormal neuronal dendritic morphology and reduced synaptic connectivity. In contrast, co-culturing FXMRP expressing neurones with healthy astrocytes prevents the development of abnormal dendritic morphology [97, 98].

7.9.4 Costello Syndrome

Costello syndrome (named so after its discoverer Jack Costello [48]) belongs to the family of the so-called RASopathies (where RAS stands for rat sarcoma) characterised by aberrant Ras signalling [205]. In this pathology, astroglial cells expressing a mutated *HRAS* (Harvey rat sarcoma viral oncogene homolog) gene demonstrate hyperactive Ras signalling, which accelerates differentiation and maturation of astrocytes, and leads to astroglial hypertrophy. This is also associated with pathological extracellular matrix and abnormal formation of neuronal networks that in turn causes cognitive and behavioural abnormalities [112].

7.10 Major Neuropsychiatric Diseases

7.10.1 Schizophrenia

In schizophrenia the wide spectrum of astroglial abnormalities is present. Conceptually, schizophrenia is associated with astroglial asthenia, atrophy, loss of homeostatic capabilities and arguably pathological remodelling, while reactive changes are not characteristic. Decrease in astroglial numbers, as well as dystrophic or swollen astroglial profiles, appear in various brain regions, including cortical and hippocampal structures [69, 163, 181, 226]. Astrocytes derived from human induced pluripotent stem cells obtained from schizophrenic patients and injected into mice, demonstrated atrophic morphology and loss of homeostatic functions [230].

Astrocytes in schizophrenia are characterised by a significant down-regulation of expression of several astroglia-specific molecules such as deiodinase type II, aquaporin-4, S100β, glutamine synthetase, plasmalemmal glutamate transporters and thrombospondin. These changes were the most prominent in the deep layers of the anterior cingulate gyrus, suggesting that a subset of astrocytes localised to specific cortical layers can be affected [231]. In the prefrontal cortex and hippocampus, a decrease in the expression of EAAT1/2 plasmalemmal glutamate transporters has been detected [16, 17, 146, 185], which may be linked to abnormalities in glutamatergic transmission. Genetic deletion of EAAT1 glutamate transporter promoted appearance of schizophrenia-like phenotypes manifested by locomotor hyperactivity and abnormal social behaviour [103, 104]. Astrocytes from rodent phencyclidine model of schizophrenia demonstrated a decrease in the expression of plasmalemmal cystine–glutamate exchanger Sxc^- [12], which modulates extrasynaptic concentration of glutamate and contributes to the biosynthesis of glutathione. Astrocytes may promote aberrant neurotransmission through synthesis and release of kynurenic acid that acts as an endogenous inhibitor of the NMDA receptor glycine binding site; kynurenic acid also blocks acetylcholine nicotinic receptors. The astroglial production of kynurenic acid is significantly up-regulated following brain infection with *Toxoplasma gondii*, which increases the risk of schizophrenia [183].

7.10.2 Mood Disorders

Astrogliopathology seems to be rather prominent in mood disorders [165, 178, 218]. The total number of glial cells and of astrocytes, in particular, is decreased in the orbitofrontal area and anterior cingulate, prefrontal, entorhinal and subgenual cortices, as well as the amygdala of the brains obtained from patients with major depression or bipolar disorder. [26, 49, 50, 149, 164]. In animals subjected to chronic stress, which instigates depressive phenotypes, GFAP expression and number of GFAP positive cells were reduced [28, 55]. Similarly, in models of attention deficit disorder and depression other astroglial markers, including aquaporin 4, astroglial connexins, astroglial plasmalemmal glutamate transporters and glutamine synthetase were all down-regulated [14, 21, 184].

Ablation of astrocytes in the medial prefrontal cortex of mice with the neuroglial toxin L-α-aminoadipic acid triggered an emergence of a depressive phenotype similar to that induced by chronic stress [13]. Exposure to chronic stress led to a down-regulation of astroglial expression of connexin 43 along with the reduction of gap junctional coupling in astrocytic syncytia. Pharmacological inhibition of astroglial connexon-based channels in the prefrontal cortex induced depressive behaviour manifested by anhedonia [201]. A similar phenotype was observed after inhibition of astroglial plasmalemmal glutamate transporters [18]. Chronic treatment with antidepressants directly affected astroglia, by increasing expression of a variety of receptors and transporters responsible for CNS homeostasis and limiting glutamate release [53, 60, 123, 171]. In conclusion, mood disorders are associated with astroglial degen-

eration and astroglial asthenia, which compromise brain homeostatic reserve and arguably synaptic transmission.

7.10.3 Addictive Disorders

Various nosological forms of addictive disorders are associated with astrogliopathies. Post-mortem analysis of the human brain samples revealed both astroglial reactivity with astroglial degeneration, and astroglial cell death with astroglial atrophy [9, 36, 72, 134, 145, 200, 225]. In the animal models of addiction with cocaine, methamphetamine and morphine, astroglial activation and increase in GFAP expression have been identified [25, 76, 84, 194]. In contrast, in the model of chronic alcoholism a decrease in GFAP expression and morphological atrophy of astrocytes were detected [75, 172]. In post-mortem tissues isolated from alcoholic sufferers, both hypertrophic GFAP positive astrocytes as well as areas with decreased GFAP expression and decreased density of astrocytes were described [52, 133].

The number of astrocytes is decreased in the prefrontal cortex of alcoholics [135]. A similar decrease in astroglial density and GFAP expression was detected in the prelimbic cortex of ethanol-preferring chronically alcoholic rats [133]. Additionally, a decrease in astrocyte density was observed in response to acute binge drinking in male (but not female) adult rats [111]. Ablation of astroglia with L-α-aminoadipic acid or uncoupling astroglial syncytia using a pharmacological inhibitor of connexin channels in the prefrontal cortex increases alcohol preference [132].

Addictive disorders are linked to astroglial plasmalemmal glutamate transport. Expression of EAAT2 as well as Sxc^- glutamate transporters is decreased in the context of alcoholism. Incidentally, total extracellular glutamate increases most likely due to an imbalance between glutamate uptake (EAAT2) and release (Sxc^-) [141, 169, 170]. Increase in the expression of EAAT2 by treatment with β-lactam antibiotic ceftriaxone decreased alcohol dependence [161, 179].

7.11 Epilepsy

In epilepsy, astrocytes undergo substantial pathological remodelling, which greatly affects their homeostatic capabilities and is linked to pathophysiology of this disease. In particular, the epileptic astroglial phenotype includes changes (mutations and/or expression levels) in ion channels, receptors and transporters [19, 196]. Abnormal electrophysiological characteristics have been observed in astrocytes isolated from patients with mesial temporal lobe epilepsy and associated sclerosis. These astrocytes, in addition, have severe impairment of intercellular coupling [19]. Astrocytes in sclerotic tissue up-regulated the expression of GFAP, suggesting thus their activation. Decrease in K^+ buffering seems to be the dominant feature of astroglial remodelling in epileptic brains, which results in an increase of extracellular K^+ concentration [124,

140]. Such an increase in extracellular K^+ can be sufficient to instigate seizures [206]. Abnormal astroglial K^+ buffering, at least in part, is linked to a significant down-regulation of inward rectifier $K_{ir}4.1$ channels. Here, decreases in $K_{ir}4.1$ current density and protein content have been found in astrocytes from the human sclerotic CA1 hippocampal area [24, 93, 94]. Genetic deletion of *KCNJ10* gene encoding $K_{ir}4.1$ channel specifically from astroglia resulted in impaired K^+ buffering, depolarisation of astrocytes, motor impairments and early death [59]. Other studies confirmed this finding by demonstrating that deletion of $K_{ir}4.1$ channels induces epileptiform symptoms in animals [196]. Mutations of *KCNJ10* gene in humans are associated with the development of SeSAME syndrome (also called EAST syndrome), an autosomal recessive disorder characterised by epilepsy, ataxia, sensorineural deafness, wasting renal tubulopathy, mental retardation and electrolyte imbalance [22, 182]. Whether the modifications of Na^+/K^+ ATPase (NKA), another critical component of astroglial K^+ buffering (NKA is primarily responsible for K^+ uptake, whereas $K_{ir}4.1$ channels for K^+ release and shuttling back to neurones [29, 114]) contribute to SeSAME, it remains to be explored. One of the forms of migraine, the familial hemiplegic migraine type 2, is however associated with loss-of-function mutation of astroglial specific α2 subunits of NKA [39]. Considering fundamental similarities of pathogenesis of migraine and epilepsy we may expect some abnormalities of astroglial NKA in the later pathology.

Epileptic astrocytes also demonstrate compromised glutamate uptake and homeostasis [51]. Deletion of the astroglial EAAT2 glutamate transporter results in an epileptiform phenotype with lethal spontaneous seizures, increased susceptibility to acute cortical injury and seizures after administration of sub-convulsive doses of pentylenetetrazole [202]. Similarly, seizures and epileptiform phenotype were triggered by pharmacological inhibition of EAAT by intracerebroventricular injections of DL-threo-beta-benzyloxyaspartate [58]. Down-regulation of glutamine synthetase was also linked to epilepsy through affecting inhibition in neuronal networks [151]. Animals subjected to long-lasting pharmacological blockade of glutamine synthetase demonstrated seizures [20, 224], whereas levels of glutamine synthetase were found to be significantly decreased in the human hippocampus and amygdala of patients with temporal lobe epilepsy [64]. Finally, loss-of-function mutations of glutamine synthetase induced severe seizures [87]. Astrocytes can also contribute to pathogenesis of epilepsy through anomalous adenosine homeostasis, resulting from modified expression of the astroglia-specific adenosine kinase, which is the key enzyme for adenosine turnover in the CNS [10, 23]. Expression of adenosine kinase is high in tissues from subjects with pharmacologically refractory temporal lobe epilepsy [10, 11, 128]. Increase in expression and activity of adenosine kinase diminishes the availability of adenosine, thus increasing neuronal network excitability and increasing probability of seizures [117, 118].

7.12 Epilogue

Since the inception of neurobiology, we have had a conceptual roller coaster ride in regards to the role of neuroglia in pathology of the brain. Two centuries ago our founding fathers of gliology had a clear vision on the active role of glia, i.e. glia is more than putty and has prominent roles in pathophysiology of the brain. Awkwardly, the twentieth century brought a different view where starring role has been solely played by neurones. This dominant neurono-centric approach has been challenged by the resurgence of neurogliopathology in the past 20 years. While we here presented the astrocyte-centric view of the brain pathology, we surely support the notion that it is the interaction between neurones and glia that underlies physiology and pathology of the brain. These two major cellular constituents interact, so that perturbing one will affect the other. Thus, only intellectually acceptable approaches to grapple with the management of the brain diseases will be those that have gestalt assets.

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Chapter 8
Neuroglia in Ageing

Alexei Verkhratsky, Robert Zorec, Jose Julio Rodriguez-Arellano and Vladimir Parpura

Abstract Ageing reduces the functional capacity of all organs, so does that of the nervous system; the latter is evident in the reduction of cognitive abilities, learning and memory. While the exact mechanisms of ageing of the nervous system remain elusive, it is without doubt that morpho-functional changes in a variety of neuroglial cells contribute to this process. The age-dependent changes in neuroglia are characterised by a progressive loss of function. This reduces glial ability to homeostatically nurture, protect and regenerate the nervous tissue. Such neuroglial paralysis also facilitates neurodegenerative processes. Ageing of neuroglia is variable and can be affected by environmental factors and comorbidities.

Keywords Ageing · Astrocyte · Microglia · NG2 cells · Oligodendrocytes

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8.1 Mechanisms of Ageing

Ageing reduces the functional capacity of all organs and systems ultimately weakening the whole organism, reducing its adaptability, wearing its defensive systems and bringing it to death through age-dependent diseases. The nervous system similarly undergoes senescence that often impairs upon cognitive abilities, affects learning capabilities and enfeebles memory. Nonetheless, brain sustains ageing with surprising tenacity; the cognitive functions attain the summit in middle age and often sustain into old age, while the decline in other systems (including skeleto-muscular, cardiovascular or endocrine) progresses much faster. What are the mechanisms of ageing and which molecular and cellular processes underlie the ageing of the brain remains a matter of intense polemics. Indeed, there are in excess of 300 theories of ageing, which highlight various pathways, many of which do contribute to this process [52].

Probably, the very first coherent theory of ageing was proposed by August Weismann [99], who considered ageing as a very natural process favoured by natural selection to prevent competition of species with their own progeny. According to this view, mechanisms of ageing could be many and they can be different in different organisms. The only common future is guaranteed termination of individual life after a presumed fulfilment of the reproductive duties. One of the widely considered pathways is the activation of endoplasmic reticulum stress/unfolded protein responses that positively correlate with longevity and negatively with fertility [86]. Another relatively old group of theories regards ageing as a result of mechanochemical deterioration of molecules and basic systems such as organelles or cell membranes; this was initially proposed as 'hysteresis of colloids' [72, 73]. The mitochondrial or free radical theory of ageing links the damage of biological systems to reactive oxygen species and regards mitochondria as the age-defining clock [29, 30]. By reducing caloric intake this may resist the rate of ageing [48]. The gene regulation theories assume that changes in gene expression define ageing process [39], while the telomere theory postulates that it is the telomere DNA localised at the end of chromosomes which determines the life span: the shortening of telomeres eventually brings to the arrest of cell replication and death [28], The inflammatory theory of ageing became popular in recent years leading to the concept of 'inflammaging' [21]. Additionally, several theories look into the role of signalling systems both at organism (e.g., neuroendocrine or immune theories of ageing [20]) and cellular (e.g., calcium theory of ageing [40, 45, 89]) levels.

8.2 Ageing of the Brain

The maintenance of cognitive capacity of the brain over most of the human's lifespan results, most likely, from prominent neuroplasticity, remarkably long development and high degree of homeostatic and protective capabilities of neuroglia. The human brain is optimised for learning, with numerous mechanisms from adult neurogenesis

(which supplies the hippocampus with new neurones [54]) and adult myelination (which lasts well into the fourth decade of human life [1, 98], while oligodendroglial progenitors are present throughout the brain across the whole lifespan and probably contribute to late-life regenerative myelination), to the highly sophisticated glymphatic system that purges the brain from toxic waste products [36], thus maintaining neural environment. Ageing affects cognition components in a rather distinct way. The age-dependent decline mainly affects the real-time processing and formation of new memories and behaviours, whereas the capacity to analyse semantic and long-term memories suffer much less [18, 35]. For example, a group of young adults were significantly better than the group of old people in recalling a list of words. However the ability of elders to use complex processing activities was indistinguishable from the youngsters [47]. This benign or physiological brain ageing is not granted to all, and age-dependent pathologies, most notably of neurodegenerative nature, affect a substantial part of population.

Age is the main risk factor for neurodegenerative diseases, which are often considered as a natural outcome of senescence process. However, there is a fundamental difference between physiological ageing and neurodegeneration. The latter reflects massive neuronal death and atrophy of the brain tissue, whereas the former is not associated with a substantial neuronal loss. The overall number of neurones is not significantly affected in physiological ageing in rodents, primates and humans [6, 12, 19, 100]. Likewise, the number and density of synapses are not significantly affected by ageing [23, 80], albeit synaptic size is reduced [56].

Factors which determine the fateful difference between physiological and pathological ageing are many. These are represented by genetic factors (the best example being familial Alzheimer's disease or Huntington disease), the environment and life style (including diet, education, mental or physical activity) and the associated pathology (such as vascular disorders and ischaemic lesions). Another fundamental factor that defines the degree of cognitive deficit of ageing and age-dependent neuropathologies is known as the cognitive reserve. The cognitive reserve is an intrinsic quality of an individual brain that determines the neurological deficit when a similar brain damage results in very different cognitive outcomes in different subjects [82, 103]. The cognitive reserve in turn is defined by (i) neuronal reserve, which is the status of neuronal networks acquired during the life span through learning and cognitive load and (ii) neuronal compensation that reflects the defensive, plastic and regenerative capacities of the individual brain. To a large extent, the neuronal compensation is defined by neuroglia, which is responsible for neuroprotection, regeneration and post-lesion remodelling of the neural circuitry. The role of neuroglia is therefore fundamental in defining physiological versus pathological senescence; the failure of glial cells to protect and sustain the neuronal networks, the neural tissue and the CNS as an organ facilitates the progression from physiological to pathological brain ageing [93].

8.3 Astroglia in Physiological Ageing

8.3.1 Morphology and Gene Profiling

Age-dependent changes in astroglial morphology and gene expression are complex and region specific. Total number of astrocytes in physiologically aged human brain does not seem to change significantly, even in centenarians [19, 59]. When it comes to astroglial morphological profiles and expression of glial fibrillary acidic protein (GFAP), which are indicative of astroglial reactivity, the data remain quite controversial. Both a decrease [7] and an increase [14] in the number of GFAP-positive astrocytes, in particular in hypothalamic areas [27], as well as astroglial atrophy and astroglial hypertrophy were observed. The volume of astroglial territorial domains has been found to almost double in 21-month-old mice when compared to 5-month-old animals [26]. Increase of GFAP expression and hypertrophy of GFAP-positive astrocytes have been described in the hippocampus of aged rodents [7, 34, 49] and humans [10, 55]. Ageing had a distinct effect on different subpopulation of astrocytes in a region-dependent manner (Fig. 8.1, [69]). The densities of GFAP-positive astrocytes in the CA1 region and dentate gyrus of the hippocampus of old (24-month-old) mice demonstrated prominent hypertrophy when compared to young (3-month-old) or adult (9-month-old) controls. To the contrary, GFAP-positive profiles of astrocytes in the entorhinal cortex of old animals were atrophic when compared to the young or adult mice. Ageing results in a substantial decrease in the number and complexity of processes of astrocytes in the entorhinal cortex. The astrocytes immunoreactive to

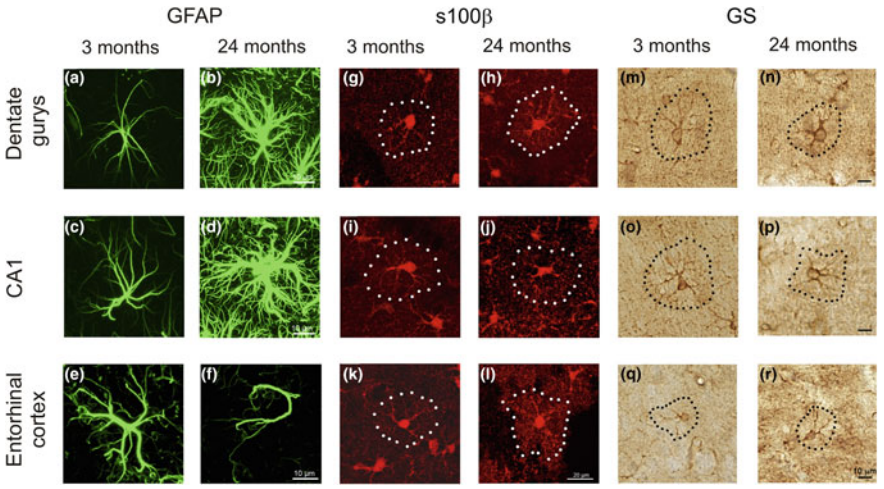


Fig. 8.1 Age-dependent remodelling of astroglial profiles in different brain areas. Confocal images showing glial fibrillary acidic protein—GFAP (a to f), s100β (g to l) and glutamine synthetase—GS (m to r) immunolabelled astrocytes in the dentate gyrus and CA1 hippocampal areas as well as in the entorhinal cortex of mice at 3 and 24 months. Modified from [69]

s100β protein were hypertrophic in the aged dentate gyrus but not in the CA1 region of the hippocampus as well as in the entorhinal cortex, whereas the profiles of a subpopulation of astrocytes labelled with glutamine synthetase were atrophic in the hippocampus with no changes in the entorhinal cortex [69]. Glutamine synthetase is a central enzyme necessary for operation of glutamine–glutamate/GABA shuttle, as well as for ammonium detoxification [70]. Suppression of expression of this enzyme may therefore affect neurotransmission and promote astroglial synthesis of GABA, an inhibitory neurotransmitter [22]. In parallel, hypertrophy of GFAP-positive astrocytes may be connected with environmental stimulation and plasticity representing the neural compensation. Exposure to the enriched environment is known for its positive effects on learning and memory, which occur in parallel with an increase in GFAP-positive astroglial profiles [68, 75].

The transcriptomic analysis of aged astrocytes similarly found a complex modification in genes expression. For example, astroglial cells from the cerebral cortex of aged mice demonstrated an increase in genes related to immune response with a decrease in expression of GFAP and genes related to neuroprotection and neuronal support [58]. Comparison of RNA-Seq from old and young astrocytes in the motor and visual cortices, hypothalamus and cerebellum revealed region specificity, with more significant changes in astroglia from the hippocampus and cerebellum [4], where astrocytes increased an expression of proinflammatory genes, genes encoding GFAP and Serpin3n, and genes linked to synaptic elimination such as complement component 3 and 4b [4]. Very similar results have been obtained in analysing the gene expression profiles of astrocytes from the hippocampus, cortex and striatum. Ageing affected hippocampal and striatal astrocytes the most with up-regulation of inflammatory genes and genes related to synaptic elimination [8]. Analysis of the gene expression profiles of different brain cells from ten brain regions of post-mortem tissue of humans, aged between 16 and 102 years, found that changes in astrocytes and oligodendrocytes were more prominent and complex compared to other cell types [81]; in particular, no age-dependent changes in neuronal gene expression pattern were identified. Again, these results indicate that functional preservation of neuroglia is critical for maintaining the ageing brain.

8.3.2 Astroglial Function

Although the data on physiology of aged astrocytes are rather limited, there are some hints for age-dependent remodelling of signalling and homeostatic profiles of astroglial cells. The resting membrane potential (around −80 mV) and membrane input resistance of astrocytes in cortical slices (from animals aged between 1 and 21 months) does not change much in ageing; if anything the input resistance is somewhat smaller in young adult mice (3–6-month-old—Fig. 8.2, [44]). Astrocytes from older mice express major types of receptors and are capable to generate ionotropic receptor-driven glial "postsynaptic" currents in response to neuronal activity [24, 44]. The density of ionotropic glutamate (AMPA and NMDA) receptors and P2X

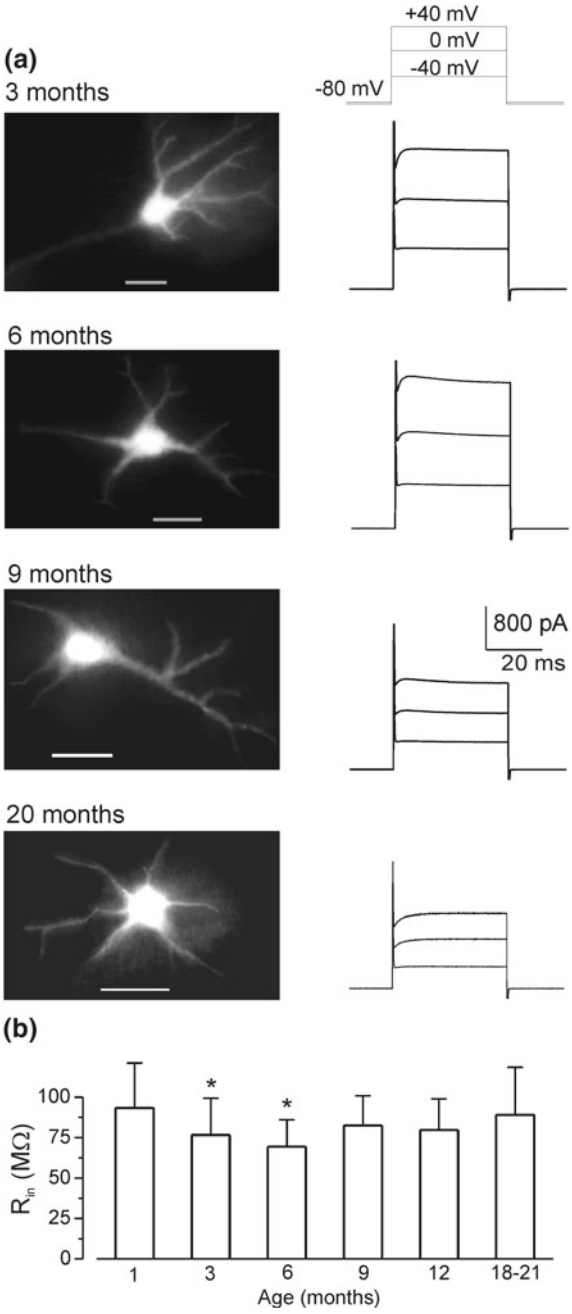


Fig. 8.2 Basic electrophysiological properties of cortical astrocytes in brain slices from mice of different ages. **a** Experiments were performed on mice expressing EGFP under control of human GFAP promoter. Representative EGFP images (*left column*) of cortical layer II astrocytes of different age groups and corresponding whole-cell currents (*right column*) evoked by depolarizing steps from the holding potential of −80 mV; the voltage protocol is shown on the top. **b** Age-related changes in the input resistance of cortical astrocytes. Data are presented as mean ± SD for 15 cells in each age group; decrease in the astrocyte R_{in} measured in 3 and 6 months in comparison to 1 month was statistically significant with $P < 0.05$ (one-way ANOVA). Reproduced with permission from [44]

purinoceptors, as well as the density of plasmalemmal glutamate transporter currents demonstrate bell-shaped age dependency (Fig. 8.3). Ionic currents generated by the above receptors and transporters are maximal in young adult (3- to 6-month-old) animals; at 9–21 months of age these currents are much smaller, although they are similar to currents recorded in 1-month-old animals [44].

Astrocytes are endowed with specific type of excitability, known as ionic excitability, which is associated with spatially and temporally organised fluctuations in the cytosolic concentration of several ions, including Ca^{2+}, Na^+, Cl^- and possibly K^+ and H^+ [95, 97]. Intracellular Ca^{2+} and Na^+ signalling are of particular importance [95, 96] being involved in regulation of numerous astroglial physiological processes such as secretion [94] or homeostatic transport [41, 71]. Neurotransmitter or synaptically induced astroglial Ca^{2+} signals are age dependent. For example, Ca^{2+} signals are the largest in young adult mice and are relatively small in old and very young animals (Fig. 8.4; [44]). This dependence may be reflected in the functional expression of astrocytic receptors. Most likely an increase in the density of receptors, as well as in the density of plasmalemmal glutamate transporters and in the amplitude of Ca^{2+} signals, occur in the period of maximal environmental stimulation associated

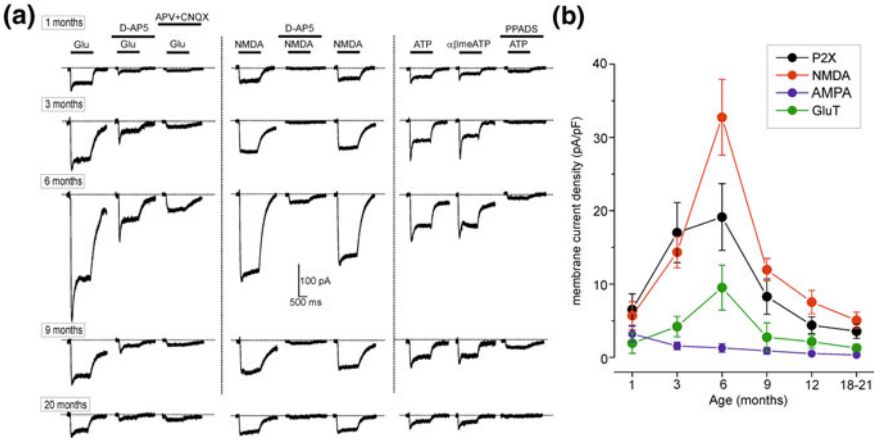
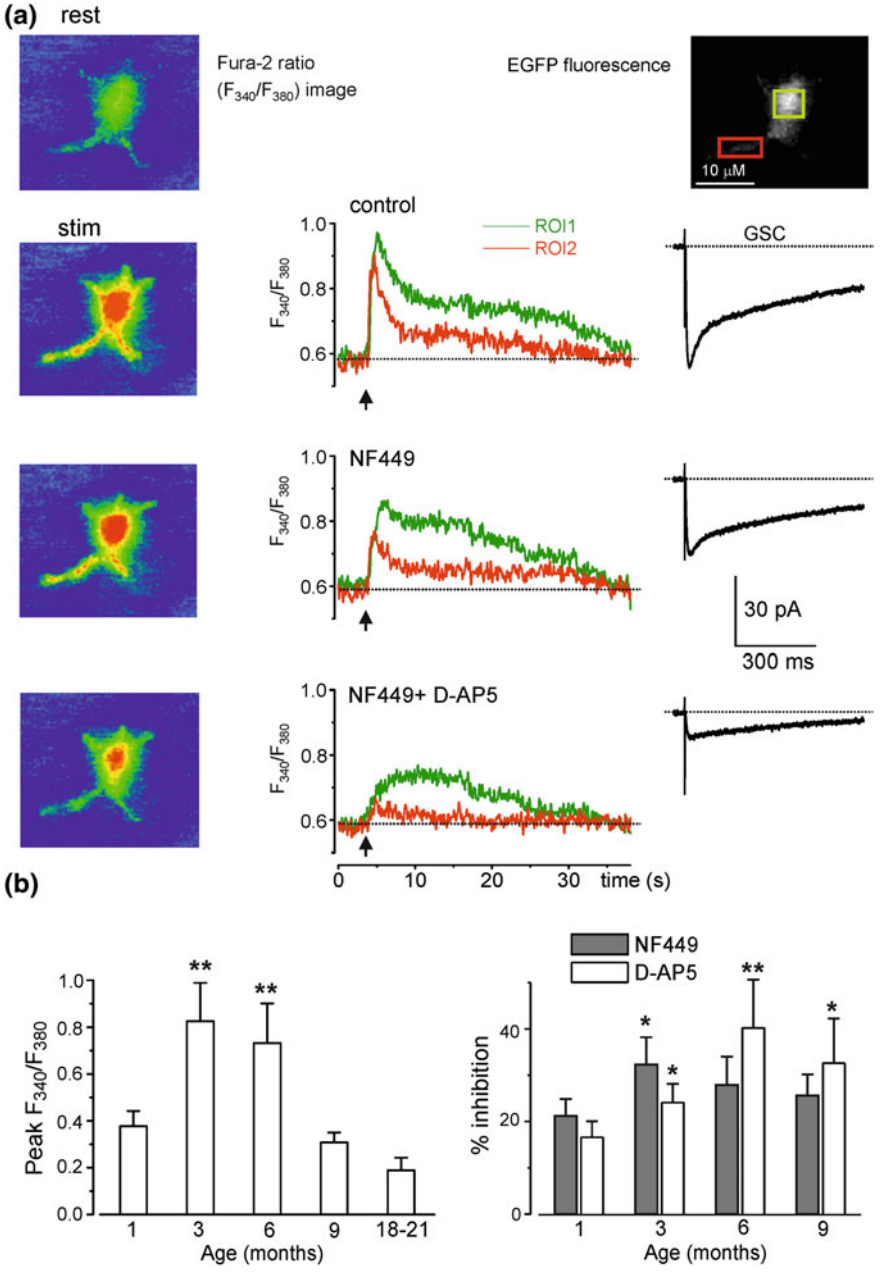


Fig. 8.3 Ageing affects the density of plasmalemmal glutamate transporters and ionotropic receptor-mediated currents in acutely isolated single cortical astrocytes. **a** Representative whole-cell currents elicited in the acutely isolated astrocytes by application of 100 μM glutamate (left column), 10 μM NMDA (middle column) and 10 μM ATP/αβmeATP (a potent and stable agonist at $P2X_1$, $P2X_3$, $P2X_{2/3}$, $P2X_{1/5}$ and $P2X_{4/6}$ receptors is also a weak partial agonist at human and mouse P2X4 receptors, but an antagonist at the rat $P2X_4$ receptor; it has little or no effect at other P2X and P2Y receptors), at holding potential of −80 mV. Glutamate- and NMDA-evoked currents were inhibited by 10 μM D-AP5, an NMDA antagonist and 30 μM CNQX, an AMPA receptor antagonist; ATP-evoked currents were inhibited by 10 μM PPADS, a selective purinergic P2X antagonist. **b** The density of currents mediated by P2X, NMDA and AMPA receptors and plasmalemmal glutamate transporters (GluT) in cortical astrocytes (mean ± SD for 9–12 cells for each age group); statistical significance of difference between average value for 1 month and corresponding values for 3 and 6 months $P < 0.02$ (ANOVA) for all types of currents


(a)
rest
Fura-2 ratio
(F340/F380) image
EGFP fluorescence
10 μM
stim
control
ROI1
ROI2
GSC
NF449
30 pA
300 ms
NF449+ D-AP5
F340/F380
time (s)
(b)
Peak F340/F380
Age (months)
NF449
D-AP5
% inhibition

◀**Fig. 8.4** Age-dependent changes in synaptically induced ionotropic Ca^{2+} signals in protoplasmic astrocytes in situ in cortical slices. **a** Cortical layer II astrocyte of 9-month-old mouse was loaded with the Ca^{2+} indicator Fura-2 in situ via patch pipette. Fluorescence images were recorded simultaneously with glial currents evoked by neuronal afferent stimulation in presence of a mixture of TBOA (GluT blocker) and CNQX in control, and after consecutive application of 10 nM NF-449 (selective antagonist of P2X receptors) and at that in a company of 30 μM D-AP5. Representative images (pseudo-colour, pipette image subtracted) and glial synaptic currents (GSC, right column) were recorded before (rest) and after stimulation as indicated. Ca^{2+} transients (middle column) are expressed as F_{340}/F_{380} ratio averaged over the corresponding regions of interest shown in the GFAP image of astrocyte (*top right*). **b** Age-related changes in the astrocytic Ca^{2+} signalling. *Left panel*, average peak amplitudes of $[Ca^{2+}]_i$ increases, induced by stimulation of neuronal afferents in cortical astrocytes of different ages. *Right panel*, average inhibitory effect of antagonists of P2X (NF449) and NMDA (D-AP5) receptors on the amplitudes of $[Ca^{2+}]_i$ increases responses in cortical astrocytes. Data are presented as mean ± SD for 3–4 cells for each age group; * $P < 0.05$, ** $P < 0.01$ one-way ANOVA compared to 1 month. Reproduced with permission from [44]

with intense learning; in younger and older ages synaptic activity is lower, which is reflected in a decrease in receptors expression.

A decrease in astroglial gap junctional coupling was found in old (20–27-month-old mice) neocortical astroglial syncytia [63]; there were no changes at earlier ages (up to 14-month-old [9]). Astrocytes in older brains down-regulate expression of aquaporin 4 (AQP4). A decrease in the density of these channels in the perivascular endfeet affects clearance of the brain parenchyma through the glymphatic pathway [42]. This decrease in AQP4 in the endfeet may be linked to the deficits in vesicular trafficking, which is the key pathway in delivery of numerous molecules to specific locations at the plasmalemma [64]. Ageing affects astroglial metabolic pathways, as an age dependent increase in oxidative metabolism was reported in older astrocytes, which may limit their ability to supply neurones with metabolic substrates [37]. There is also evidence of age-dependent alterations in astroglial ability to produce lactate and hence to operate lactate shuttle [31]. Similarly, ageing is associated with an increase in the ratio of glutamate to glutamine in the brain that indicates some aberrations in the operation of the glutamate/GABA-glutamine shuttle [16, 32]. Ageing is also associated with a decrease in the brain levels of glutathione, mainly produced in astrocytes; this limits the ability of astroglia to resist the oxidative damage to the neural tissue [17, 50].

8.4 Oligodendroglia in Physiological Ageing

The human brain has a disproportionally large white matter when compared to other mammals and even high primates [76], as indeed the white matter occupies >50% of the human brain. Additionally, the level of myelination is well developed in the grey matter [43], further demonstrating the importance of connectome to the cognition and intelligence. The anatomical prevalence of the white matter in the human brain is also associated with very long development: myelination attains its peak at ~45–47 years

of age, with a subsequent slow and yet progressive age-dependent decline [1]. Normal ageing causes rather substantial shrinkage of the white matter which diminishes by ~11%; in comparison, the volume of the grey matter is decreased by only ~3% [33]. The highest degree of age-dependent alterations of the white matter is detected in the prefrontal cortex and associative tracts [66], which suffer early in Alzheimer's disease [15]. Incidentally, these brain regions emerge late in evolution and they are the slowest to develop, which instigated a 'last in, first out' hypothesis of the white matter ageing [66, 90]. Conceptually, changes in the white matter can be considered as a valuable marker of ageing [90], and moreover, accelerated degeneration of the white matter seems to indicate development of neurodegeneration and profound cognitive decline [65, 90].

Cells of the oligodendroglial lineage represented by oligodendrocytes and their precursors (also known as NG2 glia [13]) are, arguably, the most numerous glial cells in the human brain. Cells of the oligodendroglial lineage, in contrast to astrocytes, are highly vulnerable to excitotoxicity and to oxidative stress. The oligodendroglial precursors/NG2 cells, as well as more mature oligodendroglia, express several types of ionotropic glutamate receptors (including NMDA receptors) and P2X purinoceptors, which all can mediate excitotoxic Ca^{2+} overload and cause cell death [51, 53, 74, 92]. Furthermore, oligodendrocytes are highly vulnerable to oxidative damage, which is stipulated by a rather low content of antioxidants. In particular oligodendroglial cells contain two times less of glutathione compared to astrocytes, and yet they experience six times more of oxidative stress in physiological conditions [38, 87].

Ageing is associated with a significant decrease, by up to 30%, of the total number of oligodendrocytes [19, 59]. Rather surprisingly, in monkeys the number of oligodendroglial cells has been claimed to increase with age; for example, in the visual cortex of old monkeys the number of oligodendrocytes increased by 50% [62]. Notably, these oligodendrocytes also showed aberrant atrophic morphology and a deficiency in myelin production, which defined decreased CNS myelination in old primates [62]. The age-dependent myelin deficiencies are also associated with vasculature lesions in the white matter that add strain on oligodendrocytes and promote their degeneration [3, 101]. Ageing is also associated with a diminished capacity of remyelination supported by the NG2 glia. Notwithstanding the fact that the population of NG2-oligodendroglial precursors does not change numerically in the old brain, the capacity of NG2 cells to differentiate into mature oligodendrocytes is reduced. The NG2 cells in the old brain tend to retain their precursor status, so that the time of differentiation into mature myelinating phenotype is increased by almost two times [102]. All in all, age-dependent changes in the white matter are prominent and may be the leading cause of age-dependent cognitive decline.

8.5 Microglia in the Ageing Brain

Microglia in the ageing human brain undergoes rather idiosyncratic metamorphoses, which are not present in laboratory animals. Fundamentally, human microglia gradually degenerates, thus, reducing the defensive capabilities of the senescent nervous tissue.

In animals, the ageing process results in complex changes in microglial numbers and state. In old rats, microglial numbers decreased in the nigrostriatal system and cerebral cortex [77], and remained unchanged in the hippocampus [91]. In contrast, in old rhesus monkeys the densities of microglial cells increased, while these cells showed signs of increased phagocytosis [61]. In humans, ageing is associated with dystrophy and degeneration of microglia which resulted in deterioration of neuroprotective and defensive functions of these cells [84]. Morphological features of dystrophic aged microglia include deramification, spheroid formation, gnarling and fragmentation of processes [84]. The processes of aged microglial cells are shorter with less branching and reduced arborized area; the total number of microglia seems not to change with age [11]. Microglial dystrophy and a loss of function arguably increase the vulnerability of the old brain to neurodegeneration and may facilitate evolution of age-dependent cognitive disorders, including Alzheimer's disease [83]. The age-dependent microglial dystrophy can be associated with cytoskeleton abnormalities that underlie the cytorrhexis, rupturing of cells [88]. Microglial cells can accumulate tau [5] and the aged microglia (in marmosets) were reported to contain hyperphosphorylated tau [67]; this microgliatauopathy can be a factor that initiates microglial degeneration and dystrophy [67]. The prevalence of dystrophic microglia limits the neuroinflammatory capabilities of the old brain tissue, questioning the concept of inflammaging.

There is also evidence for age-dependent microglial activation in normal ageing, especially in rodents [57, 60] and in *Macaca nemestrina* monkeys [78]. There is an overall trend of hyperreactivity of microglia in aged mice [25, 46, 79], which is strikingly different to the dystrophy and a loss of function of human aged microglia, questioning the validity of rodents as an experimental models for brain ageing.

Aged human microglial cells are represented by two morphologically distinct classes identified as dystrophic or senescent microglia and dark microglia. The dystrophic microglial cells [85] are characterised by spherical swellings of processes, dilatation of the endoplasmic reticulum and abundance of lipofuscin deposits (that emerge from incomplete lysosomal degradation and endolysosomal stress and overload). The dystrophic microglial cells have been identified both in old brains and in high densities around senile plaques of Alzheimer's diseases patients [88]. Dystrophic microglial cells have fragmented processes and have a substantially diminished activation capacity [85, 88]. The dark microglia have been defined so because of the electron-dense cytoplasm and nucleoplasm, which in electron microscopy appear as dark as mitochondria [2]. The dark microglia are also characterised by ultrathin and highly ramified processes that frequently enwrap synaptic elements, axons and dendrites. This may indicate that dark microglial cells are involved in eliminating

synapses [2]. In addition, dark microglia have altered expression of classical marker IBA1 and they do not express the $P2Y_{12}$ purinoceptor, which is considered as a marker for healthy surveillance microglia. Dark microglial cells cumulate with ageing and even more so in age-dependent pathologies [2].

8.6 Conclusions

All types of neuroglial cells undergo age-dependent remodelling which seems to be critical to define a physiological or pathological outcome of ageing process. In general, the age-dependent changes in neuroglial cells are characterised by a progressive loss of function which limits neuroprotection and regenerative potential of the neural tissue. This process of neuroglial senescence, however, is variable and most likely individually tailored by the lifestyle, environmental stress and comorbidities. Neuroglial paralysis facilitates emergence of neurodegeneration and cognitive decline, and hence a neuroglial state represents a potential therapeutic target for age-associated neurological disorders.

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Chapter 9
Astroglia in Leukodystrophies

M. S. Jorge and Marianna Bugiani

Abstract Leukodystrophies are genetically determined disorders affecting the white matter of the central nervous system. The combination of MRI pattern recognition and next-generation sequencing for the definition of novel disease entities has recently demonstrated that many leukodystrophies are due to the primary involvement and/or mutations in genes selectively expressed by cell types other than the oligodendrocytes, the myelin-forming cells in the brain. This has led to a new definition of leukodystrophies as genetic white matter disorders resulting from the involvement of any white matter structural component. As a result, the research has shifted its main focus from oligodendrocytes to other types of neuroglia. Astrocytes are the housekeeping cells of the nervous system, responsible for maintaining homeostasis and normal brain physiology and to orchestrate repair upon injury. Several lines of evidence show that astrocytic interactions with the other white matter cellular constituents play a primary pathophysiologic role in many leukodystrophies. These are thus now classified as astrocytopathies. This chapter addresses how the crosstalk between astrocytes, other glial cells, axons and non-neural cells are essential for the integrity and maintenance of the white matter in health. It also addresses the current knowledge of the cellular pathomechanisms of astrocytic leukodystrophies, and specifically Alexander disease, vanishing white matter, megalencephalic leukoencephalopathy with subcortical cysts and Aicardi–Goutière Syndrome.

Keywords Leukodystrophy · Astrocytopathy · Astrocytes

9.1 Introduction

The white matter (WM) of the brain consists of densely packed glial cells, including oligodendrocytes, oligodendrocyte progenitor cells (OPC), astrocytes and microglia, myelinated and unmyelinated axons and blood vessels with their cellular components [61, 76, 266]. The axonal tracts connect different grey matter areas to each other,

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creating functional pathways and networks [77]. Astrocytes are a heterogeneous cell population with respect to their developmental origin, morphology, function, physiological properties and environment [162, 202, 218]. They derive from radial glia cells (RGC), outer radial glial cells (oRGs), OPC and precursors situated in the spinal cord [84, 202]. Radial glial cells are multipotent progenitor bipolar-shaped cells, which reside in the embryonic ventricular zone (VZ) and are derived from the neuroepithelial cells [14, 78, 104, 116]. oRGs are a type of subventricular zone (SVZ) progenitor cells, which are generated from radial glia asymmetrical divisions in the VZ [232]. OPCs are a cell population that also express the proteoglycan NG2 and are therefore also designated as NG2-glial [269]. These cells differentiate into oligodendrocytes and astrocytes during embryogenesis [59, 179]. However, OPCs survive in the adult brain and keep their potential to differentiate into oligodendrocytes under certain conditions, such as in the case of myelin damage. OPC proliferation and differentiation are also modulated by astrocyte-derived factors [59, 120, 191].

Astroglia exist in the white and grey matter, the former having a fibrous morphology and the latter a protoplasmic morphology [13, 71, 154]. Structurally, fibrous astrocytes are constituted by intermediate filaments such as vimentin, nestin and glial acidic fibrillary protein (GFAP) [99] and express the CD44 surface receptor [139, 209]. CD44 is the receptor for the extracellular component hyaluronan that negatively regulates OPC differentiation [8, 68]. $GFAP^{-/-}$ mice show abnormal white matter architecture and impaired long-term myelination, showing the importance of this protein on the global structure and function of the white matter [137]. Considering that astrocytes develop a specific morphology and molecular profile depending on their function and location in, and within, the white or grey matter, fibrous astrocytes conceivably adapt to their surroundings, showing a selective vulnerability upon certain conditions [71, 257]. This could explain why the white matter is not always homogeneously affected in leukodystrophies.

9.2 Role of Astrocytes in Maintaining the Integrity of the White Matter

Astrocytes promote white matter integrity and homeostasis through the cooperation with other glial cells, axons and non-neural cells. They control ion-water homeostasis [55], thereby modulating neuronal activity and myelin water content [211]; provide metabolic support to neurons and other cells in the CNS by regulating the glucose metabolism and, in part, brain lipid metabolism [102, 178]; and stimulate the cerebrospinal fluid (CSF) and the interstitial fluid (ISF) flow [107, 108, 212]. Furthermore, astrocytes are involved in the formation and maintenance of the blood–brain barrier (BBB) by the release of glial-derived neurotrophic factor (GDNF), angiopoietin-1, angiotensin II, bone morphogenetic protein (BMP) signalling and microglia recruitment, therefore inducing and maintaining BBB-related functions in endothelial cells and brain homeostasis [7, 225, 258]. The BBB is essen-

tial to protect the brain against the toxicity of many substances, the entrance of pathogens and influx of ions such as K^+ and Ca^{2+} [15]. This diffusion barrier is composed of endothelial cells glued together by tight junctions, pericytes and astrocyte endfeet [1, 9, 260]. These cellular elements along with neurons and the extracellular matrix form the 'neurovascular unit' [98]. Moreover, astrocytes contact with the blood vessels inducing alterations in their shape which are crucial for proper metabolic support to neurons [186]. Astrocytic intracellular Ca^{2+} increase is responsible to initiate this process that is triggered by increased neuronal activity. Nitric oxide (NO) is an essential compound dictating whether the blood vessels undergo contraction or dilatation [87]. Astrocytes also control local blood flow in the CNS in order to coincide with neuronal metabolic demands as a response to synaptic activity [106].

Astrocytes are responsible for many basilar regulatory functions that play a crucial role in maintaining the normal physiological conditions in the CNS [16, 111, 119]. They promote proper synaptic transmission through the uptake of K^+ and glutamate [167, 256] and modulate synaptic plasticity by the release of gliotransmitters, growth factors and via astrocytic Ca^{2+} signalling [120, 171]. Furthermore, they are involved in cognition, regulation of the circadian rhythm and formation and pruning of synapses [41, 222, 269], amongst others. Astrocytes present three different phenotypes, depending on whether the CNS is under normal physiological conditions or upon injury such as inflammation. In the healthy CNS, astrocytes are 'quiescent' performing all the functions described above [69]. However, upon injury, astrocytes show an active and a reactive phenotype [120], along a spectrum spanning between hypertrophic and scar-forming cells [118]. The active phenotype is reported as the intermediary form between quiescent and reactive phenotypes, meaning that astrocytes show a mild reaction to injury [136]. When the insult persists or pathogenesis begins, this reaction gradually increases into a severe response [160].

9.3 Astrogliosis

Astrocytes become reactive when there is a disruption in the normal function of the brain such as upon injuries, inflammatory processes or demyelinating conditions [264]. This process is called astrogliosis, which constitutes a pathological hallmark of the diseased tissue. This phenomenon is heterogeneous, not an all or none event but, on the contrary, it is a gradually changing process tailored on the type of CNS injury origin and its severity [220]. As a consequence, astrocytes change their morphology, molecular profile, signalling pathways, function and even their interactions with other cells, becoming active or reactive [217, 218]. We will not discuss further all of these alterations. Despite this, it is crucial to mention the GFAP overexpression, its intracytoplasmatic aggregates in astrocytes, IFN-α upregulation and KCa3.1 potassium channel [72, 153, 176], because they characterize some of the white matter disorders that will be addressed later on. GFAP, known to regulate many cellular pathways, is present in five isoforms (GFAP α, β, γ, δ, κ) [19, 161, 185, 195, 265] with

different expressions and distributions in various pathologies, potentially shaping the outline and features of each disorder. Notwithstanding, the activity of each isoform is still not clearly defined and future research is needed to understand the functional consequences. Since the reactive astrocyte population is heterogeneous, and therefore difficult to classify in different stages, several outcomes may result from astrogliosis [78]. KCa3.1 channels play an important role in regulating ion-water homeostasis and astrocyte activation, which may be due to their upregulation in vacuolating leukodystrophies [23, 54, 197]. Astrocyte loss of function and astrogliosis can occur in different time scales with the former sometimes taking place before the latter [220]. Although they are not synonymous of each other, this is a characteristic consequence when an astrogliosis process is taking place. Loss of astrocyte coupling and inability to buffer glutamate and potassium are some of the after effects of astrogliosis [78], which impair neuronal activity and synaptic transmission by promoting an excitotoxic environment [196]. On the other hand, astrocytes along with microglia contribute to tissue repair and axon regeneration by participating in the formation of the glial scar [82, 210]. Glial scar is created when astrocytic processes surround an injured tissue, establishing a barrier to pathogens and inflammatory cells [33, 218, 220]. Hence, reactive astrocytes can play neuroprotective and detrimental roles depending on the pathological context [78].

9.4 Leukodystrophies

Two key works, carried out by Morell and Seitelberger in 1984, introduced the term leukodystrophy as a disease category providing a set of criteria for the classification. Both the authors have pinpointed the importance of oligodendrocytes, as the cells primarily affected, due to the fact that the pathogenesis of leukodystrophies was centred on myelin. Although at that time, a small number of pathologies were known and no genetic linkage had been established, their work provoked a great deal of research. This research was much sought after the introduction of more sensitive techniques such as magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS) and whole exome sequencing (WES) that greatly improved clinical diagnosis [117]. Leukodystrophies were traditionally considered myelin disorders thus, for many years, the research on white matter diseases was focused on oligodendrocytes, being these the myelin-forming cells [85, 158, 208]. The few studies that have emphasized astrocytes as important contributors to the disease mechanisms of leukodystrophies have focused on their role in regulating myelin formation and function and have always approached interactions with other glial cells from the myelin perspective [144]. However, according to a new definition and classification of leukodystrophies [249], these encephalopathies are genetic and inherited disorders affecting the white matter of the CNS as a whole. This means that they are provoked by the dysfunction of cellular mechanisms, which in health are responsible for the integrity and maintenance of the white matter, and by the impairment of the crosstalk between all white matter structural components including oligodendro-

cytes, astrocytes, microglia, blood vessels and other non-neural cells [249]. In some of these diseases, astrocytes are known to acquire abnormal and cytotoxic functions due to mutations in astrocyte-specific gene products. Alexander Disease (AxD), Vanishing White Matter Disease (VWM), Megalencephalic Leukoencephalopathy with subcortical cysts (MLC), and Aicardi Goutières Syndrome (AGS) are examples of the leukodystrophies classified as astrocytopathies meaning that astrocytes are the primarily cells affected [24, 62, 177], although the pathomechanisms of the various disorders may differ [24, 243, 249]. The impaired cell-to-cell interactions and astrocytic dysfunction, contribute to cellular, molecular and morphological alterations in the brain and, thus, to the pathogenesis of leukodystrophies.

9.5 Alexander Disease

AxD is an autosomal dominant monogenic inherited pathology caused by mutations, most of them occurring de novo, in the coding region of *GFAP* [3, 24, 134, 138, 152, 254]. *GFAP* encodes for an intermediate filament protein called glial fibrillary acidic protein (GFAP), which is only expressed by astrocytes in the CNS and is responsible for their cytoskeletal architecture [182]. This disorder presents with psychomotor impairment, spasticity, intellectual disability and possibly seizures, accompanied by other symptoms that depend on the age at onset [86, 151, 199]. Furthermore, different phenotypes have been described, according to genotype, age of onset and pathological alterations in the brain. However, AxD is currently classified only into two types—type I and type II—the former with early-onset and showing developmental delay and macrocephaly, and the later with later-onset and presenting with bulbar signs, including dysphagia and dysphonia [151, 172, 181].

Neuropathological changes in AxD include widespread degeneration of the white matter, prevailing in the frontal white matter also with cystic degeneration, and variable loss of neurons. These can be assessed through MRI and macroscopically at autopsy [74, 242]. Cerebellar and brain stem atrophy, dilation of lateral ventricles and swelling of basal nuclei and thalami are some of the features seen in type I AxD. On the other hand, ventricular garlands and atrophy of medulla oblongata and cervical spinal cord are typical of type II AxD [74, 242, 246, 247].

Several studies have evaluated the occurrence of cellular and molecular changes in the AxD brain [91, 138, 151, 182, 228]. AxD astrocytes acquire a reactive phenotype characterized by hypertrophy of terminal cell processes with intracytoplasmatic aggregates named Rosenthal fibres (RF), which are the pathological hallmark of the disease. RF are comprised of mutant GFAP, ubiquitin, vimentin, nestin, plectin, p-JNK, p62, synemin, stress proteins—hsp-27 and αB-crystallin, and the 20S proteasome subunit [110, 177, 254]. αB-crystallin plays a crucial role in maintaining rearrangement of disruptive intermediate filaments networks, as RF [125, 228]. In addition, overexpression of GFAP mutant protein, possibly resulting from the dysregulation between synthesis and degradation, leads to aberrant morphology, polyploidy and astrocytic dysfunction and modulates various

pathomechanisms of AxD [112, 228]. A previous work using transgenic mice with increased wild-type (WT) GFAP expression, showed that GFAP accumulation triggers cellular-stress and immune astrocytic responses [226, 227], by increasing the expression of complement components and macrophage-specific markers. As a result, this induces microglia and complement-dependent pathways activation [165, 166]. The complement cascade is part of the innate immune system and becomes activated when a set of soluble proteases binds to pathogens. It is initiated by a complement component, which can be either C1q (classical activation) or C3 (alternative activation). Other complement proteins become activated in a cascade, which ultimately results in the formation of a C3 protein, opsonizing subsets of synapses for elimination and leading to cellular responses such as pro-inflammatory signalling and receptor-mediated phagocytosis, among others [103, 130]. Additionally, the increase in expression of chemokines and cytokines like TNFα, IL1β and IL-6, along with the decreased expression of genes related with Ca^{2+} signalling, make astrocytes more vulnerable to the effects of stress and apoptosis [91, 182]. Additional data suggests that hyaluronan, a major constituent of the extracellular matrix known to prevent astrocyte and OPC differentiation is increased in AxD and other diseases where lack of myelin is observed. This glycosaminoglycan is mostly produced by astrocytes as its main receptor, CD44, is also expressed by these glial cells [139, 209].

9.5.1 Compromised Functions of Astrocytes in AxD

Accumulation of mutant GFAP inside astrocytic cell processes promotes the disturbance of its molecular profile, morphology, function and many cellular pathways that will alter their homeostasis and eventually normal brain physiology and interactions between cells [166].

Microglia are the resident immune cells of the central nervous system, which act as sentinels during pathological changes, responding to them by remodeling synaptic connections and their function in brain networks [126, 251]. Nonetheless, astrocytes also contribute to the regulation of the immune and anti-inflammatory responses in the CNS [42, 135, 219] through the expression of pattern-recognition receptors (PRRs). This PRR are also present in microglia and are responsible for sensing infectious agents entrance and endogenous danger signals [73]. Some studies suggest that these two glial cells are also coupled into gap junctions, this intercellular contact having the important function of modulating the activity of each other [94, 105, 219]. Specifically, astrocytes regulate migration, activation and proliferation of microglia via the release of anti- and pro-inflammatory factors [73]. On the other hand, microglia control astrocytic activation, proliferation and reactivity by the release of cytokines [174] and NO. NO induces astrocyte cell death by apoptosis, which was suggested as a mechanism that can control excessive reactivity [183]. Moreover, astrocytes and glial also dialogue in order to achieve other goals like the formation and maintenance of the BBB, [268] and the clearance of myelin debris in demyelination [216]. In AxD, astrocytes mediate microglial activation which, in return, induces stress and immune

responses in astrocytes. Several lines of evidence [165, 166] corroborate that this cell-to-cell interaction may be the reason for perpetuated inflammation, affecting the function of other cells, including oligodendrocytes. Notably, damage to these cells can result in aberrant myelin formation. In addition, loss of astrocyte coupling due to decreased expression of connexins, which are the proteins responsible for the assembly of gap junctions, is observed. A previous study showed that the lack of connexins is related to demyelinating events [145].

Proper interaction between astrocytes and oligodendrocytes is essential for the support of the white matter. Astrocytes and oligodendrocytes are coupled through A/O gap junctions, allowing intercellular communication [169]. Along with A/A gap junctions between astrocytes, this population forms the panglial syncytium [159]. This functional structure allows the transportation of substances synthesized by astrocytes to oligodendrocytes in one-way direction and may act on K^+ buffering [155, 184, 234]. Astrocytes and oligodendrocytes require energy to pursue their functions, withdrawing glucose from blood vessels through the glucose transporter 1(GLUT1) [135]. However, when glucose is low in the blood or during increased neuronal activity, astrocytes act as the main energy suppliers to oligodendrocytes and neurons [40, 178]. Since they hold the capacity to store glycogen [28], they release glycogen-derived lactate and guide its transport to neurons through astrocyte–neuron lactate transfer shuttle (ANLTS) [510, 193]. Astrocytes synthesize various cytokines, chemokines and growth factors that regulate the migration, maturation, proliferation and differentiation of OPCs [44], ultimately modulating myelination [12, 44, 61, 120]. Ciliary neurotrophic factor (CNTF) [160], brain-derived neurotrophic factor (BDNF) [156, 262], leukemia inhibitory factor (LIF) [109] are examples of proteins that increase myelination by promoting oligodendrogenesis. TGF-alpha and BMP2/4 [12] are examples of factors that hamper myelination by preventing OPCs differentiation into myelin-forming cells [37, 44, 144, 205, 206].

In addition, the ability to buffer glutamate is impaired due to the defective expression of glutamate transporters—EAAT2/GLT—[231]. Moreover, it is observed a decrease in inwardly rectifying K^+ channels due to a deficient buffer of potassium [166]. Therefore, astrocytic dysfunction in buffering glutamate and potassium, in addition to lack of astrocyte coupling may lead to cytotoxicity, followed by loss of oligodendrocytes, selective neuronal death, possible axonal dysfunction and synaptic activity impairment. Together, these astrocytic dysfunctions contribute to neuronal excitotoxicity [11, 97] and prolonged neuronal depolarization, explaining one of the possible causes of neuronal death and the occurrence of epileptic seizures in this disease.

9.6 Vanishing White Matter

Vanishing white matter (VWM) is an autosomal recessive polygenic disorder, caused by mutations in any of the five genes (*EIF2B1, EIF2B2, EIF2B3, EIF2B4* and *EIF2B5*) that encode the subunits of the eukaryotic initiation translation factor 2B (eIF2B). eIF2B is responsible for the regulation of protein synthesis [29, 132, 239,

243, 246, 247], being specifically involved in the translation of messenger RNA (mRNA) into polypeptides. Adverse conditions like thermal, chemical and oxidative stress and physical trauma trigger a cellular-stress response, which leads to inhibition of protein synthesis to preserve cellular energy and prevent cellular death. This mechanism aims at decreasing accumulation of denatured and misfolded proteins, although it is compromised when eIF2B shows to be dysfunctional and when mRNAs have peculiar ORFs that allow them to escape the inhibition of translation [43, 67, 200, 259]. This may be the possible reason behind the trigger of VWM, which is provoked by febrile infections, minor head trauma and acute fright [239, 240, 255].

Characterized, in every variant of the disease, by mild cognitive decline, occasional epileptic seizures and motor symptoms, like spasticity and cerebellar ataxia, this disease can also present other symptoms due to its broad phenotypic variation, which is influenced by genetics and environment [29, 80, 148, 180, 240, 244]. The onset of the most common variant is in childhood with an age range between 2 and 6 years [96, 201, 239]. However, the onset of the disease can vary depending on the variant of the disease. VWM is one of the most prevalent leukodystrophies in children and described as most frequent in Caucasian populations [241]. Nevertheless, one of the variants of the pathology seems to be more common among the Cree Indians with its onset in early childhood, with age at onset ranging between 3 and 9 months [80]. In other variants, the onset might be in adolescence or adulthood and is accompanied with the same symptoms described above in addition to psychiatric symptoms [148, 164, 180]. In female patients, the ovaries can also be affected [79]. Although VWM is chronic and progressive, it presents episodes of occasional seizures, increasing motor deterioration, hypotonia and loss of consciousness. Normally, the outcome is death after a few months, years or decades depending on onset age and disease severity, which are often inversely related. This means that the earlier the onset of the disease, the greater the severity thus, the younger the patient, the shorter the average life expectancy will be. The most severe variant of this disorder is the one that has an antenatal onset, in which the development of the foetus is completely compromised [246, 247].

Cellular, molecular and morphological changes are described through different stages of VWM including the pre-symptomatic phase. Some of these changes, assessed by MRI [245] consist in rarefaction and cavitation or cystic degeneration of the cerebral white matter, particularly that in the fronto-parietal lobe. Additional pathological findings include abnormally thin myelin sheaths, sometimes showing vacuolization; a certain degree of loss of myelin and axons; increased numbers of oligodendrocytes and OPC and abnormal morphology of astrocytes [194, 239, 240, 245, 246, 247]. The cause of myelin vacuolization is still not fully understood. However, a study suggests that myelin vacuolization is the result of astrocyte-specific proteins deficiency and lack of connexins expressed in those cells [55].

Cerebellum and brainstem show mild white matter signal abnormalities, the spinal cord is usually spared and the cerebral cortex is always spared. In this disorder, the brain undergoes gradual modifications, which are the result of the phenotypic variation but also of the stage of the disease. In an advanced phase, a large part of cerebral white matter ‘disappears’ by substitution with fluid with features and signal

properties similar to cerebrospinal fluid (CSF) [239, 240, 245]. The assessment of the brain metabolic changes of patients with VWM is often performed with the aid of MRS, which shows the presence of lactate and glucose, compatible with the features described above in the later stage of the disease [20, 246, 247].

9.6.1 Compromised Functions of Astrocytes in VWM

Astrocytes and oligodendrocytes are especially affected in this disorder, although it has been proven that astroglia is the determinant in VWM [62]. Like all cells in the body, both of them express eIF2B, which makes it difficult to directly assign just one of them to the pathogenesis of VWM. Nonetheless, several studies have been addressing astrocytic dysfunction as the major contributory pathomechanism for the disorder [30, 62]. Previous work has shown that VWM astrocytes present abnormal molecular profile, overexpressing the GFAP delta isoform and heat shock protein αB-crystallin [30], which may justify their abnormal morphology, dysfunction and metabolic stress. Furthermore, the proportion between GFAP delta and alpha isoform is disturbed due to the lacking of upregulation of the GFAP alpha isoform, which may be responsible for the impaired crosstalk with other cell types, including oligodendrocytes and the inability to build up a scar in cavitated lesions in the white matter [30]. Several lines of evidence suggest that scarce and disproportionate gliosis and consequent lacking of scar formation are not related with a deficient proliferation index of VWM astrocytes but, on the contrary, to astrocytic immaturity. A few studies showed increased proliferation of astrocytes in vivo and in vitro, after mechanical stress, [30, 31, 58], revealing this was not the cause for meager gliosis. In contrast, the under-expression of protein S100β, which is crucial for astrocytic differentiation [8, 31], was detected in VWM astrocytes, unveiling their immature phenotype in this disease. The lack of astrocytic maturation contributes to impaired reactive gliosis, which may redound in the development of cavitated white matter.

In addition to all of the changes in the astrocytes, the crosstalk with oligodendrocytes and OPC within this pathology is impaired, explaining the lack of myelin, a typical feature of VWM. In mice, it was demonstrated that VWM astrocytes inhibit wild-type (WT) OPC differentiation into oligodendrocytes, impeding the formation of myelin-forming cells. On the other hand, VWM OPCs are able to go through a normal differentiation process if they are co-cultured with WT astrocytes [30, 62]. This suggests that astrocytic dysfunction is primary and that oligodendrocytes are the cells secondarily affected in this disorder. One possible reason for this occurrence is the increase in expression of hyaluronan, which follows the same pattern of the activity described above for AxD thus, inhibiting OPC maturation and (re)myelination. Accumulation of a particular form of this glycosaminoglycan, the high-molecular-weight (HMW) hyaluronan, was found in the frontal white matter of patients [31], which is consistent with the white matter abnormalities in fronto-parietal lobe showed in MRI. By contrast, less HMW hyaluronan is expressed in the cerebellum, which is also compatible with it being less affected at MRI. This suggests a link between

astroglia, hyaluronan accumulation, OPC maturation and, in general, the severity of affected areas [31]. Studies using mice expressing VWM mutations showed that increased level of hyaluronan correlate with the course and severity of the pathology [62].

Astrocytes interact with neurons through the provision of trophic support [121, 221] and protection against oxidative stress [70, 147, 204] and excitotoxicity [11, 97]. Some axons are enwrapped with myelin, which is a modified plasma membrane that acts as an electrical insulator, increasing the velocity of nerve impulse propagation and decreasing energy expenditure [4]. In return, they promote the myelin formation by regulating astrocyte-derived pro-myelinating factors release and OPC differentiation. Nonetheless, a study showed that unmyelinated axons also contribute to the regulation of myelination through the vesicular release of glutamate in the white matter [267].

Another evidence that shows astrocytes as the primary cells affected in this disease is their effect on axonal pathology. Reduced axonal thickness correlates with tissue damage severity, suggesting impairment in the velocity of nerve impulse propagation and in consequence, in synaptic activity [76]. In addition to abnormalities in axonal diameter, myelin thickness and axon–myelin ratio, a recent work found an increase in the number of unmyelinated axons in the corpus callosum of VWM mice compared to WT animals [122]. The consequent increase in axonal density was showed, in the same study, to be promoted by VWM astrocytes.

In conclusion, the astrocyte dysfunction and the aberrant interaction with other glial cells and axons greatly influence the course of VWM pathogenesis

The phenotypic variation of the leukodystrophies may be influenced by astrocytes heterogeneity in terms of morphology, function, origin and even their responses to insults. Corroborating this hypothesis, evidence suggests that astrocyte location affects the abundance of GFAP [195]. Overall, it is reasonable to state that the heterogeneity of astrocytic function and its activity pattern drive the course (selective vulnerability, disease severity and repair potential) of astrocytopathies.

9.7 Megalencephalic Leukoencephalopathy with Subcortical Cysts

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) [236, 237] is an autosomal polygenic inherited disorder, caused by recessive mutations in any of two genes—*MLC1* [132] or *GLIALCAM* [142]—or dominant mutations in *GLIALCAM* [95]. The former encodes a highly hydrophobic membrane protein that shows low homology to ion channels [21, 132, 229] such as the potassium channel Kv1.1, and transporters; while the latter encodes an adhesion-like molecule [22, 113, 143] that acts as an MLC1-chaperone, as a secondary subunit of the ClC-2 chloride channel [113] and additionally as a guiding molecule to direct MLC1 to cell junctions [143].

Mutations in the MLC1 affect the majority of the population of patients with MLC with a prevalence of 80% [22, 132, 133, 157].

This leukodystrophy is marked by mild cognitive decline, epileptic seizures and motor symptoms, like spasticity and ataxia, with its onset in infancy [95, 213, 233, 236, 237]. Morphological alterations in the brain such as diffuse MRI signal changes, swelling and, eventually, atrophy of the cerebral white matter intramyelinic edema, presence of subcortical cysts mainly in the anterior temporal, frontal and parietal regions [236, 237] and, pathologically, vacuoles in myelin [175, 238] and astrocytic endfeet [32, 63, 66] are some of the main features of MLC. These were discovered through MRI, MRS [56] and brain biopsy [248].

9.7.1 Compromised Functions of Astrocytes in MLC

In the CNS, MLC1 is expressed at the A/A junctions, in astrocytic endfeet contacting the blood– and CSF–brain barriers, and in Bergmann glia of the cerebellum [21, 63, 203, 229, 230]. GlialCAM is co-localized with MLC1 at astrocytic endfeet [35, 100, 142], but is also present in oligodendrocytes processes [55, 75, 113], unlike MLC1 [6, 66, 203]. Moreover, *GlialCAM* is important for the correct targeting of MLC1 [142, 143], GlialCAM [36, 100] and the voltage-gated osmosensitive Cl-channel (CLC-2) to astrocyte junctions [113]. A previous study, using GlialCAM-null mice, showed absent expression of MLC1 and under-expression of CLC-2 revealing a direct interaction between GlialCAM and these two proteins [32, 113]. Mutations in *GlialCAM* lead to defects in trafficking and, therefore, to altered sites, which have been suggested to affect the course of the disease [32, 66, 100, 214]. Furthermore, mutations in *GlialCAM* also cause dysfunction of CLC-2, because the ability to change functional properties like rectification is impaired [114]. The functional interaction between MLC1 and ion transporters such as the sodium/potassium-ATPase pump (Na, K-ATPase) [25, 26], ion and water channels, including KCa3.1, VRAC, the transient receptor potential cation channel subfamily V, member 4 (TRPV4) [66, 128, 129], inward rectifier potassium channel 4.1 (Kir4.1), the water channel aquaporin-4 (AQP4) [128] and cell signalling pathways, among which the EGFR/ERK [129] and PLCγ1 pathways [253], are involved in several cellular processes such as proliferation, maturation, astrocyte activation and apoptosis. The dysfunctional interaction between MLC1, GlialCAM and all the proteins described above contributes to dysfunction of the white matter and, thus, to the alteration of cerebral homeostasis [65].

MLC1 is implicated in the regulation of volume alterations in astrocytes and, therefore, in the promotion of their osmotic balance [155]. In response to the disturbance of ion-water homeostasis or oxidative stress, a cascade of events follows such as astrocyte swelling and activation [34, 81]. The return to osmotic balance is achieved through the activation of volume regulated anion channel (VRAC) and calcium-activated KCa3.1 potassium channel, and EGFR/ERK and PLCγ1 pathways [253], which will modulate the activation of the regulatory volume decrease (RVD) [173, 224]. VRAC activity is focused on the control of astrocytes concentra-

tion gradient, by regulating their water flux and organic osmolytes transport, such as chloride. However, in the context of MLC, MLC1 is under-expressed in astrocytes [64] together with reduced expression of GlialCAM [192], result in defective chloride currents, hindering VRAC activity and, therefore, the adjustment of changes in the intracellular and extracellular environment [66, 155, 192]. Thus, RVD is impaired, which contributes to the long-term maintenance of swollen astrocytes. A previous study proposes a possible causal relation between disturbed fluid homeostasis and vacuolization in astrocyte end-feet [35]. Using *Clcn2*$^{-}$ mice, it has been demonstrated that ClC-2 is involved in an astrocytic vacuolation phenotype similar to MLC [18]. Findings from an earlier study, in which expression of MLC1 protein was downregulated in order to mimic the expression levels of this protein in the diseased brain, show the appearance of vacuoles in astrocytes, a phenotype not presented prior to depletion of MLC1 [63]. Furthermore, an increase in both MLC1 and GlialCAM expression is able to revert this phenotype [35]. These results suggest a correlation between MLC1 and vacuolization in astrocytes, being the former a possible cause for the latter. However, it is not known which mechanism might be involved.

The KCa3.1 are potassium channels with Ca^{+} entry-induced opening, located together with MLC1, in astrocytes processes that are part of the neurovascular unit [141]. The increase in intracellular Ca^{+} concentration is mediated by TRPV4, which demonstrate a crosstalk between different ion channels and MLC1 protein [128]. Thus, previous work suggests that MLC1 and KCa3.1 may cooperate to regulate BBB permeability, Na^{+} influx, and ion-water homeostasis [38, 129, 149]. These astrocytic functions are compromised, leading to morphological, cellular and molecular changes observed in MLC but also in many other leukodystrophies.

Astroglia clearance of K^{+} by uptake and spatial buffer is impaired in several leukodystrophies [124, 146, 256], among them MLC, due to dysfunctional interaction of several proteins [27], such as MLC1, GlialCAM, connexins [163, 261], Kir4.1 [60, 93, 207] and AQP4 [17, 92, 223]. Astrocyte function impairment leads to hampered regulation of extracellular potassium concentration ($[K^{+}]_o$) resulting in astrocyte swelling and, consequently, in the development of cerebral white matter edema [129, 248]. Furthermore, the increase in $[K^{+}]_o$, due to slow K^{+} kinetics, and defective glutamate uptake also plays a role in sustaining and spreading depolarization and, thus, in enhancing excitability followed by epileptic seizures [45, 65, 115]. Although mutations in *CLCN2* have not been found in MLC, several lines of evidence show that activation of CLC-2 is dependent on interaction with MLC1 and GlialCAM and depolarization. The formation of this tertiary complex may be important in delaying exacerbated brain activity, counteracting membrane voltage to more negative values through chloride influx [215]. The impairment of all of the astrocytic functions described above drives the course of the disease, which suggests that the cells that play the central role in MLC are the astrocytes [88].

Regarding cellular processes, WT MLC1 interferes in astrocyte proliferation by down-regulating the activation of various signalling pathways, such as ERK1/2 and PLCγ1, blocking KCa3.1 channel and promoting epidermal growth factor receptor (EGFR) degradation, all known to promote astrocyte growth. On the contrary,

mutations in MLC1 favour astrocyte proliferation. This is corroborated by evidence showing that MLC1 expression increases when cell proliferation is inhibited [63].

Studies using MLC mouse models, such as *GlialCAM*-null mice and *Mlc1*-null mice [32, 66, 75, 100] are important to better understand the cellular and molecular pathomechanisms of the disease, especially in early stages, which is difficult to assess and study in MLC patients, unveiling the pathophysiological consequences of the reduced expression in both genes, in MLC [32, 66].

9.8 Aicardi–Goutières Syndrome

Aicardi–Goutières syndrome (AGS) [2, 50] is an autosomal polygenic inherited neurodegenerative leukodystrophy, caused by mutations, most of which are recessive [187], in several genes (TREX1 [48], RNASEH2A, RNASEH2B, RNASEH2C [49], SAMHD1 [186], ADAR1 [189] and IFIH1/MDA5 [190]). For the purpose of this chapter, we will only discuss three-prime repair exonuclease 1 (TREX1) mutations for two reasons: they are the only ones in which a relationship with astrocytes has been shown and are also the most common and lethal mutations found in patients with AGS. In addition, further details regarding innate immune signalling pathways and its activation have been addressed in AxD and, for this reason, will not be explained again in this section.

Characterized by severe cognitive decline, irritability, seizures and motor deterioration symptoms, such as spasticity and dystonia, this early-onset disease presents other symptoms due to its multisystem involvement [51, 123]. Cellular and morphological changes identified by neuroimaging and other assessment tools show encephalopathy, increased levels of IFN-α in the CSF [57, 101, 131], cerebral and brain stem atrophy, absence of myelin, calcification of basal ganglia and frontotemporal swelling with presence of cysts, although not always, [47, 89, 90, 127, 140, 168, 170, 188, 235, 245] in cerebral areas that coincide with the location of cysts observed in AxD, VWM and MLC [252].

9.8.1 Compromised Functions in AGS

TREX1 prevents the cytosolic accumulation of DNA and RNA, in physiological conditions. Mutations in this gene cause impairment of exonuclease [39, 150] and exoribonuclease function [263], which leads to the incorrect recognition of DNA and RNA as antigen [46]. This triggers the activation and/or deregulation of several innate immune signalling pathways, such as the cGAS–STING pathway [83], and increased production of monocytes, cytokines, among which interferon type 1 (IFN-α,) the biomarker for the disease, GFAP and other proteins [52, 53, 198, 250]. Previous studies show that in CNS, overproduction and release of IFN signature may be attributed to astrocytes and microglia. It was demonstrated that IFN-α co-localizes

with GFAP and disrupts astrocyte-specific protein function, in addition to triggering astrogliosis [198, 250]. Regarding the mutations in the other genes, it is still unclear the contribution of astrocytes to AGS.

9.9 Conclusions

Astrocytes are a heterogeneous cell population in terms of origin, morphology, function and even their responses to brain insults. They play a role in modulating cellular and molecular mechanisms, activity and morphology of the brain. In this review, we describe how astrocytic function and factors secreted by astrocytes in addition to interaction with axons, glial cells and non-neural cells contribute to integrity and maintenance of white matter in health. We show that, in disease, astrocytic dysfunction and dysregulation of these mechanisms trigger abnormal responses in the brain, possibly also leading to leukodystrophies. In addition, we suggest that astrocytes heterogeneity influence the phenotypic variation observed in astrocytopathies. AxD and AGS are leukodystrophies, characterized by cytokines upregulation, GFAP and IFN-α, respectively. VWM and MLC are disorders also driven by an astrocytic defect in ion-water homeostasis.

Together, these conclusions suggest that astrocytes play an essential role in the pathophysiology of selected leukodystrophies, the astrocytopathies. Therefore, comprehending astrocytes physiology, function and interactions with other cells may provide new potential therapeutic targets.

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Chapter 10
Astrocytes in Motor Neuron Diseases

Chiara F. Valori, Giulia Guidotti, Liliana Brambilla and Daniela Rossi

Abstract Motor neuron disorders are highly debilitating and mostly fatal conditions for which only limited therapeutic options are available. To overcome this limitation and develop more effective therapeutic strategies, it is critical to discover the pathogenic mechanisms that trigger and sustain motor neuron degeneration with the greatest accuracy and detail. In the case of Amyotrophic Lateral Sclerosis (ALS), several genes have been associated with familial forms of the disease, whilst the vast majority of cases develop sporadically and no defined cause can be held responsible. On the contrary, the huge majority of Spinal Muscular Atrophy (SMA) occurrences are caused by loss-of-function mutations in a single gene, *SMN1*. Although the typical hallmark of both diseases is the loss of motor neurons, there is increasing awareness that pathological lesions are also present in the neighbouring glia, whose dysfunction clearly contributes to generating a toxic environment in the central nervous system. Here, ALS and SMA are sequentially presented, each disease section having a brief introduction, followed by a focussed discussion on the role of the astrocytes in the disease pathogenesis. Such a dissertation is substantiated by the findings that built awareness on the glial involvement and how the glial–neuronal interplay is perturbed, along with the appraisal of this new cellular site for possible therapeutic intervention.

Keywords Astrocytes · Motor neuron · Amyotrophic lateral sclerosis · Spinal muscular atrophy · Transgenic animal models

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10.1 Amyotrophic Lateral Sclerosis

10.1.1 A Brief Introduction

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset progressive and fatal neurodegenerative condition, caused by the demise of both cortical and spinal cord motor neurons. This extensive degeneration causes the patients to suffer from a wide array of symptoms, including dysphagia (difficulty in swallowing) and dysarthria (problems with the muscles that help produce speech) in the case of the so-called bulbar-onset ALS as well as fasciculations (muscle twitching), tremors and muscular weakness in the case of the so-called spinal-onset ALS. Diagnosis is formulated upon meeting El Escorial and Airlie House criteria, which evaluate the distribution and the progression of muscular weakness, and combine a neurological evaluation with neuroimaging and electrophysiological assessments (reviewed in [106, 291]). On the basis of these criteria, epidemiologic surveys have estimated that ALS has a prevalence of 4–6 individuals per 100,000 individuals per year, making it the most prevalent form of adult-onset motor neuron disease. The disease predominantly presents itself when the patient is 40–60 years of age, often with a rapid progression. The unfolding of the disease is monitored using different rating scales (reviewed in [106]) to examine the progression of the patient disability. Death usually occurs by a respiratory failure within 3–5 years from the symptom onset. In about 50% of patients, the decline in the quality of life is further accelerated by the onset of behavioural and linguistic abnormalities, which are monitored using an ad hoc scale, the Edinburgh Cognitive and Behavioural ALS Screen [1]. The degree of cognitive impairment might become so severe to call for an additional diagnosis of Frontotemporal Lobe Dementia in about 15% of occurrences [197, 282].

The therapeutic approach to ALS is multidisciplinary and integrates symptomatic therapies to ease muscle spasticity, excessive drooling and depression, with non-invasive assisted ventilation and gastrostomy, the latter to ensure an adequate nutritional intake. Although this strategy helps to prolong the lifespan for a few months [169], it is not aiming at slowing down the neurodegenerative process. To this end, there are only two drugs approved by the U.S. Food and Drug Administration (FDA) for the treatment of ALS: riluzole, which prolongs survival of ALS patients in late stages of the disease [65], and edaravone, a more recently approved agent, which appears to be effective in a subset of patients at early stages of the disease [97]. Additional studies are, however, required to evaluate edaravone's full potential and to assess its long-term effects [3].

The development of effective disease-halting therapies is therefore urgently needed. To successfully reach this goal, the scientific community has to move forward in the process of pinpointing molecular and cellular mechanisms driving the neurodegenerative cascade. In the vast majority of cases, the disease appears sporadically (sALS) and several epidemiological studies have identified risk factors both non-modifiable (e.g., ageing; genetic polymorphism) and modifiable (e.g., environmental factors, such as exposure to heavy metals or pesticides; viruses); the latter

including also personal behaviour (e.g., smoking and vigorous physical activity). However, approximately 5–10% of patients have a familial form of ALS (fALS), where the disease is transmitted, mainly through an autosomal dominant pattern of inheritance. In the past few years, genetic studies have been able to link the disease with mutations in several genes, thus providing an explanation for almost all affected families. Interestingly, those genes encode proteins involved in specific cellular functions, such as redox homeostasis (*Superoxide dismutase 1* (*SOD1*) [240]); RNA/DNA metabolism (*TAR DNA binding protein 43* (*TARDBP*) [123, 268], *Fused in sarcoma* (*FUS*) [144, 292], *Matrin 3* (*MATR3*) [121], *T cell-restricted intracellular antigen-1* (*TIA1*) [163], *angiogenin* (*ANG*) [95], *hnRNPA2B1* and *hnRNPA1* [133]); vesicle trafficking/autophagy (*Alsin* (*ALS2*) [314], *Optineurin* (*OPTN*) [173], *TANK-binding kinase 1* (*TBK1*) [80], *Annexin 11A* (*ANXA11*) (Smith et al., 2017)); proteostasis (*valosin-containing protein* (*VCP/p97*) [120], *Ubiquilin 2* (*UBQLN2*) [48], *Sequestosome 1* (*SQSTM1*/p62) [67], *Ubiquilin 4* (*UBQLN4*) [61]); and cytoskeletal dynamics (*Profilin 1* [309], *Kif5A* [205]).

However, the most prevalent causes of fALS are mutations in *C9orf72* [47, 232], where the genetic lesion consists of the aberrant expansion of a hexanucleotide repeat (GGGGCC) sequence localized in the intronic/promoter region of the gene. It is presently highly debated as to whether toxicity arises from haploinsufficient protein expression or from the gain of a toxic function of RNA harbouring an expanded tract (formation of foci, accumulation of dipeptide proteins due to RNA translation or sequestration of RNA/DNA binding proteins; reviewed in [220]).

Neuropathological assessment of the central nervous system (CNS) from ALS patients reveals sclerosis of the pyramidal tract as well as motor cortex atrophy and severe loss of motor neurons associated with gliosis. Notably, the hallmark of surviving motor neurons is the presence of ubiquitin-positive proteinaceous inclusions. Although the composition of these aggregates has not been entirely detailed, in 97% of occurrences inclusions are enriched in the RNA/DNA binding protein TDP-43 [201]. The mechanisms driving protein accumulation into insoluble aggregates is still under investigation, though significant attention has been given to stress granules, i.e. membrane-less cytoplasmic organelles that transiently assemble upon different types of cellular insults. Stress granules are enriched in RNA-binding proteins (including many ALS-associated proteins, such as TIA1, TDP-43 and FUS) and trap most mRNAs in a translationally silent status until stress withdrawal (reviewed in [256]). Accumulating evidence suggests that, in ALS, those organelles cannot resolve, but they aberrantly evolve into pathological aggregates (reviewed in [275]).

These observations and the growing number of ALS-linked genes coding for DNA/RNA-binding proteins set the ground for the hypothesis that aberrant RNA metabolism is playing a role in motor neuron demise along with oxidative stress, excitotoxicity, DNA damage, impaired axonal transport and mitochondrial dysfunction (reviewed in [83, 106]).

In addition to these events, occurring cell-autonomously within motor neurons, the articulated and pivotal contribution of glial cells to motor neuron dysfunction and demise has been increasingly acknowledged. In particular, neuroinflammation-related events, oligodendrocyte cell death and phenotypic changes of the astrocytes

have been reported (reviewed in [106]). Since each glial cell subpopulation is physiologically empowered to perform specialized tasks to ensure an optimal environment for neuronal survival and activity (reviewed in [301]), glia are likely to offer a distinct and specific contribution to neuronal loss under pathological circumstances. In the next sections of this chapter, we will focus on the impact of the astrocytes, first reporting histological findings describing astrocyte pathology in human specimens; then gathering genetic experiments supporting the hypothesis of their active involvement in ALS pathogenesis. Lastly, we will tackle mechanistic studies aiming at elucidating key molecular players while discussing the potential of astrocytes as a therapeutic target. With regard to microglia and oligodendrocytes, we refer the reader to other reviews addressing the role of these glial cell types in ALS [37, 145, 206].

10.1.2 Role of the Astrocytes in ALS Pathogenesis

10.1.2.1 Evidence from ALS Patients

For decades, astrocytosis has been recognized as a histological finding in ALS. In particular, neuropathologists have provided accurate morphological descriptions of this phenomenon, owing to a thorough investigation of different brain areas [125, 143, 196, 199]. Intriguingly, the characterization of astrocytosis in the spinal cord of ALS patients [253] allowed the early formulation of two key hypotheses. First, the morphology of these cells was suggesting an ongoing active process of remodelling, rather than a purely passive reaction. Second, because astrocytosis and the prevalence of dystrophic neurites were exacerbated where the corticospinal tract entered the grey matter, it was suggested a 'dying back' mechanism in the pathogenesis of ALS [253]. Moreover, the physiologically close association between astrocytes and motor neuronal cell bodies was partially lost in sALS spinal cord specimens, where a more loose interaction was reported [208]. Finally, isolated degenerating astrocytes were identified in the motor neuronal microenvironment in sALS spinal cords [172]. Although of outstanding importance, these findings depict an end-stage scenario in ALS, while it is of primary relevance to gain information about earlier events. The development of the glial [^{11}C]-PBR28 [6, 7, 321] or, even better, of the astrocyte-specific [11C](L)-deprenyl-D2 PET [119] radioligands, coupled to recent advances in neuroimaging techniques, paved the way to detect the development and localization of astrocytosis in vivo. These tools are indeed expected to enable the correlation between astrocytosis and neuronal loss during disease progression in patients. Furthermore, they hold the potential to monitor the efficacy of glial-targeted therapies.

Abnormal morphological changes are not the only neuropathological evidence of astrocyte involvement in ALS, but molecular and functional abnormalities were also reported. For example, hyaline [127] and ubiquitinated protein inclusions were shown not to be an exclusive hallmark of motor neurons, but were observed also in the astrocytes [31, 172, 177, 217]. In fALS-*SOD1* and sALS cases such inclusions were

described to be enriched in misfolded SOD1 protein [76], despite early appraisals reporting reduced or absent SOD1 [208] and increased SOD2 immunoreactivity [26]. Taken together, this amount of evidence suggests that astrocytes themselves are subject to stress. Among others, oxidative stress conditions were proposed to strike the astrocytes, as indicated by their increased expression of several proteins induced by reactive oxygen species (ROS). These proteins include the calcium-binding protein S100beta [183]; cyclooxygenase 2 (COX-2) [166]; iNOS and nitrotyrosine [251]; and nNOS [8] in sALS patients, together with different advanced glycation and lipoxidation end products in fALS cases with SOD1 inclusions [127, 257–259]. However, spinal cord astrocytes have been described to counteract these insults by mounting a protective response via the upregulation of the c-Jun N-terminal kinases (JNKs) and the nuclear translocation of the nuclear factor kB (NF-kB) [182].

The early observation that ALS patients show abnormally elevated concentrations of the excitatory amino acids glutamate, aspartate and their metabolites in their cerebrospinal fluid (CSF) [244] led to the core discovery that astrocytes are functionally impaired, as they display reduced expression of the high-affinity plasmalemmal glutamate transporter EAAT2 [31, 243]. This protein is critical to quench glutamatergic synaptic signalling, thereby preventing excitotoxicity and modulating the energy metabolism in response to neuronal activity (reviewed in [236]). Given these critical roles in preserving brain homeostasis, it is not surprising that EAAT2 dysfunction could contribute to the pathogenesis of a growing number of CNS disorders (reviewed in [273]). In addition, glutamatergic signalling in astrocytes was reported to be perturbed as a consequence of the upregulation of several metabotropic glutamate receptors (mGluR1alpha, mGluR5, mGluR2/3) in the spinal cord of ALS patients [12, 172].

10.1.2.2 Generation and Characterization of Transgenic Animal Models

The evidence gathered from human post-mortem material collectively supports the hypothesis that the ALS-causing mechanisms are hitting also the astrocytes. However, neuropathological assessments of autoptic tissues allow to describe only the end-stage situation and do not permit to unveil whether astrocytic lesions can be considered just as ‘collateral damage events’ or whether astrocyte malfunctioning directly and critically contributes to neuronal demise. To shed light on these issues and to reproduce the complexity of the CNS, one needs to model the disorder in intact organisms, such as transgenic animals. These were first made possible by the milestone discovery of mutations in the *SOD1* gene in a subset of fALS [240]. The earliest and most extensively characterized model of ALS is a transgenic mouse that ubiquitously expresses high amounts of the mutant human SOD1 protein (hSOD1) carrying a single amino acid substitution at position 93, where glycine is substituted by alanine ($hSOD1^{G93A}$; [102]). These mice develop an early onset and quickly progressing phenotype consisting of a rapid escalation of tremors, muscular weakness, motor impairment and, finally, premature death. Histological analysis has revealed

substantial motor neuron loss, presence of ubiquitin-positive inclusions in the surviving motor neurons as well as in glial cells [177, 217, 242, 270], astrocytosis and microgliosis [105], thus mimicking the human condition. A remarkably similar phenotype could also be described in mice overexpressing hSOD1^{G37R} [308] or hSOD1^{G85R} [31], where glycine at position 37 or 85, respectively, was replaced by arginine. Intriguingly, the latter strain was reported to develop astrocytic SOD1- and ubiquitin-positive inclusions as the earliest indication of the disease. Such observation has two implications: first, it suggests that astrocytes are directly damaged by mutant human SOD1 expression; second, it supports the hypothesis that motor neuron demise may be a consequence of glial dysfunction [31]. To test this theory, the astrocyte-specific GFAP promoter was used to drive the expression of mouse SOD1^{G86R}, the murine orthologue of hSOD1^{G85R}. Although these mice displayed astrocytosis, they failed to develop motor impairment. Furthermore, histological assessments did not reveal motor neuron loss or microgliosis, thus suggesting that the restricted expression of mutant SOD1 in the astrocytes is not sufficient to cause neurodegeneration [93]. Subsequent genetic experiments, however, argued against this early conclusion. In particular, thorough histological analyses of chimeric mice revealed that wild-type motor neurons display pathological ubiquitinated protein inclusions when surrounded by mutant SOD1-expressing non-neuronal cells. Complementary, the survival of mutant SOD1-expressing neurons was prolonged in the presence of wild-type non-neuronal cells [41]. In keeping with this evidence, selective ablation of different mutant SOD1s from astrocytes slowed down disease progression and extended survival in ALS mouse models [303, 310]. Transplantation studies provided further evidence in support of the hypotheses that ALS astrocytes are intrinsically neurotoxic and that healthy cells retain their neuroprotective phenotype in an ALS environment. Briefly, transplanting hSOD1^{G93A}-expressing astrocyte precursors in the spinal cord was reported to induce an ALS-like phenotype with motor neuron degeneration in wild-type rats [213], while introducing wild-type astrocyte precursors extended the lifespan in hSOD1^{G93A} rats [153]. More recently, the tremendous advancements in the field of stem-cell technology enabled researchers to perform analogous transplantation experiments with human cells. In particular, induced pluripotent stem cell (iPSC)-derived glial precursors from a SOD1^{D90A} fALS patient [38] or a sALS patient [228] transplanted into the spinal cord of wild-type mice predominantly differentiated into astrocytes and induced both motor impairment and histological signs of motor neuron degeneration. Complementary to this evidence, iPSC- or human embryonic stem-cell-derived healthy donor glial precursors transplanted in the spinal cord of hSOD1^{G93A}-overexpressing mice at the early symptomatic stage ameliorated the mouse phenotype [116, 137].

Although these studies provide indisputable evidence that astrocytes play a role in the pathogenesis of ALS, they almost exclusively focus on the ALS-*SOD1* subtype. Therefore, it is reasonable to postulate that their relevance might be restricted to this specific form of the disease. The continuous development of newer models aiming at recapitulating other familial forms, such as ALS-*TDP-43*, is meant to allow additional investigations on astrocyte dysfunction in order to determine whether they represent core mechanisms of ALS pathogenesis. An important contribution to

the comprehension of this issue came from the generation of transgenic rats with astrocyte-specific inducible expression of human TDP-43 harbouring the substitution of methionine to valine at position 337 (hTDP43^{M337V}) [279]. Upon transgene induction, the animals underwent a rapid deterioration of their motor functions with motor neuron loss, accumulation of ubiquitinated inclusions in the astrocytes, astrocytosis and microgliosis [279]. Since it has been proposed that TDP-43 mutations lead to a loss of its function, it is particularly relevant that mice with TDP-43 knockdown predominantly occurring in the astrocytes developed motor dysfunction, electromyographical abnormalities, paralysis and spinal cord motor neuron loss [311]. Furthermore, neuromuscular junction abnormalities were present in fruit flies with expression of different ALS-associated TDP-43 variants in various glial populations, including the astrocytes [64].

From this large amount of evidence, it clearly emerges that fALS-associated proteins can trigger astrocyte dysfunction, and their aberrant phenotype is critical to induce motor neuron demise. Furthermore, healthy astrocytes retain their protective abilities even in a disease microenvironment, an observation that has tremendous implications for the development of new cell therapies. One may hypothesize that implanting healthy cells should halt the disease progression in ALS patients. To explore this therapeutic option in the clinical practice, a phase I/IIa clinical trial (ClinicalTrials.gov Identifier: NCT03482050) has recently started (April 2018) recruiting patients to assess not only safety and tolerability but also disease progression rates upon intrathecal administration of human stem-cell-derived astrocytes.

10.1.2.3 Intrinsic Dysfunctions of the Astrocytes

To fully exploit the potential of astrocytes as targets for ALS therapy, it would be extremely valuable to understand not only the molecular mechanisms underlying astrocyte abnormalities but also how their communication with motor neurons is impaired. Several lines of research are trying to give an answer to the following key questions: are astrocytes directly suffering from fALS-driven toxicity? are they releasing toxic substances or, when stressed, do they become unable to provide trophic and metabolic support to neurons?

As previously mentioned, ubiquitinated protein inclusions were identified in the astrocytes of both human post-mortem material and transgenic animal models of ALS [31, 127, 136, 172, 177, 217]. The biological relevance of such inclusions has remained elusive until our group demonstrated that cells harbouring ubiquinated protein aggregates are degenerating astrocytes expressing also activated caspase-3, a marker of apoptosis. Damaged astrocytes appear at the pre-symptomatic stage, prior to the loss of motor neuronal cell bodies, and their number ramps up during disease progression [172, 242]. Remarkably, it has been recently demonstrated that the accumulation of such apoptotic astrocytes is also shortening the lifespan of hSOD1^{G93A} mice [136], thus consolidating the hypothesis that neuroglia degeneration is an active driver of disease progression. Furthermore, in hSOD1^{G93A} mice at the end stage, it has been shown that activated astrocytes overexpress some BH3-only members of

the Bcl-2 family proteins, which are normally channelling detrimental signals from mitochondria into an apoptotic pathway of cell death [58, 140]. However, those cells do not simultaneously express further markers of apoptosis nor display aberrant morphologies, thus suggesting that such proteins might possess other functions than apoptosis induction [58, 140], or they might be marking cells which are committed to die, but have not yet fully executed the cell death program. Finally, evidence of astrocyte degeneration came from the characterization of human astrocytes differentiated from TDP43^{M337V} [255] and VCP^{R191Q} (arginine to glutamine substitution in position 191) or VCP^{R155C} (arginine to cysteine substitution in position 155)—expressing inducible pluripotent stem cells (iPSCs) [104].

From a mechanistic point of view, the observation that apoptotic astrocytes are located in the immediate neighbourhood of glutamatergic terminals suggested to investigate whether hSOD1^{G93A} astrocytes were susceptible to glutamate toxicity. In vitro experiments demonstrated that the viability of ALS astrocytes is unaffected under standard culturing conditions. On the contrary, glutamate triggers astrocyte apoptosis by activating its metabotropic receptor 5 (mGluR5) followed by downstream aberrant calcium (Ca^{2+}) release from the intracellular stores [172, 242] (Fig. 10.1), a phenotype recently linked to the downregulation of protein kinase C isoform epsilon [297]. Interestingly, in the past few years, the evidence of abnormal Ca^{2+} signalling in ALS astrocytes has been confirmed and extended to other experimental settings by several independent groups. In particular, in hSOD1^{G93A} expressing astrocytes, it has been discovered a chain of events triggered by the excessive activity of the store-operated Ca^{2+} entry mechanism, which leads to Ca^{2+} overfilling of the endoplasmic reticulum (ER), thereby producing an augmented Ca^{2+} release from the ER in response to ATP stimulation [5, 128] (Fig. 10.1). The overexpression of Connexin-43 [5], a molecule responsible for the formation of gap junctions and hemichannels within the astrocyte network, has been also implicated in the elevation of intracellular Ca^{2+} levels. Moreover, sustained Ca^{2+} increases deriving from the extracellular environment have been observed in the astrocytes upon treatment with recombinant mutant SOD1 [187].

Curiously, degeneration is not the only consequence of the overexpression of ALS-associated proteins in the astrocytes. Another subpopulation of astrocytes with high proliferation rate could be identified in hSOD1^{G93A} transgenic rats [52] and mice [39]. It is yet to mention that in vivo ablation of proliferating astrocytes did not ameliorate the ALS phenotype in different mouse models [152], thus suggesting that the contribution of such glial cell population to disease pathogenesis is likely limited.

In the wake of recent discoveries pointing at stress granules as precursors of pathological lesions in ALS, dynamics of these membrane-less organelles has been investigated in relevant neuronal populations as well as in the astrocytes, thus uncovering their distinctive behaviour. In particular, it has been recently demonstrated that astrocytes resolve stress granules with faster kinetics than neurons [131].

Finally, to provide a comprehensive and unbiased picture of the repercussions of ALS-associated proteins on the astrocyte homeostasis, different groups have set out to investigate transcriptional, translation and metabolic changes either in cell

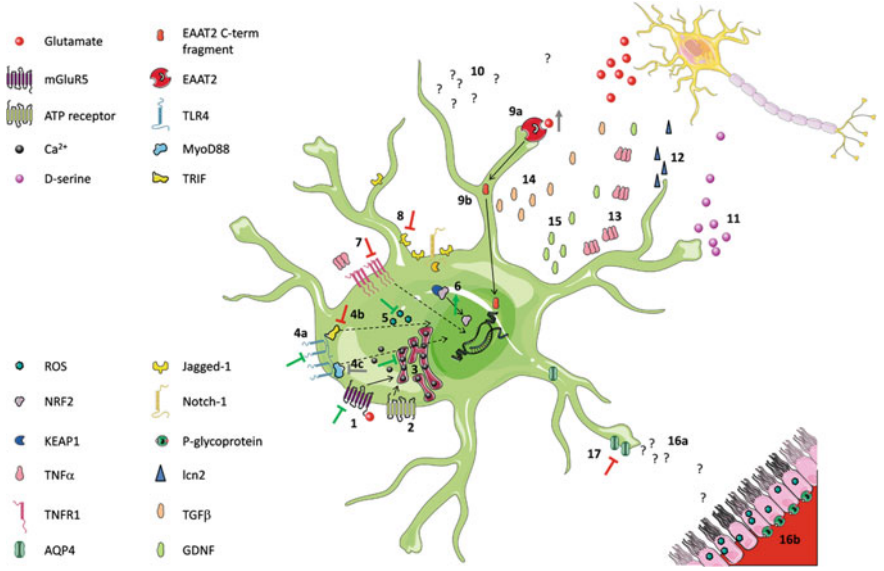

Fig. 10.1 Aberrant pathways in ALS astrocytes: a window of therapeutic opportunity. In ALS astrocytes, several signalling pathways are intrinsically aberrant. Activation of mGluR5 (1) or ATP receptors (2) leads to abnormal calcium signalling (3) and apoptosis. Moreover, upregulated TLR4 (4a) signals through its adaptor TRIF (4b) to counteract cell demise or through MyoD88 (4c). Accumulation of reactive oxygen species (ROS; 5) and subsequent activation of the transcription factor NRF2 (6), which is released from its KEAP1 inhibitor, are also hallmarks of ALS astrocytes. Upregulation of TNFR1 (7) and the Notch ligand Jagged-1 (8) have also been described. In terms of cross-talk with neurons, ALS astrocytes display reduced expression of the glutamate transporter EAAT2 (9a), which become abnormally SUMOylated and cleaved (9b). The excitotoxic milieu is then worsened by the release of an unidentified proteinaceous factor (10) and D-serine (11). Toxicity triggered by factors including lipocalin (lcn2; 12), TNFα (13) and TGFβ (14) is opposed by the release of the neurotrophic GDNF (15). Astrocytes also contribute to the impairment of the BBB by releasing unidentified neuroinflammatory factors (16a) that lead to the upregulation of the transporter P-glycoprotein (16b). Finally, upregulation and mislocalization of the water channel aquaporin-4 (AQP4; 17) is also contributing to BBB leakage. Therapeutic intervention (inhibition, blunt arrow; activation, pointed arrow) on these pathways proved to be beneficial (green), detrimental (red) or ineffective (grey) on disease progression. Details and references in the text

culture [113, 165] or in animal models along the disease progression [14, 69, 271]. In cell culture, among the differentially expressed genes, there is an enrichment in those encoding for secreted proteins. More specifically, in response to pathogenetic TDP-43 expression, neuroprotective mediators are downregulated whereas neurotoxic factors, such as lipocalin-2 (*lcn2*) and chitinase-3-like protein 1 (*Chi3l1*), are upregulated [113]. Furthermore, several metabolic pathways become dysregulated in response to glutamatergic stimuli or in co-culture with motor neurons, particularly the cellular shuttling of lactate between astrocytes and motoneurons [165]. Astrocytes captured from hSOD1^{G93A} mice display an analogous metabolic dysregulation as well as a shift from a neuroprotective towards neurotoxic phenotype already at the

pre-symptomatic stage [69]. Furthermore, their phenotype shows further changes along disease progression, when ALS astrocytes display faulty cholesterol homeostasis due to defective nuclear translocation of the sterol regulatory binding protein 2 (SREBP2), along with a pro-inflammatory phenotype characterized by enhanced phagocytosis and lysosomal activity [14]. Importantly, upregulation of the inflammatory process and downregulation of metabolism has been observed also in hSOD1^{G37R} astrocytes [271]. The peroxisome proliferator-activated receptors (PPARs) and the liver X receptors (LXRs) have been proposed as master regulators of the observed transcriptional change [271], although nuclear translocation of PPARs does not seem to enhance in spinal cord astrocytes from another mutant SOD1 mouse model of ALS (i.e. hSOD1^{G93A}) during disease progression [18].

These latest 'omics' studies not only describe how astrocytes adapt to the expression of noxious proteins but shed some light on how their interactions with neighbouring cells may be affected, in terms of both (i) receiving and transducing signals coming from the extracellular environment and (ii) providing an adequate response. These abilities are particularly critical in the context of astrocyte-motor neuron crosstalk. Thus, in the next paragraph, we will discuss how they might have lost their capacity to maintain a supportive microenvironment for motor neurons while gaining neurotoxic properties.

10.1.2.4 Astrocyte-Motor Neuron Communication Is Impaired in ALS

The evidence outlined so far suggests that the expression of ALS proteins in astrocytes affects their bidirectional communication with motor neurons. Many independent groups have demonstrated that ALS astrocytes are toxic to the motor cells in mice [25, 50, 69, 132, 198, 225, 280] and, more importantly, in human co-culture systems [49, 103, 167, 180, 231, 267]. Moreover, such toxicity appears to be mediated by one or more soluble factors, as conditioned medium from ALS-*SOD1* (hSOD1^{G93A} or hSOD1^{G86R}) or ALS-*TDP-43* (hTDP43^{A315T}) astrocytes is sufficient to induce motor neuron hyperexcitability, mitochondrial damage with enhanced oxidative stress and activation of a deadly c-Abl signalling cascade [81, 238, 239]. The toxicity of conditioned medium from hSOD1^{G93A}-expressing astrocytes has been confirmed also in vivo through its chronic administration in rat lumbar spinal cords, where it rapidly induces motor dysfunction and motor neuron loss [230]. These findings have been further expanded by exploring the toxicity of the astrocyte medium from either mouse cells expressing hFUS^{R521G} (position 521: arginine to glycine substitution) [132] or from iPSC-derived astrocytes harbouring a repeat expansion in the *C9orf72* gene [164].

These observations globally prompted the hunt for neurotoxic factor(s), and several molecules have been subsequently implicated in the harmful cascade, fuelling an extensive scientific debate along the last years. Astrocytes are well known to release a wide variety of bioactive molecules via the activation of an array of mechanisms (reviewed in [300]). The most obvious candidate to investigate in the context of ALS was initially the excitatory amino acid **glutamate**, a molecule whose

implication is supported by several findings. First, it is well known that excessive glutamatergic stimulation triggers neuronal demise through a distinctive mechanism of cell death named 'excitotoxicity'. Second, in ALS patients, astrocytes display reduced expression and function of the astrocyte-specific glutamate plasmalemmal transporter EAAT2 [31, 243], a condition that leads to CSF accumulation of glutamate [244]. Importantly, deficient expression of EAAT2 (GLT-1 in rodents) has been consistently reported in several animal models of both ALS-*SOD1* [31, 100, 112, 215, 243] and ALS-*TDP-43* [51, 279] (Fig. 10.1). As yet, there is no common consensus about the pathway that mediates such depletion, and a large number of potential mechanisms have been implicated. Early evidence excluded that EAAT2 downregulation had a genetic origin, as ALS patients do not segregate *EAAT2* mutations [9, 117]. Recently, it has been discovered that the transcription factor Yin Yang 1 (YY1) is a negative regulator of *EAAT2* expression [126]. In hSOD1^{G93A} mouse astrocytes, a detrimental signalling cascade is set in motion by the overexpression of astrocytic elevated gene 1, leading to nuclear accumulation of YY1 and subsequently reduced transcription of *EAAT2* [315]. Furthermore, other groups have scrutinized all the steps affecting the maturation and the stability of *EAAT2* primary transcript, starting from the occurrence of alternative splicing events. Thus, Lin et al. demonstrated the accumulation of aberrant mRNA species that limits the availability of a translationally competent transcript, either by its enhanced degradation or through a dominant-negative effect [160]. However, subsequent studies revealed that those mRNA species are not specific for ALS cases, as they can be detected also in cohorts of controls and patients affected by other CNS disorders [99, 111, 181]. Another important post-transcriptional modification of the mRNA may be RNA editing. Flomen et al. detected an aberrant editing event taking place in *EAAT2* primary transcripts, specifically in affected areas of the CNS of ALS patients. This modification generates an alternative polyadenylation site that shortens the mRNA half-life [72]. Finally, *EAAT2* mRNA could be regulated also by signalling from neurons. In particular, glutamate released upon neuronal activation induces the transcription of *EAAT2* [313] and modulates its mobility into the membrane [4]. Intriguingly, transcriptional activation is mediated by the expression of the nuclear protein Kappa-B Motif-Binding Phosphoprotein (KBBP, [313]) the murine homolog of the heterogeneous nuclear RNA/DNA-binding protein K (hnRNP K), a protein recruited by RNA foci in ALS patients with *C9orf72* hexanucleotide expansion [43] and showing a complex regulatory interplay with TDP-43 [194, 195].

However, glutamate might not be the only molecule released by neurons and acting on the astrocytes to modulate EAAT2 activity. MicroRNA 124a (miR-124a) is an additional interesting candidate. It has been reported that this noncoding RNA is enclosed in exosomes released by neurons and internalized by the astrocytes, where it positively regulates EAAT2 expression [193]. In the context of ALS, miR-124a is downregulated [193, 320], an event that can possibly cause the loss of *EAAT2*. MiR-124a downregulation might have even a broader impact on the astrocyte phenotype, as it also regulates the expression of the transcription factors *Sox2* and *Sox9,* which govern astrocyte differentiation [320]. Importantly, astrocytes themselves are competent for the release of exosomes (reviewed in [300]) and, although an early report

did not unravel any abnormalities in the profile of miRNAs released from hSOD1^{G93A} astrocytes [122], further studies are needed to investigate this topic thoroughly.

The studies discussed so far mainly focused on the possible modulation of EAAT2 expression and function by acting at various levels of its transcript metabolism. However, its activity can be limited also at the protein level by post-translational modifications. For example, EAAT2 was reported to be cleaved by caspase-3 [28] and subsequently SUMOylated on the resulting C-terminal fragment [89] (Fig. 10.1). In hSOD1^{G93A} astrocytes and spinal cord, caspase-3 cleaves EAAT2 after an aspartic acid in the position 504, thus inactivating the transporter [28]. More recently, hSOD1^{G93A} mice have been crossed with knock-in animals, where the endogenous *EAAT2*$^{WT/WT}$ has been replaced with the *EAAT2*$^{D504N/D504N}$ variant (position 504: aspartic acid to asparagine substitution), which destroys the caspase-3 consensus sequence and makes the protein resistant to cleavage without altering its properties as a transporter. The resulting mice display delayed disease progression and increased lifespan, in the absence of significant neuroprotection, thus demonstrating that EAAT2 inactivation drives the late phase of the disease [241]. Furthermore, in cell cultures and hSOD1^{G93A} spinal cords, the SUMOylated C-terminal fragment redistributes to the nucleus of astrocyte, where it triggers glial- and neurotoxicity [75, 89]. More recently, it has been demonstrated that also a fraction of the full-length EAAT2 protein is SUMOylated, and this latter modulates its insertion into the cell membrane. Interestingly, the extent of this post-translational modification is unaffected by the expression of hSOD1^{G93A} in astrocytes and does not change during the course of ALS in mice [74].

From a therapeutic standpoint, EAAT2 dysfunction is a particularly attractive target and several approaches have been attempted to rescue its downregulation, hoping to significantly halt disease progression. A validation of this hypothesis came from early studies in transgenic mice. In particular, genetic overexpression of EAAT2 itself [100] or of its modulator Peroxisome Proliferator-Activated Receptor γ Coactivator 1α (PGC1α; [156]) in hSOD1^{G93A} mice improved their motor functions. More recently, EAAT2 overexpression was achieved through intraspinal injection of a viral vector driving its expression specifically in the astrocytes at the symptomatic stage of hSOD1^{G93A}. Unfortunately, despite successful and sustained astrocyte transduction, no prolonged survival was recorded [154]. In parallel, attempts to identify small drugs able to pharmacologically enhance EAAT2 activity gained momentum from the seminal discovery that β-lactam antibiotics increase EAAT2 expression. In particular, the cephalosporin ceftriaxone gave extremely promising results in terms of lifespan extension when chronically administered to hSOD1^{G93A} mice [245]. Since antibiotics have been long used in clinical practice, a phase I/II clinical trial was promptly started and demonstrated that ceftriaxone has a very good safety profile and can reach the CNS in therapeutic amounts in ALS patients [23]. It was therefore quite disappointing when ceftriaxone failed to provide any therapeutic benefit in a phase III clinical trial aiming at assessing its efficacy [45]. Despite this shortcoming, other drugs have been investigated in preclinical testing such as harmine [155], LDN/OSU-021320 [42, 139] and, more recently, MC1568 [146]. The latter drug is an inhibitor of class II histone deacetylases (HDACs), enzymes regulating gene tran-

scription by shaping the epigenetic landscape and involved in a growing number of neurodegenerative conditions (reviewed in [276]). In primary cultures of mouse glia, MC1568 administration increased both EAAT2 expression and its SUMOylation, thereby failing to boost transport activity. Nevertheless, upon chronic administration in hSOD1^{G93A} mice, this drug sustained EAAT2 expression and activity, thus inducing a transient improvement of the motor function. Unfortunately, this beneficial effect was only temporary, as the treatment was unsuccessful in extending the lifespan [32, 146]. Taken together, these studies suggest that post-translational modifications play a crucial role in tuning EAAT2 function. Therefore, it is tempting to speculate that drugs that promote glutamate transport should be associated with a SUMOylation inhibitor to achieve a successful therapeutic outcome in ALS. Intriguingly, glutamate accumulation is also a feature of hTDP-43^{WT} overexpressing mice, even in the absence of detectable EAAT2 loss. In this specific model, the amino acid accumulation is attributed to astrocyte malfunction and it is coupled with reduced glutamate metabolism [109].

The evidence of a role for excitotoxic stress in the pathogenesis of ALS was further reinforced by the discovery that ALS astrocyte release D-serine, an amino acid necessary for the full activation of neuronal N-methyl-D-aspartate (NMDA) glutamatergic receptors [249, 250]. Intriguingly, genetic analysis of an ALS family could identify a single loss-of-function mutation (position 199: aspartate to tryptophane) in the gene coding for the enzyme D-aminoacid oxidase (DAO), deputed to the catabolism of D-serine [190]. Furthermore, co-culture experiments demonstrated that the expression of this hDAO^{D199W} mutant in astrocytes is sufficient to trigger impaired autophagy, ubiquitinylated protein accumulation and, ultimately, motor neuron cell death [190, 221]. In vivo, hDAO^{D199W} overexpressing mice developed motor dysfunction and age-dependent motor neuron loss [138]. Curiously, crossing these animals with hSOD1^{G93A} overexpressing mice had only a modest worsening of the phenotype in females, while the severity of motor dysfunction was unaffected in males [138]. In addition, two different viral-mediated gene therapy approaches, boosting DAO expression, proved to be neuroprotective and led to extended lifespan in hSOD1^{G93A} mice [161, 304]. Finally, an intriguing link between D-serine overproduction and EAAT2 dysfunction has been proposed in hSOD1^{G93A} expressing astrocytes by showing aberrant expression of the protein interacting with C kinase 1 (PICK1), an interacting partner of both EAAT2 and serine racemase (i.e. the enzyme converting L-serine into D-serine) [73]. Further functional studies are, however, necessary to clarify the mechanistic relevance of such early observation in the context of ALS pathogenesis.

Astrocytes can modulate excitotoxicity also by regulating the composition of AMPA receptors on motor neurons, thus tuning their vulnerability to glutamatergic stimuli. In particular, they control the expression of the GluA2 subunit, the presence of which renders AMPA-type glutamate receptors impermeable to calcium influx from the extracellular environment under physiological conditions and, therefore, it increases the resistance to the glutamatergic insult [266]. Remarkably, hSOD1^{G93A} [290] and hFUS^{R521G} [132] astrocytes lose their ability to induce upregulation of GluA2 in neurons which, in turn, become more vulnerable to excitotoxic stimuli.

Increased production of **ROS** has been extensively investigated as another possible culprit of astrocyte-mediated neuronal toxicity, and various species have been detected in different familial subtypes of ALS [35, 81, 167, 189, 238, 239, 294] (Fig. 10.1). Consistently, increasing astrocytic antioxidant activity through pharmacological or genetic approaches showed neuroprotective potential [35, 167, 189, 223, 294].

A particularly appealing target was identified in the transcription factor nuclear erythroid 2-related factor 2 (NRF2), a master regulator of the oxidative stress response in the astrocytes. The regulation of this molecule and its target genes has been described in detail elsewhere [158]. Briefly, under physiological conditions, NRF2 is sequestered in the cytoplasm by its inhibitor Kelch-like ECH associated protein 1 (KEAP1) (Fig. 10.1). Upon chemical or oxidative insult, KEAP1 is rapidly degraded and NRF2 can translocate to the nucleus where it competes with Broad-complex, tramtrack, bric-à-brac (BTB) and CNC homology 1 (BACH1) to interact with small Maf proteins. Such interaction leads to the formation of heterodimers that can bind to the antioxidant response element (ARE) in the promoter region of its target genes, ultimately boosting their expression. Several studies of human genetics [21] as well as investigations performed in both cell and animal models [56, 94, 135, 188, 194, 224, 229, 278, 302] and in human post-mortem material [15, 248] converge to suggest that the NRF2 signalling is affected in ALS and could be exploited as a therapeutic target. Several compounds apt at sustaining NRF2 activity were identified in vitro and, subsequently, tested in hSOD1^{G93A}-expressing mice. Regrettably, the outcome of these treatments revealed only a modest improvement in motor performance and, in some cases, in survival [68, 175, 202]. In keeping with this, NRF2 overexpression in neurons through genetic manipulation [296] or viral-mediated gene therapy [200] could not extend lifespan in ALS mice. Furthermore, direct NRF2 ablation in hSOD1^{G93A} mice did not accelerate disease progression [101]. However, selective NRF2 activation in astrocytes had a clear neuroprotective effect in co-cultures [53, 293, 295] and in vivo, extending the lifespan of hSOD1^{G93A} mice [294]. Taken together, this amount of evidence has profound implications for the design of disease-modifying therapies based on tackling oxidative stress, namely, the therapeutic agent should specifically target the astrocytes. Furthermore, one should consider that not every target giving promising results in astrocyte cell cultures is confirmed in animal models [223]. When oxidative stress escapes the control systems, it can exert various detrimental effects by an autocrine/paracrine mechanism. For example, it was reported to cause defective glutamate homeostasis by reducing EAAT2 activity, an event that has been demonstrated in cells expressing different ALS-associated mutant hSOD1 [284–286]. These early findings set the ground for a novel gene therapy approach that was recently developed. Specifically, lentiviral vectors were designed to deliver *EAAT2*, *Glutamic dehydrogenase2 (GDH2)* and *NRF2*. In hSOD1^{G93A} astrocytes co-cultured with the motor neuron-like NSC-34 cells, only the simultaneous administration of these three vectors to glial cells conferred protection against a glutamatergic insult. Moreover, the cocktail ameliorated motor dysfunction, delayed onset and prolonged survival in vivo [20].

Since activation of astrocytes is part of the inflammatory response in the CNS, a number of **neuroinflammatory molecules** have also been investigated as putative neurotoxic agents in the astrocyte-driven neuronal demise. First, attention was given to the eicosanoid class and, in particular, to the arachidonic acid-derived metabolites prostaglandin (PG) D_2 and E_2, whose receptors are upregulated in hSOD1^{G93A} astrocytes and neurons [49, 141, 157]. Notably, delayed disease onset and prolonged survival were obtained by crossing ALS mice with animals devoid of the PGE_2 receptor EP2 [157]. Mechanistically, it has been speculated that pre-stimulation with low doses of PGE_2 can induce its EP2 receptor, thereby exposing neurons to the toxic action of a subsequent prostaglandin challenge [141]. More recently, the expression of the catabolic enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) was reported to increase with the disease progression in the spinal cord of hSOD1^{G93A} mice, and its immunoreactivity was associated with astrocytes in the white matter [191]. This suggests an unbalance between the synthesis and degradation of PGE_2 in the spinal cord. Remarkably, arachidonic acid serves as a precursor also for another class of inflammatory mediators, namely, leukotrienes. In this biochemical metabolic cascade, it is processed by different lipoxygenases to generate intermediates known as 12- and 15-hydroxyeicosatetraenoic acid (12-HETE; 15-HETE), which were reported to accumulate in the brain of hSOD1^{G93A} mice with the disease progression. Importantly, pharmacological treatments with nitrosylated fatty acids, such as nitro-oleic acid (NO_2-OA), prevented 12-HETE accumulation and improved motor function of ALS mice [283]. Remarkably, this compound is activating NRF2 signalling in hSOD1^{G93A} astrocytes, thereby blocking their toxicity towards neurons in cell cultures [53] and in vivo [283]. These findings suggest that drugs simultaneously tackling different aspects of astrocyte malfunction might grant a better rescue of their toxic phenotype.

A particularly interesting neuroinflammatory and neurotoxic factor is the secreted lcn2 protein, which has been implicated in a wide variety of cellular processes within the CNS, both under physiological and pathological conditions (reviewed in [118]). In the context of ALS pathogenesis, lcn2 is strongly upregulated in the astrocytes in rodent models of different subtypes of fALS, namely, ALS-*SOD1*, ALS-*TDP-43* and ALS-*FUS* [24, 113, 279] (Fig. 10.1). In vitro studies allowed depicting a mechanistic loop where neuronal expression of ALS-associated TDP-43 and FUS mutants not only induce the astrocytic release of lcn2, but also sensitize neurons to lcn2 toxicity [24]. Further investigations are certainly needed to identify the missing pieces of this puzzle, though the reported observations seem to suggest that testing an lcn2 receptor antagonist could be a possible strategy to halt ALS progression.

Another class of inflammatory molecules that could mediate neurotoxicity is that of cytokines. hSOD1^{G93A} astrocytes were in fact reported to release interferon γ (IFNγ), which triggers a detrimental signalling cascade on motor neurons expressing the lymphotoxin-β receptor (LT-βR) and the adapter Light [2]. Interestingly, disrupting this axis by genetic ablation of *Light* [2] or by infusing IFNγ neutralizing antibodies in the CNS ameliorates the phenotype of hSOD1^{G93A} mice [210]. More complex is the role played by the cytokine Tumor Necrosis Factor α (TNFα; Fig. 10.1) which, in addition to its roles as immunomodulatory factor (reviewed in [124]), is

also critically modulating neurotransmission (reviewed in [246]). Regarding its specific involvement in ALS, astrocytes expressing hFUS^{R521G} were recently described to display enhanced release of TNFα, coupled to perturbed AMPA receptor trafficking and neurotoxicity in co-cultures, thus revealing a deleterious paracrine action of astrocytic TNFα in ALS-*FUS* [132]. Yet, in both transgenic hSOD1^{G93A} co-cultures and mice, this pleitropic cytokine was reported to trigger opposite effects, depending on the TNF receptor (TNFR) engaged. Thus, activation of TNFR2 was shown to trigger motor neuron loss [280], whereas we demonstrated that stimulation of TNFR1 in the astrocytes can be beneficial for motor neurons by prompting the expression and secretion of the glial cell line derived neurotrophic factor (GDNF, [30]), a powerful neuroprotective agent in ALS and several other CNS disorders (reviewed in [114]) (Fig. 10.1). More specifically, we reported that this neuroprotective response is completely abolished by genetic ablation of TNFR1 in vitro and in vivo. In hSOD1^{G93A} mice and in sALS patients, we showed progressive upregulation of the entire TNF-TNFR1-GDNF axis. We, therefore, speculated that disrupting this protective cascade by transferring hSOD1^{G93A} mice on a TNFR1 knockout background would prevent astrocytes from mounting a neuroprotective reaction, thus exacerbating neuronal loss and shortening lifespan. Consistent with this hypothesis, disease onset was not anticipated in hSOD1^{G93A}mice lacking TNFR1, whilst disease progression was accelerated and neuronal loss became more severe [30]. The relevance of sustained release of glial-derived GDNF has been recently corroborated by a study describing the positive impact on the disease course of the implantation of human progenitor cells overexpressing GDNF and differentiating into astrocytes [277]. Thus, there is great hope that a phase I/IIa clinical trial (ClinicalTrials.gov Identifier NCT02943850), aimed at assessing the safety of such cells in ALS patients, will give positive results and will be quickly transferred to the clinic to determine the efficacy of this approach.

Growth factors are another category of mediators that was surprisingly reported to play a role in the toxic interplay between astrocytes and motor neurons in ALS. The earliest evidence of their involvement came from the investigation of the transcriptome of astrocytes extracted through laser capture microdissection from presymptomatic hSOD1^{G93A} mouse spinal cord sections. In particular, upregulation of nerve growth factor β (β-NGF) was observed, and extensive in vitro characterization demonstrated that ALS astrocytes release toxic amounts of the immature form of this neurotrophin. This latter activates an apoptotic cell death program in motor neurons upon interacting with its receptor p75 (p75^{NTR}) [69]. Intriguingly, in symptomatic hSOD1^{G93A} mice, motor neurons display strong immunoreactivity for the fibroblast growth factor 1 (FGF-1), which activates its receptor FGFR1 on astrocytes to mediate the release of NGF [34]. A possible explanation for this toxic effect of NGF came from a very recent paper showing that the neurotrophin undergoes nitration and glycation in the spinal cord of hSOD1^{G93A} mice. These post-translational modifications grant NGF aberrant contacts with the receptor for advanced glycation end products (RAGE), which interacts with p75^{NTR} to activate a toxic cascade [134]. However, boosting the NRF2-mediated antioxidant response in astrocytes prevented NGF-induced motor neuron death [224]. An unbiased assessment of the transcriptome of hSOD1^{G93A} astrocytes identified a core role also for Transforming Growth

Factor β (TGFβ) signalling in ALS [225] (Fig. 10.1). In hSOD1^{G93A} mice, TGFβ1 was shown to be upregulated in the astrocytes and indirectly drive disease progression and motor neuron cell death by reducing the protective component of microglia and T-cells [63]. Moreover, astrocyte-derived TGFβ1 was reported to increase cell stress directly in human motor neurons by inhibiting autophagy, which in turn causes pathological protein inclusions [281].

Besides releasing a wide array of neurotoxic molecules, ALS astrocytes can be deleterious to neurons also by failing their duties as supportive cells. As already mentioned while discussing the dysregulation of glutamatergic homeostasis, ALS astrocytes lose their ability to sustain GluA2 expression on motor neurons, thus favouring the influx of calcium ions through AMPA receptors [132, 165, 290]. Furthermore, hSOD1^{G93A}-expressing astrocytes were shown to reduce metabolic support from lactate release [69]. More recently, evidence started to accumulate that ALS pathogenesis is also characterized by impaired processing of 'help-me' signals that neurons launch to astrocytes, either because the message itself is impaired or because astrocytes fail to react adequately. For example, the fALS-associated protein angiogenin (Ang) is a motor neuron-secreted RNAse that is internalized by the astrocytes [264]. Within astrocytes, Ang cleaves RNA to trigger in return a neuroprotective response characterized by the release of several trophic proteins [263]. Intriguingly, the ALS-associated mutant Ang^{K40I} (position 40: lysine to isoleucine substitution) fails to enroll this neuroprotective response from astrocytes because it is devoid of RNA cleavage activity, despite being correctly released and internalized [264]. Furthermore, Tyzack et al. have shown that ephrin type B receptor 1 (EphB1) is upregulated in injured motor neurons, which activate a potent ephrin-B1-mediated neuroprotective response in the astrocytes. Yet, the EphB1-ephrin-B1 pathway was shown to be disrupted in hSOD1^{G93A} mice and in iPSC-derived astrocytes from a SOD1^{D90A} patient, thus implicating that astrocytes fail to support motor neurons in ALS [287]. Finally, a supporting environment can be maintained upon the concomitant expression of major histocompatibility complex class I (MHCI) molecules on motor neurons and their receptor killer cell immunoglobulin-like receptor KIR3DL2 on the astrocytes. However, this axis seems to be disrupted in ALS, as motor neurons from hSOD1^{G93A} mice and from both sporadic and familial cases display reduced MHCI expression because of the action of a soluble factor released by ALS astrocytes [267].

In the previous paragraphs, we have reported the current evidence on the molecular mediators implicated in astrocyte-induced motor neuron toxicity as well as on the loss-of-function astrocytes can experience in ALS. Those studies have been complemented by others, aiming at elucidating the cellular pathways that become aberrant within astroglial cells, thereby forcing astrocytes to acquire a toxic phenotype. For example, abnormal activation of Jagged-1/Notch signalling was recently reported in reactive astrocytes in the spinal cord of hSOD1^{G93A} mice and sALS patients. Astrocyte-specific inactivation of Jagged-1 intensified the activation of Notch signalling and accelerated the disease progression in mice, thus suggesting that Notch overactivation contributes to ALS pathogenesis and it is mitigated by the upregulation of Jagged-1 in reactive astrocytes (Fig. 10.1) [207]. The toll-like receptor 4 (TLR4)

signalling cascade is another pathway that captured particular attention, as this receptor controls the expression of various proteins through the activation of its downstream effector NF-kB (reviewed in [88]). There is evidence indicating that TLR4 and its endogenous ligand, the damage-associated molecular pattern molecule High Mobility Group Box-1 (HMGB1), are upregulated in the astrocytes and microglia during disease progression in hSOD1^{G93A} mice [149] (Fig. 10.1). TLR4 signalling is transduced along two different pathways depending on two diverse interaction partners, the myeloid differentiation factor 88 (MyoD88) and the TIR domain-containing adaptor inducing interferon-β (TRIF) (Fig. 10.1). Intriguingly, MyoD88 ablation in hSOD1^{G93A} mice does not modify the disease course, while hSOD1^{G93A} animals lacking TRIF have a shorter lifespan and display a higher number of aberrant astrocytes. This suggests that TRIF plays an important role in protecting the microenvironment surrounding motor neurons [136]. In keeping with this, we recently demonstrated that activation of TLR4 (and RAGE) by HMGB1 can trigger the production of the trophic factors GDNF and brain-derived neurotrophic factor (BDNF) by the astrocytes, which are likely to exert neuroprotective activities [29]. Furthermore, different groups have investigated the potential role of the TLR4 and RAGE downstream transcription factor NF-kB in ALS astrocytes. This protein complex is not only at the crossroad of many inflammatory pathways, but it is also directly activated by different proteins implicated in fALS, including TDP-43 [272], Ubiquilin 2 [227], hnRNPA1 [107] and FUS [288]. Human astrocytes derived from sALS and fALS patients were shown to differentially express an array of cytokines, whose expression is driven by NF-kB, thus suggesting the involvement of this transcription factor in the disease [103]. Furthermore, preserved motor function was observed in TDP-43 overexpressing mice [272] and prolonged survival was obtained in hSOD1^{G93A} and hSOD1^{G37R} mice, upon pharmacological treatment with the NF-kB inhibitor Withaferin A [218], also able to block the TLR4-TNFα axis in the astrocytes [171]. Although these papers suggest that targeting NF-kB might be a useful therapeutic tool in ALS, early evidence demonstrated that this factor should be specifically targeted in cells others than the astrocytes. In particular, crossing hSOD1^{G93A} mice with animals that express the dominant-negative IkBα, under the control of the GFAP promoter, did not affect disease onset or progression [44]. These results were then replicated by Frakes et al., who demonstrated that overexpression of another repressor of the NF-kB signalling, through gene therapy or genetic manipulation in the astrocytes, did not rescue motor impairment nor extended survival in hSOD1^{G93A} mice [79]. However, a very recent investigation shed light on the role of astrocytic NF-kB activation in vivo, in the hSOD1^{G93A} mouse model of ALS. Taking advantage of astrocyte-restricted conditional expression of constitutively active NF-kB, Ouali Alami and collaborators revealed a multifaceted and stage-specific response of the transcription factor [211]. While prolonged NF-kB activation in the astrocytes was shown to accelerate disease progression, its activation in the pre-symptomatic phase induced neuroprotective effects on motor neurons [211]. The most straightforward conclusion arising from these observations is that, when planning NF-kB modulation for therapeutic purposes, one should carefully consider the stage of the disease.

10.1.2.5 Astrocyte Faulty Regulation of the Blood–Brain Barrier

The astrocyte social network does not exclusively include neurons and other glial cells, but comprises additional cell types and structures, notably constituting the blood–brain barrier (BBB). This is a highly specialized formation consisting of microvascular endothelial cells, pericytes and perivascular astrocytes. Serving as a highly guarded border, the BBB carefully regulates the exchange of substances between the bloodstream and the brain parenchyma through different mechanisms (reviewed in [159]). As demonstrated in several studies (reviewed in [209]), astrocytes are regulators of different BBB functions owing to the localization of their endfeet around the capillaries. In the context of ALS, evidence of BBB leakage has been provided both in hSOD1^{G93A} mice and in sALS [84–86, 186, 269, 306, 318], and it has been associated with the overexpression of transporter proteins, such as the P-glycoprotein [36, 84]. Of note, this phenomenon seems to be secondary to aberrant signalling from astrocytes. In particular, both mouse hSOD1^{G93A} and human SOD1^{A4V} astrocytes were reported to trigger NF-kB translocation and P-glycoprotein upregulation through enhanced oxidative stress, while in astroglial cells from a patient expressing FUS^{H517Q} mutation, the mechanism was described to involve the release of pro-inflammatory factors [229]. Clarifying in detail these pathways would be of outstanding importance to identify new potential targets for therapeutic intervention. Interestingly, riluzole was found to be a substrate of the P-glycoprotein and to modulate the activity of other transporters [184, 185]. Thus, a formulation of riluzole and verapamil, a blocker of voltage-dependent calcium channels [62] that can reduce P-glycoprotein activity, was placed under development in order to block the efflux of riluzole from the CNS parenchyma. This cocktail of drugs gave promising results in cell culture experiments [312].

Astrocytes are likely to contribute to BBB dysfunction in ALS also via the upregulation of aquaporin-4 (AQP4; [16, 46, 203, 305]), an important regulator of both the brain water homeostasis (reviewed in [82]) and the glymphatic system, i.e. a CNS cleansing system that interacts with and complements the BBB (reviewed in [299]). Interestingly, AQP4 upregulation is also associated with its mislocalization in hSOD1^{G93A} mouse and ALS patient spinal cord, but not in other models of gliosis, thereby suggesting that this regulation is not part of a generic inflammatory response, but it is caused by some yet unidentified ALS-specific events [46, 305] (Fig. 10.1). In support of this hypothesis, TDP-43 depletion in astrocytes was shown to cause AQP4 overexpression, though it reduced surface levels [131]. While restoring the BBB function appears an interesting therapeutic approach in ALS, an early attempt to pursue this strategy proved disappointing. Thus, the generation of hSOD1^{G93A} transgenic mice lacking AQP4 led to the rescue of BBB leakage, but also to an earlier onset of the disease and shortened lifespan [305]. This result suggests that the extent and the timing of AQP4 depletion are critical to ensure a therapeutic effect, as AQP4 overexpression might also play a beneficial role clearing up toxins that would otherwise accumulate into the CNS parenchyma.

10.2 Spinal Muscular Atrophy

10.2.1 A Brief Introduction

Spinal muscular atrophy (SMA) is a progressive neurodegenerative disease caused by the loss of spinal motor neurons and affecting approximately 1 in 6000 to 1 in 10000 live births [298]. In the vast majority of occurrences (~96%), the disease is caused by deletions or gene conversion events in the *Survival of Motor Neuron 1 (SMN1)* gene on chromosome 5q13 [151], causing a reduction in the levels of its translated product, the SMN protein. This genetic defect is transmitted as an autosomal recessive trait and leads to an extremely variable clinical presentation (reviewed in [274]). SMA patients are subclassified into 5 categories on the basis of the age of onset and symptom severity. Briefly, in the most aggressive form of the disease, i.e. SMA type 0, muscular weakness is already evident at birth, when respiratory difficulties can be observed and lead to death within few weeks. SMA type I, also known as Werdnig-Hoffmann disease, appears within 6 months of age. Affected children never reach the ability to sit unaided and die within 3 years of age. SMA type II (or Dubowitz syndrome) has an age of onset between 6 and 36 months of age and a milder severity. Patients live to adulthood with substantial motor disabilities, but their life expectancy is reduced due to respiratory complications. SMA type III (Kugelberg Welander disease) appears at around 3 years of age and does not shorten life expectancy, although the quality of life is reduced by significant muscular weakness. SMA type IV is an adulthood onset disorder and it is the mildest form of the disease, leading to some degree of motor disability without affecting life expectancy. Since a unique genetic defect causes such dramatically different clinical phenotypes, it was soon speculated that other genes might act as disease modifiers. The architecture of the 5q13 locus supports this hypothesis. This is an unstable region of the human genome, characterized by duplications and inversions generating a variable number of copies of a more centromeric gene, called *SMN2*. This evolutionary backup gene is almost identical to *SMN1*, differing principally in a single nucleotide at the beginning of exon 7. However, this point variant has a deep impact on SMN expression as it leads to exon 7 skipping. The resulting transcript is translated in a shorter protein with an alternative C-terminus, conventionally referred to as SMNΔ7, which is unstable and rapidly degraded. Only 10% of *SMN2* primary transcript is processed with exon 7 inclusion and can be translated into the full-length stable SMN protein (reviewed in [260]). Consequently, the number of *SMN2* copies that SMA patients carry critically tunes residual SMN protein expression, thereby modulating disease severity. Importantly, many different animal models could be generated by reducing the expression of the endogenous *SMN* gene (reviewed in [60]).

On the basis of this evidence, it has clearly emerged that even a small increase in SMN expression could lead to substantial therapeutic benefit. Thus, several approaches have been developed to achieve this goal [96]. The first strategy aims

at delivering the whole SMN cDNA through viral-mediated gene therapy. Since the initial proof-of-principle study in transgenic mice [13], a substantial advancement into the development of an effective therapy came from the discovery of the adeno-associated virus serotype 9 (AAV9) as vector apt to cross the BBB [77]. This approach has been successfully exploited in transgenic mice [11, 19, 55, 78, 90, 91, 179, 235, 289] and larger animal models of the disease [57, 115, 179], obtaining a tremendous impact in both preserving animal motor functions and extending lifespan. These promising preclinical findings paved the way to a phase I clinical trial whose outcome suggests that a single injection of therapeutic virus leads to improved motor abilities and extends life expectancy in SMA type I patients [176]. Thus, the restoration of SMN levels by means of AAV9-based gene therapy (Zolgensma®) has been recently approved by the U.S. FDA for the treatment of pediatric patients, less than 2 years of age. Complementary, the existence of the *SMN2* gene in humans was found to offer an exceptional opportunity for an alternative approach to raise SMN expression, taking advantage of the development of antisense oligonucleotide (ASO) technology as a toolbox to modulate protein expression (reviewed in [233]). Extensive studies on the molecular mechanisms regulating *SMN2* primary transcript splicing [260] led to the development of Nusinersen (Spinraza™), an ASO able to prevent exon 7 skipping, thereby allowing full-length SMN expression. In two recent clinical trials, this drug was administered to children with type II and type III SMA (Phase I study; NCT01494701; NCT01780246; [40]) and, later, in patients with infantile-onset SMA (phase II study; NCT01839656; [70]). Promising signs of efficacy in motor function were observed, which have encouraged the design of sham-controlled, phase III clinical studies for in infantile- and late-onset SMA (NCT02193074; NCT02292537; [71, 178]). The overall findings of these studies have supported the recent approval of Nusinersen by the U.S. FDA for treatment of 5q SMA, followed by the European Medicine Agency (EMA) and by other national drug management authorities worldwide (Canada, Japan, Brazil, Italy and Switzerland).

Despite these advancements, many questions still deserve to be addressed in order to be able to develop even more effective therapies to cure SMA patients. First, are there other tools to modulate SMN expression, for instance, by regulating its stability? Second, what are the molecular mechanisms linking SMN deficiency to motor neuron demise? Evidence from genetic studies and animal models of the disease have highlighted the possibility that other genes may play a critical role in modulating SMA severity in either SMN-dependent (i.e. *Uba1*) or SMN-independent ways (i.e. *Plastin3*; reviewed in [307]). It is, therefore, crucial to understand the molecular mechanisms driving SMA pathogenesis. Many hypotheses can be formulated on the basis of the growing number of cellular functions in which SMN has been involved so far (reviewed in [261]). The SMN protein is composed of 294 amino acids that are arranged in functionally distinct domains. Each of them specifically mediates the interaction with nucleic acids or with a plethora of other proteins, either in the nucleus or in the cytoplasm. Based on this, the functions proposed for SMN include, but are not limited to, trafficking and remodelling of small nuclear ribonucleoproteins (snRNPs), modulation of RNA/DNA metabolism, signal transduction pathways

affecting actin-cytoskeletal remodelling, and endocytosis and autophagy. Among the different cellular formations, the neuromuscular junction emerges as a trans-cellular structure that is particularly vulnerable to reduced expression of SMN (for a specific review on the topic see [27]).

Finally, 'non-5q' SMA is an umbrella term to group a minority of cases (~ 4%) who develop the disease in the absence of mutations in *SMN1*. These patients often display symptoms other than proximal muscular atrophy and show a diverse pattern of inheritance. Only recent advances in whole-genome sequencing allowed to identify a growing number of causative genes (reviewed in [222]).

10.2.2 The Role of Astrocytes in the Pathogenesis of 5q SMA

Although reactive gliosis has been reported upon neuropathological assessment of 5q [10, 87, 142] and some forms of non-5q SMA [54, 108], the actual role played by astrocytes in the pathogenesis of these disorders has not been specifically addressed for a long time. Only in recent years, evidence has been accumulated that suggests a causal role for astrocyte dysfunction in the pathogenesis of 5q SMA, whilst its implication in non-5q SMA remains neglected.

The possibility that SMA may be a non-cell autonomous disease arose from the observation that astrocytosis precedes the loss of motor neuron cell bodies in vitro and in vivo [174, 219] as well as from the phenotypic analysis of several transgenic mice where SMN expression was modulated in motor neurons. In one instance, reduced SMN expression in motor neurons (and oligodendrocytes) was achieved by crossing Olig2-Cre expressing mice with others harbouring a *floxed* version of SMN exon 7 [216]. The progeny of this breeding displayed a mild SMA-like phenotype with no reduction in lifespan, in sharp contrast with the aggressive and fatal phenotype exhibited by mice where SMN expression was systemically reduced [216]. Complementary, several studies in different mouse models of SMA investigated the benefit of selective SMN reintroduction in motor neurons. These investigations failed to show a complete rescue of the detrimental phenotype [92, 148, 162, 170, 212]. Taken together, this early evidence strongly argues in favour of the theory that toxicity triggered by SMN deficiency has a non-cell autonomous component. To directly address this hypothesis, SMN expression was selectively reintroduced in the astrocytes, in a transgenic mouse model of the disease, by viral-mediated gene therapy [234]. In this study, Rindt et al. designed a vector where the SMN coding sequence was under the control of the GFAP promoter, and was further modified to harbour the consensus sequence of a miRNA selectively expressed by motor neurons, in order to prevent any SMN expression in those cells. Importantly, mice injected with such vector displayed enhanced lifespan, reduced neuromuscular junction pathology and diminished gliosis [234], thus demonstrating that targeting astrocyte dysfunction

can be beneficial to control SMA. Yet, the exact molecular mechanisms mediating astrocyte involvement remain to be fully elucidated.

The earliest evidence that SMN deficiency might be directly perturbing astrocyte physiological functions came from the discovery that, both in a human astrocytoma cell line treated with siRNA against *SMN1* as well as in astrocytes from the spinal cord of SMA mice, there is an upregulation of Jagged-1, a Notch ligand. Furthermore, in the surrounding motor neurons, Notch receptors are correspondingly upregulated with evidence of activation of the downstream signalling cascade [33]. Other studies took advantage of the possibility of culturing astrocytes from mouse models of SMA and, more recently, from the differentiation of SMA patient-derived iPSCs into astrocytes. Using these models, abnormal calcium signalling was reported in both human [174] and mouse cells [319]. Specifically, in human SMA astrocytes, basal calcium concentrations were increased, while the cell response to ATP stimulation was reduced in comparison with non-SMA controls [174]. In striking contrast, mouse SMA astrocytes did not display any difference upon resting conditions, whereas their response to stimuli was enhanced [319]. Taken together, this amount of evidence suggests that SMN deficiency is intrinsically detrimental for the astrocyte homeostasis. But what is the impact on the cross-talk with neurons? Early experiments aiming at assessing the endurance of iPSC-derived motor neurons revealed reduced survival of cells differentiated from patient tissues [59, 247], thus suggesting that SMN deficiency can lead to cell-autonomous cell death. However, those early cultures contained significant contamination of astrocytes, which might have contributed to cell demise. Indeed, later refinement of the differentiation protocol allowed to obtain homogenous motor neuron populations, and these revealed comparable survival rates between cells from SMA patients and healthy controls [262].

This evidence supports the hypothesis that SMA astrocytes might trigger motor neuron sufferance either by losing their supporting functions or by gaining toxic activities. Consistent with these latter hypotheses, human SMA astrocytes were reported to display reduced release of the trophic factor GDNF [174]. Furthermore, co-culture experiments provided evidence that SMA astrocytes can induce neurotoxicity by releasing miR-146a, although follow-up studies are still necessary to elucidate the exact mechanism triggered by this molecule [262]. Conversely, mouse SMA astrocytes did not show reduced support to motor neurons in a model of contact co-cultures, although neurons exhibited a lower density of synaptic contacts [319]. Since this detrimental effect on motor neuronal function was not replicated in non-contact co-cultures, it was postulated that it may be caused by an antigen exposed on the cell membrane. In keeping with this view, SMA astrocytes were shown to exhibit reduced surface expression of Ephrin B2 [319]. Finally, another group provided evidence that also SMA astrocyte conditioned medium can lead to neuronal sufferance in terms of reduced neurite outgrowth by decreasing the release of the Monocyte Chemoactive Protein 1 (MCP1) [168].

10.3 Discussion/Perspective

As extensively documented in the previous sections, astrocytes appear to play complex and critical roles in both ALS and SMA, thereby emerging as key elements in the pathogenesis and progression of motor neuron diseases. Many aspects driving their detrimental effects appear to be intermingled. First, their own homeostasis seems to be directly disrupted by respective disease proteins. Secondly, they sense and react to the distressed status of their neighbouring neurons, becoming reactive. Multiple evidence suggests that 'reactive astrocytosis' can lead to both loss of support to motor neurons and gain of new aberrant toxic functions. However, a comprehensive consensus on the molecular mechanisms disrupting the astrocyte–neuron crosstalk in motor neuron degeneration is still missing and needs to be pursued. To achieve this goal, several lines of investigation are currently underway.

To start with, the majority of findings concerning the role of astrocytes in the pathogenesis of ALS have been inferred using mutant SOD1 overexpressing models of the disease. It is only very recently that this horizon has been widened including evidence from other molecular subtypes of the disease. The same need applies, even with stronger urgency, to SMA research. In this context, the investigations on the astrocytes are still an underappreciated topic, limited to the 5q-linked form of the disease, while no mechanistic studies have been conducted in non-5q SMA. Furthermore, in ALS and SMA, the field is currently progressing along parallel, unconnected paths and no side-by-side experiments have been so far presented. Yet, some analogies and differences start emerging (summarized in Table 10.1). The more refined this type of analysis will be, the earlier it will be possible to distinguish shared from distinctive pathways and envision suitable therapeutic strategies. For instance, we have discussed findings describing the dysregulation of intracellular calcium homeostasis in the context of both ALS and SMA. This is a particularly intriguing evidence, considering that calcium concentrations regulate several signalling cascades coupled with correct neuronal interplay (reviewed in [98]). An interesting difference between the two diseases relates to the neuroprotective factor GDNF. We have demonstrated that, in ALS, its production is not only retained, but even boosted, during disease progression. By contrast, human SMA astrocytes displayed reduced GDNF expression and release, thus suggesting that this event is not specifically caused by gliosis, but it is rather due to a disease-specific dysregulation. Further studies are needed to prove whether restoring GDNF expression in astrocytes in animal models of SMA would be sufficient to sustain neuroprotection.

Another point for discussion stems from the collective interpretation of the evidence gathered so far, namely, the assumption that astrocytes located within the same CNS region are *bona fide* a homogenous population. However, recent studies (reviewed by [17]) challenged this view. It is now believed that, although astrocytes express an array of core transcripts that provide them with the ability to fulfil their housekeeping duties towards neurons, they express also distinct subsets of transcripts making them apt to tune the activity of specific neuronal populations even within individual brain regions. Intriguingly, single-cell transcriptome analysis within the

Table 10.1 Analogies and differences of the astrocyte features in ALS and SMA

	ALS		SMA	
Correcting the pathogenic genetic alteration in astrocytes ameliorates the phenotype in animal models of the disease	Selective ablation of mutant SOD1 from astrocytes slows down disease progression and prolongs survival	Yamanaka et al. [310], Wang et al. [303]	Selective restoration of SMN expression in astrocytes by viral-mediated gene therapy prolongs survival of SMA mice	Rindt et al. [234]
	In chimeric mice, motor neurons expressing mutant SOD1 resist the pathogenic insult when surrounded by non-transgenic glial cells	Clement et al. [41]		
	Transplanting non-transgenic astrocyte precursors ameliorates the phenotype of $hSOD1^{G93A}$ rats	Lepore et al. [153]		
	Transplanting human iPSC- or embryonic stem cell-derived astrocyte precursors ameliorates the phenotype of $hSOD1^{G93A}$ mice	Kondo et al. [137], Izrael et al. [116]		
Disease-astrocytes are toxic to motor neuron	In chimeric mice, non-transgenic motor neurons display signs of cellular stress when surrounded by mutant SOD1 expressing glia	Clement et al. [41]		

(continued)

Table 10.1 (continued)

	ALS		SMA	
	Transplanting hSOD1^{G93A} astrocyte precursors triggers motor neuron loss in non-transgenic rats	Papadeas et al. [213]		
	Transplanting human iPSC-derived ALS astrocyte precursors triggers motor impairment in non-transgenic mice	Chen et al. [38], Qian et al. [228]		
	Mouse ALS astrocytes are toxic to motor neurons in co-cultures	Ferraiuolo et al. [69], Di Giorgio et al. [50], Nagai et al. [198], Bilsland et al. [25], Phatnani et al. [310], Tortarolo et al. [280], Kia et al. [132]	Mouse SMA astrocytes do not trigger motor neuron demise in co-cultures, but the number of synaptic contact is reduced	Zhou et al. [319]
	Human ALS astrocytes are toxic to motor neurons in contact co-cultures	Di Giorgio et al. [49], Marchetto et al. [167], Haidet-Phillips et al. [103], Meyer et al. [180], Re et al. [231], Song et al. [267]	Human SMA astrocytes are toxic to motor neuron in contact co-cultures	Sison et al. [262]
	Conditioned medium from ALS-astrocytes is sufficient to trigger motor neuron toxicity in vitro and in vivo	Kia et al. [132], Fritz et al. [81], Rojas et al. [238, 239], Ramirez-Jarquin et al. [230], Madill et al. [164]	Conditioned medium from SMA astrocytes triggers reduced neurite outgrowth in motor neurons	Martin et al. [168]

(continued)

Table 10.1 (continued)

	ALS		SMA	
Astrocytes display an abnormal intracellular calcium homeostasis	Abnormal calcium accumulation and release from intracellular stores upon different stimuli	Martorana et al. [172], Vergouts et al. [297], Kawamata et al. [128], Almad et al. [5], Milosevic et al. [187]	In human iPSC-derived SMA astrocytes, the intracellular calcium concentration is increased, upon standard culturing conditions, and the response to ATP stimulation is reduced	McGivern et al. [174]
			Mouse SMA astrocyte do not display any difference in the resting calcium concentration, but their response to ATP stimulation is enhanced	Zhou et al. [319]
GDNF supply to motor neurons	During disease progression, enhanced stimulation of the TNF-TNFR1 axis prompts augmented GDNF release from ALS astrocytes	Brambilla et al. [30]	Human iPSC-derived SMA astrocytes display reduced release of GDNF	McGivern et al. [174]
Activation of the Notch signalling pathway	Notch ligand Jagged-1 is abnormally overexpressed in $hSOD1^{G93A}$ expressing mouse astrocytes. Preventing this event has detrimental consequences on the phenotype	Nonneman et al. [207]	SMN depleted astrocytes display enhanced expression of Jagged-1 in vitro and in SMA mice	Caraballo-Miralles et al. [310]

mouse cortex and the hippocampus has revealed the presence of two subpopulations of astrocytes (defined as type 1 and type 2; see [317]). In the ventral spinal cord, adult astrocytes display a regionally distinct phenotype, enabling their classification into three populations (VA1, VA2 and VA3), which develop from progenitors allocated in defined domains [110]. Functionally, the expression of domain-specific proteins is pivotal to ensure correct motor neuron circuitry [192] and electrophysiological properties [130]. It is likely that such diversity is the result of an intricate interplay between the unfolding of an intrinsic patterning program and the microenvironment, which is modulated by inputs from surrounding neurons and other glial cell populations (reviewed in [66]). This landscape becomes even more complex with ageing or when a neuroinflammatory insult strikes. Astrocytes then mount a phenotypical switch toward a reactive status, which limits or exacerbates neuronal loss, depending on the experimental context [316]. It is, therefore, tempting to speculate that implementing these findings to address the role of astrocytes in the pathogenesis of ALS and SMA might have a tremendous impact in several ways. For instance, a different transcriptional profile might explain (i) why some astrocytes undergo cell sufferance and death in the context of ALS and others 'simply' develop a reactive phenotype; or (ii) which cells mount a protective response and which ones exacerbate neurodegeneration. The challenge is now to link a specific astrocyte subpopulation defined by distinct molecular features under physiological conditions with their fate in the context of disease development, an issue that would probably need computational models of gliosis to be addressed. Also, since we have previously discussed that astrocytes are a potential therapeutic target, the pre-clinical investigation should be refined including the possibility that only specific subpopulations of astrocytes should be manipulated. In keeping with this, the efficiency of transplant studies might also be perfected by infusing only the most beneficial subtype of cells. Finally, an aspect that should be carefully considered is the impact of physical activity on astroglial cells. Several lines of evidence indicate that enriched environment and physical exercise importantly contribute to reduce reactive astrocytosis [129, 147, 237, 252, 254] and to alleviate the neuroinflammatory response in various animal models of injury and disease [22, 150, 204, 214, 226]. This supports the view that rehabilitative training can be used to favour the morphological remodelling and to improve the functional performance of the astrocytes. What remains to be clarified is whether this is a general issue or whether it is effective on specific astrocytic subpopulations.

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Chapter 11
Astroglia in Alzheimer's Disease

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Abstract Alzheimer's disease is the most common cause of dementia. Cellular changes in the brains of the patients suffering from Alzheimer's disease occur well in advance of the clinical symptoms. At the cellular level, the most dramatic is a demise of neurones. As astroglial cells carry out homeostatic functions of the brain, it is certain that these cells are at least in part a cause of Alzheimer's disease. Historically, Alois Alzheimer himself has recognised this at the dawn of the disease description. However, the role of astroglia in this disease has been understudied. In this chapter, we summarise the various aspects of glial contribution to this disease and outline the potential of using these cells in prevention (exercise and environmental enrichment) and intervention of this devastating disease.

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11.1 Senile Dementia—The Outcome of Pathological Ageing

Dementia as a medical term was introduced in the first century AD by Aulus Cornelius Celsus in his fundamental discourse *De Medicina* [50] to characterise major cognitive impairments of the mankind. The term dementia originates from the prefix "de" (meaning "out of"), the stem "ment" ('mind') and the suffix "ia" (diseased condition). Historically, this term was used in a very broad sense to indicate chronic cognitive impairments associated with psychotic symptoms such as delusions or hallucinations. However, dementia was not associated with age-dependent cognitive decline, although from the very dawn of medical observations, these impairments were considered as an essential and inevitable part of ageing process. Already in the seventh century BC, Pythagoras defined the advanced ages of human life as "senium" when "the system returns to the imbecility of the first epoch of infancy" [26]. From Aristotle to Lucretius and Galen, the ageing was considered to be associated with mental decline, impossible to arrest or recuperate [26, 38, 121, 302]. This gloomy outlook was not, however, shared by Cicero who believed in selective development of a senescence-dependent cognitive decline: "senile imbecility does not occur in all old men, but only in those of feeble mind" [54].

Over centuries the mental weakness of old people was defined as senility, idiocy, morosis, dotage, etc.; in 1794, the term dementia was formalised by Philippe Pinel and this term was officially recognised by the French Law [291]. At the end of the nineteenth century, the definition of senile dementia became widespread and underlying histopathology became to be scrutinised. The specific lesions, the plaques (then known as *miliary foci*), were discovered by Block and Marinesco in the brain of old epileptic patient [36], and subsequently these plaques were observed in the post-mortem tissues of patients suffering from senile dementia by Redlich and by Otto Fischer [77, 225]. In 1903, Max Bielschowsky developed an improved version of the Golgi silver stain that allowed visualisation of neurofibrilles [30]. Using this new technique, Alois Alzheimer (in 1906) was able to visualise neurofibrillary tangles in the post-mortem brain of Mrs. Augusta D, whom he first seen in 1901 in Frankfurt with the symptoms of confusion, delusions and dementia. These tangles displayed extraordinary thickness and often merged into dense bundles reaching the surface of a neurone [7, 26]. The brain of Augusta D also contained senile plaques, and thus the case of early dementia associated with appearance of senile plaques and pathological neurofibrillary tangles has been reported in 1907 [7]. This was a rather unique description, which differed from the widespread dementia of the early twentieth century associated predominantly with neurosyphilis or vascular ischemic brain damage. The disease was named "Alzheimersche krankheit" by Emil Kraepelin in the 8th edition of his immensely influential textbook on Psychiatry (*Psychiatrie:*

Ein Lehrbuch fuer Studierende und Arzte). Kraepelin defined this new disease as a rapidly progressive, early-onset dementia distinct from the senile dementia [136]; for the history, people involved, histological details and controversies see [27, 109, 172].

Alzheimer's disease (AD) was rarely diagnosed in the first half of the twentieth century and was generally regarded as a rare pathology that affected relatively young persons. Only in 1960, the AD histopathology was related to the sporadic cases of age-dependent (i.e. senile) dementia and the notion of AD as senescent-associated pathology had been developed [244, 245, 290]. It seems also that the pandemic of the senile dementia observed in recent years has evolved over the last century. Detailed physiological investigation of organs and systems of an extended population (826 subjects) of elderly (80–100 years of age) inhabitants of the UK performed in 1889 revealed surprisingly little changes in their cognitive status. Furthermore "… the brain in many held out as well or better than other organs - which may be regarded one of the bright rays, if not the brightest, in the centenarian landscape" [111]. Indeed, in this study dementia was observed only in 2 out of 74 centenarians. This contrasts remarkably with our times, when >50% of people older than 85 demonstrate signs of severe cognitive impairment [343]. Of course, evolution of epidemiological changes may be interpreted from many angles, and yet it is impossible not to speculate that an increased environmental toxicity, changes in diet and mounting social pressure have contributed to a rise of sporadic AD in the modern population.

Increased prevalence of senile dementia at advanced age parallels increase in life expectancy. Modern definition regards AD as a severe neurodegenerative disorder associated with specific histopathological markers represented by (i) focal extracellular deposits of fibrillar β-amyloid generally known as neuritic or senile plaques in the brain parenchyma and the walls of blood vessels, and (ii) intraneuronal accumulation of neurofibrillary tangles composed of abnormal hyperphosphorylated tau filaments [39, 70]. AD affects specific brain regions associated with learning and memory, including the basal forebrain, the hippocampus and the neocortex. Clinical symptoms of AD are manifested by a progressive decline of cognitive functions including short- and long-term memory [185]. At advanced stages of the disease, clinical presentation of AD is complicated by a variety of behavioural disturbances including agitation, irritability, anxiety, delusions and depression [165].

Conceptually, two forms of AD are defined: (i) early-onset or familial Alzheimer's disease (FAD) and (ii) late-onset or sporadic AD, or SAD [35]. Epidemiologically, the late-onset SAD accounts for the absolute majority (95–99%) of AD cases in people above 65 years of age. The familial variant of AD is associated with mutations in three genes encoding for amyloid precursor protein (APP), presenilin-1 (PS-1) and presenilin-2 (PS-2), which are inherited in an autosomal dominant manner [23, 257, 294]. In contrast to SAD, which is linked to an old age, the familial AD occurs in much younger group of patients between 40 and 50 years old; the FAD is characterised by a rapid progression and idiosyncratic clinical manifestation. Anatomical and histopathological progression of AD begins from early degeneration of cholinergic neurones in the nucleus basalis of Meynert and septum. In parallel, the accumulation of intraneuronal β-amyloid and formation of neurofibrillary tangles develop,

which ultimately leads to an emergence of senile plaques [182]. Deterioration of neuronal networks begins with synaptic damage and malfunction which affects CNS plasticity; these changes occur prior to the formation of senile plaques and neurofibrillary tangles and prior to neuronal death [251, 255]. In addition, this process suppresses neurogenesis, which further impairs neuronal plasticity [231, 237]. Apart from the cholinergic system, AD pathology impairs other major neurotransmitter systems including noradrenergic, serotonergic and dopaminergic [153, 234, 348].

11.2 Experimental Animal Models of Alzheimer's Disease

Alzheimer's disease, similar to other neurodegenerative diseases, is a specific disease of humans; animals as a rule do not develop AD-like pathology [289]. Experimental study of AD therefore requires the development of animal disease models which are capable of faithful reproduction of single or multiple subsets of neuropathological, histological, cellular, behavioural or biochemical alterations resembling those seen in classical AD [48, 91].

11.2.1 Old Animals

The very first models of AD were represented by aged animals [28, 29, 289]; several species from rodents to primates have demonstrated atrophy and death of basal forebrain neurones expressing choline acetyltransferase or nerve growth factor [60, 78, 248]. In monkeys, alterations of cholinergic neurones were even associated with β-amyloid depositions [289]. In addition, old animals showed not only a cholinergic dysfunction but also a concomitant alteration of other neurotransmitter systems such as the monoaminergic, peptidergic or serotonergic [15, 176, 198, 234].

11.2.2 Lesions

The global lesion models of AD (Table 11.1) relied on destruction of certain brain areas. At the beginning, the electrolytic lesions were used; these caused diffuse damage of several brain areas and lacked specificity [156, 289]. In the majority of global lesion models, the non-selective excitotoxic toxins such as NMDA, ibotenic acid, quisqualic acid, quinolinic acid, colchicine and other alkaloid substances were employed [289]. Injections of these substances triggered cell death with consequent neurological dysfunctions including impaired cognition. The global lesion models also employed injections of alcohol, which is toxic to cholinergic neurons [13, 289]; or injections of β-amyloid peptides, which produces multiple alterations of corticobasal neurones affecting acetylcholine release and cholinergic receptors [88, 211].

Table 11.1 Summary of lesion models of Alzheimer's disease. Modified from [232]

Lesion	Cholinergic	Non-cholinergic	Neuropathology	References
Electrolytic	Yes	Yes	Neuronal death	[156, 293]
Excitotoxins (NMDA, Ibotenic acid, Quisalic acid)	Yes	Yes	Neuronal death	[69, 330]
Quinolinic acid	Yes	Yes	Neuronal death	[37]
Colchicine	Yes	Yes	Neuronal death	[258]
Alkaloids	Yes	Yes	Neuronal death	[65]
AF64A	Yes	No	Neuronal death	[53, 97, 318]
192Ig-G saporin	Yes	No	Neuronal death	[325, 327]
Alcohol	Yes	Yes	Neuronal death	[13]
β-Amyloid	No	No	Cholinergic Dysfunction	[88, 211]

As mentioned above, a loss of cholinergic neurones is a prominent feature of AD [18]. With this in mind, animal models, which employed specific lesioning of cholinergic neurones, were developed. Among these, the most relevant were the rodent models with lesions in the nucleus basalis magnocellularis, which is the equivalent of the nucleus of Meynert in humans [214, 322], in the diagonal band of Broca and the septum [289] (Table 11.1).

Specific cholinergic lesion models used toxins, which affected only cholinergic neurones in the brain regions relevant to AD, including septum, nucleus basalis magnocellularis and the diagonal band of Broca, but did not impair non-cholinergic neurones [289, 325]. For example, the AF64A cholinotoxin, which binds to the high-affinity choline uptake system, was injected. Alternatively, the immunotoxin 192 IgG-saporin that binds selectively and irreversibly to low-affinity nerve growth factor receptor interrupting cholinergic neuronal protein synthesis was employed. Both techniques lead to selective impairment and death of cholinergic neurones [289].

Similarly, the noradrenergic system can be lesioned in rats by the injection of the construct, consisting of antibody against dopamine-β-hydroxylase, the enzyme converting dopamine to noradrenaline, and saporin [215], a ribosome-inactivating plant lectin extracted from *Saponaria officinalis* (Caryophyllaceae) [17, 151]. This technology allows a selective and gradual lesioning of noradrenergic neurones in the brain stem nucleus locus coeruleus, the primary site of noradrenaline production in the CNS [75]. Upon injection into the LC, this immunotoxin binds dopamine-β-hydroxylase, which is not only localised mainly in the cytosol, but also at the plasma membrane surface of noradrenergic neurones [271, 321]. Due to its structure, saporin cannot enter the cell [56], but when coupled to a carrier molecule (for example, an antibody), is able to specifically bind a surface antigen protein (such as dopamine-β-hydroxylase, in this case), the toxin gains access to the cytosol and binds to the ribosomal 60S subunit, interfering with protein synthesis, and soon leading to cell

death [326]. In initial anatomical investigations, the immunotoxin, infused into the lateral ventricles of either adult [331] or developing rats [57], produced specific and dose-dependent depletions of locus coeruleus neurones, with no effects on other cholinergic, dopaminergic or serotonergic neuronal populations [153]. The possibility to induce a partial or total noradrenergic loss (by varying the injected dose) makes this immunotoxic approach an ideal model to study events within the noradrenergic projection system, as they occur during age-related demise of locus coeruleus in humans [329].

11.2.3 Transgenic Animals

The AD models described above, although triggering neuronal death with consequent cognitive impairments, did not mimic histopathology and temporal progression of the disease. In the last two decades, an alternative and much more effective approach of using transgenic technologies have produced numerous models of familial forms of AD, which have been widely employed in experimental research. These transgenic animal models replicate several neuropathological features of AD (Table 11.2 and [91, 142, 232]) and they are based on mutated genes isolated from patients with various forms of familial AD. The very first transgenic animal carrying mutant APP and showing an AD-like pathology was developed in 1995 [83]. In this model, known as PDAPP, several pathological hallmarks of AD have been identified, including extracellular β-amyloid deposits, dystrophy of neurites, astrogliosis and memory impairments. Memory impairments, however, did not show any correlation with β-amyloid load [83]. The next transgenic AD mouse model, designated as Tg2576 mice, harboured APP_{swe} (Swedish K670 N/M671L) mutation; this model developed numerous senile plaques in parallel with learning and memory impairments, which begun to develop from 9 months of age onwards [110]. The next generation of transgenic models carried double mutation of APP gene; such a model known as APP23 demonstrated some (~14%) neuronal death in the hippocampus [72, 272]. The next step was to combine mutated APP and PS genes; co-expression of $PS1_{dE9}$ with APP_{Swe} resulted in an AD mouse model characterised by accelerated β-amyloid deposition and memory deficits but without tangle formation [250]. These developments culminated in creation of 5xTG AD mice, designated as Tg6799; these animals carry a single human APP Swedish K670N/M671L double mutation as well as the Florida I716V mutation, and the London V717I mutation, along with PS with double M146L and L286V mutations. These mice develop amyloid depositions as early as 2 months of age [200].

In parallel to the animal models with increased β-amyloid production, the pathological tau models also have been created; the first being produced in 1995. In this model, the hyperphosphorylated tau was accumulated in neuronal somata and dendrites, although neurofibrillary tangles were never developed [90]. The next tauopa-

Table 11.2 Transgenic mouse and rat models of Alzheimer's disease

Transgenic mouse and rat models	Neuropathology	References
APP_{751SL}	Plaques	[32]
APP/Ld/2	Plaques	[183]
APP_{Swe}	Plaques	[72]
APP Swedish, 695 K670N M671L	Plaques	[272]
$PS1_{M146L}$	Diffused plaques	[32]
$APP_{751SL}/PS1_{M146L}$	Plaques	[32]
$APP_{SWE}/PS1_{dE9}$	Plaques	[250]
APPSwedish and $PS1_{M146L}$	Plaques	[115]
APP_{695SWE}	Plaques	[110]
APP_{V717F}	Plaques	[67]
K670N/M671L and V717F	Plaques	[115]
APP Swedish, 695 K670N-M671L and Indiana V717F	Plaques	[72]
APPSwedish and V717F	Plaques	[51]
V337 M	Tangles	[280]
4R/2 N	Tangles	[281]
Tau_{P301L} (4R,2-,3-)	Tangles	[159]
P301L	Tangles	[89]
Tau_{P301L}	Tangles	[12]
P301S/G272V	Tangles	[252]
P301S	Tangles	[6]
G272V, P301L, R406W	Tangles	[72]
Endogenous tau KO	Tangles	[9]
P301L TET-off	Tangles	[224]
7TauTg	Tangles	[113]
Tg2576 × JNPL3 (APP_{SWE})	Plaques and Tangles	[158]
Tg2576 and VLW	Plaques and Tangles	[228]
3xTg-AD	Plaques and Tangles	[202]
Tg478	None	[79]
Tg1116	None	[79]
Tg478/Tg1116	Plaques	[79]
Tg 478/1116/11587	Plaques	[79]
K670M/N671L	Plaques	[132]

thy model, over-expressing Tau_{P301L}, did develop neurofibrillary tangles without β-amyloid pathology and neuronal loss [159].

In 2003, the triple transgenic AD mice (3xTg-AD) was created combining the mutants of the three major implicated genes; these animals harbour the mutant genes for APP_{Swe}, for presenilin $PS1_{M146V}$ and for Tau_{P301L} [201, 202]. These animals demonstrated temporal- and region-specific Aβ and tau pathology, which resembles that seen in the human AD brain. Additionally, the 3xTg-AD animals also displayed plaques and tangles, and also showed reduced long-term potentiation in the hippocampus along with functional and cognitive impairments seen as deficient spatial and long-term memory [201, 202]. These pathological changes progress in an age-related manner; most importantly functional deficits precede the appearance of histological hallmarks. Cognitive deficits in the 3xTg-AD model correlate with the accumulation of intraneuronal Aβ [44, 177]. Moreover, at the cellular level, changes in astroglial subcellular vesicle traffic contribute to the pathophysiology of AD [268, 269].

11.3 Neurodegenerative Diseases 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. The aetiology of neurodegeneration is complex and multifaceted. Neurodegeneration can have a genetic background or it can be instigated by acute trauma, by chemical poisoning, by metabolic insufficiencies or by infectious attacks, as well as by vascular abnormalities, or by sporadic accumulation of genetic/biochemical errors of unknown nature. At the early stages, neurodegeneration as a rule affects synaptic contacts in the brain tissue, thus causing early cognitive deficits. The early stages of neurodegeneration are of course of specific significance, because during this early phase the pathological process can be arrested or even reversed, thus offering the hope for preventing the cognitive decline.

Cellular and molecular mechanisms of underlying initiation and progression of neurodegenerative diseases are highly complex, which makes it almost impossible to identify a single leading cause. At the level of cellular biochemistry, neurodegeneration is frequently linked to aberrant handling of proteins, which promotes intra- or extracellular accumulation of abnormal proteins such as, for example, β-amyloid, tau or α-synuclein [116]. At a more systemic level, however, neurodegeneration reflects a generalised failure of brain homeostasis, which results in a functional and structural decline in the connectivity of neural networks, thus ultimately destroying information processing. Neurodegeneration starts from functional impairment of synaptic connectivity and synaptic plasticity which leads to a neurotransmission misbalance; these processes stipulate early cognitive deficiency. With further progression of the neurodegenerative process, the structural abnormalities develop, trigger disappearance of synapses and death of neural cells, ultimately resulting in a generalised

atrophy of the brain accompanied with profound cognitive deficiency [133, 207, 254, 282].

The neuroglia provides for the birth, maintenance and demise of synapses, as well as for overall homeostasis of the nerve tissue, these functions being summarised in a concept of the astroglial cradle [303, 304]. Thus, these non-neuronal cells likely represent the main cellular element shaping the progression of neurodegenerative processes. The generally acknowledged and prevailing point of view considers neurones as main substrates of neurodegeneration, and it is generally assumed that failures in neuronal protein synthesis and/or direct neuronal damage caused by various factors constitute the leading mechanism of neurodegenerative pathologies. These neurone-centric doctrine has been challenged in the past decade, with considerable attention re-routed to neuroglia, which being primary cells responsible for the brain homeostasis and defences, fundamentally contribute to an overall homeostatic failure promoting neurodegeneration [40, 45, 106, 212, 235, 238, 242, 300, 307, 310, 311, 315].

11.4 Astroglial Atrophy and Astrogliosis in Neurodegenerative Diseases

Astrogliopathology in neurodegenerative diseases includes reactivity and astroglial atrophy, asthenia and loss of function. These processes develop in a stage-specific manner and contribute to pathological progression; frequently astroglial asthenia develops at early stages of the disease, whereas at the advanced stages an emergence of disease-specific lesions (for example, senile plaques) and death of neurones instigates astroglial reactivity [14, 306, 308, 317]. Pathological changes in astroglia evolve in parallel with microglial responses. Microglial reactions, at least in the context of human disease, are represented by either activation (that may contribute to neuroinflammatory progression) or microglial paralysis with loss of neuroprotective capabilities, which all contribute to brain atrophy. Cells of oligodendroglial lineage are also affected, which leads to a failure in myelination and atrophy of brain connectome.

11.4.1 Neurodegeneration Following Toxic Brain Injury

Astrocytes play the leading role in chronic neurodegeneration following the brain poisoning by toxic agents. The core mechanism underlying this astroglial-dependent neurotoxicity, which leads to a substantial neuronal death, is linked to a failure of astroglial glutamate uptake. Glutamate clearance from the extracellular space is mainly accomplished by astroglial Na^+-dependent plasmalemmal glutamate transporters; astrocytes specifically express two types of these glutamate transporters, the

excitatory amino acid transporters 1 and 2 (EAAT1 and 2). This glutamate uptake is fundamental for astroglia-mediated neuroprotection against glutamate excitotoxicity; suppressing of astroglial glutamate uptake greatly increases neuronal damage following exposure to glutamate [58]. Astroglial glutamate uptake is usually impaired in neurodegeneration and can be considered as one of the common mechanisms of this process [126].

Exposure to heavy metals triggers neuronal death underlying condition known as heavy metal toxic encephalopathy, which manifests itself by impaired cognition and psychotic symptoms. This neurotoxicity results from astroglial homeostatic failure; heavy metals are accumulated by astroglial cells, thus damaging pathways responsible for glutamate homeostasis and catabolism. In methylmercury-induced encephalopathy known as Minamata disease (the name derives from the Japanese city of Minamata where massive poisoning with methylmercury occurred in 1950s, see [174]). When in astrocytes, methylmercury inhibits glutamate, glutamine and cystine transporters which compromises glutamate homoeostasis [194, 339]. Resulting increase in extracellular glutamate concentration triggers neuronal death underlying clinical symptoms that include cognitive decline, impaired vision and hearing, as well as motor symptoms. Similarly, astrocytes, endowed with capacity manganese transport system, emerge as a main target for manganese toxicity. Again, increased manganese in astroglial cells suppresses astroglial glutamate uptake with subsequent excitotoxic neuronal damage [260]. Similarly, astrocytes appear as a primary target for other heavy metals, such as arsenic, lead and cadmium, which all reduce expression of glial fibrillary acidic protein (GFAP) and trigger astroglial apoptosis, thus reducing astroglial homeostatic presence [223]. In aluminium toxic encephalopathy (symptoms of which include cognitive deficits and speech alterations), astrocytes are again the main targets. Aluminium accumulated by astrocytes impairs plasmalemmal glutamate transporters as well as gap junctions and causes astrocytic death [275]. Likewise, astroglial loss through apoptotic death plays a leading role in the encephalotoxic damage caused by cypermethrin, a synthetic II pyrethroid insecticide [173].

11.4.2 Wernicke Encephalopathy

Wernicke encephalopathy is a pathoanatomical substrate for Korsakoff syndrome, symptoms of which include ante- and retrograde amnesia, apathy and confabulation [135, 323]. This type of encephalopathy is essentially rapidly progressing malignant thalamo-cortical neurodegeneration. The pathological mechanism of Korsakoff–Wernicke syndrome is primarily associated with acute failure in astroglial glutamate uptake resulting from ~60 to 70% decrease in expression of EAAT1 and EAAT2 glutamate transporters. This remarkable decrease in plasmalemmal glutamate transporters expression has been identified in post-mortem human samples, as well as in the rat thiamine deficiency model of the disease [104, 105]. In addition to decrease in EAAT1/2 expression, astrocytes demonstrated signs of atrophy including decrease

in GFAP morphological profiles, as well as decrease in expression of glutamine synthetase (GS) and GAT-3 GABA transporter.

11.4.3 The Human Immunodeficiency Virus-1 (HIV-1)-Associated Dementia (HAD)

In the nervous system, the HIV-1 virus primarily infects and propagates in microglial cells. Microglia contributes to neuronal death by releasing various neurotoxic factors [123, 171], including Nef protein [267]. In HAD, astroglial cells develop both astrodegeneration and reactive astrogliosis. In the basal ganglia, astrocytes undergo a serious loss with the degree of astroglial death correlated with the degree of cognitive impairments [283]. In the entorhinal cortex and in the hippocampus, astrocytes show prominent reactivity [295].

11.4.4 Non-AD Dementia

The non-AD dementia is represented by many disorders including fronto-temporal lobar degeneration, Pick's disease, Cockayne syndrome, juvenile neuronal ceroid lipofuscinosis (JNCL) or Niemann-Pick type C disease. Astroglial contribution to these disorders is complex with signs for astroglial atrophy and astroglial apoptotic death [42, 320] as well as for astroglial reactivity, which is particularly prominent in the frontal and temporal cortices of patients with fronto-temporal dementia [124]. In thalamic dementia, a profound astrogliosis likely represents a key pathophysiological factor [221]. There is evidence indicating uncoupling of astroglial syncytium and aberrant activity of astroglial connexin hemichannels in JNCL [43], whereas early astroglial reactivation was reported in animal models of Niemann-Pick type C disease [222].

11.4.5 Amyotrophic Lateral Sclerosis (ALS)

Astrocytes play fundamental role in the pathogenesis of hereditary familial ALS associated with the mutation of the human superoxide dismutase 1 (hSOD1) gene. In hSOD1/G93A, associated animal models of ALS astrocytes undergo atrophy, pathological remodelling loss of function and cell death. These astroglial changes precede neuronal abnormalities and the emergence of clinical symptoms [241, 242]. The key pathogenetic factor linked to the neurotoxicity is represented by deficient astroglial glutamate uptake. Selective silencing of *hSOD1* gene in astrocytes in animal model delays ALS progression [337].

11.4.6 Parkinson's Disease

The role of neuroglia in emergence and progression of Parkinson's disease remains to be fully elucidated. There are some indications for microglial activation in relevant brain regions; this activation being possibly linked to neurotoxicity [63]. This microglial response, however, can be secondary, being triggered by neuronal death [108]. In 6-hydroxydopamine (6-OHDA) animal model of Parkinson's disease, inhibition of microglial activation was found to be neuroprotective [152]. Astrocytes have been considered to provide neuroprotection to dopaminergic neurones, based on in vitro experiments [180, 181]. In primary neuronal–glial co-cultures, astrocytes were shown to convert L-DOPA, the immediate precursor of dopamine, from neurotoxic to neurotrophic substance, and hence astroglia can be crucial for L-DOPA substitute therapy [179].

11.5 Astrocytes in Alzheimer's Disease

Alzheimer's disease is characterised by progressive neurodegeneration and an occurrence of specific histopathological markers represented by (i) focal extracellular deposits of fibrillar β-amyloid (also called neuritic or senile plaques) in the brain parenchyma and in the walls of blood vessels, and by (ii) intraneuronal accumulation of neurofibrillary tangles composed from abnormal hyperphosphorylated tau filaments. The initial neurodegenerative events in AD appear in the transentorhinal cortex, which subsequently spread to the entorhinal cortex and hippocampus. At later stages of the disease, the neurodegenerative process disseminates through the temporal, frontal, and parietal lobes [284, 285]. At these late stages, the grey matter undergoes severe damage with a profound loss of neurones and synaptic contacts and generalised atrophy of the brain parenchyma; this atrophy includes both white and grey matters. Contribution of neuroglia to the histopathology of Alzheimer's disease has been initially suggested by Alois Alzheimer himself; Fig. 11.1 shows original drawings of Alzheimer depicting pathologically modified glial cells of a senile plaque [8]. The role of astrocytes in the pathogenesis and progression of AD remains to be fully characterised, primarily because of the lack of longitudinal studies assessing the status of astroglia at different stages of the disease. From analyses of human post-mortem tissues, there has been generally agreed that at the late stages of the disease there are prominent reactive astrogliosis and inclusion of astrocytes into senile plaques [106, 193, 235].

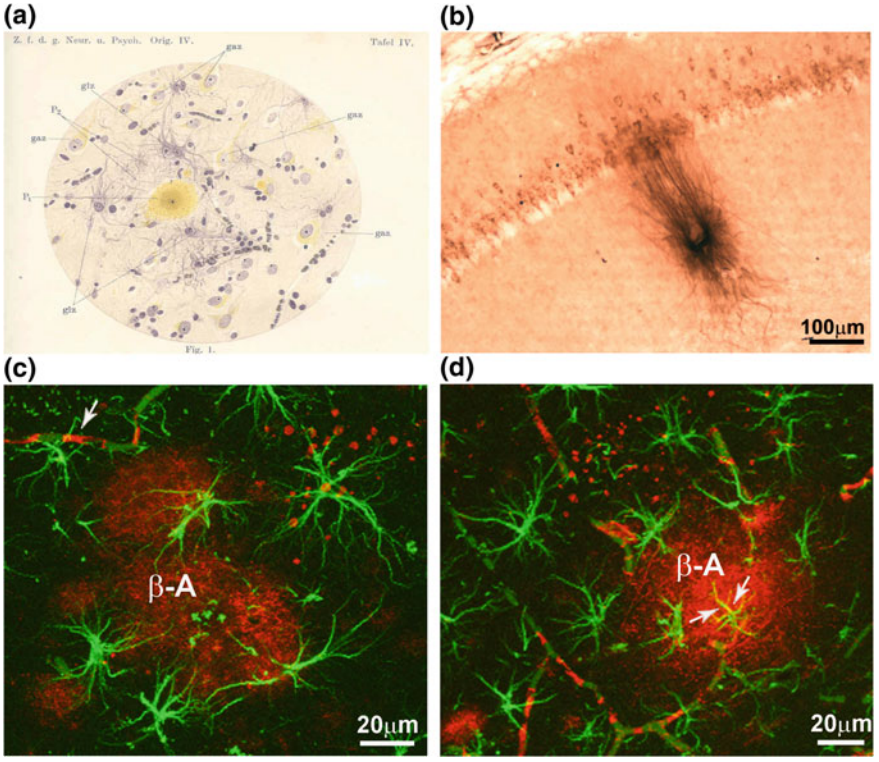

Fig. 11.1 Glial cells in AD **a** Alois Alzheimer´s drawing illustrating the glial reaction (astro- and/or micro-gliosis and hypertrophy) in a pathological brain containing senile plaques. Abbreviations: *gaz*, ganglionic cell—i.e. neurone; *glz*, glial cell, P, central part of the plaque; P_2, peripheral part of the plaque. From [8]. **b** Photomicrograph showing the presence of β-amyloid within the pyramidal neurones of the hippocampal CA1 area as well as the presence of a plaque in 12 months 3xTg-AD mice. **c**, **d** Confocal images showing GFAP-positive (green) reactive astrocytes surrounding β-amyloid plaques (β-A red; **c**). **d** Reactive astrocytes (green) and an astrocyte showing cytoplasmic β-amyloid accumulation (indicated by arrows; co-localisation is in yellow) near a neuritic plaque (red). Modified and adapted with permission from [235]

11.5.1 Astrocytes and β-Amyloid

The prevailing views on AD pathogenesis associate disease evolution with progressive accumulation of β-amyloid protein in the brain parenchyma and formation of senile plaques [87, 100, 122, 134]. Recently, however, the β-amyloid hypothesis became the subject of extensive criticism [49, 98, 99, 226]. Production of β-amyloid is mostly associated with neurones although there are several reports indicating the role of astrocytes in this process through either direct β-amyloid production or through deficient clearance.

Astroglial contribution to the clearance and degradation of β-amyloid has been suggested some 15 years ago [96, 195]. Reactive astrocytes associated with senile

plaques in the transgenic AD mouse model expressing mutant APP were found to express a zinc-dependent metalloendopeptidase neprilysin, an enzyme capable of degrading β-amyloid [11]. In experiments in vitro, cultured astroglial cells obtained from healthy mice were shown to accumulate exogenous β-amyloid. In contrast, this ability was absent in astrocytes isolated from the brains of APP AD model [333]. Similarly, astroglial β-amyloid accumulation was detected in cells from the entorhinal cortex of AD patients [192]. Conversely, β-amyloid was almost never detected in astrocytes from 3xTg-AD mice (Fig. 11.1d, [203]).

Astroglial contribution to β-amyloid production is not fully characterised. Neurones, which express β-amyloid producing enzymes β- and γ-secretases, were for a long time considered to be the main source for β-amyloid [143]. Indeed, healthy astrocytes seem not to express β-secretase; nonetheless, its expression can be induced by conditions of chronic stress or neuroinflammatory environment, thus adding astroglia to amyloidogenesis [33, 82, 117, 157, 205, 243, 342]. Expression of β-secretase was detected in reactive astrocytes emerging following immune lesion of cholinergic septohippocampal afferents or an occlusion of the middle cerebral artery [243]. Similarly, expression of β-secretase was found in reactive astrocytes in AD mice models expressing mutant human APP; these models, for example, included Tg2576, K670N-M671L APP or APP V717I mutations [101, 107, 243]. Of note, an increase in production of APP was characterised in a rat model of chronic neocortical astrogliosis, induced by grafting a foetal 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 E4 [166].

11.5.2 Astrogliosis in AD

Astroglial reactivity, generally characterised by an increase in expressions of GFAP, vimentin or s100β protein, has been detected in post-mortem tissues from AD patients [19, 92, 178, 189]. No obvious correlation between GFAP levels, degree of astrogliosis and β-amyloid load was detected [261]. Similarly, no differences in GFAP expression were found between the brains obtained from cognitively sound and demented patients [324]. Reactive, hypertrophic astrocytes, associated with senile plaques and perivascular β-amyloid deposits, are also observed in the brains of AD mice models (Fig. 11.1, [204, 235, 306]). It is important to highlight that astrogliosis in AD is never associated with the scar formation and it does not hamper the physiological non-overlap of astroglial territorial domains. It can be classified therefore as isomorphic or mild astrogliotic response. In the context of AD, the astrogliotic response can be triggered by various molecules, such as β-amyloid, molecules released from damaged cells or certain cytokines and chemokines. Soluble β-amyloid was found to initiate reactive astrogliosis in astrocytes in vitro [64]. This may be associated with certain intracellular signalling events, including, for example, Ca^{2+} signals. Such signals are indeed generated by exposure of cultured astrocytes to β-amyloid [2, 3,

94]. Treatment of cultured astrocytes with β-amyloid also resulted in inhibition of glutamate uptake, which can contribute to pathological progression [168].

11.5.3 Astroglial Atrophy in AD

Pathological changes of astrocytes in the AD pathology are not limited to astrogliotic response; it seems that astrogliosis occurs at later stages of the disease, with reactive astrocytes being mainly associated with senile plaques. Recent studies of transgenic AD mice models revealed a profound astrodegeneration that occurs at the early stages of AD progression [20, 203, 306].

Total number of astrocytes (labelled with antibodies against GFAP, s100β or GS) did not show any age-dependent variations in 3xTg-AD mice of 3–24 months of age [203, 204]. There are complex region- and disease stage-specific morphological changes in astrocytes in 3xTg-AD mice (Figs. 11.2, 11.3 and 11.4). At the early (i.e. pre-plaque) stages of the AD, astrocytes in the entorhinal cortex, prefrontal cortex and hippocampus demonstrate signs of morphological atrophy [139, 203, 204,

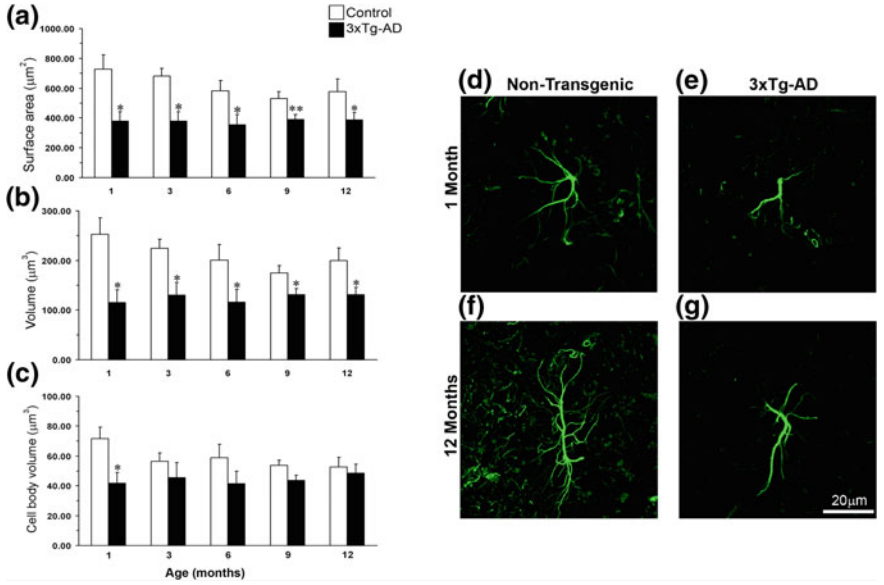

Fig. 11.2 Astroglial atrophy in the entorhinal cortex (EC) of 3xTg-AD mice. Comparison of astrocytic GFAP surface area and volume in the EC of non-Tg and 3xTg-AD animals of different ages. The histograms show a comparison of **a** surface area, **b** total cell volume and **c** somata volume in the EC at the ages of 1, 3, 6, 9 and 12 months between 3xTg-AD and non-Tg animals. Results are means ± S.E.M. (*$p < 0.05$ compared with the age-matched non-Tg control). Confocal micrographs show astrocytic atrophy in 3xTg-AD at 1 month (**e**) and 12 months (**g**) compared with the control animals (**d**, **f**). Reproduced with permission from [338]

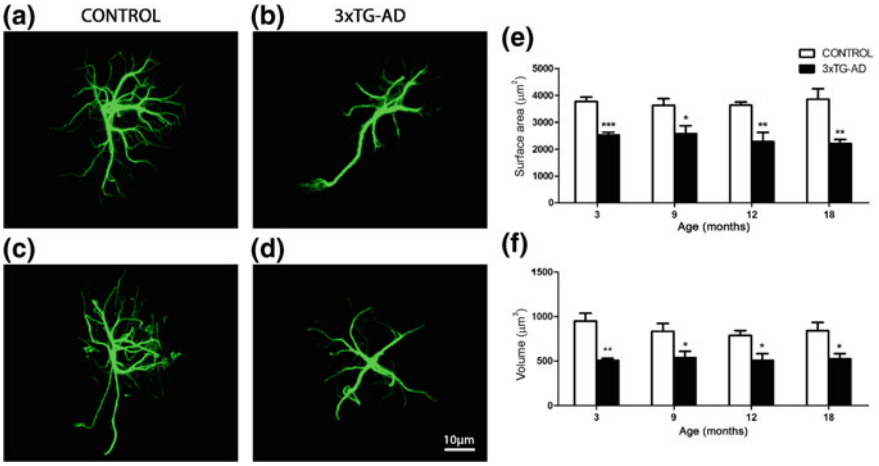

Fig. 11.3 Astroglial atrophy in the prefrontal cortex of 3xTg-AD mice. Confocal images showing morphology of GFAP-positive astrocytes in control non-Tg animals and astrocytic atrophy in the 3xTg-AD animals at 3 months (**a** and **b**, respectively) and 18 months (**c** and **d**, respectively) in the prefrontal cortex. Bar graphs showing the decreases in the surface area and volume (**e**, **f**) in 3xTg-AD mice when compared with control animals. Bars represent mean ± SEM. Reproduced with permission from [139]

338]. Astroglial atrophy develops first in the entorhinal cortex (from 1 month of age, Fig. 11.2); next, it occurs in the prefrontal cortex (3–4 months of age, Fig. 11.3) and finally in the hippocampus (6–9 months of age, Fig. 11.4). 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 labelled with GS antibodies in the hippocampus and in the prefrontal cortex, but not in the entorhinal cortex. Morphological atrophy of astrocytes was manifested by reduced expression of GFAP-rich cytoskeleton (surface and volume coverage) and decreased somata volume, as well as number and branching of cell processes. Very 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 [20, 21]. Astroglial atrophy was subsequently confirmed in human material, in astrocytes derived from pluripotent stem cells isolated from patients with family and sporadic AD (Fig. 11.5, [119, 184]).

At the later stages of AD pathology in hippocampi of 3xTG-AD animals (12–18 months; at this time neurofibrillary tangles also start to develop in neurones), formation of plaques and accumulation of extracellular β-amyloid initiates reactive astrogliosis. Numerous hypertrophic astrocytes accumulate exclusively around senile plaques and β-amyloid inundated blood vessels (Figs. 11.6 and 11.7; [203, 230, 316]). This astroglial hypertrophy is characterised by an increased volume and surface of both astrocyte somata and processes, which can increase their size up to 70% (Fig. 11.3). At the same time, astrocytes positioned away from the senile plaques retain their atrophic morphology (Fig. 11.6). In contrast to the hippocampus, accu-

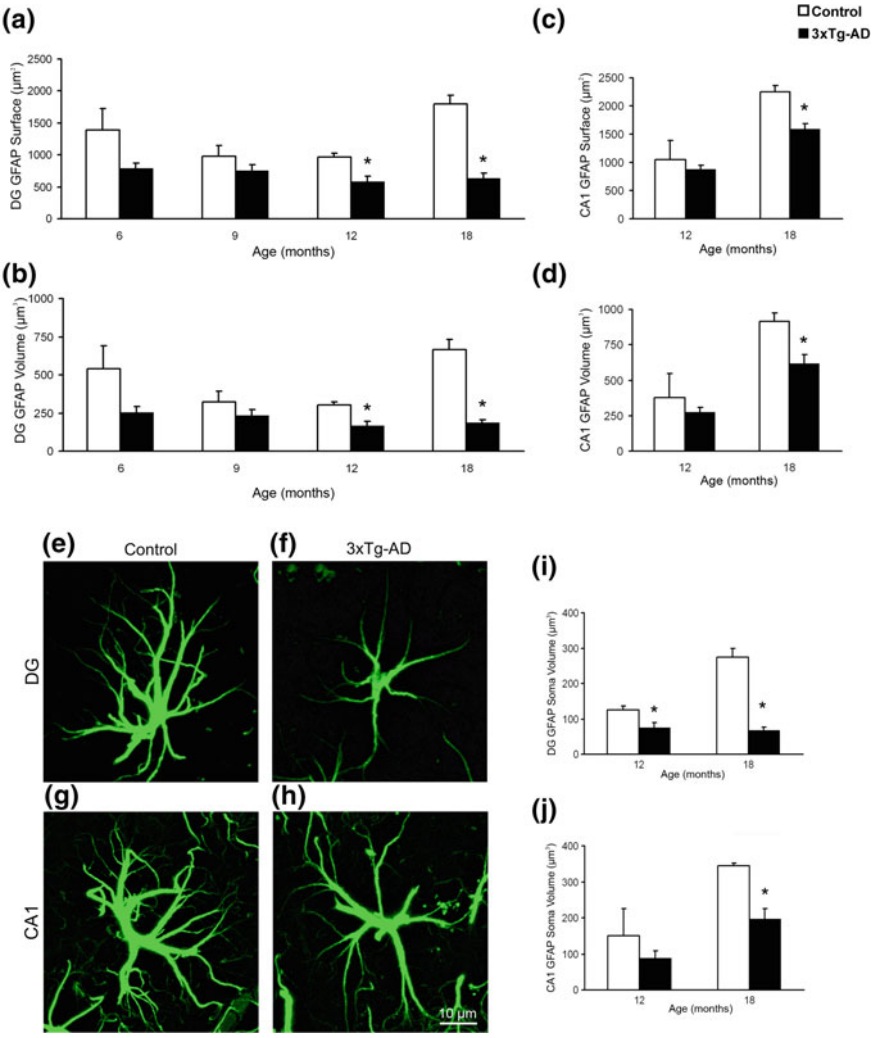

Fig. 11.4 Astroglial atrophy in the hippocampal areas of 3xTg-AD mice. Bar graphs showing the significant decrease in surface area, volume, and soma volume of GFAP-positive astrocytes in the dentate gyrus (DG) (**a**, **b**, **i**) and the CA1 region (**c**, **d**, **j**) of the hippocampus of the 3xTg-AD mice when compared with control animals. Bars represent mean ± SEM ($p < 0.05$). (**g**–**j**). Confocal micrographs illustrating the astrocytic atrophy in 3xTg-AD mice in the DG (**f**) and CA1 (**h**) compared to control animals (**e** and **g**). Reproduced with permission from [203]

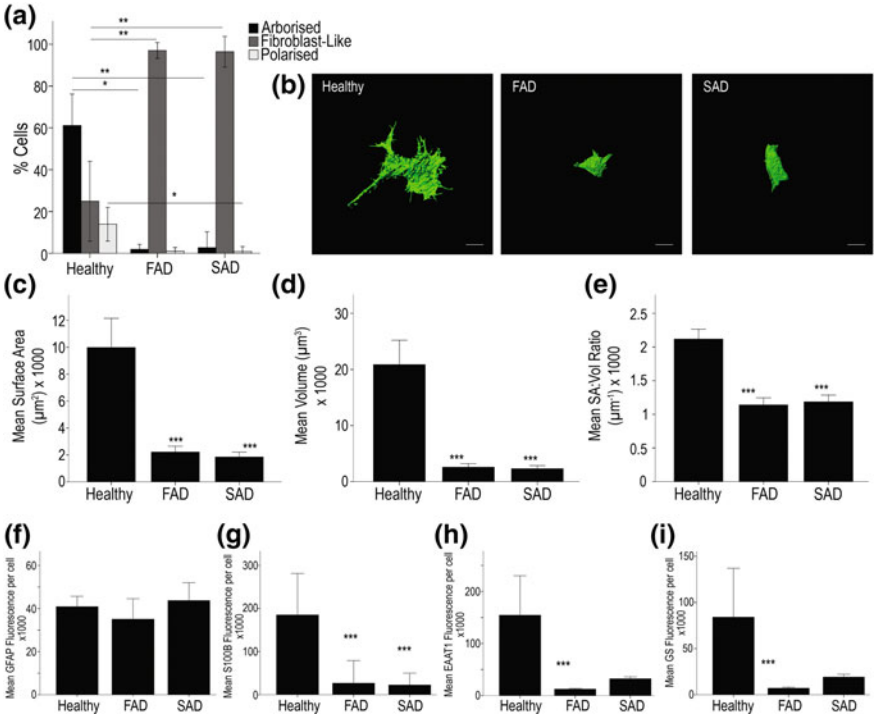

Fig. 11.5 Astrocytes derived from *PSEN1* M146L FAD and *ApoE4*$^{+/+}$ SAD patients exhibit significant atrophy when compared to those from healthy patients. **a** Exemplar 3D isosurface renders constructed from serial confocal z-stacks display clear differences in cell size and overall morphology (**b**). Scale bar = 10 μm. Quantification of cells using these renders by way of surface area (**c**), cell volume (**d**) and SA:Vol ratio (**e**) reveal significant differences in all aspects of cellular morphology between healthy and diseased astrocytes. Quantification of mean fluorescence intensity per immunoreactive cell reveals no significant difference in GFAP staining intensities between AD and control astrocytes (**f**) but S100B, EAAT1 and GS intensities are reduced in both FAD and SAD cells (**g**, **h** and **i**, respectively). Asterisks on graph; ***$p < 0.001$, **$p < 0.005$, *$p < 0.05$. Reproduced from [119]

mulation of β-amyloid and formation of senile plaques do not induce reactivity of astrocytes neither in the entorhinal nor in the prefrontal cortex (Fig. 11.7, [139, 338]). Deficient reactivity of astrocytes may determine the specific vulnerability of different brain regions to AD-type pathology. Atrophy of astrocytes at the early stages of AD may have important functional consequences. The decrease in astroglial complexity may affect synaptic coverage and homoeostatic support as well as functional performance of the neuronal–glial–vascular unit. This in turn can affect connectivity in neural network, reduce synaptic strength and disturb synaptic plasticity thus contributing to cognitive deficits.

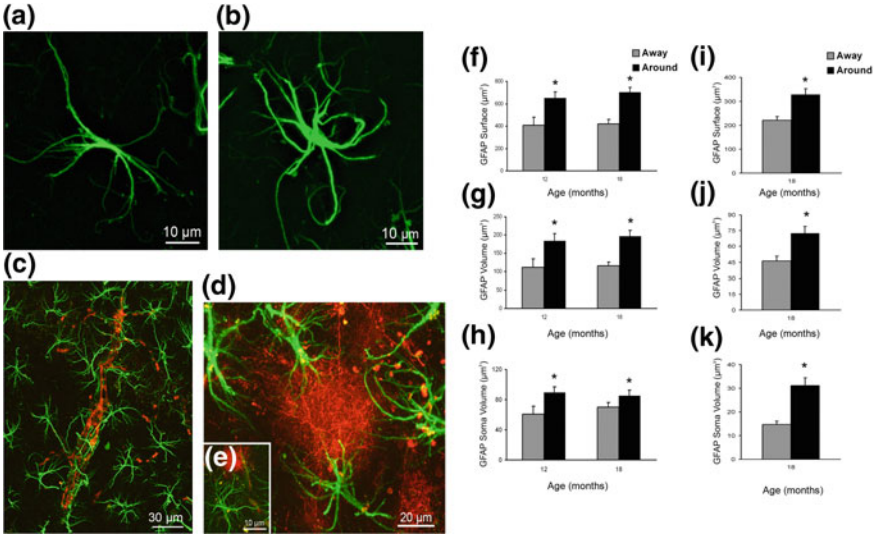

Fig. 11.6 Concomitant astroglial atrophy and astrogliosis at the advanced stages of AD-like pathology in 3xTg-Ad mice. **a**, **b** Confocal images of hippocampal preparations dually labelled by GFAP and by anti-β amyloid monoclonal antibody illustrating differential changes in GFAP profiles in astrocytes distant to the plaques (**a**) and associated with the β-amyloid plaques (**b**). **c**–**e** Confocal dual labelling images (GFAP in green and β-amyloid in red) in 3xTg-AD mice showing the accumulation of astrocytes around the β-amyloid plaques and vascular β-amyloid deposits. Astrocytes surrounding β-amyloid plaques (**d**, **e**) and β-amyloid deposits around a blood vessel (**c**), undergo astrogliosis. **f**–**k** Bar graphs showing GFAP-positive astrocytic surface area (**f**), volume (**g**) and somata volume (**h**) differences between astrocytes located around the β-amyloid plaques (Aβ) and those distant to the plaques in the CA1 of 3xTg-AD animals. **i**–**k** Similar astrocytic surface area (**i**), volume (**j**) and somata volume (**k**) differences are observed in the DG at 18 months of age. Bars represent mean 6 SEM ($p < 0.05$). Reproduced with permission from [203]

11.5.4 Loss of Astroglial Homeostatic Support Contributes to Early Cognitive Impairments

Atrophic changes in astrocytes, characterised in several AD animal models as well as in stem cells derived astrocytes appear as general diminution of astroglial territories, of astroglial coverage of neuronal membranes and overall decrease in astroglial homeostatic support. Arguably, this atrophy and loss of function of astrocytes, which occur early in the disease progression, may contribute to the disease pathophysiology. Atrophic astrocytes provide less synaptic coverage with deleterious consequences for synaptic transmission associated with compromised ion and neurotransmitter homeostasis or reduced local metabolic support; astroglial asthenia also results in decreased neuroprotection [232, 238, 316, 317]. All these changes are likely to weaken synaptic transmission and affect synaptic plasticity, thereby being responsible for initial cognitive deficiency observed at the early stages of AD.

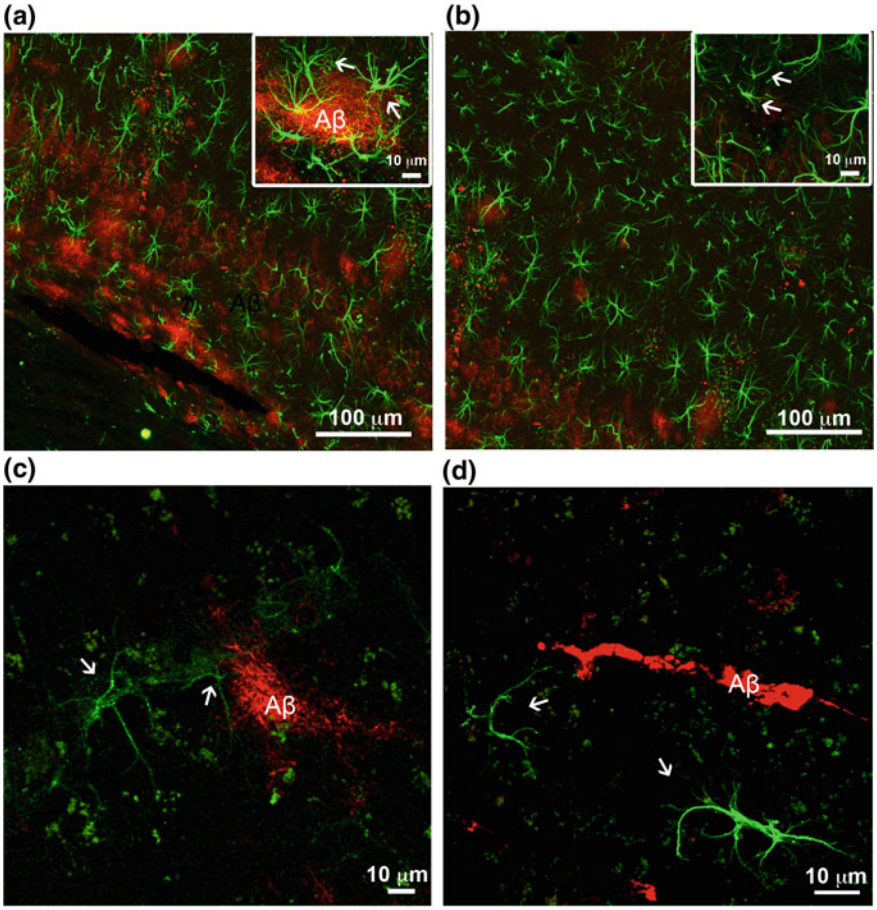

Fig. 11.7 β-Amyloid depositions trigger gliotic response in associated astrocytes in the hippocampus but not in the entorhinal cortex. **a**, **b** Confocal images of hippocampal preparations labelled by GFAP (green) and β-amyloid (red) illustrating differential changes in GFAP profiles in astrocytes in close association with Aβ plaques (**a**) and atrophic profiles of astrocytes (arrows) distant from β-amyloid deposits (**b**) in 3xTg-AD mice. **c**, **d** Confocal dual labelling images (GFAP in green and β-amyloid in red) showing the absence of reactive response of astrocytes in the entorhinal cortex of 3xTg-AD mice around perivascular vascular β-amyloid deposits (**c**) and β-amyloid plaques (**d**). Modified and reproduced with permission from [300, 338]

Early cognitive deficits are the very first symptoms of AD, which start to emerge decades before the occurrence of specific histopathology [55, 282]. Loss or impairment of cognitive capacities reflects reduced synaptic connectivity due to decreased synaptic function and synaptic loss [347]. Decrease in number of synapses indeed was found to be the earliest morphological change in AD [282]; and moreover the degree of synaptic loss correlates with the severity of dementia [61, 247]. Atrophy of astroglial perisynaptic processes may indeed underlie synaptic loss at the

early stages of AD. Furthermore, astrocytes are fundamental for synaptogenesis and synaptic maintenance; furthermore, astroglial plasmalemmal transporters control local concentrations of ions and neurotransmitters, most notably glutamate, that may contribute to local excitotoxicity [73, 292, 303]. Astroglial asthenia also impairs metabolic support accomplished by lactate shuttle [213]. Astrocytes are also critical for maintaining normal neurotransmission by supplying neurones with glutamine that is an indispensable precursor for both glutamate and GABA. Impairment of all these fundamental functions associated with astroglial atrophy and loss of function may be considered as a primary cause for distorted synaptic connectivity and early cognitive deficits in AD [300, 306, 316].

11.5.5 Neurovascular Unit in AD

Clinical evolution of AD is almost invariably associated with vascular deficiency and pathologies of the blood–brain barrier [276, 277]. It is well documented that the blood flow is significantly reduced in the brains of patients with AD, with these vascular deficits being prominent already at the early stages of the disease [24, 345]. These functional deficits stem from substantial remodelling of vascularisation in the brains altered by AD pathology [74]. Brain vessels are controlled by both neuronal and astroglial inputs [112, 346]. Astrocytes are central integrating elements of neurovascular units that bridge brain parenchyma with local circulation. By secreting various agents astrocytes target pericytes, vascular smooth muscle cells and endothelial cells, thus contributing to functional hyperaemia and regulating the blood–brain barrier [191, 279, 346]. Astroglial atrophy as well as reactivity may differentially remodel the neurovascular unit, even that can occur at both early and late stages of the disease and can contribute to cognitive abnormalities and neuronal damage.

11.5.6 AD and Astroglial Metabolic Support

Metabolic deficiency of the brain is a common feature of AD. Functional brain imaging demonstrated a progressive loss of utilisation of glucose in patients with different stages of AD; deficits in brain metabolism are present already at the very early stages of the disease, having thus diagnostic significance [187, 229]. Exposure of cultured astrocytes to β-amyloid impairs cellular metabolism, although both decrease [209, 264] and increase [5] of glucose utilisation were detected. Likewise, both decrease [34, 160] and increase [31, 264] of the activity of glucose metabolism enzymes were described in post-mortem AD brains.

11.5.7 Deficient Astroglial Reactivity Defines Susceptibility of Brain Tissue to AD Pathology

Astroglial atrophy and asthenia in AD also lead to a loss of their defensive function [300]. As has been alluded previously, in experiments on 3xTg-AD mice, reactive astrocytes were accumulated around senile plaques and form perivascular β-amyloid deposits [203, 204]. These hypertrophic astrocytes are specifically associated with extracellular β-amyloid deposits, whereas astrocytes distant to the plaques remain atrophic (so in this sense astroglial atrophy emerges at the early stages of AD and is complimented by astrogliosis at later stages, when specific lesions develop). In contrast, in entorhinal and prefrontal cortices, extracellular β-amyloid accumulation does not trigger astrogliotic response (Fig. 11.7, [139, 338]) indicating failure of astroglial neuroprotection.

There are several lines of evidence demonstrating that reactive astrocytes are neuroprotective in the context of AD. For example, the Tg2576 mice (that harbour the APP_{Swe} mutation—see [110]) demonstrate early and prominent astroglial reactivity which correlates with relatively slow development of AD. Furthermore, senile plaques in these animals are resembling human β-amyloid deposit being represented by fleecy, granular, cored and diffused amyloid plaques [336], Incidentally, the Tg2576 mice display certain similarities with the prodromal stage of AD known in humans as mild cognitive impairment [16]. Astroglial capabilities to mount astrogliotic response change with age. The density of reactive astrocytes changes with age. In old Tg2576 mice, GFAP staining demonstrated prevalence of atrophic astrocytes with fewer reactive astroglial cells, which may be related with increased AD pathology in ageing [300]. Inhibition of reactive astrogliosis in the AD mouse model significantly increased β-amyloid load and exacerbated pathological progression [137].

The in vivo brain imaging of astrocytes uses PET detection of ^{11}C-deuterium-L-deprenyl (^{11}C-DED) that binds to MAO-B in the astrocytes [81]. When using a multi-tracer PET detecting ^{11}C-PIB (marker of fibrillar β-amyloid), ^{18}F-FDG (marker cerebral glucose metabolism) and ^{11}C-DED (marker of astrogliosis), the highest binding of ^{11}C-DED (which reflects maximal reactivity of astrocytes) was observed in patients with mild cognitive impairment (MCI) and high levels of fibrillar amyloid plaques in the brain (PIB+) reflecting prodromal AD [46]. Decrease in astroglial reactivity parallels the switch from MCI to full-blown AD with senile dementia again demonstrating the neuroprotective role of astrogliotic remodelling (Fig. 11.8 and [300]).

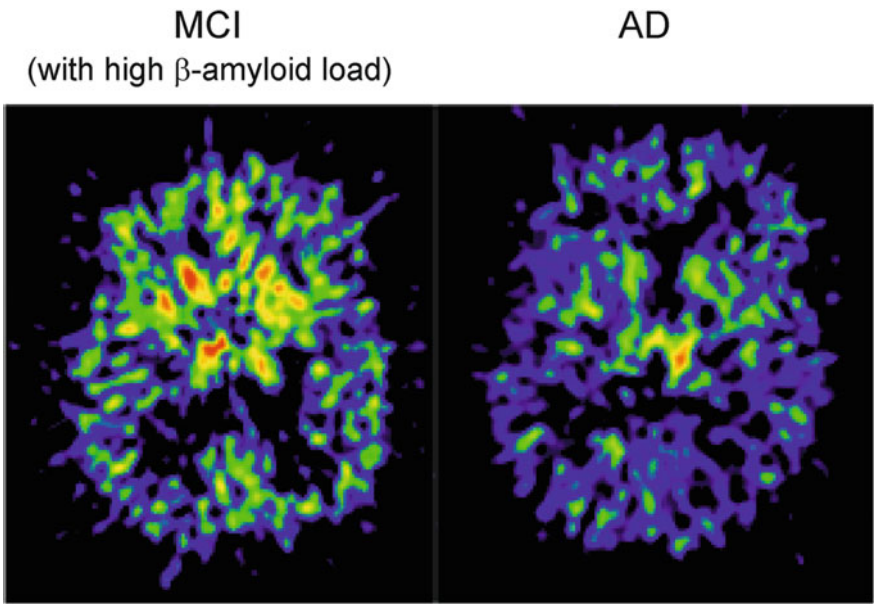

Fig. 11.8 Failure in astroglial reactivity defines the switch between mild cognitive impairment and senility in AD. Prominent astrogliosis in the brain of patient with mild cognitive impairment associated with high β-amyloid load (*Left panel*) in comparison with patient with Alzheimer's disease (*Right panel*). Representative images of ^{11}C-d-deprenyl binding (that reflects MAO-B expression in astrocytes) were obtained by position emission tomography. The MCI patient also showed high presence of fibrillar amyloid plaque as measured with ^{11}C-PIB (the status that could be identified as a prodromal AD). The PET scans show sagittal sections of the brain at the level of basal ganglia. Colour scale indicates red = very high, yellow = moderately high, green = high, blue = low ^{11}C-d-deprenyl binding. Photo courtesy of A. Nordberg, Karolinska institutet. Reproduced with permission from [300]

11.6 Astroglial Calcium Signalling in AD

11.6.1 Ionic Signalling as a Substrate of Astroglial Excitability

Astroglial excitability is based on spatially and temporally controlled fluctuations of intracellular concentration of ions, most notably of Ca^{2+} and Na^{+}, although recently the signalling role for K^{+} and Cl^{-} begun to be considered [130, 240, 246, 256, 313, 314, 328]. Astroglial Ca^{2+} signalling is the most studied; astroglial Ca^{2+} responses have been discovered in the late 1980s [71, 299], and are implicated in various signalling functions.

Physiological stimulation has been demonstrated to trigger Ca^{2+} signals in astrocytes in vitro, in situ and in vivo [22, 66, 129, 131, 256]. Astroglial calcium signalling has a spatio-temporal hierarchical organisation: at the cellular level, astrocytic Ca^{2+}

signals are classified into local Ca^{2+} microdomains, intracellular propagating waves, global Ca^{2+} signals and Ca^{2+} oscillations [95, 196, 259, 265]. These distinct forms of Ca^{2+} signals reflect operation of different mechanisms. Global Ca^{2+} signals and propagating Ca^{2+} waves originate from Ca^{2+} release from the endoplasmic reticulum Ca^{2+} store; this release is primarily mediated by inositol 1,4,5 trisphosphate ($InsP_3$) receptor type 2, ($InsP_3R2$). Local Ca^{2+} microdomains in contrast are often generated by Ca^{2+} entry through ionotropic receptors, transient receptor potential channels, store-operated Ca^{2+} entry (SOCE) or reversed Na^+/Ca^{2+} exchanger [305]. Astroglial Ca^{2+} signals regulate several cellular processes, including secretion, metabolism and astroglial reactivity. Astroglial Na^+ signalling is much less characterised, although basic parameters of Na^+ transients evoked by physiological stimulation have been described in experiments in cultured cells and in astrocytes in brain slices [86, 127, 128, 148–150, 227, 344]. Astroglial Na^+ signals regulate many homeostatic plasmalemmal transporters, thus coordinating neuronal activity with astroglial support [130, 240].

11.6.2 Aberrant Calcium Signalling in AD

The fundamental role of Ca^{2+} in regulation of cellular survival and cell death inspired the "calcium hypothesis of ageing and neurodegeneration" formulated by Zaven Khachatirian [125] who based this hypothesis on experimental studies of Philipp W. Landfield [146, 147]. This Ca^{2+} hypothesis posits that ageing neurones experience increased Ca^{2+} influx during depolarisation, which elevates cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), thus triggering excitotoxicity. Subsequent studies revealed that physiological neuronal ageing is associated with much subtle alterations of neuronal Ca^{2+} extrusion, which, although capable of handling normal Ca^{2+} loads, fail to clear excessive Ca^{2+} influx. This deficit in Ca^{2+} handling stipulates higher vulnerability of old neurones to the periods of high activity [286, 287, 312, 334]. In neurodegenerative diseases (including AD), Ca^{2+} homeostatic machinery is, however, seriously compromised, and hence these disorders have been regarded as "chronic calciumopathies" [273, 274]. Almost nothing is known about changes in Ca^{2+} homeostatic machinery, resting Ca^{2+} handling, and Ca^{2+} signalling in aged astrocytes. There are several isolated reports demonstrating a decrease in evoked astrocytic Ca^{2+} signals in mice aged 16–21 months, when compared to adult animals [144, 145].

11.6.3 Exposure to β-Amyloid Disturbs Astroglial Ca^{2+} Signalling

Whether β-amyloid is indeed a causal factor in AD or a mere epiphenomenon, exposure to it affects astroglial Ca^{2+} dynamics. Experimental studies in vitro in primary

astroglial cultures demonstrated acute effects of β-amyloid on Ca^{2+} signalling. Resting $[Ca^{2+}]_i$ significantly (2–3 times) increased in astrocytes exposed to β-amyloid (in concentrations ranging between 100 nM and 5 μM) for several hours [103, 161]. These findings, however, have not been universally confirmed; several investigations found that incubations of cultured astrocytes with 100–200 nM of β-amyloid (or its toxic fragment β-amyloid$_{25\text{-}35}$) for 48–72 h did not significantly change resting $[Ca^{2+}]_i$ [47, 288].

Acute exposure to β-amyloid triggered oscillations of $[Ca^{2+}]_i$ transients in cultured astrocytes and in astrocytes in organotypic slices [2–4, 52, 114, 163]. These acute effects, however, were not always observed and several studies have not noticed such acute effects [47, 288]. Treatment of cultured astrocytes with 1 μM of β-amyloid$_{1\text{-}40}$ induced $[Ca^{2+}]_i$ elevations only in 17% of all the cells, whereas application of β-amyloid$_{25\text{-}35}$ triggered Ca^{2+} signals in 36% of all astrocytes [114]. In primary cultured rat newborn astrocytes, application of 1 μM of β-amyloid$_{25\text{-}35}$ induced $[Ca^{2+}]_i$ transients in 27% of primary cultured rat newborn astrocytes; at 2–5 μM ~60% of astrocytes responded with $[Ca^{2+}]_i$ transients [270]. Of note, low concentrations of β-amyloid apparently stimulate astroglial Ca^{2+}-permeable α7 nicotinic cholinoreceptors, which resulted in Ca^{2+} influx and generation of Ca^{2+} responses [154, 216].

11.6.4 Pathological Ca^{2+} Signalling in AD Astrocytes In Vitro

Analysis of $[Ca^{2+}]_i$ dynamics in astrocytes isolated from several mouse models of AD also demonstrated aberrant Ca^{2+} signalling [162, 163]. Abnormally large Ca^{2+} signals have been detected in astrocytes isolated from newborn 3xTg-AD mice, indicating intrinsic alterations of Ca^{2+} homeostatic cascades [239]. Astrocytes isolated from 3xTg-AD mice in particular showed increased store-operated calcium entry (SOCE) [239]. Cultures of astrocytes isolated from 3xTg-AD animals also demonstrated aberrant kinetics of ATP-induced Ca^{2+} signals and Ca^{2+} oscillations [269]. Further analysis revealed that these aberrations are most likely associated with expression of mutant PS1 presenilins residing in the endoplasmic reticulum [269]. In the Tg5469 AD mouse which over-expressed APP, the SOCE was not changed, whereas deletion of APP caused an inhibition of store-operated Ca^{2+} entry [164]. This inhibition may be associated with down-regulation of expression of either TRPC1 or Orai 1 channels.

11.6.5 Pathological Ca^{2+} Signalling in Astrocytes In Vivo

Imaging astroglial $[Ca^{2+}]_i$ dynamics in vivo in the brains of AD animal models reliably demonstrated aberrant, hyperactive $[Ca^{2+}]_i$ dynamics, which is fundamentally similar to neuronal hyperexcitability routinely observed in AD-like experimental pathology [350]. Aberrant hyperactive $[Ca^{2+}]_i$ oscillations have been observed in reactive astrocytes associated with senile plaques. High levels of resting $[Ca^{2+}]_i$,

pathological Ca^{2+} oscillations and long-projecting propagating Ca^{2+} waves have been identified in plaque-associated astrocytes in the brains of APP/PS1 mice [138]. Emergence of astroglial Ca^{2+} hyperactivity was also suggested to be linked with abnormal purinergic signalling in reactive astrocytes. There are claims that reactive astrocytes release excessive amounts of ATP through connexin hemichannels. This ATP, acting in autocrine manner, activates astroglial P2Y purinoceptors, which mediate pathological Ca^{2+} signalling [62]. An increased frequency of astroglial Ca^{2+} oscillations was also observed in AD animals in the pre-plaque stage, and these abnormal $[Ca^{2+}]_i$ dynamics coincided with the instability of vascular tone probably indicating that astrocytes in their ability to regulate local blood flow [278].

11.6.6 Astroglial Ca^{2+} Signalling Toolkit Is Remodelled in AD

The AD as a chronic pathology leads to a substantial remodelling of astroglial Ca^{2+} signalling toolkit. Chronic exposure of cultured astrocytes to β-amyloid as well in vivo AD pathology (in model animals) changes expression of various components of Ca^{2+} homeostatic/signalling system; these molecules include, for example, metabotropic and ionotropic receptors, intracellular Ca^{2+} channels, store-operated Ca^{2+} channels and Ca^{2+} sensors [162, 163, 309].

Exposure of astroglial cultures to 10–30 μM β-amyloid$_{1\text{-}40}$ for 48–72 h resulted in an increase of the amplitude of $[Ca^{2+}]_i$ transients in response to stimulation of metabotropic glutamate receptor mGluR5. This augmentation of metabotropic Ca^{2+} signalling was a consequence of an up-regulated expression of mGluR5 detected at both mRNA and protein levels [47]. This was corroborated in another series of experiments which demonstrated that 24–72 h exposure of cultured astrocytes to 100 nM–20 μM of oligomeric β-amyloid increased expression of mGluR5 [93, 94, 161]. This up-regulation of mGluR5 expression was suppressed by the inhibitors of calcineurin and Nf-κB (nuclear factor κ-light-chain-enhancer of activated B cells) [161]. Similar increase in expression of mGluR5 was detected in astrocytes in the animal AD models and in post-mortem human tissues. Increased levels of mGluR5 protein were found in the post-mortem hippocampal preparations obtained from AD patients at advanced (Braak V–VI) stages of the disease [47, 161]. Incubation of astrocytes with nanomolar (0.1–100 nM) concentrations of β-amyloid$_{1\text{-}42}$ for 24–72 h increased the expression of several subunits of nicotinic cholinoreceptors including α7nAChR, α 4nAChR and β2nAChR [335]. Similarly, increased levels of α7nAChR were identified in the post-mortem brain tissue of patients with sporadic AD and familial AD associated with the Swedish APP mutation [341].

Another important class of molecules affected by AD progression is represented by intracellular Ca^{2+} release channels. Exposure of cultured astrocytes to 125 nM of Tat-ProADAM10$_{709\text{-}729}$ peptide (this peptide inhibits production of β-amyloid$_{1\text{-}40}$ and β-amyloid$_{1\text{-}42}$) for 72 h leads to an increased expression of $InsP_3R1$ [93]. Sim-

ilarly, up-regulation of expression of $InsP_3R1$ and $InsP_3R2$ mRNA was detected in astrocytes in vitro which were exposed to 100 nM oligomeric β-amyloid$_{1-42}$ [161]. Pathological remodelling of Ca^{2+} homeostatic and signalling cascades differ between different brain regions. Expression of InsP3R1 is increased in healthy hippocampal astrocytes exposed to β-amyloid, but remains unchanged in astrocytes from the entorhinal cortex [94]. However, β-amyloid did not affect expression of $InsP_3R1$ in astrocytes isolated from 3xTg-AD animals, indicating that exogenous β-amyloid and over-expression of mutated AD-related genes share common molecular pathways that cause deregulation of Ca^{2+} homeostasis. In post-mortem studies, however, generalised decrease in the expression of $InsP_3Rs$ was detected in all brain regions including frontal, parietal and entorhinal cortices and the hippocampus [102, 141, 340]. These studies did not, however, discriminate between cell types. Many other components of Ca^{2+} signalling system are affected by AD; these include components calpain-10 [85], NFAT (Nuclear factor of activated T-cells) [1], NF-κB [93], calcineurin [93, 199], L-type calcium channels [59] and store-operated Ca^{2+} channels [239]. All in all 32 genes associated with Ca^{2+} signalling were found to be affected in the transcriptome of astrocytes microdissected from patients with different Braak stages of AD. It appeared that expression of several isoforms of calmodulin kinase CaMKII, two isoforms of calmodulin, plasma membrane Ca^{2+}-ATPases, ryanodine receptors and InsP3Rs, was decreased at advanced (Braak V–VI) stage when compared with early (Braak I–II) stage of the disease [262].

11.6.7 Ca^{2+} Release and Astroglial Reactivity

As has been alluded before, astrogliosis is a prominent component in certain brain regions in the context of AD; reactive astrocytes associate themselves with senile plaques in human tissue and in the brains of AD animal models, arguably forming a defensive barrier protecting neural networks [106, 306]. Suppression of astrogliotic response (for instance, by genetic deletion of GFAP and vimentin) exacerbates β-amyloid load and facilitates plaques dissemination [137]. Astroglial reactivity, however, is different in different regions of the brain. Prominent astroglial reactivity is observed in the hippocampus, whereas the emergence of senile plaques and β-amyloid depositions does not trigger astrogliosis in entorhinal and prefrontal cortices of AD mice models. Underlying molecular mechanisms might be linked to a deficient Ca^{2+} signalling in astrocytes from different brain regions.

In the AD context, one of the most relevant signals instigating astroglial reactivity is β-amyloid, and indeed exposure of astroglia to β-amyloid in vitro or in situ triggers astrogliosis [4, 306]. As has been described above, β-amyloid also evokes $[Ca^{2+}]_i$ elevation. It appears that β-amyloid-induced Ca^{2+} signals originate from Ca^{2+} release from the endoplasmic reticulum Ca^{2+} store and these Ca^{2+} signals are directly linked to the initiation of astrogliotic response. Suppression of Ca^{2+} release from the ER with pharmacological tools effectively inhibits astrogliosis induced by β-amyloid in both cultured astrocytes and astroglia in organotypic slices [4]. The causal role of

Ca^{2+} release in astroglial reactivity was directly demonstrated: deletion of $InsP_3R2$ effectively suppressed astrogliotic activation [120]. Sensitivity of astrocytes from different brain regions to β-amyloid is different: β-amyloid up-regulates expression of molecules providing for ER Ca^{2+} release in hippocampal but not in entorhinal astrocytes [94]. This may explain the absence of astrogliotic defensive response in astrocytes from cortical regions, which renders these parts of the brain vulnerable to the AD pathology [162, 300].

11.7 AD Pathology Affects Astroglial Vesicular Trafficking and Secretion

Astrocytes are secretory cells, being a part of CNS-wide "gliocrine" system [301]. Astrocytes are known to secrete ~200 molecules, many of which are released through exocytosis of secretory vesicles [349]. Intracellular astroglial vesicles are also fundamental for delivery of various molecules [298] such as ion channels, membrane receptors and transporters, as well as major histocompatibility complex II (MHC-II, [296]) and EAAT 2 [266], to the plasma membrane. Vesicular traffic is controlled by sophisticated molecular cascades, which in turn are regulated by increases in $[Ca^{2+}]_i$ [220, 266]. Changes in $[Ca^{2+}]_i$ differentially regulate motility of distinct vesicles types. Increases in $[Ca^{2+}]_i$ reduce the motility of vesicles carrying peptides, such as atrial natriuretic peptide, while accelerating motility of vesicles containing vesicular L-glutamate transporter VGLUT1 [218–220]. Proteolytic enzymes stored in endolysosomes may contribute to the development of AD. One of these proteases is represented by the insulin-degrading enzyme (IDE), which, when secreted into the extracellular space, may degrade β-amyloid. Astrocytes are the main cell type which produces and releases IDE [68, 263]. It has been hypothesised that in AD the capacity of secreting IDE is reduced, leading to an increase in β-amyloid, which involves a reduction in autophagy-based lysosomal secretion of IDE [263].

Astrocytes from 3xTg-AD mice demonstrated an aberrant vesicular traffic. Spontaneous mobility of peptidergic and endolysosomal vesicles as well as the ATP-evoked, Ca^{2+}-dependent, vesicle mobility was all diminished in AD astrocytes (Fig. 11.9). Transfection of healthy rat astrocytes to express familial AD-associated mutated presenilin 1 (PS1M146V) caused very similar impairment of peptidergic vesicle trafficking. The stimulation-dependent peptide secretion from single vesicles was less efficient in 3xTg-AD and PS1M146V-expressing astrocytes than in healthy controls. The impaired vesicle dynamics and reduced evoked secretion of the signalling peptides both may contribute to the development of AD [269].

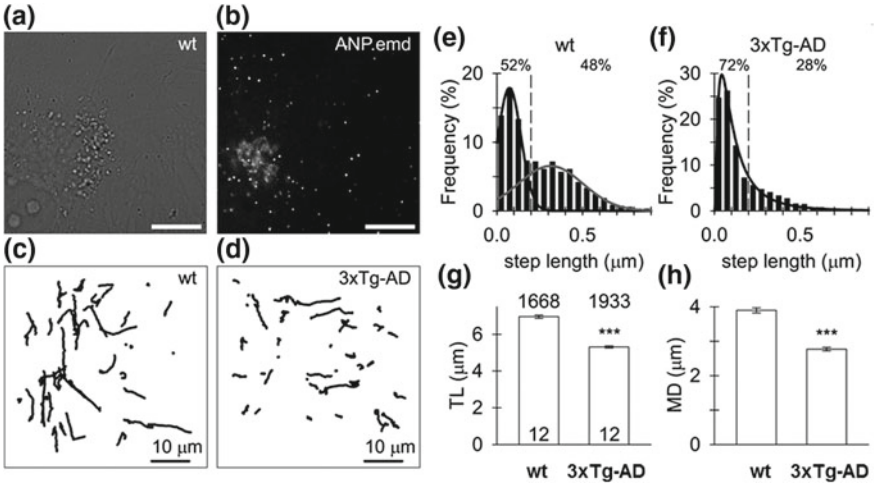

Fig. 11.9 Decreased spontaneous mobility of peptidergic vesicles in 3xTg-AD astrocytes. **a** Live cultured wild-type (wt) astrocyte under DIC optics and **b** the confocal image of the same cell expressing fluorescent peptide atrial natriuretic peptide-emerald green (ANP.emd), stored in individual vesicles, observed as bright fluorescent puncta; scale bars, 10 μm. **c** Vesicle tracks ($N = 50$) obtained in a 15-s epoch of imaging representative control (wt) and **d** 3xTg-AD astrocytes expressing ANP.emd, respectively. Note less elongated vesicle tracks in the 3xTg-AD astrocyte. **e**, **f** Frequency histogram of the step length in spontaneously moving vesicles in wt ($N = 5025$, **e**) and 3xTg-AD ($N = 5072$, **f**) astrocytes. The data were fitted with the function $f = a \times \exp(-0.5 \times (x/x_0)/b)^2/x$, where $a = 17.88 \pm 0.00$, $b = 0.07 \pm 0.00\ \mu m^{-0.5}$, $x_0 = 0.07 \pm 0.00\ \mu m$ (black curve) and $a = 6.53 \pm 0.13$, $b = 0.19 \pm 0.01\ \mu m^{-0.5}$, $x_0 = 0.31 \pm 0.01\ \mu m$ (grey curve) in wt astrocyte, and with the function $f = a \times \exp(-0.5 \times (\ln x/x_0)/b)^2/x$, where $a = 1.96 \pm 0.04$, $b = 0.92 \pm 0.02\ \mu m^{-0.5}$, $x_0 = 0.10 \pm 0.00\ \mu m$ (black curve) in 3xTg-AD astrocyte. The vertical dashed line indicates the step length of 0.2 μm obtained close to the intersection of distributions (black and grey curve) in wt astrocytes to discriminate small (<0.2 μm) from large (≥0.2 μm) steps. Note the higher proportion (%) of smaller steps lengths in the 3xTg-AD astrocyte indicated by the absence of the second mode distribution seen in wt astrocytes. **g** Track length (TL), **h** maximal displacement (MD),note substantially diminished TL, MD in 3xTg-AD astrocytes. The numbers above the top of the bars (mean ± SEM) indicate the number of vesicles analysed; the numbers at the bottom of the bars indicate the number of cells analysed; "***"—indicates p values < 0.001. Modified with permission from [269]

11.8 GABAergic Astrocytes in AD

In the healthy young CNS, astrocytes contribute to GABAergic transmission through (i) supplying glutamine, needed for GABA biosynthesis in neuronal terminals and (ii) removing ~20% of all released GABA by dedicated plasmalemmal transporters GAT-1 and GAT-3. After being transported into the astrocytes, most of GABA is catabolised by GABA transaminase (GABA-T) to succinate, which is subsequently utilised for production of ATP [253, 305, 319]. Due to this energy-oriented catabolism, the concentration of GABA in the cytosol of astrocytes is rather low. Ageing and neurodegeneration, however, significantly affect astroglial GABA metabolism; concentration

of GABA in astrocytes in elderly [155], in patients with AD [118, 332] and in transgenic AD models [41, 118, 332], is significantly higher. This increase is particularly prominent in reactive astrocytes associated with senile plaques in AD animal models; intracellular GABA concentration in these AD reactive astrocytes is several times higher than in age-matched controls and is very similar to neuronal GABA content [41, 118, 332]. These changes in astrocytic GABA content in reactive astrocytes are accompanied with an up-regulation of expression of GABA producing enzyme glutamic acid decarboxylase GAD67 as well as with an increase in expression of astroglia-specific monoaminoxidase-B (MAO-B) [118]. At the same time, expression of glutamine synthetase is specifically down-regulated in reactive astrocytes surrounding senile plaques in the hippocampus and prefrontal cortex of 3xTg-AD mice (Fig. 11.10 and [204]). Thus reactive astrocytes acquire machinery to synthetase GABA either from glutamate (through GAD67 and increased glutamate availability due to the loss of glutamine synthetase) or from putrescine through MAO-B pathway [84]. Furthermore, there is an increased glutamatergic neuronal activity around senile plaques [206]; this conceivably increases astroglial glutamate uptake and availability of cytosolic glutamate for conversion to GABA cytosolic glutamate concentration glutamate transport into astrocytes Astroglial GABA may potentially be released from astrocytes by diffusion through Bestrophin-1 Cl^- channels or through reversed GAT3 transporters (Fig. 11.11, [84]). The emergence of GABAergic astrocytes may represent yet another defensive response; as GABA release from astroglia may counteract neuronal hyperexcitability by an increase of tonic inhibition [84].

11.9 Astrocytes as Therapeutic Targets in AD

Neuroglia is yet to be considered as a fundamental target for novel therapeutic agents for neurological disorders and neurodegenerative diseases in particular. It is conceivable that by modulating the status of astrocytes, by reversing or halting astrocytes degeneration and asthenia or by modulating astroglial reactivity, the course of AD can be altered and the disease can be delayed or cognitive alterations reversed. Several possible strategies that may affect astroglial pathology have recently emerged.

11.9.1 Lifestyle Changes May Reverse Astrodegeneration

Recent experiments have demonstrated that environmental modifications such as sensory stimulation, dieting or usage of food supplements may affect AD progression and at the same time change astroglia morphology and revert astroglial atrophy. Experiments on APP and 3xTg-AD mice models revealed that chronic exposure of these animals to physical activity and/or to enriched environment reversed morphological atrophy of astrocytes, increased GFAP expression and normalised GFAP-positive astroglial profiles (Fig. 11.12); most importantly, these astroglia-specific

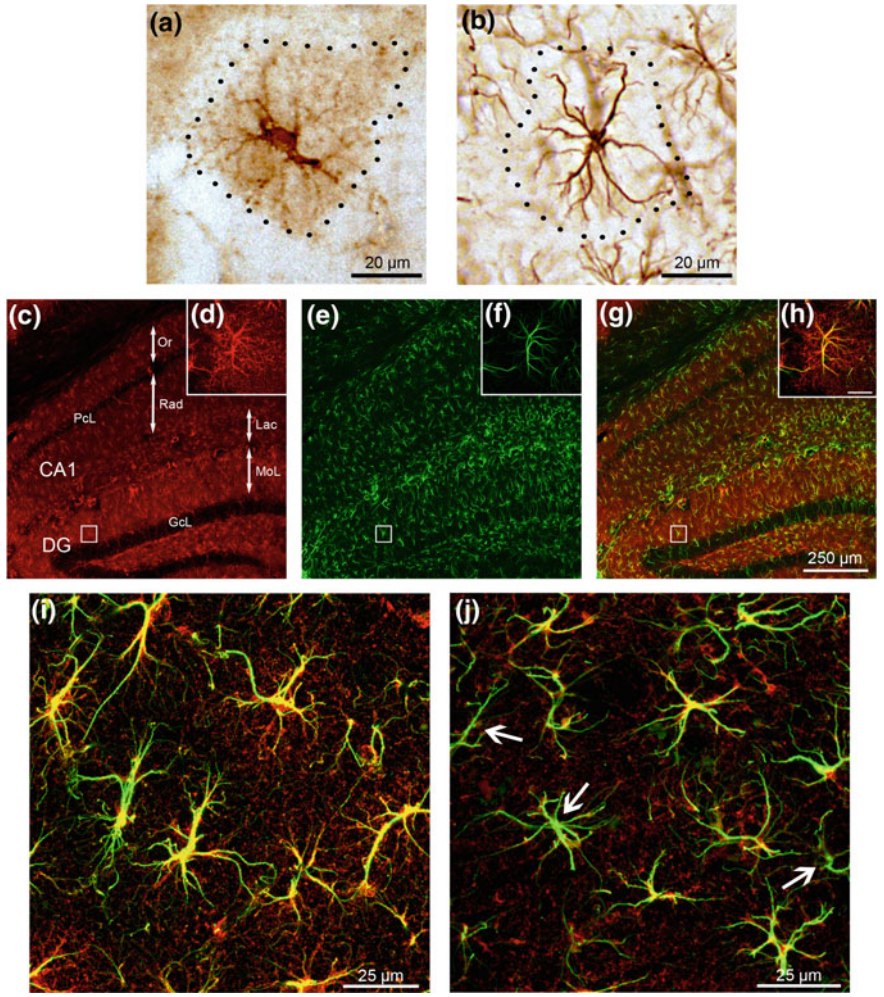

Fig. 11.10 Down-regulation of glutamine synthetase (GS) expression in hippocampal astrocytes in 3xTg-AD mice. **a**, **b** Light microscopy images of GS—(**a**) and GFAP—(**b**) positive astrocytes. **c**, **e**, **g** Confocal images of hippocampal preparation labelled for GS (**c**, red), GFAP (**e**, green) and their co-localisation (**g**, yellow). **d**, **f**, **h** High magnification confocal images illustrating the co-expression of GS and GFAP. **i**, **j** Ubiquitous co-expression of GS and GFAP in wild-type control mice (**i**) and down-regulation of GS expression (astrocytes lacking GS are indicated by arrows) in 3xTg-AD mice (**j**). DG, dentate gyrus; GcL, granule cell layer; MoL, molecular layer; Lac, stratum lacunosum moleculare; Or, stratum oriens; PcL, pyramidal layer; Rad, stratum radiatum. Modified and reproduced with permission from [204]

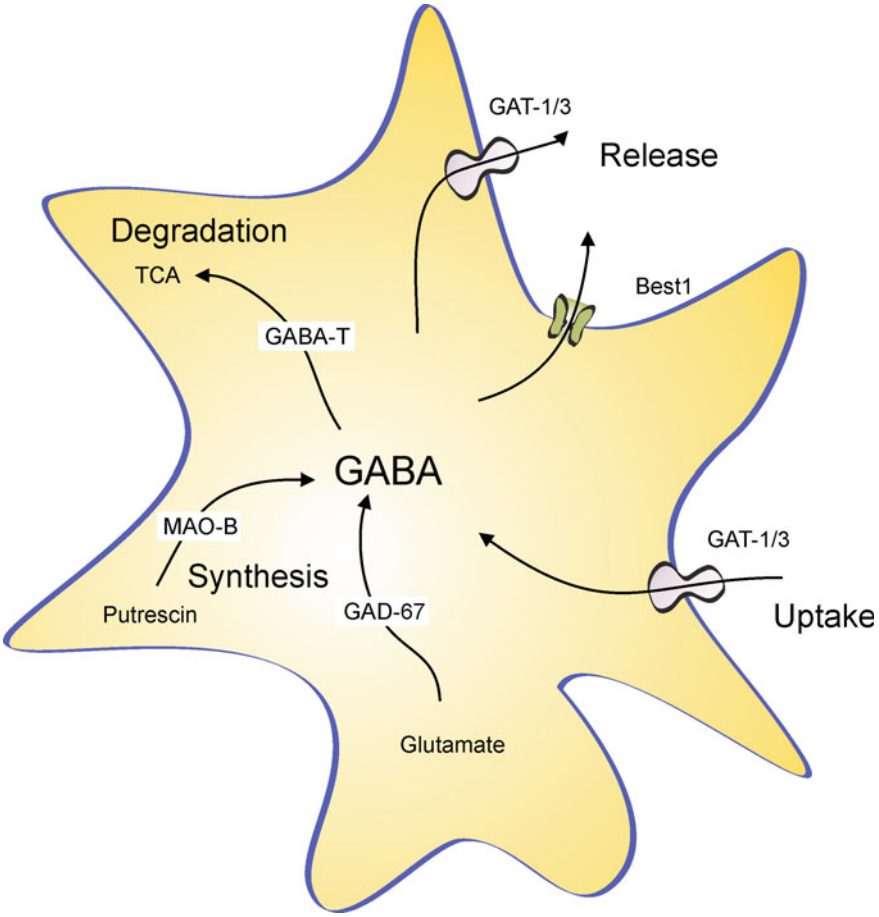

Fig. 11.11 GABAergic reactive astrocytes in AD. See text for explanation. Abbreviations: GAT1/3 GABA transporters 1 (SLC6A1) and 3 (SLC6A11); Best1—bestrophin 1 anion channel 1; GABA-T—GABA transaminase; TCA—tricarboxylic acid (Krebs) cycle; MAO-B—Monoamine oxidase B; GAD67—glutamate decarboxylase. Modified from [84]

changes developed in parallel to a decrease in β-amyloid load [20, 236]. Incidentally, environmental stimulation also improved neurogenesis which is impaired in the AD [231, 233]. Another AD model, the 5xFAD mice chronically treated with polyunsaturated fatty acid 2-hydroxy-docosahexaenoic acid similarly rescued astroglial atrophy, restored adult neurogenesis and improved cognitive performance [76]. Treatment with specific diets may also affect ageing and AD progression. It is well appreciated that caloric restriction exerts prominent positive effect of lifespan of several species and may boost cognitive resilience of the brain [80, 169, 170, 175]. It appeared that caloric restriction induces growth of astroglial perisynaptic processes, thus extending synaptic coverage, preventing glutamate spillover, improving K^+ buffering and glu-

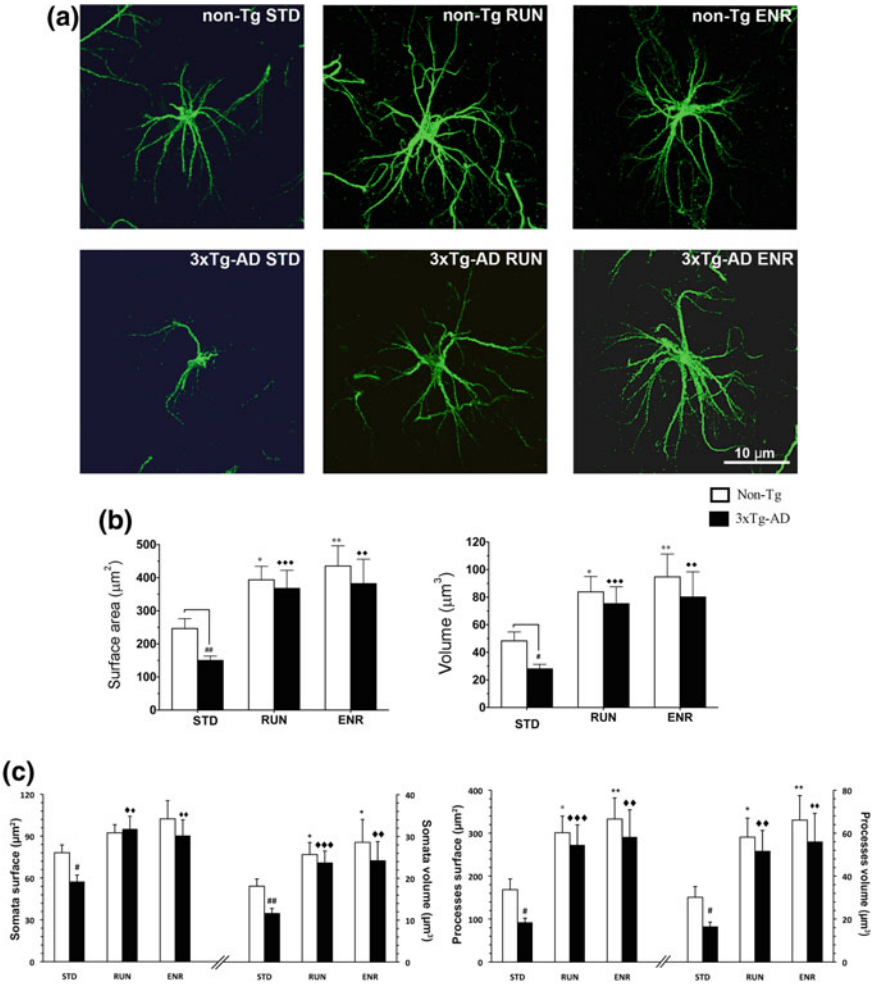

Fig. 11.12 Environmental stimulation (enriched environment, ENR and physical activity, RUN) reverse morphological atrophy of astrocytes seen in the dentate gyrus isolates from 3xTg-AD mice. GFAP-immunoreactivity of astrocytes in the DG of non-Tg and 3xTg-AD animals housed in different conditions. **a** 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-immunoreactivity of 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 with permission from [236]

tamate uptake from the synaptic cleft, thus ultimately enhancing synaptic plasticity [217].

11.9.2 Preventing Neurodegeneration by Adrenergic Astroglial Excitation

Noradrenergic innervation of the CNS is provided by projections of adrenergic neurones localised in the brainstem nucleus locus coeruleus. This small nucleus is located near the fourth ventricle and, in humans, comprises around 50,000 neurones [188]. Diffuse innervation by projections of locus coeruleus neurones reaches practically all parts of the brain and the spinal cord [25]. The locus coeruleus neurones are vulnerable to oxidative stress; apparently, they are lost in ageing and they are first to die during neurodegeneration including AD [75, 167, 190, 249]. Astrocytes, being universally sensitive to noradrenaline, represent the major target for deficient noradrenergic innervation and interfering with astroglial adrenergic mechanisms may be therapeutically relevant [348].

Astroglial sensitivity to noradrenaline, released from locus coeruleus neuronal projections, is mediated by both α- and β-adrenoceptors linked, respectively, to cytosolic Ca^{2+} signalling [66, 131] and cyclic AMP (cAMP) cascades [297]. In the in vivo experiments in the awake mice, the vast majority of astrocytes generated synchronous $[Ca^{2+}]_i$ signals in response to noradrenaline released from locus coeruleus projections [22, 66, 210]; of note neurones did not generate Ca^{2+} responses to the same stimulation [208]. This difference reflects upon much higher density of adrenoceptors in astrocytes when compared to neurones [10]. Degeneration of locus coeruleus neurones associated with ageing most certainly impairs adrenoceptors–medicated astroglial excitability, which may be linked to the cognitive decline [348]. Consequently, preventing death of locus coeruleus neurones or boosting astroglial adrenergic excitability may represent a valid therapeutic strategy [348]. Alternative possibilities may involve drugs, such as deprenyl, that limit noradrenaline catabolism in astrocytes.

Transcranial direct current stimulation (tDCS) was used with positive effects including memory enhancements, accelerated motor function rehabilitation, alleviation of depressive symptoms and decelerated progression of cognitive impairments in AD patients [140, 197]. The mechanism of action of tDCS is astroglial and noradrenergic. It has been revealed that tDCS induces a massive increase in astroglial $[Ca^{2+}]_i$ which has been suppressed by the ablation of noradrenergic neurones or by the inhibition of α_1-adrenoceptors [186]. Alternative possibilities may involve drugs that limit noradrenaline catabolism in astrocytes such as, for example, deprenil.

11.10 Conclusions

Astroglial contributions to the pathophysiology of AD are complex and range from early astroglial atrophy, which limits homeostatic support and may cause synaptic weakness and early cognitive decline, to astroglial reactivity, which seems to protect the CNS against AD-associated pathology and limits the spread of β-amyloid load. Astroglia may also undergo pathological remodelling, in which astrocytes may acquire new functions such as, for example, secreting GABA. Specific manipulations with astroglia may represent a valid therapeutic approach for treating neurodegenerative disorders including AD.

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Chapter 12
Oligodendroglial Cells in Alzheimer's Disease

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Abstract Oligodendrocytes form the myelin that ensheaths CNS axons, which is essential for rapid neuronal signalling and underpins the massive computing power of the human brain. Oligodendrocytes and myelin also provide metabolic and trophic support for axons and their disruption results in axonal demise and neurodegeneration, which are key features of Alzheimer's disease (AD). Notably, the brain has a remarkable capacity for regenerating oligodendrocytes, which is the function of adult oligodendrocyte progenitor cells (OPCs) or NG2-glia. White matter loss is often among the earliest brain changes in AD, preceding the tangles and plaques that characterize neuronal deficits. The underlying causes of myelin loss include oxidative stress, neuroinflammation and excitotoxicity, associated with accumulation of Aβ and tau hyperphosphorylation, pathological hallmarks of AD. Moreover, there is evidence that NG2-glia are disrupted in AD, which may be associated with disruption of synaptic signalling. This has led to the hypothesis that a vicious cycle of myelin loss and failure of regeneration from NG2-glia plays a key role in AD. Therapies that target NG2-glia are likely to have positive effects on myelination and neuroprotection in AD.

Keywords Oligodendrocyte · Oligodendrocyte precursor cell · OPC · NG2-glia · Myelin · Axon

12.1 Introduction

Alzheimer's disease (AD) is characterized by a loss of neurones and synapses, with an associated progressive decline in cognitive function and dementia [56]. The pathological hallmarks of AD are deposition of amyloid-β (Aβ) plaques and neurofibrillary tangles of hyperphosphorylated tau, although their specific roles in neuronal demise remain unclear [52]. AD is classified as a neurodegenerative disorder, but glial cells

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are intricately involved in disease progression [46]. Indeed, disruption of white matter (WM) is a major element in AD and, in many cases, myelin disruption may precede overt neuropathology [6]. Indeed, loss of myelin can result in axonal and neuronal degeneration [51]. The causes of myelin disruption in AD are likely to be complex and to include oxidative stress, together with glutamate, iron and metabolic dyshomeostasis [42, 55, 60]. Furthermore, Aβ may be directly toxic to oligodendrocytes and their progenitors [19, 30, 43]. In addition, astrocytes and microglia are markedly altered in AD (Chaps. 12 and 14), which will impact on oligodendrocyte degenerative changes and regeneration from oligodendrocyte progenitor cells (OPCs), or NG2-glia, which are responsible for the life-long generation of oligodendrocytes (Chap. 5). Studies in human post-mortem tissue and mouse models provide evidence that NG2-glia are altered in AD, which may be indicative of reduced regenerative capacity [21, 43, 62]. The self-renewal and differentiation of NG2-glia is modulated by synaptic activity [13], and is considered to underpin adult oligodendrogenesis and experience-dependent or 'adaptive' myelination, which plays a critical role in neuronal network remodelling and learning [25, 28, 38, 58]. On top of this, myelin is required for the integrity and survival of axons [51], due at least in part to axonal metabolic support provided by oligodendrocytes [1]. Thus, neurones and their axons, together with oligodendrocytes and their myelin, are interdependent functional units, whereby loss or disruption of one affects the others. On top of this, a decline in NG2-glia self-renewal results in a vicious cycle of neuronal disruption, myelin loss, and failure of regeneration (Fig. 12.1) [45]. Hence, novel therapies for rejuvenating oligodendrogenesis in the ageing brain have the potential for neuroprotection in AD.

12.2 Evidence of WM Disruption in Human AD

Magnetic resonance imaging (MRI) and post-mortem studies have demonstrated reduced WM volume and alterations of WM microstructure in AD [6, 41]. WM abnormalities correlate with phosphorylated tau 181/β-amyloid 42 [17], and it appears late myelinated regions are more vulnerable to myelin breakdown and AD pathology compared to areas that meylinate earlier, referred to as 'neuropathologic retrogenesis' [5, 9]. Moreover, analysis of human samples enriched for high AD risk (APOE ε4 and parental history of AD) suggests that WM degeneration is an early pathological feature of AD [27]. Indeed, WM changes are detectable in preclinical AD and may precede overt neurodegenerative changes [23], suggesting oligodendrocyte disruption and myelin loss may be primary events in AD pathology [41]. Nonetheless, some degree of WM abnormalities in AD is associated with cortical neurodegeneration [36], and a number of studies have emphasized the importance of vascular disease in the development of WM abnormalities in AD [10].

Post-mortem analyses support widespread abnormalities in myelin and oligodendrocytes in AD [41], and genomic analyses have identified that oligodendrocyte genes are dysregulated in AD and are associated with AD risk variants, such as BIN1 and

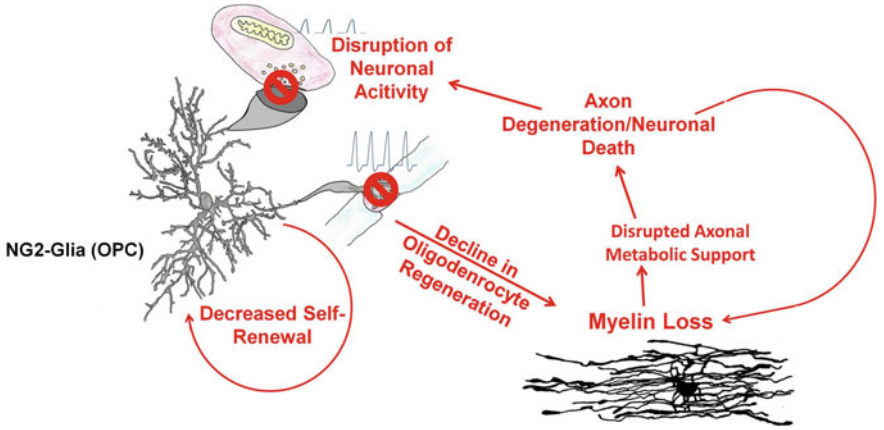

Fig. 12.1 Disruption of NG2-glia has a negative impact on myelin replacement in AD. NG2-glial cells regenerate myelinating oligodendrocytes throughout life, which is essential for replacing myelin lost through pathology and providing new myelin in response to new life experiences. NG2-glia contact neurons at synapses, the sites of neurotransmission, and nodes of Ranvier, the sites of action potential propagation along axons. Neuronal activity helps drive self-renewal and differentiation of NG2-glia, termed 'adaptive myelination', which is important for neural circuit remodelling and learning. Disruption of synaptic signalling is a key factor in AD and would have adverse effects on NG2-glia, which are disrupted in AD. In this scenario, loss of myelin and oligodendrocytes results in disruption of their metabolic and trophic support for axons, resulting in axonal demise and neurodegeneration, all of which are aggravated by reduced regenerativae capacity of NG2-glia . This vicious cycle may be a key factor in the pathogenesis of AD and therapies that promote oligodendrocyte regeneration and myelination are likely to have important neuroprotective effects, and vice versa

GOT2 [37]. In the human brain, mature oligodendrocytes are the main cells expressing BIN1, which has diverse functions in membrane remodelling, implicating it in myelin disruption in AD [16]. Myelin degeneration has been demonstrated throughout frontal and temporal lobes in AD [29], and biochemical analyses of post-mortem WM revealed decreased myelin basic protein (MBP), myelin proteolipid protein (PLP), cyclic nucleotide phosphohydrolase (CNPase) and cholesterol in AD [47]. Post-mortem analyses have demonstrated a loss of Olig2+ oligodendrocyte lineage cells and NG2-glia in AD [7, 43]. Myelin injury in AD cortex is associated with axon degeneration and amyloid plaques [61], and focal loss of oligodendrocytes in AD is associated with Aβ plaque cores [40]. A recent study has indicated that Olig2+ and NG2-glia associated with Aβ plaques exhibited a 'senescence-like' phenotype and suggested a role for Aβ-induced OPC cell senescence in cognitive deficits in AD [62]. Thus, myelin deficiencies are evident in AD and are associated with senescence of NG2-glia and loss of oligodendrocytes.

12.3 Oligodendrocyte and Myelin Changes in Animal Models of AD

Studies in animal models of AD support human evidence that oligodendrocyte disruption and myelin loss is an early event in AD pathology [46]. A number of studies provide evidence of Aβ toxicity in oligodendrocytes [18, 19], and myelin disruption correlates with the earliest appearance of Aβ accumulation in the 3×Tg-AD mouse model [20]. Similarly, studies in the APP/PS1 mouse model of AD demonstrate oligodendrocyte differentiation is disrupted [57], together with downregulation of MBP, shrinkage of the corpus callosum, increased NG2-glia and behavioural deficits [21]. Recent studies in the 5xFAD mouse model of AD demonstrate myelin deficits occur at an early stage and progress with ageing [24], with evidence that subcellular accumulation of Aβ drives axonopathy and myelinopathy [14]. However, there are contradictions between mouse models and human AD, for example, Olig2+ cells and NG2-glia are increased in APP/PS1 mice, but are decreased in human AD pathology [7, 43]. In addition, in vitro studies indicate NG2-glia engulf Aβ peptides and degrade them by autophagy [32], and provide evidence that Aβ promotes oligodendrocyte differentiation, maturation and survival [44]. Thus, Aβ may not directly cause oligodendrocyte loss and myelin disruption in AD, but instead may be related to OPC-mediated repair mechanisms.

12.4 Mechanisms of Oligodendrocyte and Myelin Disruption in AD

The molecular mechanisms leading to myelin loss in AD have not been elucidated, but are likely to include oxidative stress, neuroinflammation and excitotoxicity [41]. Multiple studies implicate Aβ in oligodendrocyte dysfunction and myelin breakdown, which could be due to direct Aβ toxicity in oligodendroglial cells or oxidative stress [19, 31, 59], but equally could be related to OPC-mediated repair mechanisms [44]. It is important to note that clinical trials targeting the removal of Aβ plaques did not prevent progressive neurodegeneration and cognitive decline in AD patients [26]. Moreover, early changes in oligodendrocytes and myelin precede Aβ deposition in the 3xTg mouse [20]. Hence, the primary importance of Aβ as causative in oligodendrocyte and myelin pathology is no less clear than its role in neuronal pathology [50]. It also seems that WM disruption and myelin loss in AD is associated with tau hyperphosphorylation [35], and maybe triggered by the formation of NFTs, with oxidative stress as a common factor [11].

A feature that is common to both AD and oligodendrocyte pathology is dysregulation of glutamate signalling and Ca^{2+} dyshomeostasis [22, 46]. Oligodendrocytes and myelin express ligand-gated channels that are permeable to Ca^{2+}, including glutamate receptors (Chap. 5). Prolonged activation of NMDAR triggers oligodendrocyte death and myelin destruction [39, 49], and this can be partly offset by the NMDAR receptor

blocker memantine [4]. Interestingly, NMDAR contributes to synaptic dysfunction in AD and this can be alleviated by treatment with memantine [33]. These studies raise the possibility that the effects of memantine on oligodendrocytes and myelin may play an important role in its neuroprotective effects in AD. Moreover, pathological changes in oligodendrocytes and/or myelin may compromise their trophic support of axons [1]. Hence, therapies that protect oligodendrocytes are also likely to be neuroprotective.

In addition to its role in pathology, glutamatergic signalling is also implicated in the regulation of NG2-glia self-renewal and differentiation (Chap. 5). Glutamate released from electrically active axons acting on AMPAR promotes proliferation and differentiation of NG2-glia [12, 53], which is important for neural circuit remodelling and is gradually lost with ageing [25]. Altered glutamatergic synaptic signalling is a major component of AD [15], which would impact on oligodendrogenesis [45]. In addition, ablation of NG2-glia causes deficits in glutamatergic neurotransmission and depressive-like behaviour in mice [8, 48]. Thus, the changes in NG2-glia observed in AD [7, 21, 43] are likely to impact upon information processing in multiple ways.

The pivotal role of Glycogen Synthase Kinase-3 (GSK-3β) in the formation of Aβ plaques and NFTs has identified GSK-3β as a key factor in AD progression and a relevant therapeutic target [34]. In addition, activation of Wnt signalling through inhibition of GSK-3β, a key negative regulator of Wnt signalling, is able to protect against Aβ toxicity and ameliorate cognitive performance in AD [54]. It is noteworthy, therefore, that we have identified a persistent role for Wnt signalling in driving oligodendrogenesis in the adult brain [3]. Moreover, Wnt signalling declines in ageing and targeting Wnt signalling with GSK3β inhibitors can rejuvenate the regenerative capacity of the ageing brain [2]. These studies indicate that manipulating the GSK3β-Wnt signalling pathway may be a potential treatment for promoting myelination and neuroprotection in AD.

12.5 Concluding Remarks

In summary, oligodendrocyte and myelin disturbances are pathological features in AD and may even precede and predict overt neuropathology [5, 6]. Although the molecular mechanisms leading to myelin loss in AD have not been elucidated [41], there is an age-related decline in myelination that is accelerated in AD and the loss of axonal trophic support provided by oligodendrocytes is likely to contribute to neurodegenerative changes. It seems unlikely that it will be possible to determine unequivocally whether myelin loss is secondary to or is a primary contributor to neuronal demise. However, this may be a moot point, since oligodendrocytes and the axons they myelinate are interdependent units, and loss or disruption of one will have adverse effects on the other. In addition, myelin loss will be aggravated by

the apparent age-related decline in NG2-glial cell regenerative capacity, most likely associated with the disruption of neuronal signalling [45]. Thus, novel therapies for rejuvenating oligodendrogenesis in the ageing brain, such as targeting GSK3β-Wnt signalling, have the potential for neuroprotection in AD.

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Chapter 13
Microglia in Parkinson's Disease

Margaret S. Ho

Abstract Microglia are the most abundant immune cells in the central nervous system (CNS), where they interact with neurons and exhibit a wide array of functions in physiological and pathological conditions. Physiologically, microglia mediate synaptic pruning and remodeling crucial for neural circuits and brain connectivity. In pathological conditions such as neurodegeneration in the Parkinson's disease (PD), microglia are activated, migrated to the injury site, and prone to engulf debris, sense pathology, and secrete possible pro- and anti-inflammatory factors. Microglia mediate responses such as inflammation and phagocytosis associated with neurodegeneration and are pivotal players in exacerbating or relieving disease progression. This chapter provides an overview on microglial function in the neurodegenerative disease—Parkinson's disease (PD). An overview on the pathology of PD will first be given, followed by discussion on receptors and signaling pathways involved in microglia-mediated inflammation and phagocytosis. Mechanism of how microglia contribute to PD by inflammation, phagocytosis of α-Synuclein (α-Syn), and interaction with PD genes will also be discussed.

Keywords Microglia · Neuroinflammation · Parkinson's disease · Phagocytosis · Alpha-synuclein

13.1 Introduction

As the most abundant immune cells residing in the central nervous system (CNS), microglia are small cells intertwining with neurons both physically and functionally, exhibiting a wide array of functions in physiological and pathological conditions. Microglia display differential density in various brain regions, with different combinations of markers underlying their regional identity and distinct functional roles [37]. This distributional difference, dynamic behavior, and unique cellular features

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have made them significant brain cells that receive substantial attention and merit in-depth exploration.

Microglia in physiological conditions mediate a variety of brain functions such as synaptic pruning and remodeling. Neuron-microglia bidirectional signaling is particularly crucial for neural circuits and brain connectivity [98, 114, 142]. Upon pathological trigger, microglia migrate to the injured site and act as a double-edged sword to relieve or exacerbate the injury. Decades of study have indicated that microglia doing these jobs are in two major states, resting and activated, distinguishable by the forms of their morphology. While microglia constantly surf around the environments and sense pathology in their resting state [93], they transit to an activated state once the nervous system is under detrimental attack and becomes pathological. Transition from the resting to activated state requires complex regulation, thus allowing microglial activation to be under tight control [55]. Despite this common bipartite categorization, it is generally believed that different targets and receptors tune microglial responses in a continuous manner and multiple forms of activation state exist [43, 116, 137].

Intriguingly, activation of microglia is often associated with neurodegeneration, a degenerative process underlying the ultimate pathology of neurodegenerative diseases. Distinct from resting microglia, activated microglia are often of amoeboid morphology, short processes, enlarged soma, and de novo expression of cell surface receptors. They are prone to engulf debris, sense pathology, and secrete possible pro- and anti-inflammatory factors that exacerbate or relieve disease progression. Thus, the activation profile of microglia is often an important indicator for and reflects neuronal dysfunction in neurodegenerative diseases. In this chapter, we will discuss microglial function in the neurodegenerative disease Parkinson's disease (PD). An overview of the pathology of PD will first be given, followed by a discussion on receptors and signaling pathways involved in microglia-mediated inflammation and phagocytosis. How microglia contribute to the occurrence of PD pathological hallmarks such as dopaminergic (DA) neuron death and formation of α-Synuclein (α-Syn)-containing Lewy bodies (LB) aggregates and mechanisms pertaining to PD gene function will also be discussed.

13.2 Parkinson's Disease

As the second most common neurodegenerative disorder, PD is clinically characterized by symptoms such as resting tremor, bradykinesia, postural instability, accompanying non-motor symptoms like cognitive impairment and autonomic dysfunction. Inside the brain, a series of neuropathological changes appears throughout the course of PD development, ultimately leading to the diagnostic hallmark: the aggregation of intracellular inclusions named Lewy bodies (LBs) and Lewy Neurites (LNs) and the loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (*SNpc*).

This progressive brain pathology can be staged by the LB appearance in different regions of the brain [12, 13], with initial detection of LB in the periphery such as dorsal motor nucleus of the glossopharyngeal and vagal nerves or the olfactory bulb [74], followed by the appearance in the *SNpc* DA neurons in mid-stage, then the rostral propagation to other parts of the brain.

While most of the PD cases are sporadic, studies on rare familial cases offer the strength of identifying possible genetic causes for PD. The central component for LB and LN, α-Synuclein (α-Syn), is the gene product from *SNCA* (*PARK1*)—the first *PARK* gene identified in the studies of rare familial PD cases. Not only that genome-wide association (GWAS) studies have identified *SNCA* SNPs as risk variants for sporadic PD [112, 118], the missense *SNCA* mutation A53T [104], along with many others and duplications in the *SNCA* locus, have all been shown to associate with PD [2, 18, 66, 73, 101, 119, 141]. Interestingly, PD-associated mutations of α-Syn confer differential self-aggregation properties [19, 21, 28, 34, 35, 38], implicating that mutant α-Syn with altered propensities are potentially toxic and more prone for aggregation in disease conditions.

13.3 Microglia in PD

How microglia contribute to PD pathology remains to be an important area of study for researchers centering on the perspective of non-cell-autonomous regulation of neurodegeneration. Although no affirmative connection between DA neuron death and microglial activation has been established yet, microglial activation is thought to be a significant part of the disease process integrated either as a cause or consequence [11, 56]. Some of the earlier studies have provided evidence that microglia are involved in PD. First, the human leukocyte antigen gene (HLA-DRA) expressed specifically in microglia has been identified by a genome-wide association (GWAS) study as a genetic risk factor for late-onset PD [40]. p.R47H variant of microglial triggering receptor expressed on myeloid cells-2 (TREM-2) is also associated with PD [106]. These results suggest that microglia-specific regulation of PD progression exists. In addition, positron emission tomography (PET) studies show that microgliosis is an early and sustained response of PD [6, 33, 122]. Reactive microglia have also been detected in toxin-induced and transgenic mouse models of PD [22, 46, 84, 111]. Brains with an injection of the Gram-negative bacterial endotoxin lipopolysaccharide (LPS), a toxin that specifically induces microgliosis, show signs of DA neuron loss in the substantia nigra (SN). In toto, these findings suggest that microglial activation correlates with PD progression and induces DA neuron toxicity and death. Finally, studies on regional density of microglia revealed that they are prominently distributed in SN and striatum, where microglia exhibit a region- and stage-specific release of cytokines and mediators, thereby affecting DA neuron death [37, 76]. Taken together, these observations indicate a pivotal role for microglia in PD disease progression and raise interests in studying their functional roles during PD pathology (Fig. 13.1).

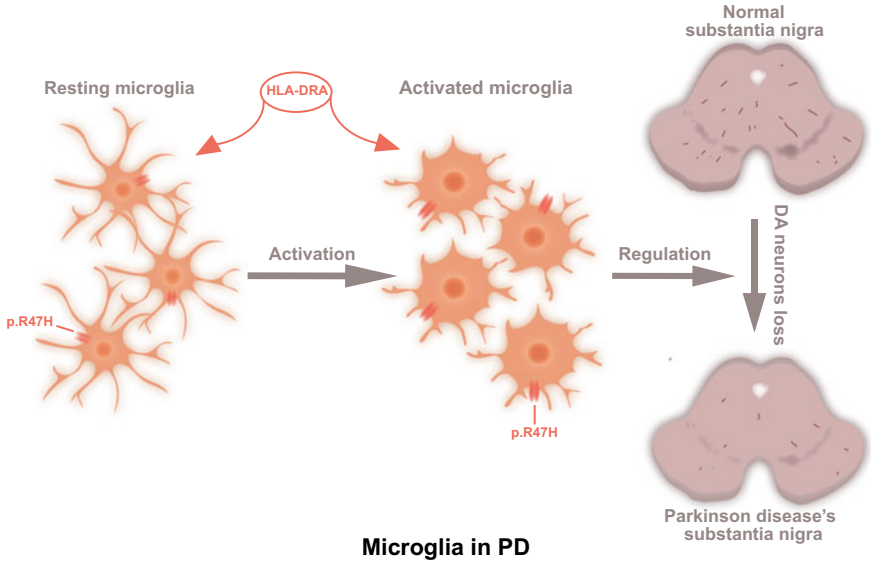

Fig. 13.1 Microglia in PD. Microglia transit from resting state to activating state when the nervous system undergoes pathological attack such as DA neuron loss in PD. A number of observations have dictated a pivotal role for microglia during this process, such as the identification of a microglial specific gene HLA-DRA by GWAS associated with PD, p.R47H variant of TREM-2 associated with PD, and microgliosis as an early and sustained response of PD shown by PET studies

13.4 Microglial Receptors in PD

Similar to other immune cells, CNS microglia express pattern recognition receptors (PRRs) that respond to pathogen-associated molecular patterns (PAMPs) and recognize invading pathogens for host defense immune mechanisms. One type of microglial PRRs, toll-like receptors (TLRs) [132, 136], are single-pass transmembrane proteins with an N-terminal extracellular ligand recognition domain carrying leucine-rich repeats [83] and the C-terminal intracellular Toll-interleukin 1 receptor (TIR) domains transforming extracellular recognition into an intracellular response [15, 48, 140]. TIR domains interact with adaptor molecules such as MyD88, TRIF, and TRAM in response to various stimuli. For instance, α-Syn or Pam3CSK4, a synthetic triacylated lipopeptide, activates TLR2-mediated downstream signaling via the adaptor MyD88 and a co-receptor, either TLR1 or TLR6. Upon α-Syn activation of the TLR1/2 receptor, interaction between MyD88 and TIR domain first activates the kinase activity of the interleukin-1 receptor-associated kinase (IRAK) complex [75], which in turn interacts with and activates the TNF receptor-associated factor 6 (TRAF6) via its K63-linked auto-ubiquitination. These sequential events lead to the activation of the transforming growth factor β-activated kinase-1 (TAK1) complex and the release of IKKs, which mediate IκBα degradation and the ultimate pro-

duction of pro-inflammatory cytokines through MAPK activation and the nuclear translocation of NF-κB, JNK, and p38 (Figs. 13.1 and 13.2) [57, 58, 125].

Microglial TLR1/2 has been shown to be central to the α-Syn pathogenesis: an important therapeutical target for analysis [4]. First, the expression level of microglial TLR2 is elevated in patients of incidental Lewy Body disease (iLBD) which equals to a prodromal Braak stage 1–3, suggesting that elevated TLR2 level correlates early microglial activation response in PD [25]. Next, α-Syn activates microglial TLR1/2 in different experimental systems including BV-2 microglia, primary mouse microglia, or human microglia [7, 23, 60]. Similarly, medium from α-Syn over-expressing SH-SY5Y cells containing oligomeric α-Syn activates microglia in a TLR2-dependent manner [60, 61]. Upon TLR1/2 activation, microglia release pro-inflammatory cytokines tumor necrosis factor-alpha (TNFα) and interleukin (IL)-1β in a MyD88-dependent manner [23, 124, 143]. On the other hand, activated microglia also release anti-inflammatory cytokines, pointing to a diverse functional output upon α-Syn activation of microglial TLR1/2. Taken together, these results suggest that microglial TLR1/2 is a direct α-Syn target and the subsequent TLR1/2-activated signaling pathways participate in PD progression by releasing cytokines that tune the degree of neuroinflammation.

Furthermore, mice lacking the fractalkine receptor CX3CR1 show extensive loss of tyrosine-hydroxylase (TH)-positive neurons in the MPTP-induced PD mouse model [17]. CXCL-CX3CR1 signaling is also involved in a 6-hydroxydopamine (6-OHDA) rat model of PD, where CX3CL1 was found to suppress microglial activation and reduce neuronal loss [96]. It has also been shown that mice lacking CX3CR1 exhibit reduced α-Syn-mediated inflammatory response and microglial phagocytosis, further strengthening the importance of CXCL-CX3CR1 signaling in PD [128].

13.5 Microglia-Mediated Neuroinflammation in PD

The very first evidence that inflammation is involved in PD came from the observation that pro-inflammatory mediators such as TNFα, IL-1β, and IL-6 were detected in elevated levels in the cerebral spinal fluid (CSF) and brains of PD patients, particularly in the striatum [87, 88, 91]. The elevation of cytokine levels is part of the microglial activation and recruitment (microgliosis) process that starts early, accompanies neurodegeneration, and persists throughout the course of PD [50, 51, 65]. When activated microglia induces neuroinflammation, they either exhibit the M1 neurotoxic phenotype or the M2 neuroprotective phenotype [42, 63, 89, 105]. In the scenario of activated M1-like microglia, these cells often adopt an amoeboid morphology, are highly capable to phagocytose and remove apoptotic cell debris, and release massive pro-inflammatory factors such as IL-1β, IL-12, TNFα, and inducible nitric oxide synthase (iNOS). Microglial release of these factors often couples with DA neuron loss in PD. On the contrary, M2-like activated microglia are of thin cellular bodies and ramified processes, and secrete anti-inflammatory cytokines including IL-4, IL-13, IL-10, TGFβ, and neurotrophic insulin-like growth factor 1(IGF-1) to

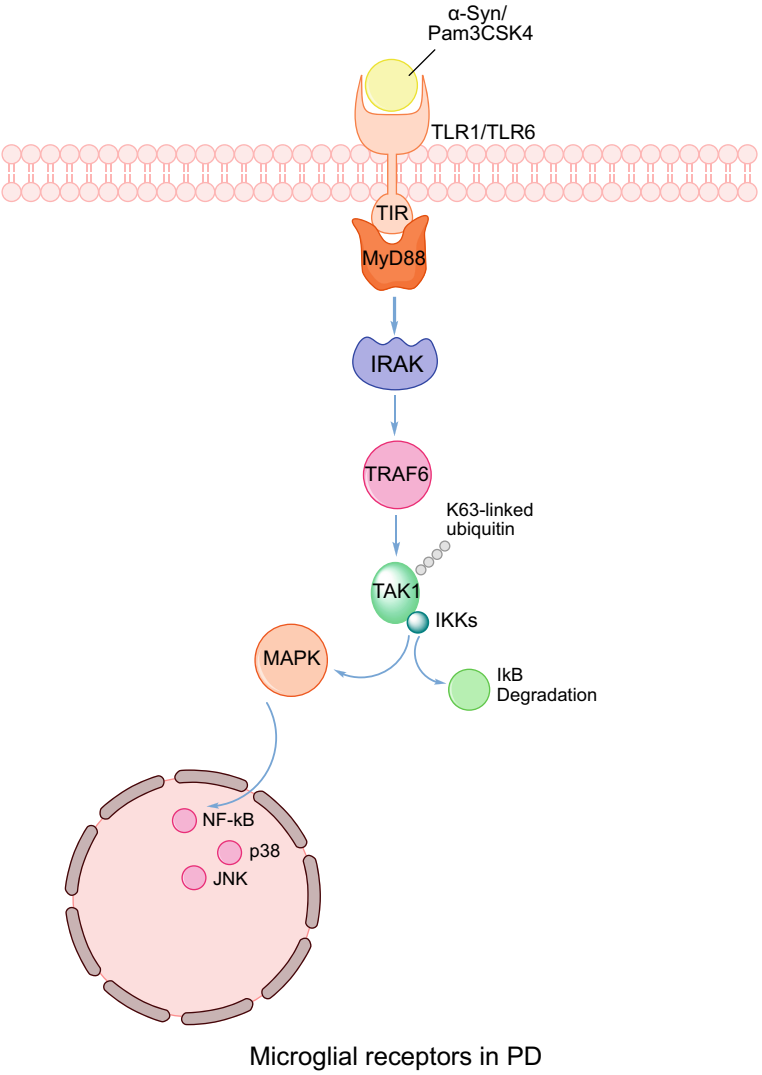

Fig. 13.2 Microglial receptors in PD. An example of microglial receptor TLR1/6 and its downstream pathway was illustrated. TIR domains of TLRs interact with adaptor molecules such as MyD88, TRIF, and TRAM in response to various stimuli. Ligands such as α-Syn or Pam3CSK4 activate TLR2-mediated downstream signaling via the adaptor MyD88 and a co-receptor, either TLR1 or TLR6. Upon α-Syn activation of the TLR1/2 receptor, interaction between MyD88 and TIR domain first activates the kinase activity of the interleukin-1 receptor-associated kinase (IRAK) complex, which in turn interacts with and activates the TNF receptor-associated factor 6 (TRAF6) via its K63-linked auto-ubiquitination. These sequential events lead to the activation of the transforming growth factor β-activated kinase-1 (TAK1) complex and the release of IKKs, which mediate IκBα degradation and the ultimate production of pro-inflammatory cytokines through MAPK activation and the nuclear translocation of NF-κB, JNK, and p38

ease inflammation and accelerate repair. Thus, microglia-mediated inflammation has double-sided effects in terms of relieving and exacerbating disease progression [139]. At one end, the inflammatory response might be beneficial by promoting neuron survival, whereas, on the other hand, the production of neurotoxic factors might also enhance the neurodegeneration. It is noteworthy to mention that microglia-released pro- and anti-inflammatory molecules coexist in the early stage of PD and their expression profiles change over time, suggesting that dynamic regulation of microgliosis correlates with PD progression [102, 113].

Interestingly, prominent microgliosis is detected in various toxin-based models of PD such as 6-OHDA, MPTP, and rotenone [80, 81, 94, 120, 133, 138] as well as transgenic models of PD based on α-Syn. Microgliosis in α-Syn transgenic models occurs early in the stage and precedes DA neuron death, suggesting that cell death is not necessarily a prerequisite for microglial activation [71, 85, 123]. Based on these findings, it has been suggested that signals inducing microgliosis and inflammation might be released from the toxic α-Syn protein aggregates or the degenerated neurons, making an increasing number of microglial cells reactive and migrate to the injury site to defend the progressively degenerating environment.

13.6 Microglial Activation by α-Syn

Microglia-mediated inflammation is regulated by PD risk factors such as DJ-1, LRRK2, and α-Syn. For instance, lacking LRRK2 attenuates inflammation via inhibiting p38 MAPK and NF-κB pathways [59, 86]. α-Syn, as mentioned above, positively regulates microglial inflammatory responses [124, 143]. These findings provide the molecular link between microglia-mediated neuroinflammation and PD pathology. Given that some of these factors might be neuronal specific, bidirectional signaling between neurons and microglia is therefore established as an extremely important theme in PD disease progression.

During PD pathology, α-Syn is secreted to the extracellular space from neurons and detected in the extracellular biological fluids in PD patients [79, 129]. Clear evidence shows that extracellular α-Syn directly activates microglia [124, 143]. This activation has significant consequences. First, it is part of a key event for fully shifting activated microglia to exhibit a pro-inflammatory phenotype [3, 127]. Next, α-Syn-induced microglial activation promotes α-Syn phagocytosis via microglial FcγR receptor and subsequently activates a series of pro-inflammatory events such as nuclear translocation of NFγB p65 and elevated release of cytokines, potentiating the loss of DA neurons and chronic neurodegeneration in PD [16, 64, 69, 72, 124].

Results from studies on the form of α-Syn that activates microglia were contradictory. Different forms of α-Syn exhibit different effects on microglial phagocytosis and inflammatory activation. Pathogenic form of α-Syn, such as $\alpha\text{-Syn}^{A53T}$, triggers pro-inflammatory microglial response and impairs phagocytosis [46, 108]. In a different study, however, $\alpha\text{-Syn}^{A53T}$ is implicated in promoting phagocytosis [109]. Physiological α-Syn, on the other hand, inhibits inflammation yet promotes

phagocytosis [3]. It is generally believed that monomeric α-Syn promotes phagocytosis whereas oligomeric α-Syn acts in an opposite way [100], yet other studies also indicate enhanced microglial phagocytosis by fibrillar and C-terminal truncated α-Syn [29]. Aggregated α-Syn has been shown to inhibit microglial phagocytosis by activating SHP-1 via interaction with FcγRIIB, and is more potent in mediating microglial release of TNFα and IL-1β [20, 47]. Taken together, α-Syn conformation and its pathogenic form play pivotal roles in regulating microglial phagocytosis and subsequent activated inflammatory response, accompanying neurodegeneration in PD.

In addition to the aforementioned TLRs, α-Syn interacts with a number of different microglial receptors for potentiating phagocytosis and inflammatory responses. For instance, in response to α-Syn, the Prostaglandin E receptor subtype 2 (EP2) regulates α-Syn phagocytosis and CD11b-mediated microglial activation [54]. α-Syn also interacts with CD11b to activate NOX2 through Erk1/2 kinase activation and RhoA-dependent pathway to direct microglial migration [49, 134]. Furthermore, α-Syn, in its monomeric or pathogenic mutant form, interacts with the scavenger receptor CD36 to regulate microglial activation and TNFα release [123, 124]. Another receptor associated with α-Syn is the protease-activated receptor (PAR-1), working in a paracrine manner initiated by the secretion of matrix metalloproteinases [69]. The microglial purinergic P2X7 receptor is also implicated in α-Syn-mediated microglial activation via PHOX activation [53]. Taken together, these receptors receive signals from α-Syn and trigger different cascades of signaling pathways within microglia to activate inflammatory responses, creating the diversity in microglial outputs upon pathological trigger as PD progresses.

13.7 Microglial Phagocytosis in PD

Microglia mediate phagocytosis of apoptotic cells, unfolded proteins, or neuronal debris, a process carried out by the resting microglia in the developing brain or the reactive microglia in pathological conditions such as PD [116, 117]. Interestingly, phagocytosis has been considered beneficial associated with the anti-inflammatory function of microglia, raising the interest in studying cellular machinery mediating this process. A list of receptors has been shown to mediate microglial phagocytosis, including TLRs, the scavenger receptors CD14, TAM (Tyro3, Axl, and Mer) receptor, and TREM-2 [31, 41, 121, 131]. First, microglial phagocytosis of α-Syn is impaired in the absence of TLR4, suggesting TLR4 is involved in microglial α-Syn uptake. Alternation of TLR4 signaling modulates pro-inflammatory responses and ROS production, and promotes neurodegeneration [29, 121], whereas treatment of TLR-4 agonist also protects the survival of transgenic α-Syn overexpressing mice [131]. These findings suggest that TLR4 promotes microglial clearance of α-Syn, thus playing a beneficial role in controlling α-Syn spread and PD progression.

TREM-2 is another microglia-specific receptor that mediates phagocytosis of apoptotic neurons [126]. Alternation in TREM-2 expression affects phagocyto-

sis and subsequent microglia-mediated inflammatory responses by regulating pro-inflammatory gene transcription. TREM-2 is considered neuroprotective as increased levels of TREM-2 enhances microglial phagocytosis and decreases pro-inflammatory responses by regulating TLR4-mediated activation of NF-κB signaling [107].

Expression of the scavenger receptor Mannose Receptor C-Type 1 (MRC1) is decreased in an MPTP mice PD model, suggesting MRC1-mediated microglial phagocytosis is crucial for PD progression. Like TREM2, increased MRC1 expression (thus MRC1-mediated phagocytosis) is also beneficial as the increased MRC expression is part of the peroxisome proliferator-activated receptor gamma (PPARγ)-mediated mechanism of neuroprotection [68]. It is noteworthy mentioning that despite the supporting evidence from TREM-2 and MRC1 that microglial phagocytosis is beneficial for PD, other evidence has suggested phagocytosis contributes to neurodegeneration [5]. For instance, loss of TAM phagocytic receptor slightly extended survival of α-Syn^{A53T} overexpressing mice, suggesting TAM-mediated microglial phagocytosis promotes neurodegeneration and accelerates animal death [31] (Fig. 13.3).

Microglial phagocytosis is also regulated by PD risk factors such as DJ-1, α-Syn, and LRRK2 [20, 78, 82, 92, 100]. For instance, DJ-1 regulates microglial phagocy-

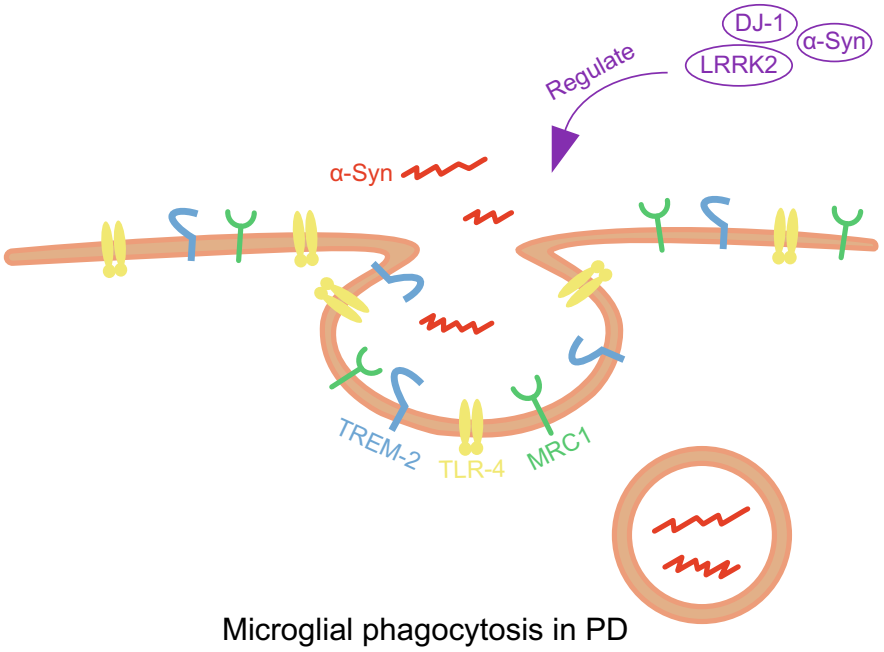

Fig. 13.3 Microglial phagocytosis in PD. Using α-Syn as an example, the receptor-mediated microglial phagocytosis were illustrated. TLR4, TREM-2, and MRC1 are receptors that mediate microglial phagocytosis during PD. This process is under tight regulation by other PD factors such as LRRK2, DJ-1, and α-Syn

tosis of α-Syn via autophagy and in an LC3 (microtubule-associated protein 1A/1B-light chain 3)-dependent (LAP) manner [52, 92], whereas α-Syn is both a regulator and a substrate for microglial phagocytosis. How α-Syn contributes to microglial activation and phagocytosis is discussed above, and microglial phagocytosis of α-Syn is summarized in the next section.

13.8 Microglial Phagocytosis of α-Syn

Previous studies have indicated that microglial phagocytose α-Syn [10, 70]. Some of the receptors functioning in microglial phagocytosis and activation, like TLR2 and TLR4, have been implicated in α-Syn uptake and α-Syn-mediated activation [29, 60, 121, 130]. While TLR4 is required for both microglial activation and phagocytosis of α-Syn [29, 121], TLR2 mainly receives signals from oligomeric α-Syn, but not monomeric or fibrillar α-Syn [60]. TLR2 activation is also crucial in neurons to decrease the uptake and autophagy of α-Syn, promoting neuronal α-Syn accumulation [26, 62]. These findings suggest that signaling cascade initiated by TL2 might be different in neurons and glia, and contribute differently to the disease. Moreover, microglia have been observed in vitro to uptake α-Syn-containing exosomes released by oligodendrocytes via macropinocytosis [30]. In addition to phagocytosis and macropinocytosis, other clathrin-independent routes such as monosialoganglioside (GM1)-dependent lipid rafts have also been shown to mediate microglial uptake of α-Syn [99]. Reduced expression of DJ-1, another PD risk factor, reduces cell surface lipid raft expression in microglia and impairs their ability to uptake soluble α-Syn [92].

13.9 Microglia, LRRK2 and PD

Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are the most common cause of familial PD and a risk factor for sporadic PD [44, 67, 97]. In the immune system, LRRK2 expression in monocytes is increased upon inflammation and the release of pro-inflammatory mediators like IL-1β, TNFα, and IFNγ [32]. Upon microglial activation by LPS in the brain, LRRK2 expression is also increased in a TLR4-dependent manner [86]. It is generally believed that LRRK function correlates with its phosphorylation level on Serine residue 935 (Ser935) [27, 115], a site in which phosphorylation level is crucial for microglial activation and induces inflammatory response in PD. It has also been shown that PD-associated mutations of LRRK2 at position R1441 reduce PKA-mediated LRRK2 phosphorylation and prevent its binding with the adaptor protein 14-3-3 binding [90], suggesting that mutations in LRRK2 associated with PD could affect LRRK2 phosphorylation, hence its kinase activity and cellular function.

One of the major LRRK2 funtions is to regulate the autophagy/lysosome degradation pathway. LRRK2 is localized on the autophagosome vesicles as shown by immune-electron microscopy and biochemical approaches [1, 36, 115]. Membrane localization of LRRK2 on autophagic and lysosome-related vesicles indicates that LRRK2 plays a pivotal role in regulating their function [8, 9]. LRRK2 also interacts with membrane proteins on these vesicles such as Rab7 (late endosomes) and Lamp2A [24, 45, 77, 95]. These results suggest that LRRK2 is involved in different steps of autophagy/lysosome pathway and its activity alters the degradative activity, development, or final maturation of these different vesicles.

Interestingly, the PD-associated LRRK2 mutation, G2019S, in its kinase domain results in an upregulation of LRRK2 kinase activity [39, 135] and has been implicated in autophagic dysfunction. Cells expressing $LRRK2^{G2019S}$ consistently exhibit increased autophagic vesicles or marker expression [14, 103, 110], possibly due to an increase in autophagic flux or an arrest in autophagosome/lysosome fusion. It is possible that defects in the autophagy/lysosome pathway caused by increased LRRK2 activity result in insufficient degradation of accumulated protein such as α-Syn, disrupting α-Syn proteostasis and underlying the mechanism of PD.

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Chapter 14
Astrocytes in Huntington's Disease

Michelle Gray

Abstract Huntington's disease (HD) is a dominantly inherited neurodegenerative disease that results in motor, cognitive and psychiatric dysfunction. It is caused by a polyglutamine repeat expansion mutation in the widely expressed HTT protein. The clinical manifestations of HD have been largely attributed to the neurodegeneration of specific neuronal cell types in the brain. However, it has become clear that other cell types, including astrocytes, play important roles in the pathogenesis of HD. The mutant HTT (mHTT) protein is present in neuronal and non-neuronal cell types throughout the nervous system. Studies designed to understand the contribution of mHTT expression in non-neuronal cell types to HD pathogenesis has lagged considerably behind those focused on neurons. However, the role of astrocytes in HD has received more attention over the last 5–10 years. In this chapter we present an overview of HD and our current understanding of astrocytic involvement in this disease. We describe the neuropathological features of HD and provide evidence of morphological and molecular changes in mHTT expressing astrocytes. We review data from animal models and HD patients that implicate mHTT expressing astrocytes to the progression of HD.

Keywords Astrocytes · Huntingtin · Excitotoxicity · BDNF · Cholesterol

14.1 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 [47]. George Huntington published the first clinical description of HD in 1872 in his paper

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"On Chorea" in which he described sufferers of this disease quite accurately [64]. 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 [64]. Remarkably, George Huntington published this classic work just 6 years after Gregor Mendel published his famous study of pea plants (in 1866) without any knowledge of it, but he was clearly able to identify its pattern of inheritance.

These descriptions of the disease, which now bares Huntington's 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, 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 [59]. HD patients also display changes in learning and working memory [122]. They have difficulties in learning new information and in memory recall [94].

14.2 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 [103]. HD is caused by a repeat expansion in the highly conserved huntingtin (*HTT*) gene [52]. The triplet repeat, cytosine–adenosine–guanine (CAG), is found in exon1 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 3,144 amino acid protein [52, 149]. 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 [104, 112, 121]. When the CAG repeat expansion is greater than 40, the carriers will develop HD [4, 52, 149].

The average age at onset in HD is in the mid-forties and is inversely correlated to the length of the CAG repeat expansion in HD [36, 149]. The CAG repeat length can explain nearly half of the variability in the age at onset for HD [142] with additional modifiers present in the genome accounting for another significant portion. Recently, age at onset genetic modifiers have been identified that are involved in DNA repair and DNA damage mechanisms including those that could contribute to increasing the length of the CAG repeat in somatic cells and germ cells (mismatch and base excision repair) [26, 48, 73].

14.3 Huntington's Disease Neuropathology

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 the disease (Fig. 14.1), [135]. HD disease grades are determined postmortem-based on the degree of striatal degeneration, with modest degeneration assigned as Grade 0 and the most severe atrophy and degeneration assigned to Grade 4 with ~95% of neurons lost in the striatum [137]. Grade 0 brains are determined microscopically since no sizeable changes are observed at a macroscopic (whole brain) level. At this stage, neuronal loss (30–40%) is observed in the tail of the caudate nucleus. In Grade 1 tissue, the body and tail of the caudate nucleus degenerate and then the tissue progresses in a caudal to rostral and dorsal to the ventral gradient. There are also differences between the interspersed MSNs based on neurochemical and anatomical features, with the MSNs expressing enkaphalin and dopamine receptor D2/Drd2 (and projecting to the globus pallidus externa) being lost earlier than MSNs expressing dopamine receptor D1/Drd1 and substance P (projecting to the globus pallidus interna and the substantia nigra pars reticulata) loss after Drd2 MSNs [109, 137]. While the MSNs are the most degen-

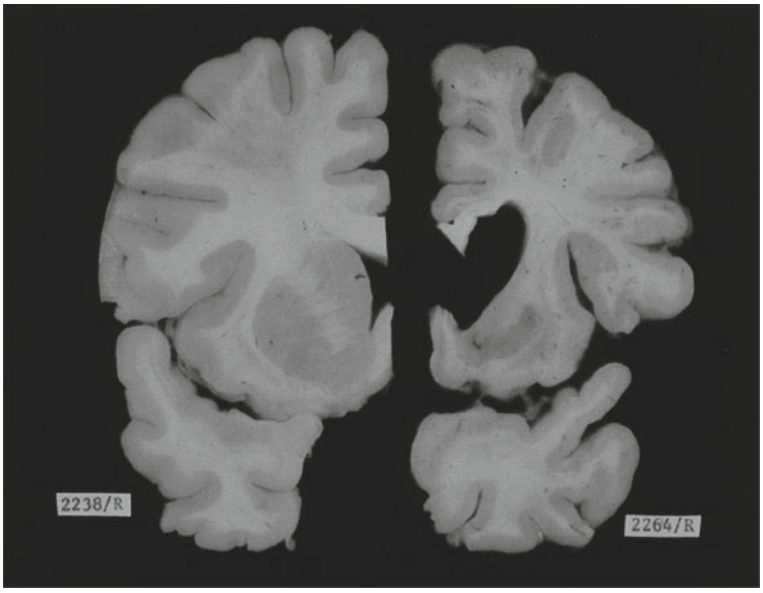

Fig. 14.1 Postmortem human brain at the level of the caudate-putamen. Coronal brain sections taken through the caudate-putamen of a normal (left) and a Huntington's disease patient (right). The Huntington's disease brain on the right shows degeneration of the caudate nucleus adjacent to the lateral ventricle, which has enlarged in response to the striatal atrophy. Courtesy of J.-P. Vonsattel. Reproduced from Alexi et al. (2000), with permission from Elsevier

erated cell type in the striatum, there is increasing evidence from postmortem HD patient tissue that parvalbumin-positive interneurons in the caudate and putamen are decreased in number as disease progresses [108]. Interestingly, prior to a decrease in number, these cells appear shrunken and have decreased expression of PARV [108]. A recent study of aged transgenic HD monkeys (5 years) revealed a significant decrease in the number parvalbumin-positive interneurons in the caudate nucleus and putamen as compared to control [71].

Magnetic resonance imaging, positron emission tomography, and computed tomography have been used to image brains of presymptomatic *HTT* mutation carriers. Atrophy of the striatum was shown prior to overt motor symptoms and a clinical diagnosis of HD [8]. These studies also helped to confirm degeneration of extrastriatal regions, with the cortex as the next most affected brain region in HD. There is significant cortical atrophy that can be detected early in disease [110, 111] and degeneration of cortical pyramidal neurons especially those in cortical layers III, V, and VI, including those that project directly to the striatum [56, 57]. The 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 [70, 125, 133].

One prominent feature found upon neuropathological examination of HD patient tissue is the presence of astrogliosis [39, 137] (Fig. 14.2). 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). In Grade 0 brains, there is no significant astrogliosis in the caudate nucleus [136, 137]. The reactive astrogliosis can be seen most predominantly in the tail of the caudate nucleus at Grade 1 coincident with neuronal loss. In another study, astrogliosis was assessed in striatal tissue from all disease grades using GFAP immunohistochemistry. These studies reveal that increased 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 [39]. 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 (nucleus accumbens). Since the dorsal striatal astrogliosis is so early in the disease process (Grade 0–1), 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 the disease progresses, the reactive phenotype becomes more severe with hypertrophic and overlapping protrusions from the astrocytes (Fig. 14.2) [39]. Neuronal loss and reactive astrogliosis are present predominantly in the tail of the caudate nucleus.

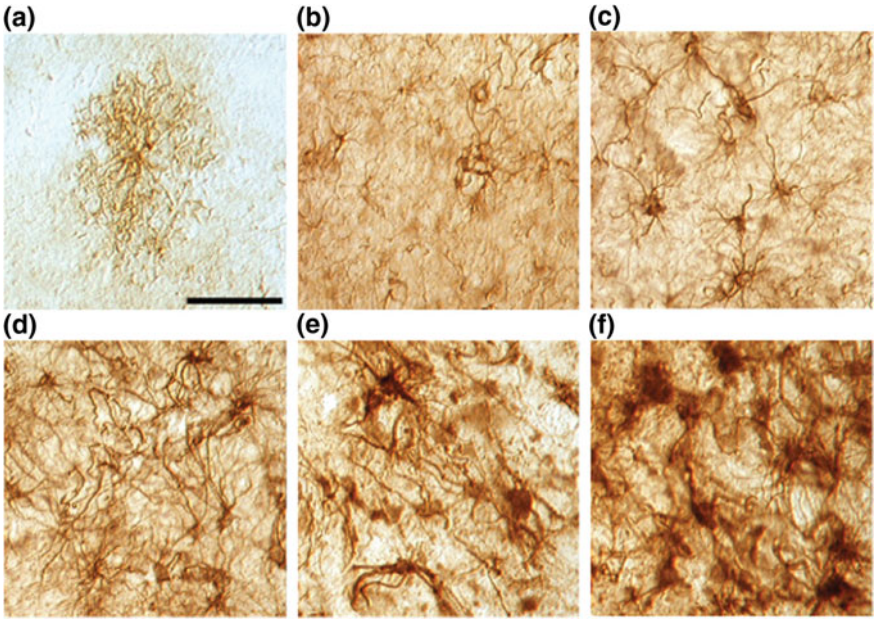

Fig. 14.2 Astrogliosis in HD patient striatal tissue. GFAP immunohistochemistry in 50 μm tissue sections from the caudate nucleus in non-neurological control (**a**), and in increasingly severe HD specimens, Grades 0–4, Grade 0 (**b**), Grade 1 (**c**), Grade 2 (**d**), Grade 3 (**e**), and Grade 4 (**f**) 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 [39]; Oxford University Press

14.4 Excitotoxicity in Huntington's Disease

14.4.1 Excitotoxicity and Neurons

There are multiple 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 [27]. This mechanism had been hypothesized for HD many decades ago. In HD this mechanism has primarily focused on the corticostriatal synapse, with the pre- and postsynaptic neuron receiving the most attention. The MSNs in the striatum receive glutamatergic excitatory input from both the cortex and thalamus [43, 44]. 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 [1, 10, 72]. 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 [28, 87]. 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 nonhuman primates [11, 42, 113, 147]. 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 as an important mechanism in HD.

The excitotoxicity hypothesis is further supported by data from multiple HD mouse models. There is an 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 wild-type mice, although as the disease progressed in these models, this phenotype did not persist in older mice [49, 147]. Furthermore, there is increased glutamate level in the striatum of YAC128 mice upon cortical stimulation [67], although other studies suggest no such change in young YAC128 mice not yet displaying behavioral or neuropathological features of HD [30, 91]. These data support an altered NMDAR function early in the course of disease in these mouse models, and that altered striatal NMDAR signaling likely contributes to the deficits seen in HD.

14.4.2 Excitotoxicity and Astrocytes

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) [7, 29]. In situ hybridization studies on HD brain tissue reveal a decrease in EAAT2 mRNA labeling that correlated with disease severity [7]. In the tissue, a decrease in the number of cells expressing EAAT2 mRNA can be seen in the remaining tissue of both the caudate and putamen, although the putamen seemed to have a 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 [39]. 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 postsynaptic cells, but also the astrocyte. The role of this transporter in astrocytes is to remove glutamate from the synaptic cleft after it is released [31, 86]. 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 corticostriatal inputs, the ability to efficiently uptake glutamate from the synaptic space is of vital importance.

14.5 Huntingtin Expression and Mutant Huntingtin Aggregation

The *HTT* RNA is found throughout the nervous system. In situ hybridization used to localize the RNA found the transcript throughout the brain. Neurons express a small amount more *HTT* RNA than other cell types. It is interesting that the neurons most susceptible to neurodegeneration in HD, the MSNs, do not display higher levels of *HTT* RNA expression than neurons in other brain regions. It can be found in nonneuronal cell types as well. The HTT protein is found in all of these cell types with the levels similar in all of the cell types as a whole [72, 115, 117, 119, 148].

Like 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 R6 mouse models harboring an expanding CAG repeat stretch within exon1 of a human *HTT* transgene [32]. These inclusions were identified as neuronal intranuclear inclusions containing HTT and ubiquitin prior to neurological phenotypic development in these animals. Subsequently, these inclusions were identified in the neurons of HD patients [35]. The initial descriptions of these inclusions in HD tissue, largely identified them as neuronal in nature and cytoplasmic/neuropil with a few intranuclear locations, and found primarily in gray matter (with EM48 antibody) [55]. The largest number of inclusions identified in patient tissue in those studies were observed in the deeper cortical layers, with many fewer and much smaller inclusions found in the striatum. More recent analysis of inclusions and aggregates in postmortem tissue from HD patients with an antibody that recognizes aggregated mHTT (S829), showed that mHTT nuclear inclusions are found in all nervous system cell types—neurons, astrocytes, oligodendrocytes, and microglia [65].

14.5.1 Expression and mHTT Aggregation in Human Astrocytes

RNA in situ hybridization for *HTT* identified positive signals in astrocytes from normal brain tissue [72]. 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 [119], 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 [18, 39, 65, 118, 119]. Recent work has confirmed with the S829 antibody that the frequency of nuclear inclusions in astrocytes was much lower than that observed in neurons. They also identified nuclear inclusions in the astrocytes that were less frequent than neuronal nuclear inclusions [65].

14.5.2 Expression and mHTT Aggregation in Mouse Astrocytes

The HTT protein is also found in astrocytes from mice [18, 74]. 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 wild-type 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 wild-type HTT [25]. This data is in line with previous studies showing that wild-type HTT plays a role in central nervous system development and neuronal survival [82, 107]. The wild-type 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 wild-type 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. 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 [105, 118, 146]. 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 [19, 118]. In addition, in a mouse model expressing a fragment of m*HTT* only in astrocytes, driven by the human GFAP promoter, there are aggregates in the astrocytes in the cortex, striatum, brainstem, and spinal cord [18]. Further analysis of various mouse models expressing mHTT using the S829 antibody reveals the presence of nuclear inclusions in astrocytes in those models [65].

14.6 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 [17, 51, 143]. 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 demonstrate 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 an m*HTT*-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 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 [53, 54]. In another model conditionally expressing a different m*HTT* fragment in medium spiny neurons under the control of the Darpp32 promoter (DE5 mice), there are late-onset motor abnormalities but no evidence of neurodegenerative changes [20], whereas mice expressing this fragment throughout the brain showed extensive neuropathological changes [146].

14.7 Astrocyte Changes in Huntington's Disease Mouse Models

14.7.1 Astrogliosis in Mutant Huntingtin Expressing Models

The examination of astrogliosis, mHTT aggregation, and excitotoxicity has been performed in many different mouse models expressing different forms of the mutated *HTT* gene. These mouse models have not only revealed phenotypes that are observed in HD patients but they have also uncovered mechanisms that were not previously implicated for astrocytic contribution to HD pathogenesis. Progressive astrogliosis has been observed in postmortem tissue of increasing grade from HD patient brains. The reactive astrocyte phenotype has also been observed in many of the mouse models expressing mHTT. Neuropathologically, these models display varying degrees of pathological changes, with regional 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, expressing mHTT throughout the nervous system in both neurons and glia, there is significant astrogliosis in the cortex and striatum [54]. 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 [53, 54]. 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 [39]. 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, thus reinforcing 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 [80].

One of the most widely used mHTT expressing mouse models, R6/2, which express an exon1 fragment with an expanded CAG repeat, does not exhibit significant astrogliosis as indicated by morphological changes in the cortex nor striatum as the animal ages [85, 126]. There is also not a significant upregulation of the GFAP level in the mice. Additional analysis of the somatosensory cortex specifically did not reveal significant astrogliosis [138]. There is no significant astrogliosis identified in the knock-in Q175 model nor in the human mHTT expressing YAC128 or BACHD models [50, 60, 126].

Multiple monkey models have been generated for use in understanding HD pathogenesis [23, 145]. A study describing the characterization of two monkeys created via lentiviral gene transfer was recently published. One monkey contained exons 1–10 of the m*HTT* gene with 70 CAG repeats under the control of a minimal human *HTT* promoter and the other monkey contained human *HTT* exon1 with 29 CAG repeats and is regulated by the human polyubiquitin C promoter. A study of these two types of monkeys at 5 years of age revealed significant astrogliosis in the model [71]. The caudate nucleus and putamen contained an increase in astrocytes that were heavily GFAP-stained. The increase was more pronounced in the monkey that contained only exon1 with 29 CAG repeats. The control monkey did not have much GFAP positive staining in these regions (Fig. 14.3).

Although it is not completely understood why astrogliosis is not a striking feature in all of the animal models expressing mHTT, there is astrogliosis in some of the models. The lack of significant astrogliosis does not negate the possibility that the astrocytes are not completely normal in those models. Nonetheless, some of the mouse models demonstrate that the reactive astrocyte phenotype can be elicited from the 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.

14.7.2 GLT-1 in mHTT Expressing 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 the astrocytic contribution to HD. The loss of GLT-1 in HD could be of critical consequence given the important role of GLT-1 in maintaining glutamate homeostasis at the excitatory synapse. 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 [12, 118, 126]. The decrease in *GLT-1* mRNA levels seem progressive as a decrease can be seen in R6/2 animals

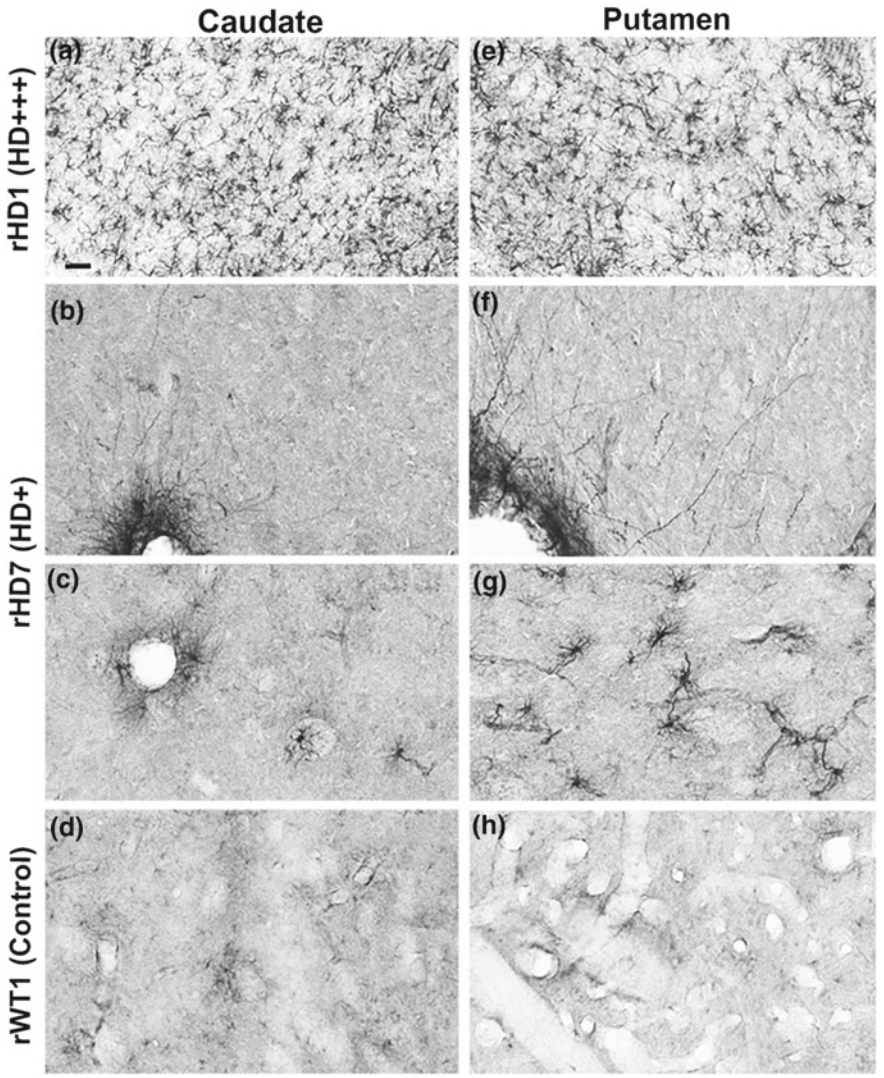

Fig. 14.3 Astrogliosis in the brains of 5 year old mHTT expressing monkeys. (**a**–**h**) GFAP labeling in the caudate nucleus (CD; **a**–**d**) and putamen (PU; **e**–**h**) of the two transgenic HD monkeys (rHD1, rHD7) and the control animal (rWT1). There is a large density of GFAP labeled astrocytes in the CD and PU of the most severely affected HD monkey (rHD1-**a**, **e**) compared with the least affected HD monkey (rHD7; **b**, **c**; **f**, **g**) and the control (**d**, **h**). Scale bar in A: 30 μm in A and E; 20 μm in (**b**–**d** and **f**–**h**). Edits to the figure legend were made to decrease space. Reproduced from [71], under a Creative Commons Attribution 4.0 International License. https://creativecommons.org/licenses/by/4.0/legalcode

in 6-week-old animals, in both cortex and striatum and further declines at 12 weeks [12]. In addition to mRNA levels in the R6/2 mice, there is a significant reduction in the protein level of GLT-1 during 11–12 weeks of age [12, 100], although there is a likely decrease in protein levels early as well; it does not reach statistical significance [118]. Glutamate uptake is decreased in the striatum of the R6/2 transgenic model [12, 38, 79, 118]. 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 [118]. This result is likely given the slower progression in this full-length mHTT mouse model that exhibits no obvious neuropathological changes at this age [80].

The YAC128 model expresses full-length human mHTT and recapitulates aspects of HD neuropathology and behavior that becomes progressively worse as the animal ages [60, 120]. There does not appear to be a decrease in the levels of GLT-1 protein in the brain of these mice even as the disease progresses. Interestingly, glutamate uptake was decreased in the striatum of these mice as early as 3 months of age; it is also seen at 12 months in the cortex [63]. EAAT2 was identified in a proteomics study of palmitoylation [68], 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 [63]. 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.

BACHD mice express full-length human mHTT from a Bacterial Artificial chromosome that has been modified to contain a mixed CAA–CAG repeat. This is a slowly progressing mouse model with behavioral and neuropathological changes that are similar to those observed in HD patients [50]. The neuropathological changes manifest at about 1 year of age with a decrease in the striatal volume and robust mHTT aggregation. Western blot analysis of protein extracted from the cortex and striatum revealed no significant change in the levels of GLT-1 at 6 or 12 months [144].

The knock-in line zQ175 (human HTT exon1) is a slowly progressing mHTT expressing mouse model. Robust phenotypic changes are observed at 1 year of age. Examination of GLT-1 expression in this line revealed a significant reduction of the GLT-1 protein at 10–12 months of age [126]. Interestingly, *GLT-1* mRNA was only reduced in homozygous Q175 mice at 10 months of age [126]. Another study revealed a significant reduction in the level of the *GLT-1* transcript at about 10 months of age in these mice [88]. In addition, glutamate transport activity is altered in the zQ175 mice that are thought to critically depend on or be caused by a decrease in Kir4.1 conductance in the HD models [37]. It is interesting, though not surprising, that the degree of GLT-1 loss or dysfunction is variable across the models expressing different forms of mHTT. Some of these models express full-length mHTT (knock-in or transgenic) driven by endogenous promotor regions that give rise to a much slower progressing HD-like phenotype, while others express truncated forms of mHTT driven by minimal *HTT* gene promotors or other neuronal promotors that seem

to produce a more rapid progression of HD-like phenotypes. The differences in the genetic construct used in these various models could contribute to some of the variability observed in the animals.

Mice generated using the human GFAP promoter to specifically express an mHTT exon1 fragment carrying 160Q repeat in astrocytes (GFAP-HD) [18]. 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 [18]. In mice that expressed an 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 [39]. The level of the GLT-1 protein that was assessed by immunohistochemistry in the mHTT positive astrocytes 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 [39]. 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 decrease in GLT-1 levels in the mouse models includes 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 [18]. This reduction in this model is likely due to the 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 [18].

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 [39]. 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 improved some of the motor deficits found in the R6/2 mice [90]. However, use of ceftriaxone must be approached with caution as it has been found to affect long-term depression in the hippocampus [96] and impairs prepulse inhibition [13]. Nonetheless, while the appropriate approach to take is yet to be determined, increasing the levels of GLT-1 in HD may have beneficial effects on glutamate uptake deficit and motor impairment.

Another study using primary cultured astrocytes expressing an N-terminal fragment of mHTT (HTT-552), observed a significant decrease in the level *GLT-1* mRNA

as well as the GLT-1 protein. The authors tested whether enhancing autophagy with rapamycin, a known autophagy activator, would have beneficial effects on clearing mHTT protein and thus increasing GLT-1 protein levels. When the cultures were treated with rapamycin, there was a significant increase in the levels of GLT-1 and also increased glutamate uptake in the astrocytes [24].

14.8 Glutamate Release from Mutant HTT Expressing Astrocytes

Much of the study in HD on astrocytes centers around the decrease in EAAT2/GLT-1 level, the ability of the astrocyte to take up excess glutamate from the synaptic cleft, and on 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 [58]. They are capable of releasing glutamate through various mechanisms, including but not limited to Ca^{2+}-dependent vesicular exocytosis [99]. Glutamate released from astrocytes has been shown to act on extrasynaptic NMDARs to modulate neuronal excitability and synaptic transmission [5, 41]. 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 effect on the levels of Ca^{2+}-dependent glutamate release [74]. 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 [62]. Mechanical stimulation of astrocytes allows one to assess the exocytotic release of glutamate [93]. Mechanical stimulation of full-length mHTT containing cortical astrocytes from BACHD mice resulted in an increase in the level of glutamate that was released into the extracellular space near the solitary astrocytes as compared to wild-type astrocytes [74]. 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 [97]. While these BACHD cells responded to mechanical stimulation with a rise in cytosolic Ca^{2+} levels, there was not a significant difference between wild-type 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 wild-type astrocyte, suggesting that other mechanisms are involved in causing 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.

14.9 BDNF and mHTT Expressing Astrocytes

The motor abnormalities originate due to degeneration of the MSNs in the striatum. These cells receive trophic support from the cortex in the form of the brain-derived neurotrophic factor (BDNF) [3]. A lack of BDNF trophic support can increase a neuron's susceptibility to cell death and it is crucial for the survival of MSNs [9, 21]. The MSN does not produce BDNF, but receives it from other sources. There is a decrease of BDNF levels in HD patient striatum that is also recapitulated in some of the animal models expressing mHTT [149–152]. This decrease is attributed not only to transcriptional dysregulation caused by mHTT in the cortex but also to a decrease in BDNF transport to the striatum [46, 77]. Astrocytes play important roles in providing trophic support in the nervous system. They are able to synthesize and release neurotrophic factors including glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and brain-derived neurotrophic factor (BDNF) [16, 78, 92]. Astrocytes can release BDNF through exocytosis [99].

Astrocyte expression of mHTT impairs multiple normal aspects of astrocyte function. The expression of mHTT (100Q) in astrocytes by adenoviral vector in cultured rat primary astrocytes caused a decrease in the level of BDNF found in the astrocyte-conditioned media as compared to conditioned media from cells expressing HTT with a short (18Q) polyQ with a retention of BDNF within the astrocytes [139]. Analysis of the BDNF transcript level revealed a decrease in the transcript, thus indicating that the presence of mHTT repressed BDNF transcription in the astrocytes [140]. Using two mouse models: one is a full-length knock-in mouse model where mHTT is expressed in all cells of the nervous system, including astrocytes; and another is where expression of a truncated mHTT fragment is driven by the GFAP promoter. Hong et al. showed a reduction in secreted BDNF from mHTT expressing astrocytes and it was shown to likely be due to impaired docking of BDNF containing vesicles in astrocytes [61]. Thus, mHTT expression in astrocytes is capable of interfering with multiple mechanisms that would contribute to a decrease of BDNF levels in astrocytes.

There clearly is a deficit in BDNF generated and released by mHTT containing astrocytes; thus, these cells are contributing to the BDNF deficit observed in HD patient brains suggesting that these cells are good targets for therapeutic intervention. Adenoviral astrocyte specific expression of BDNF using the GFAP promotor injected into the striatum of R6/2 mHTT expressing model there was a delay in the onset of motor abnormalities in the model [6]. In addition, glatiramer acetate, which is known to increase BDNF expression in T cells [69, 81, 124] was used to treat R6/2 mice and YAC128 mice and increased the expression of BDNF in the brains of the mice and also decreased the neurodegenerative changes observed in these mice [106]. Together these studies implicate another mechanism whereby mHTT in astrocytes could contribute to the pathogenesis of HD.

14.10 Cholesterol Changes in Huntington's Disease

Brain cholesterol is required for nervous system development and to maintain proper nervous system function [34]. The most cholesterol-rich organ in the body is the brain and all cholesterol found in the brain is synthesized in the central nervous system [128]. In the adult brain, astrocytes are essential for de novo cholesterol synthesis [15, 101]. Cholesterol homeostasis is maintained in the brain by de novo synthesis and export of excess cholesterol from the brain to the plasma in the form of 24-hydroxycholesterol (24-OHC) [15, 83]. In humans, mutations in genes that affect cholesterol synthesis or transport can result in neurodegeneration and abnormal brain formation [102, 134]. The mutations in these genes also impair cognitive function [102].

Studies in HD patient postmortem tissue have revealed changes in cholesterol metabolism with increased striatal neuronal cholesterol membrane accumulation [132]. There is a decrease in the enzymes involved in cholesterol synthesis in post-mortem HD brain tissue. The levels of mRNA expression for genes involved in cholesterol synthesis, including HMG-CoAR and 7-DHC reductase and lanosterol 14 a-demethylase, are reduced in postmortem striatal and cortical tissue [132]. One group reported an increase in cholesterol levels in postmortem HD brains [33]. This contradictory data is likely due to the differences in methodology used to assess cholesterol content in tissue. Plasma levels of cholesterol are decreased in late-stage HD patient plasma [75]. Plasma levels of 24-OHC change as the disease worsens in HD patients. In plasma from premanifest HD patients (prior to overt motor deficits), the 24-OHC levels are normal as compared to controls. However, in HD patients that display motor deficits, the level of 24-OHC levels are reduced [75, 76]. In general, there is a decrease in the level of cholesterol synthesis enzymes in postmortem HD brain tissue and also a reduction in the level of 24-OHC that is transported out of the nervous system.

There is significant data from mHTT expressing mouse models that demonstrate changes in cholesterol levels in the brain and plasma. While there are numerous studies now focused on cholesterol and its contribution to the pathogenesis observed

in HD, some of this work is contradictory. However, it must be recognized that this work has been performed in different animal models with various polyQ lengths, with mHTT driven from different promoters, and with different timeframes for HD-like disease manifestation. Thus, while some of the data seems contradictory, it highlights that the cholesterol story in HD is highly complicated and in need of further investigation to ascertain what is truly happening during disease progression. The R6/2, YAC72, YAC128, HdhQ111/111, and zQ175 [116, 129, 130], all have reduced brain cholesterol levels. Another study in YAC72 mice suggests they have increased cholesterol striatal neuron accumulation with increased cholesterol content [127]. There is data from mouse models that clearly show reduced expression of genes involved in cholesterol synthesis, like HMG-CoAR, CYP51, and 7-DHC-reductase, just like what is observed in HD patient brain [132]. The effect on the expression of these genes may be due to the interaction of HTT with specific transcription factors like Sp1, which is known to coordinate sterol regulatory element-binding protein (SREBP) that activates gene transcription when cholesterol levels are low [114]. Plasma levels of 24-OHC are decreased in zQ175, YAC128, and R6/2 mice as the disease progresses in those mHTT expressing models [116]. It has been shown that mHTT expressing astrocytes in a culture that secreted less cholesterol into the medium and conditioned media from these astrocytes (and media depleted of lipoproteins) did not support neurite outgrowth nor synaptic activity when compared to cholesterol supplementation or conditioned media from normal astrocytes [131]. Due to the significant amount of data in support of cholesterol changes in HD, a study using cholesterol-loaded nanoparticles that were able to cross the blood–brain barrier were injected intraperitonealy, and localized to glial and neuronal cells [131]. The nanoparticles improved electrophysiological deficits, cognitive dysfunction, and increased decreased synaptic proteins [131].

14.11 Genetic Manipulation of Astrocytic mHTT Expression in Mice

Numerous studies have been performed in mice expressing mHTT specifically in astrocytes as well as studies focused on their contribution to HD pathogenesis in the context of mHTT expressing neurons. An important study to demonstrate that astrocytes expressing mHTT were important for neurodegeneration in HD was performed in cell culture. Co-culturing of adenoviral-mediated expression of a fragment of mHTT in astrocytes in culture with normal neurons, resulted in increased neuronal cell death in culture [118]. Studies in a mouse model where mHTT with 160Q was expressed under the control of the human glial fibrillary acidic protein (GFAP) promoter, revealed abnormal neurological phenotypes as the animals aged and mHTT aggregation within astrocytes. Furthermore, those mice had motor dysfunction, a decrease in body weight, and premature death [18]. These mice also had a decrease in the level of GLT-1 attributed to a decreased interaction of the transcription fac-

tor Sp1 with the promoter region of the GLT-1. Interestingly, breeding a transgenic mouse with a GFAP promoter-driven fragment of mHTT with 98Qs to the transgenic mouse model N171-82Q that contains the mHTT fragment only in neurons revealed an exacerbation of the HD-like phenotypes observed in the N171-82Q model, thus revealing that the mHTT expressing astrocytes are important targets in HD [19].

Another mouse model expressing a fragment of mHTT was developed using lentiviral vectors with a pseudotype (Mokola) that drove expression specifically in striatal astrocytes. This model showed increased astrogliosis with a concomitant decrease in the expression level of GLT-1 and GLAST. There was also a reduction in dopamine and adenosine 3′,5′-monophosphate-regulated phosphoprotein (32 kDa) DARPP-32, a protein highly enriched in MSNs, and a marker of neuronal loss and dysfunction [39]. This study reveals non-cell autonomous toxicity of mHTT expressing astrocytes on the MSNs in this model. A study using AAV2/5 viral vectors expressing mHTT N171-82Q with the Gfa2(B)3 promoter or the Chicken-β Actin (CBA) in the striatum of mice, revealed that when mHTT was expressed alone in astrocytes, less severe motor phenotypes were observed in the mouse than when mHTT was expressed in both neurons and astrocytes [89]. Using this system, the authors demonstrated minimal changes in the mice when the vectors were targeted to astrocytes in the striatum. Interestingly, when both neurons and astrocytes expressed mHTT, the number of mHTT aggregates was increased and the level of DARPP-32 decrease was exacerbated [89]. This work suggests that the mHTT expressing astrocytes within the striatum are contributors to the disease although mostly in the context of mHTT expressing astrocytes.

The BACHD mouse model is a conditional transgenic full-length human mHTT expressing mouse model with exon1 that contains the expanded repeat flanked by loxP sites [50]. The genetic design of the model allows assessment of cell-type-specific contributions to HD pathogenesis. It has been used to explore the necessity of fl-mHTT expression within the vulnerable MSN and cortical pyramidal neuron population in HD [141]. None of the other models are designed to be able to assess whether mHTT expression in astrocytes is necessary for HD phenotypic progression when modulated only in astrocytes and not changed in neurons. A study using this BACHD model bred to two different GFAP-CreER^{T2} lines [22, 45], reveals that the Cre expression is sufficient to decrease mHTT levels in the brains of the BACHD mice [144]. Using a reporter mouse model, it showed the recombination was highly selective for astrocytes in the cortex and striatum [84]. The fl-mHTT was decreased in the animals after weaning and behavioral assessments were taken as the phenotype progressed in the animals from 2–12 months of age. This study revealed the reducing mHTT in the astrocytes at 2 mths did not affect the onset of the behavioral phenotypes in the BACHD model. However, the decrease of mHTT in the astrocytes decreased disease progression. The BACHD/GFAP-CreER^{T2} expressing mice showed behavioral phenotypes that were better than the BACHD mice at 6 and 12 mths of age, indicating a slowing of the behavioral phenotypes. In addition, the BACHD/GFAPCreER^{T2} mouse brain weight increased and the striatal volume significantly improved over what was observed in the BACHD mice alone. In agreement with the change in the neuropathology observed in the mice, the electrophysiological

deficits displayed by the BACHD MSNs at 12 mths were restored to normal in the MSNs in the BACHD/GFAPCreER^{T2} mice. Together, the behavioral, neuropathological, and electrophysiological rescue observed in the BACHD/GFAP-CreER^{T2} mice, reveal the significant contribution to the disease progression in this model. This has important implications for therapeutic development in HD. If targeting disease onset is not feasible, targeting mechanisms at play in astrocytes may slow the progression of disease in HD patients.

14.12 Concluding Remarks

The importance of astrocytes to HD pathogenesis is starting to gain more focus and acceptance. While the presence of reactive astrocytes has been found in postmortem 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 remain to be completely determined. Interestingly, many of the mouse models expressing mHTT do not fully recapitulate this neuropathological feature. 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 posttranslational modification of the receptor are likely important mechanisms that could contribute to excitotoxicity. Mouse models expressing mHTT do not all demonstrate a broad decrease in the level of GLT-1. However, it is important to note that changes on the microcircuit level may not be as obvious when using techniques such as Western blot to assess protein levels of this highly expressed protein. However, if more sophisticated imaging modalities are employed, one may be able to visualize cell-specific changes. The 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. However, another study suggests that there is no change in astrocyte glutamate release in the striatum of a different mouse model using a glutamate sensor to measure glutamate levels [66]. Although neurotransmitter uptake/levels have been widely studied, some of the additional processes that astrocytes are involved in have not been fully explored in HD although increased efforts along these lines are underway. These processes including the release of glutamate [74], astrocyte roles in the inflammatory response [14, 123], and sleep (also abnormal in HD) [40, 95], need further exploration in HD. These studies will require the use of advanced imaging modalities, manipulation of normal HTT and mHTT in both cell and animal models, and novel methodologies for introducing agents specifically to astrocytes. Many of these studies can likely be performed using the many genetic models of expressing mHTT in all the cell types or only in astrocytes that have been generated to date. However, proper performance and interpretation of these studies will require the cooperation of expert glial biologists and those focused on the elucidation of pathogenic mechanisms in HD.

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Chapter 15
Induced Pluripotent Stem Cell-Derived Astroglia: A New Tool for Research Towards the Treatment of Alzheimer's Disease

Rebecca Atkinson-Dell and Lisa Mohamet

Abstract Despite over a century of research into Alzheimer's disease (AD), progress in understanding the complex aetiology has been hindered, in part, by a lack of human, disease relevant, cellular models, reflected in an inability to translate results from animal studies to successful human therapies. Induced pluripotent stem cell (iPSC) technology, in which somatic cells are reprogrammed to pluripotent stem cells, creates an ideal physiologically relevant model as they maintain the genetic identity of the donor. These iPSCs can self-renew indefinitely in vitro and have the capacity to differentiate into any cell type, opening up new discovery and therapeutic opportunities. Despite a plethora of publications indicating the generation and utility of iPSC-derived neurones for disease modelling to date, in comparison only a limited number of studies have described generation of enriched astroglia from patients with early- or late-stage onset of AD. We recently reported that iPSC-astroglia derived from these patients are capable of mimicking a wide variety of deficits in homeostatic molecular cascades, intimately associated with AD, that are routinely observed in vivo. This review examines the opportunities and limitations of this innovative technology in the context of AD modelling and uses for preclinical discovery to improve our success for an efficacious therapeutic outcome.

Keywords Alzheimer's disease · Induced pluripotent stem cell · Astroglia · Neurodegeneration · Therapeutics

15.1 Introduction

Despite over a century of research into Alzheimer's disease (AD), progress in understanding AD complex aetiology has been hindered in part, by a lack of human, disease relevant, cellular models reflected in an inability to translate results from animal studies to successful human therapies. In the 1980s Glenner and Wong [18] identified one of the central components of the disease, namely, beta-amyloid protein

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plaques that were considered key to triggering neuronal cell damage. Later in the same decade, researchers discovered a second key component, tau protein tangles to cause neuronal cell degeneration. In 1987, Pfizer began clinical trials of the first specific drug, Tacrine, to target the symptoms of AD, which was later approved by the FDA in 1993. Yet, to date, there is still no drug or treatment that will cure AD or any other form of dementia. Despite a number of potential treatments in the pipeline, current treatment paradigms fall into two categories: acetylcholinesterase inhibitors or N-methyl-D-aspartate (NMDA)—receptor antagonists, both of which, only help treat symptoms, have modest clinical efficacy and do not treat the underlying cause(s) of AD. Moreover, no new AD drug treatments have been approved since 2003. Data shows that between 2002 and 2012, the attrition rate for novel AD drugs was 99.6%, attributed to a lack of clinical efficacy [11, 12].

Human pathology is dominated by the late-onset or sporadic form of AD (SAD), which disease variant does not display significant Mendelian genetic bias. At the same time, absolute majority of cell- and animal-based models of AD employ mutant genes isolated from the clinically rare and dominantly inherited familial AD (FAD), characterised by early onset. Whilst the use of transgenic animal models of AD is crucial to preclinical drug development and may recapitulate some key human AD biological components, such as amyloidogenesis, neuronal loss and cognitive deficits, which are the hallmarks of AD in humans, are rather limited in animal models [23]. Subsequently, there is a compelling need for superior complex human models that offer confidence in disease relevance and improved clinical translation.

Induced pluripotent stem cells (iPSCs) offer a promising advantage over existing models for humanising drug development earlier in the pipeline and offering the potential to provide a cellular-based model for research, preclinical drug efficacy and toxicity. This chapter will focus on highlighting the progress and achievements of human iPSC-derived astroglia in AD modelling, their limitations and future challenges and opportunities both in research and translation to the clinic.

15.2 Astroglia and Their Role in Health and Disease

In order to understand the pathophysiology of any disease, one must comprehensively understand the role and contribution of any cellular component(s) in a typical physiological setting. Astroglia (astrocytes) are an abundant cell type in the central nervous system (CNS) and are heterogeneous in structure and molecular profile. A single astrocyte creates a distinct non-overlapping territory that encompasses thousands of synapses. Their extensive branches and fine processes allow direct communication over long distances, as well as indirect communication through secretion of chemokines and cytokines. To this end, astrocytes are the major homeostatic cells of the CNS, executing their diverse functions at molecular, cellular, tissue and organ levels; furthermore, astrocytes contribute to several systemic functions such as regulation of Ca^{+} balance, energy nd food intake [20, 75, 81]. Multiple homeostatic pathways expressed in astroglial cells represented, for example, by membrane trans-

porters for neurotransmitters [89], a complex system of secretion of neuromodulators, neurotransmitter precursors and hormones [72, 77, 76] or aerobic glycolysis producing energy substrates [53] maintain CNS functional activity and provide essential neuroprotection. Astrocytes are also a significant component of the neurovascular unit as their endfeet processes terminate directly onto cerebral vessels, regulating cerebral blood flow according to metabolic demand.

Human astroglia have distinct features over those of rodents, in both their morphological and molecular heterogeneity. For example, the protoplasmic and fibrous astrocytes of the human brain are substantially (10–20 times) larger and protoplasmic astrocytes are markedly more complex than the astrocytes of rodents. As a result, a human protoplasmic astroglial cell covers ~20 times more synapses than the same cell in the rodent brain [49, 50]. In addition, human brains contain several unique types of astrocytes, which are absent in non-primate CNS. An abundant type of human astroglia is represented by interlaminar astrocytes [3, 10]. The second type of astroglia, found only in humans and higher primates, are polarised astrocytes. Further heterogeneity is revealed by the relative protein expression profiles human astroglia. Whilst all of the human astroglial subtypes outlined above are positive for canonical astrocyte marker, glial fibrillary acidic protein (GFAP), their expression of other astrocyte-associated proteins such as the calcium-binding protein, s100B, excitatory amino acid transporters (EAA) 1 and 2 and glutamine synthetase (GS) vary markedly [50, 63]. Indeed, interlaminar astrocytes, are unique in their reactivity to antibodies against the extracellular matrix receptor, CD44 [2, 63]. The significant differences that exist between rodent and human astrocytes and regional specificity represent another obstacle in understanding translational pathophysiology of human astroglia.

15.3 Current Human Cellular Models

Largely due to technical limitations, we still know relatively little about a major cell type of the brain, how it develops and their functional properties in both health and disease. Traditional cell-based approaches utilise assays based on primary cells, which have restricted use due to limited supply and/or transformed and immortalised cell lines, such as human brain microvascular endothelial cells or SH-SY5Y neuroblastoma cell line, which fail to offer a physiologically relevant in vitro model that captures the specific genomic information of the patient, and certainly are unable to capture the biology for complex diseases of ageing with environmental and genetic risk factors, such as AD.

Human astrocyte development comprises two distinct types: first, a foetal, proliferative astrocyte progenitor and an adult non-proliferative mature astroglia [86]. The current state-of-the-art involves isolation of the progenitor cell, which can be subsequently matured in vitro. However, these cells can quickly become quiescent in long-term cultures, and when cultured in the presence of serum show a morphological phenotype markedly different from those in vivo. For example, an eloquent compara-

tive study on human microglia demonstrated significant transcriptional and enhancer remodelling of microglia when transitioned from the brain to in vitro culture [19]. A more recently developed technique known as 'immunopanning' provides improved isolation and purification of mature human astrocytes. The immunopanned cells were used to assess gene expression signatures in human and mouse astrocytes, showing that only a third of the genes most enriched in human astrocytes were expressed in mouse [86]. Of further significance is that the authors also demonstrated functional hominid distinctions to rodents highlighted by differences in astroglial responses to exogenous glutamate. Therefore, current studies on models employing rodent cells should be considered with caution when their application is to human health and disease. Nonetheless, these types of invasive methods for human adult astrocyte sample collection remain challenging [86].

Advances in RNA-sequencing and single-cell biology have not only allowed us to gain insights into the role of astroglia, but also demonstrated marked gene expression profiles dependent on their origin, development and environmental niche [47, 85, 86]. This further highlights the limited biological relevance of culturing isolated cell types, in monolayers. The 3D structure of the CNS comprises a cellular component (neurones, astrocytes, microglia, oligodendrocytes) and an extracellular matrix component with an integral role in facilitating cell–cell interactions, cell viability, cell morphology, cell differentiation and ultimately influencing disease advancement. Consequently, bioengineering of applicable 3D in vitro culture matrices, scaffolds and co-culture systems, to recapitulate complex in vivo modelling are a current focus within the field; however, challenges outlined above still remain.

Together, these limitations underscore a pressing need for new technologies that can replicate human (patho)physiology, at scale, to provide means for improved disease modelling, and a better understanding of human astrocyte development and their role in disease. Thus, efforts to study astrocyte physiology should be directed as much as possible to the most physiologically relevant system: the intact human brain.

15.4 Induced Pluripotent Stem Cell Technology—A Humanised Platform to Study Health and Disease in a Dish

A ground-breaking study by Evans and Kaufman [16] demonstrated generation and in vitro propagation of mouse embryonic stem (ES) cells. It took a further decade, for the field to understand the key factors critical to support ex vivo culture of human ES cells and in 1998 Thomson et al. [68], defined the necessary culture conditions and transcription factors critical for the maintenance of pluripotency. A pluripotent stem cell is defined as a stem cell that has the capacity, given the appropriate cues, to form any of the >200 cell types in the human body. These cells also possess the ability to self-renew in vitro and therefore, potentially permitting an infinite supply of human

cells that can be matured to any cell type(s) of interest. Pluripotent stem cells comprise an expanding number of different cell types, but largely speaking comprise two main cell types: ES cells, active during early development, and induced pluripotent stem cells (iPSCs) that are artificially generated in vitro, using nuclear reprogramming. Nuclear reprogramming, by which nuclei of differentiated somatic cells are reprogrammed by injection into an undifferentiated cell type to induce a pluripotent or embryonic-like cell state, was first described by Gurdon et al. [22], in which an enucleated oocyte was successfully injected with the nucleus from a somatic cell to create a cloned Xenopus. This scientific breakthrough led to mammalian cloning of the infamous Dolly, the sheep [80]. These seminal studies led Takashi and Yamanaka to identify the key transcriptional regulators, namely, octamer-binding transcription factor-3/4 (Oct3/4), sex-determining region Y-box 2 (Sox-2), kruppel-like factor-4 (KLF-4) and c-Myc (now referred to as 'Yamanaka factors') to successfully reprogram adult human dermal fibroblasts cells to the pluripotent stem cell state and the first to report an 'induced' pluripotent stem cell (iPSCs) [64]. A number of publications thereafter have reported various cocktails of transcription factors able to reprogram a variety of adult cell types [68, 79], including keratinocytes [46], peripheral blood mononuclear cells [44, 64] to human urine-derived cells [78, 88]. This innovation revolutionised the stem cell field as it circumvented ethical apprehension associated with the use of human ESCs, which ultimately required destruction of human embryos. Importantly, it has opened new therapeutic opportunities, creating an ideal physiologically relevant model; whereby cells can be derived from any individual; are genetically identical to the donor; can self-renew indefinitely and have the capacity to differentiate into any cell type. Therefore, providing a platform that offers significant advantages over existing models, by delivering the only source of clinically relevant, healthy and diseased human-cell types amenable to various regenerative medicine applications and the ability to study 'disease in a dish' (Fig. 15.1).

Over the last decade, researchers have focused on improving the efficiency of reprogramming by introduction of alternative delivery of episomal, non-integrating and more recently, the use of small molecules for chemical reprogramming and reducing the likelihood of tumorigenicity, making them potentially safer for patient-specific cellular therapy [1]. Moreover, transdifferentiation (or direct conversion), which permits reprogramming of somatic cells to mature committed cell types or multipotent progenitors was first demonstrated by direct conversion of astrocytes into neurones by exogenous expression of four neural transcription factors [6, 26]. The advantages of transdifferentiation over reprogramming are two folds: first, it permits reduced timelines for generation as negates the necessity for generation of a pluripotent cell stage, and second maintains the 'age' of the cells from the donor source. Cells undergoing reprogramming to an ES–cell-like state could effectively wipe the age of the cells and this is particularly relevant for diseases of ageing. The disadvantage of transdifferentiation however, is that it results in progeny that are non-proliferative or possess limited multipotency. For the purposes of this review, we will focus on the use of iPSC-derived cell types and therefore refer the reader to an excellent review of alternative reprogramming strategies [21]. iPSC-derived astroglia for modelling human development.

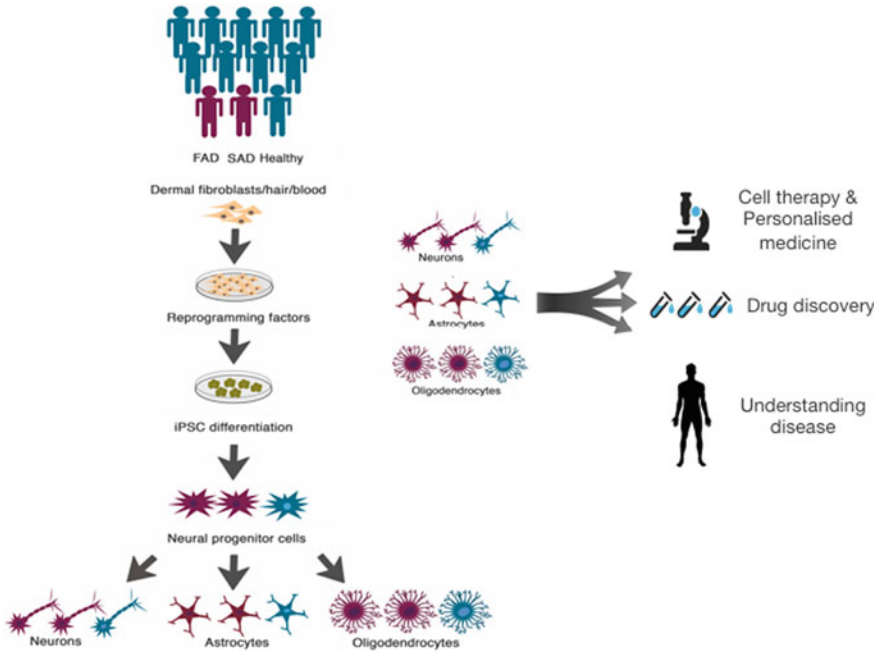

Fig. 15.1 Principles of stem cell technology. Human iPSCs, non-invasively generated from somatic cells (e.g., skin fibroblasts), have the capacity to self-renew indefinitely, can generate all cells of the body and retain the genetic information of the donor. Robust generation of specific mature neural cells (such as astroglia) from iPSCs created from patients with brain pathologies offers an unrivalled platform for the study of human brain disorders in vitro, including the screening of potential novel therapeutics, more accurate disease modelling and in cellular and personalised therapy. FAD: Familial AD; iPSC: Induced pluripotent stem cell; SAD: Sporadic form of AD. Reproduced with permission from [48]

In order for us to learn about pathophysiology of any disease, one must understand the physiology of that cellular system in health. Despite a significant number of publications on iPSC-derived neurones in the last decade, by comparison, only a limited number of studies to date have described homogenous generation of enriched astroglia from healthy patients (Fig. 15.2). More recently, the use of iPSC-derived models has permitted a snapshot of the development of healthy astroglia. A number of groups have developed modified protocols and methods to generate human astroglia from various sources of pluripotent stem cells [14, 34, 37, 43, 57, 61, 66]. The majority of these publications describe modifications of media composition; extracellular matrix, seeding density and timings of morphogen(s) (detailed later), that result in significant improvements in differentiation efficiency, functionality and maturity of iPSC-derived astroglia. Current methods exploit the gliogenic switch observed from in vivo developmental cues, whereby iPSC-astroglia can be derived from either patterned neural progenitor cells or from committed glial lineage [55, 58].

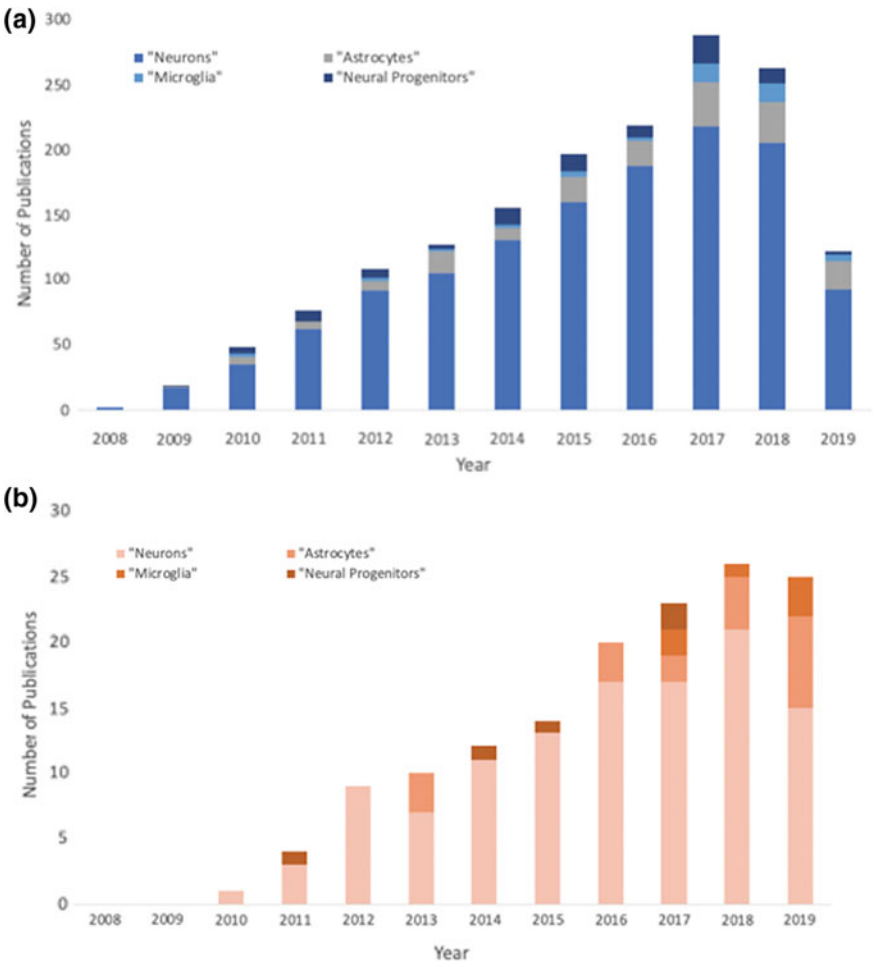

Fig. 15.2 Publication data on original research papers using induced pluripotent stem cells between 2008 and 2019 (May). Analysis of the search terms, title and abstract only publication search terms: ('induced pluripotent stem cells' and "neurons/astrocytes/microglia/neural progenitors") (**a**—blue bars) and ('induced pluripotent stem cells' and 'Alzheimer's Disease' and "neurons/astrocytes/microglia/neural progenitors") (**b**—red bars) for research papers published on NCBI database (PubMed) between 2008 and 2019

Typically, the first step to establish an iPSC-astroglia platform is to generate an intermediate neural progenitor cell (NPC) population that can be expanded and cryopreserved. These cells retain a multipotent, lineage-restricted and differentiative capacity. This recapitulates known cues from in vivo development and patterning that is achieved along the neural tube in primitive neuroepithelial cells, which give rise to different classes of glia. Broadly speaking, there are two predominant methods for the derivation of NPCs from pluripotent stem cells: first, via an embryoid body

intermediate (a 3D cluster of stem cells that mimics embryo development) either in the presence or absence of SMAD inhibition, or via a monolayer-based method with dual SMAD inhibition [62]. Specifically, undifferentiated iPSCs are dissociated and plated in culture medium comprising Noggin to inhibit bone morphogenetic protein (BMP) pathways and SB431542, an antagonist of transforming growth factor β (TGFβ) signalling, and these cells are cultured in suspension or plated onto adherent matrix (e.g., laminin) (2D culture) to promote neuroectodermal lineage commitment. The emergence of columnar epithelial neural rosette structures (polarised cells) (10-15 days post initiation of differentiation) is then selected and expanded for a number of passages and directed to astroglia progenitor formation by the addition of specific cytokines/growth factors in defined culture conditions (detailed below).

Astroglia progenitors derived from human iPSCs was first described by Hu et al. [28], who expanded precursors with mitogens thought to increase oligodendrocyte transcription factor 2 positive progenitors and reduce the development of post-mitotic neurones (e.g., cAMP, T3, insulin growth factors (IGFs), platelet-derived growth factor (PDGF)). Krencik and Zhang [37] were one of the first to demonstrate that repression of neurogenesis in neuroepithelial cells that could be accomplished by regular dissociation of neural rosettes, and this triggered differentiation of the cells into glial progenitors. Subsequently, neuroepithelial cells were expanded in suspension (known as neurospheres) and triturated weekly to reduce neuron differentiation and promote gliogenesis. Following around 90 days in culture, emergence of predominant astroglia progenitors was observed by their positive demarcation of S100B and CD44. These cells have limited self-renewal capacity and can be cryopreserved and expanded for several passages. Terminal astroglial differentiation was achieved by the removal of exogenous mitogens to prohibit mitosis and supplementation of medium with ciliary neurotrophic factor (CTNF) to promote gliogeneic gene expression. CTNF and other members of interleukin (IL)-6 type cytokines have been shown to induce Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signal transduction to activate phosphorylation of STAT3. However, this protocol is labour intensive and time-consuming (~180 days). Shaltouki et al. [61] demonstrated generation of functional astrocytes within in 60–90 days in culture using defined, xeno-free conditions from both iPSC- and ESC-derived NPCs. However, this method resulted in 20–30% contamination with unwanted cell types. Recently published data has shown that transient expression of the nuclear factor 1A (NF1A) was sufficient to induce conversion of iPSC-neural stem cells to astroglia within 5 days. Chromatin remodelling and GFAP promoter demethylation permitted generation of region-specific or reactive astrocytes [66].

An important consideration for any cellular ex vivo model is how does it recapitulate in vivo identity and function. Astroglia morphological changes are closely correlated to their activated or resting status but are typically identified by their stellate profile. Lessons learned from in vivo development also help us to identify astrocyte subtypes. To date, numerous studies, including our own, have demonstrated iPSC-derived astroglia exhibit a number of characteristics to those in vivo; for example, are immunoreactive for the canonical markers GFAP and S100B. More recent efforts to find a comprehensive and definite set of standardised markers to

delineate mature astrocytes have described additional markers such as aquaporin-4 GS, glutamate transporters GLT-1and GLAST1; Aldehyde Dehydrogenase 1 Family Member A1 (ALDH1A1) [30, 33, 36, 38, 66]. We recently demonstrated highly efficient generation of enriched populations of mature human cortical astroglia (with less than 5% of neuron contamination) from a seemingly healthy donor iPSC within 30 days of induction from cortical neural stem cells (NSCs). Based on the method described in Shaltouki et al. [61], NSCs were exposed to CTNF, to activate downstream JAK/STAT signalling that transcriptionally activates astrocyte-specific loci, GFAP and S100B; BMP2; epidermal growth factor (EGF) and insulin, all of which have been shown to drive astroglial commitment and proliferation. We assessed a panel of known functional astrocyte markers namely, GFAP, GS, S100B and EAAT1 comparable to those reported in the literature for adult astrocytes in vivo [30].

A known limitation of using pluripotent stem cells models is that often the terminally differentiated cell types resemble more foetal-like phenotypes and the above protocols, as well as others, report expression profiles consistent with an immature astrocyte phenotype [14, 31, 60, 61]. More recently, a comprehensive study of different culture conditions for the derivation of human iPSC-derived astrocytes from 30 independent donors generated as three independent cohorts (both healthy and diseased samples) showed derivation of astrocytes expressing classical markers, S100B, GFAP, GLAST, Vimentin, ALDH1L1 and APOE using a commercially available media within 30 days. It must be noted that the authors however, found significant differences in robustness of generation and heterogeneity of expression. Transcriptional profiling of these iPSC-derived astrocytes and primary human foetal astrocytes from two brain regions (cerebral cortex and midbrain) and comparisons to in vivo human astrocyte transcriptomic metadata revealed that iPSC-astrocytes are highly analogous to primary foetal astrocytes. Differential expression of genes associated with regulation of neuronal maturation, such as synapse or ion channel formation was underpresented in iPSC-astrocytes, whereas signals promoting extracellular cell adhesion and interaction were upregulated compared to human in vivo astrocytes [67].

Of further interest, is the examination of astroglia regional subtypes throughout the CNS. During neuroepithelial differentiation the cells can undergo directed specification to regional progenitor cells for the generation of different neuronal subtypes. This is also thought to be true for astroglial subtypes, and therefore addition of exogenous cues will determine patterning of astroglia, for example, sonic hedgehog (SHH) directs the formation of ventral astroglia, whilst retinoic acid (RA) derived progenitors exhibit spinal cord phenotypes and those without additional morphogens display characteristic cortical astroglia. However, it is important to note that a significant limitation in our knowledge of markers to specifically identify each astroglial subtype remains controversial. For example, classical delineation of astrocyte identity is by GFAP positivity, particularly relevant for reactive astrocytes, yet some evidence shows that a subpopulation of non-reactive astrocytes do not express GFAP [38], this is confounded by a lack of clearly defined markers that are able to delineate their identity.

Astrocytes may adopt either a quiescent state with protoplasmic morphology, characterised by low GFAP and high GLT1, or a fibrous, reactive phenotype characterised by high GFAP and low GLT1 and traditional culture systems reflect the latter [84]. Therefore, a robust cell model of mature quiescent astrocytes would be beneficial to progress studies of human neural function. TCW et al. [67] showed that iPSC-astrocyte gene expression was closely clustered to those of a quiescent state rather than reactive astrocytes. However, some caution should be given to the interpretation as comparisons were made to datasets from murine astrocytes due to a lack of availability of human samples.

15.5 Astroglia in AD Pathogenesis

Diseases of the CNS can be generally defined as a homeostatic failure of nerve tissue, and hence they are directly associated with the functional performance of homeostatic astroglia. Since these pathologies are the result of neuronal death, a neuro-centric focus in the search for mechanisms and therapeutic approaches has prevailed for some time. The classical neurocentric view of neurodegeneration has, in recent years, been challenged with a plethora of information to support the role of astroglia in non-cell-autonomous mechanisms in neurodegenerative diseases. Astrocyte pathology contributes to all types of neurological disorders and this contribution is complex and disease specific. In AD, astroglia undergoes complex and regionally specific pathological changes. A decrease in astroglial profiles (indicative of atrophic changes) at the early stages of disease progression have been observed in studies on transgenic animals harbouring AD-related mutant human genes [4, 5, 39, 52, 82]. At the later stages of disease, emergence of senile plaques elicited astroglial reactivity, with hypertrophic cells accumulating around the plaques [27, 52]. In the human brain, astrogliosis is prominent at the early stages of the disease, but decreases in advanced stages of AD, when overall glial paralysis contributes to severe brain atrophy [56, 73]. In summary, various aspects of astrogliopathic changes contribute to the progression of AD, and conceivably, astrocytes define both early cognitive deficits due to deficient synaptic support, whilst astroglial reactivity defines resilience of nervous tissue to the pathology. Please refer to preceding chapters for in-depth reviews of the contribution of astroglia to the pathophysiology of AD.

15.6 iPSC-Derived Astroglia for AD Modelling

Despite a plethora of publications indicating the generation and utility of iPSC-derived neurones for disease modelling to date, in comparison only a limited number of studies have described consistent generation of enriched astrocytes from patients with AD [17, 29, 34, 35, 51] (Fig. 15.2). Generation of functional astroglia from healthy iPSCs has previously been reported to be time-consuming, with further

limitations in purity (summarised above), which denotes a significant challenge in delineating autonomous contribution of astroglia in disease pathogenesis. Although studies of cell-autonomous pathobiology of human astrocytes derived from patient stem cells are in the nascent state, several lines of evidence show that these astroglia retain some pathological disease signatures, including AD (Fig. 15.3). Importantly,

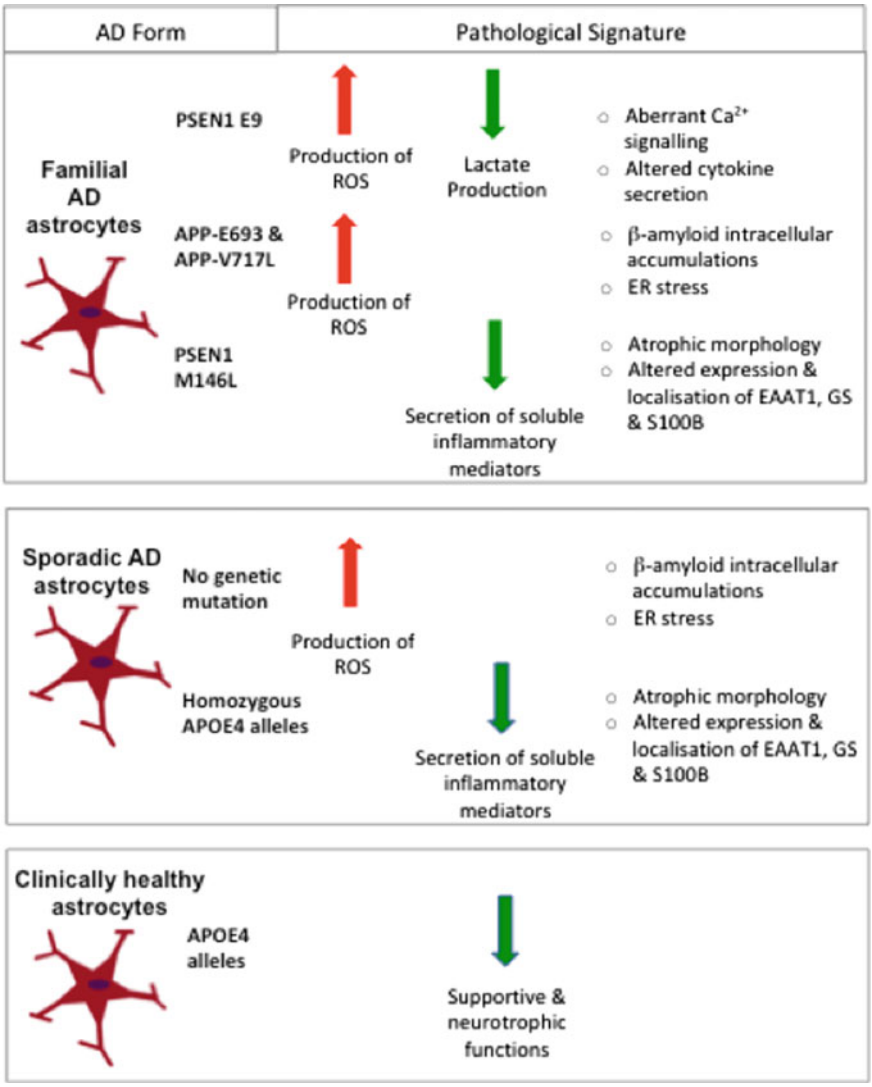

Fig. 15.3 Comparison of AD pathological signatures in iPSC-derived astroglia. AD modelling in iPSC-astroglia derived from patients with either late-stage (sporadic) or early-onset (familial) forms of the disease and a summary of key pathological changes from [30, 34, 51, 87]

advances in genome engineering technologies, such as CRISPR/Cas9, allows us to engineer parental cells to generate control or reference cells that are almost biologically identical (apart from the amino acid changes used to introduce or repair the genetic mutation). This allows researchers to minimise the clonal and donor variations exhibited in iPSC-derived cell types, permitting robust endpoints analyses. Availability of such cells is becoming more accessible as the field expands, with large consortia such as European Stem Cell Bank Initiative and academic groups providing open access.

We recently reported that human astroglia reprogrammed from dermal fibroblasts of a 53-year-old male donor with type III early-onset FAD (bearing an M146L mutation in the presenellin-1 gene, *PSEN1*) and from an 87-year-old female clinically affected with late-onset SAD (homozygous for the four allele of apolipoprotein E, $ApoE4^{+/+}$; the single-largest genetic factor determining SAD risk) exhibited pathological phenotypes when compared with iPSC-derived astroglia reprogrammed from a healthy control [30]. This pathological signature comprised (i) mislocalisation and abnormal expression of mature astrocyte markers, (ii) compromised astrocyte heterogeneity and (iii) astroglial atrophy. Astrocytic dysgenesis, manifested as an almost complete loss of processes and overall reduction in cell size was significant (Fig. 15.4); a finding that strongly correlates with observations of morphological astroglial atrophy in early-stage AD pathology in mouse models [52, 73]. In contrast, astrocytes derived from iPSCs isolated from patients with fronto-temporal dementia show hypertrophic morphology [24], further indicating disease-specific glial metamorphoses. A recent study using iPSC-derived astrocytes reprogrammed from a patient exhibiting a PSEN1 E9 mutation, known to cause FAD, supported our

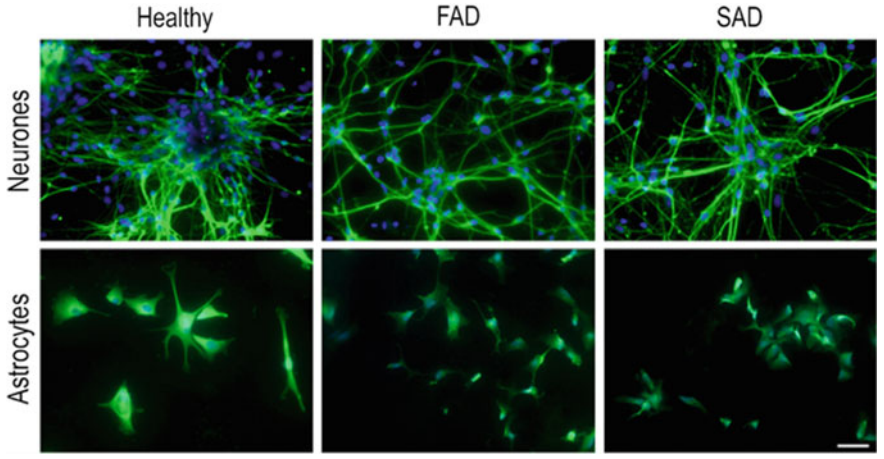

Fig. 15.4 Comparison of healthy control, FAD and SAD patient-derived βIII-tubulin immunoreactive neurones and GFAP immunoreactive astrocytes. Whilst early neuronal appearance is indistinguishable across the groups, AD astrocytes show markedly reduced heterogeneity of morphology and striking atrophy compared to healthy cells. Scale bar = 50 μm. Reproduced with permission from [30]

initial observation of a lack of difference in the differentiation potential of AD and control (healthy) iPSC-derived astrocytes. However, the author reports that stellate morphology was observed across both healthy- and AD-derived astrocytes. This discrepancy may be down to significantly different culture methods, reflecting a more mature phenotype or indeed a donor/mutation-specific phenotype [51]. It must be noted that our study was based on non-isogenic comparisons with a single clone per donor and although, the only publication to date, to provide an in-depth morphological and immunocytochemical characterisation of iPSC-astrocytes in AD, additional quantitative data must be performed to provide more conclusive evidence.

Distribution of astroglial markers was similarly aberrant in cells derived from AD patients in our study. For example, S100B in AD iPSC-derived astroglia was almost entirely confined to large nuclear inclusions, in contrast to healthy iPSC-derived astroglia, which show typical cytoplasmic localisation (Fig. 15.4). This was accompanied by significant decrease in total S100B expression in AD astroglia when compared to control astroglia. Since S100B is known to interact with various cytoskeletal components, this phenomenon may represent a novel and early mechanism underlying SAD- and FAD-induced astrocytic atrophy. Mislocalisation was also observed for glutamine synthetase in both FAD and SAD astroglia, whilst the glutamate transporter EAAT1 was misplaced only in SAD iPSC-derived astroglia. Furthermore, the expression levels of both EAAT1 and glutamine synthetase were decreased only in FAD-derived astroglia [30]. Intriguingly, other iPSC-based studies have shown that astroglia derived from iPSCs from SAD and FAD backgrounds exhibit no significant deficit in their overall abilities to sequester extracellular glutamate compared to controls [34, 87]. Whether these findings are a result of the application of glutamate assay concentrations being orders of magnitude higher than predicted physiological [59], and hence masking nuanced alterations in uptake, or whether they imply that it is the processing rather than gross uptake of glutamate that is altered in AD, remains to be established.

Three human isoforms of apolipoprotein E (ApoE) exist; with homozygous expression of the E4 alleles conferring significantly increased risk for late-onset SAD in contrast to the E2 isoform, which has been reported to confer a protective mechanism against AD. Whilst it is known that ApoE contributes to neuronal health by transporting the cholesterol required for cellular repair, synaptic plasticity and dendritic spine integrity [8], the precise mechanisms by which the ApoE isoforms contribute to AD pathogenesis or protection are not fully understood. Astroglia generated from iPSCs derived from APOE4 donors secrete active ApoE, but exhibited profound hypolipidation in astrocytes $ApoE4^{+/+}$ SAD-prone background compared to $ApoE3^{+/+}$ controls [87], consistent with findings in mice and humans [25, 83]. Thus, human iPSC-derived astrocyte models provide an excellent platform to study relative contribution of its isoforms to AD progression.

One of the most impactful and early pathological characteristics of AD is progressive loss of synapse/synaptic function albeit through a yet unknown mechanism. Of further interest is that iPSC-derived *ApoE4* astrocytes show an impaired ability to support neuronal survival and synaptogenesis, compared to controls; indicating the loss of neurotrophic and neuroprotective functions [87]. This functional deficit

appears independent of neuronal contact or glutamate scavenging, hence implicating alterations in the astrocytic secretome. An increasing area of interest in AD progression is neuroinflammation, in which the cytokine mileu is thought to drive amyloid deposition and hyperactivation/recruitment of microglia. It can be characterised by the accumulation of pro-inflammatory cytokines such as IL-6, IL-1β and tumour necrosis factor (TNFα) amongst others. Pro-inflammatory astrocyte reactivity contributes to glutamate imbalance and release of mediators that cause synapse death [15]. A number of studies have observed altered cytokine secretion from iPSC-astrocytes derived from AD patients, compared to be healthy. Interestingly, Oksanen et al. [51] showed partial rescue of this phenotype using a γ secretase inhibitor, indicating a role for amyloidosis in neuroinflammation. In our study, we revealed significant alteration of the constitutive secretion of the pro-inflammatory mediators IL-8 and membrane cofactor protein-1(MCP-1) in FAD- and SAD iPSC-derived astroglia when compared to seemingly healthy controls [30]. However, it must be noted that this was constitutive secretion and not under disease-relevant stimulatory conditions. These observations may further indicate a potential glial paralysis that has been postulated to be a fundamental factor in the evolution of AD [73]. It is thought that Ca^{2+} homeostasis could play a role in neuroinflammation, and astroglia derived from human iPSCs are capable of generating spontaneous Ca^{2+} signals [58], although proper physiological characterisation of these cells is yet to be produced. A recent study goes some way to address some of the questions around functional iPSC-derived astroglia Ca^{2+} activity [67].

At the earliest stages of AD pathology, activated reactive astrocytes are predominant in the molecular layer of the cerebral cortex and close to amyloid plaques. A recent study has also shown that in conjunction with microglia, reactive astrocytes form a 'net' over the amyloid-beta plaques and astrocytic processes invade the plaque, directly interacting with amyloid-β (Aβ) protein [7]. Accumulation of Aβ oligomers in intracellular organelles has been described in some, but not all, iPSC-derived SAD- and FAD-astrocytes, concomitant with the induction of both ER and oxidative stress [34]. This was recently corroborated in PSEN1 E9 mutant iPSC-astrocytes, but a discrepancy in Aβ1-42 secretion was seen between these studies [87]. Specifically, astrocytes derived from patients with the *APP*-E693Δ mutation demonstrated large inclusions of Aβ, which were shown to co-localise to the endoplasmic reticulum, early endosomes and lysosomes, whilst those from *APP*-V717L patients showed no accumulation at all. This finding sheds new insight into disease heterogeneity, both within and between SAD and FAD, and is supportive of our own findings of subtle variations at the cellular level between the two forms of the disease [43].

A new paradigm in the field is the link of neurodegenerative disease as a metabolic disorder. This hypothesis addresses some of the most important features of AD that include mitochondrial dysregulation, oxidative stress and diminished cerebral glucose metabolism [70, 65]. Glucose hypometabolism in the brain appears early in the genesis of AD [71], and in fact presents a common phenomenon with other neurodegenerative diseases [4]. Mitochondrial dysfunction, which is strongly associated with age-related neurodegeneration, is particularly prevalent in AD [5, 39]. Metabolic changes in the cerebrospinal fluid (CSF) of AD patients includes ele-

vated methionine (MET), 5-hydroxyindoleacetic acid (5-HIAA), vanillylmandelic acid, xanthosine and glutathione have been linked to accumulation and abnormal tau metabolism [82]. In addition, decreased blood flow in specific brain areas and reduction of glucose transporters at the blood-brain barrier were also shown to contribute to the hypometabolic state in AD [27]. As a result, a significant correlation between diminished cerebral glucose metabolism and cognitive performance has been shown in AD patients [56, 74]. In line with these findings, AD is closely linked to dysfunction in the regulation of energy metabolism, production of radical oxygen species (ROS) and mitochondrial defects [74]. Recently, PSEN1 mutant iPSC-astroglia exhibited oxidative phosphorylation versus glycolytic metabolism seen in healthy iPSC-astroglia. However, γ-secretase inhibitor treatment was unable to reverse mitochondrial metabolism, indicating this is likely to be independent of Aβ induced pathophysiology [51].

In summary, astroglia derived from iPSCs are capable of mimicking in vitro, a wide variety of deficits in homeostatic molecular cascades intimately associated with AD, that is routinely observed in vivo and in patients. Collectively, these results show that iPSC-derived astrocytes secrete elevated levels of Aβ1-42, show altered Ca^{2+} homeostasis, reactive oxygen species/mitochondrial metabolism switching thus providing a platform for preclinical screening. To this end, Thorne et al., utilised human ES-derived astrocytes to execute a phenotypic assay for compounds that prevent oxidative stress. This study established a scalable system to support high-throughput screening of over 4000 compounds in stem cell-derived astrocytes [69].

15.7 Therapeutic Strategies Towards Neuroregeneration

The WHO reports the global burden of neurological diseases that affects up to 1 billion people and accounts for 12% of all deaths worldwide and 72% of this total burden is caused by four disorders: cerebrovascular disease, epilepsy, dementia and migraine. The global cost of mental health conditions alone was estimated at US$2.5 trillion in 2010, with a projected increase of >US$6 trillion in 2030 and in Europe alone, the total cost of diseases of the brain is estimated at €800 billion per year. A significant contributing factor to one of the largest medical burdens currently facing public health is a lack of knowledge as to the cause(s) of such diseases and pathways that could be manipulated to reduce their progression. A gap in the translation from existing animal models to success in human trials has been identified as a major factor in lack of effective therapeutic interventions in a number of diseases, including AD. The exponential increase in the expected number of patients presenting with AD in particular, not only represents a major area of unmet medical need, but it also represents a significant market opportunity. For example, to delay the onset of AD by 5 years could save $50 billion in annual healthcare costs in the US alone. There have been no new drug approvals for treatment of AD since 2003 and existing treatment paradigms fall into two categories: acetylcholinesterase inhibitors or NMDA receptor antagonists, both of which, only help treat symptoms, have modest clinical efficacy

and do not treat the underlying cause(s) of AD. Data shows that between 2002 and 2012, the attrition rate for novel AD drugs was 99.6%, attributed to a lack of sufficient target engagement or adverse toxicity. The development of new AD drugs to market are confounded by our incomplete understanding of AD, but also challenges in the clinical setting as overt clinical symptoms are not evident until the latter stages of the disease; meaning that many therapeutics may be refractive. The field is moving towards treating patients earlier in AD progression, but expose associated risks with drug dosing failures and side effects, which are yet still poorly defined. As of 2018, there were 112 agents in the current pipeline for AD treatment distributed across early and late stages. The mechanism of action of the majority of these therapeutics is disease modifying [11]. However, recent trials such as Lanabecestat that failed at phase II/III, pharma giants Eli Lilly and Astra Zeneca announcing the end of two phase III trials for similar β-secretase inhibitors and more strikingly, Pfizer ending all drug discovery programmes into neurological diseases clearly shows that a new strategy towards neurological disease discovery and patient treatment is critical.

Previously, astrocytes were not considered as targets for neurodegenerative drug discovery, as such disorders were previously viewed as neuronal in their pathology. However, progress in astroglia research has revealed key functional roles of astrocytes in the CNS and their neuroprotective or neurotoxic attributes in disease states. The advent of novel technologies available (including improved human cellular models such as iPSCs) enables phenotypic high-throughput compound and genetic screening campaigns for drug discovery. Development of therapeutics towards astrocytes, or perhaps more importantly, targeting both astrocytes and neuron degeneration may provide new opportunities to generate efficacious and ultimately disease-modifying medicines for an ever-growing unmet medical need. The shift from a neuron-centric view to one that incorporates, not only astrocytes, but also other key cells of the CNS (e.g., microglia), is a crucial step into rejuvenating neurodegenerative drug discovery to treat disease.

15.8 Limitations

The promise of iPSCs to revolutionise regenerative medicine has become clear in the preceding decade by enabling generation of patient-specific cells for cell- and disease-specific pathogenesis modelling and for cell-based therapeutic advances. However, careful consideration of the limitations such as, chromosomal instability, genetic differences between donors and epigenetic memory from the parental cell means that thorough characterisation is necessary prior to use as a cellular model. Furthermore, significant challenges to their use in the clinic exist such as the use of oncogenes (e.g., c-Myc) for reprogramming that could lead to tumours, viral transduction reactivation and incomplete differentiation of iPSCs. The limitations of current models are further highlighted when specifically applied to neuroglia. Very little is known about pathological neuroglial phenotypes in the context of human AD, again largely due to limitations of animal models and lack of access to human

samples. This is even more pronounced as remarkable differences between rodent and human astrocytes, hinder in-depth characterization of translational pathophysiology of human astroglia. However, as a note to the reader, it should be emphasised that cultured astrocytes discussed during this chapter represent a simplified model relative to that of astrocytes in the CNS, whereby additional interactions with other cell types and matrix components are likely to influence the astrocytic phenotype. Though, these highly purified human astrocyte culture models and combined precision genome editing represent a unique system to delineate the autonomic responses of astrocytes to be defined stimuli/matrix/co-cultures in both healthy and AD-affected cells in an unparalleled manner.

15.9 Future Perspectives

'Humanisation' of neuropathological research is the main challenge which theoretical medicine is facing. A large majority of neurological diseases do not have an effective cure and with only symptomatic therapies available at best. There are classes of neurological disorders, which do not develop in animals (rare occurrences of neurodegenerative phenotype in lemurs or in some canines remain exceptions), and hence require development of artificial animal models. These are commonly produced in model organisms such as mice, zebrafish and *Drosophila* due to their amenability for genetic manipulation. However, the brains of these animals are not even remotely close to the brain of humans, their lifespan is significantly shorter and their social interactions are much inferior and fundamentally distinct to humans. These limitations underlie the slow progress of neurological therapies.

Another salient revolution developing over last decade concerns a fundamental shift in the understanding of cellular pathophysiology of the brain. The classical paradigm that regards neurones as the cell-autonomous substrate of neuropathology has shifted towards neuroglial mechanisms, that, by virtue of homeostatic and defensive capabilities, seems to determine the resistivity of nervous tissue to pathological insults and chronic neuropathologies. Evolution of astroglia from lesser mammals to humans is remarkable and human astroglia are unique in their complexity compared to rodents. Poor translation from animal models to clinical outcomes has severely limited the development of effective therapeutics for neurological disorders to date, not least AD which remains incurable. The idiosyncratic astroglia of the human brain might be the key to better understanding of uniquely human neurological diseases; hence the development of such human-based models as described here to more accurately study such diseases is essential. Emerging evidence using iPSC-astroglial models is beginning to uncover subtle variations in individual molecular and cellular phenotypes not only between but also within FAD and SAD classifications. Invariably these models pave the way towards the stratification of patient treatment regimes and personalised medicine. Arguably, the most influential development in stem cell culture is the ability to derive in vitro tissues termed organoids that capture more of the complexity of 3D tissues such as multicellular components and functional char-

acteristics of organs. This is particularly relevant for the study of neurodegeneration as it possible to generate cerebral organoids [9, 45, 13, 32, 40–42].

Generation of brain organoids from human pluripotent stem cells exploits EB formation, which permits differentiation into cells of the three lineages, closely mimicking the developing brain. The outer layer forms ectodermal properties, latterly forming neural progenitor cells. Culture of these EBs in matrigel (an extracellular matrix preparation) promotes self-organisation (the defining feature of organoids versus a spheroid/3D culture) and polarisation of neuroepithelium. Subsequent propagation in bioreactor culture induces luminal structures resulting in a fluid-filled cavity. A principal study demonstrated the use of such cerebral organoids for AD modelling; generating iPSC-derived organoids from patients with FAD, recapitulated some of the key hallmarks of AD pathology (including hyperphosphorylated tau and amyloid aggregation). Of further relevance, following treatment β- and γ-secretase inhibitors, FAD organoids showed significantly reduced Aβ and tau pathology [54]. This demonstrates an exciting opportunity for organoids to increase the translational and clinical predictivity of neurological disease discovery and personalised medicine.

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