

**ROLE OF THE INWARD RECTIFYING POTASSIUM CHANNEL, $K_{IR4.1}$,
IN ASTROCYTE PHYSIOLOGY AND NEURONAL EXCITABILITY**

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ABSTRACT

BILJANA DJUKIC: Role of the Inward Rectifying Potassium Channel, $K_{ir}4.1$,
in Astrocyte Physiology and Neuronal Excitability
(Under the direction of Ken D. McCarthy, Ph.D.)

During neuronal activity extracellular potassium concentration ($[K^+]_{out}$) becomes elevated and if uncorrected causes neuronal depolarization, hyperexcitability, and seizures. Clearance of K^+ from the extracellular space, termed K^+ spatial buffering, is considered to be an important function of astrocytes. Results from a number of studies suggest that maintenance of $[K^+]_{out}$ by astrocytes is mediated by K^+ uptake through $K_{ir}4.1$ channels. Furthermore, a missense variation in the $K_{ir}4.1$ gene is linked to seizure susceptibility in mice and humans. To study the role of this channel in astrocyte physiology and neuronal excitability we have generated a conditional knockout (cKO) of $K_{ir}4.1$ directed to astrocytes via human GFAP promoter, *gfa2*. $K_{ir}4.1$ cKO mice die prematurely and display severe ataxia and stress-induced seizures. Histological analysis of $K_{ir}4.1$ cKO brain and spinal cord revealed white matter vacuolization suggestive of oligodendrocyte pathology. Immunostaining studies confirmed removal of $K_{ir}4.1$ from cKO astrocytes and oligodendrocytes, indicating that these cell types arise from a common GFAP-expressing precursor. Passive astrocytes in $K_{ir}4.1$ cKO hippocampus appeared normal in morphology and coupling ability; however, we observed a significant loss of complex astrocytes suggestive of $K_{ir}4.1$ role in astrocyte development. Whole-cell patch clamp revealed large depolarization (>35 mV) of $K_{ir}4.1$ cKO astrocytes and oligodendrocytes. Complex cell depolarization appears to be a direct

consequence of $K_{ir}4.1$ removal. In contrast, passive astrocyte depolarization seems to arise from an indirect process that may involve a change in Na^+/K^+ -ATPase function. $K_{ir}4.1$ cKO passive astrocytes displayed a marked impairment of both K^+ and glutamate uptake induced by neuronal stimulation. Surprisingly, membrane and action potential properties of CA1 pyramidal neurons, as well as basal synaptic transmission due to single pulse stimulation appeared unaffected, while spontaneous neuronal activity was reduced in the $K_{ir}4.1$ cKO. However, increased synaptic stimulation (100 pulse train) revealed greatly elevated (>20%) post-tetanic potentiation and short-term potentiation in $K_{ir}4.1$ cKO hippocampus. Our findings implicate that through its involvement in astrocyte development and K^+ buffering, $K_{ir}4.1$ participates in the modulation of synaptic strength thereby modulating neuronal spontaneous activity and synaptic plasticity.

To my parents, Živko and Zorka Đukić

Voli vas vaš Mile

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LIST OF ABBREVIATIONS

(e)GFP:	(enhanced) green-fluorescent protein
[ion] _{in} :	intracellular ion concentration
[ion] _{out} :	extracellular ion concentration
4-AP:	4-aminopyridine
ACSF:	artificial cerebrospinal fluid
ALS:	amyotrophic lateral sclerosis
AMPA:	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AQP4:	aquaporin-4
ATP:	adenosine triphosphate
BBB:	blood-brain barrier
BDNF:	brain derived neurotrophic factor
Ca _v :	voltage-gated Ca ²⁺ channel
CBX:	carbenoxolone
CNP:	2,3-cyclic-nucleotide-3-phosphodiesterase
CNS:	central nervous system
EAAT:	excitatory amino acid transporters
E _K :	equilibrium K ⁺ potential
E _{Cl} :	equilibrium Cl ⁻ potential
(f)EPSP:	(field) excitatory postsynaptic potential
GABA:	γ -aminobutyric acid
GDNF:	glia-derived neurotrophic factor
GFAP:	glial fibrillary acidic protein

GLAST: glutamate-aspartate transporter
GLT-1: glutamate transporter-1
GluT: glutamate transporter
GS: glutamine synthetase
iGluR: ionotropic glutamate receptor
IP3: inositol-1,4,5-trisphosphate
IPSC: inhibitory postsynaptic current
K_{2P}: two-pore K⁺ channel
K_a: transient A-type K⁺ channel
K_{Ca}: Ca²⁺-gated K⁺ channel
K_{dr}: delayed rectifying K⁺ channel
K_{ir}: inward rectifying K⁺ channel
K_v: voltage-gated K⁺ channel
LTD: long-term depression
LTP: long-term potentiation
MBP: myelin-basic protein
mGluR: metabotropic glutamate receptor
Na_v: voltage-gated Na⁺ channel
NMDA: N-methyl-D-aspartate
OUA: ouabain
PLP: proteolipid protein
PPF: paired-pulse facilitation
PTP: post-tetanic potentiation

R_m : membrane resistance
RVD: regulatory volume decrease
SD: spreading depression
(s)EPSC: (spontaneous) excitatory postsynaptic current
STP: short-term potentiation
SVZ: subventricular zone
TBOA: threo- β -benzyloxyaspartate
TEA: tetraethylammonium
TGF β : transforming growth factor- β
TNF α : tumor necrosis factor- α
TTX: tetrodotoxin
 V_m : membrane potential
VRAC: volume-regulated anion channels
VZ: ventricular zone

CHAPTER I. GENERAL INTRODUCTION

1. GLIA THROUGH HISTORY: FROM “NERVENKITT” TO “TRIPARTITE SYNAPSE”

For more than a century the awe striking ability of our nervous system to process an enormous variety of stimuli enabling us to function and interact within the complex environment was solely attributed to the information processing capability of neurons. This “neurocentric” view started changing only a decade or two ago when due to the overwhelming evidence glial cells, which are about 10-times more numerous than neurons, were finally acknowledged as an integral part of the central nervous system (CNS) information processing network.

In 1846, Rudolf Virchow described the “connective tissue” of the brain that penetrates the entire brain mass, filling the spaces between nerve cells and their fibers and separating them from the blood vessels (reviewed in Somjen, 1988). He named this substance “nervenkitt” (literally meaning “nerve putty”) which was later coined as neuroglia (glia meaning “glue” in Greek). Virchow and later Dieters recognized that “nervenkitt” contained cells, but the primitive staining techniques of that time did not allowed for their morphological characterization (Virchow, 1858; Dieters, 1965). Several decades later, improved staining techniques, such as Golgi’s silver impregnation, for the first time enabled clear identification of different cell types within the brain (Golgi, 1885). Using this technique, Andriezen described two types of cells, fibrous and protoplasmic glia found in white-matter and gray-matter, respectively (Andriezen, 1893). Soon after, Ramón y Cajal

classified fibrous and protoplasmic glia as astrocytes due to their similar star-like shape (Ramon y Cajal, 1909, 1913). He also introduced gold impregnation which further improved study of glial morphology and was able to identify another group of non-neuronal cells lacking processes, the “third element” cells (the first two elements being neurons and astrocytes; Cajal, 1913). Cajal’s disciple, Del Rio-Hortega visualized processes of “third element” cells by silver carbonate stain and categorized them into oligodendrocytes and microglia (1942). Therefore, Del Rio-Hortega is responsible for the classical division of neuroglia into macroglia, including astrocytes and oligodendrocytes derived from the ectoderm, and microglia which he believed were of mesodermal origin (reviewed in Somjen, 1988). Once glial cells were morphologically characterized and differentiated from neurons, numerous speculations about their function emerged. In a truly visionary paper for that time, Lugaro (1907) speculated that glial cells a) guide the migration of developing neurons by chemotaxis, b) keep the cerebral interstitial fluid habitable for neurons, c) detoxify blood before it enters brain, d) deliver nutrients from blood to neurons, e) remove toxic waste products of neuronal metabolism, f) insulate nerve fibers and fiber bundles, g) act as phagocytes in clearing of dying neurons, and h) remove compounds secreted by nerve endings to terminate their action. Remarkably all of these glial functions have indeed been confirmed and several are reviewed in *Astrocyte Functions* (page 13).

Unfortunately, Lugaro’s paper and speculations about the importance of glia were lost in the thrill generated by the discovery of neuronal electrical excitability and advancement of electrophysiology preparations and techniques. What followed was the “glial dark-age” or the age of “neuron doctrine” which stressed the individuality of neurons and the belief that they were the sole functional units of the brain (Sherrington, 1906). Determined to unveil the

mystery of neuronal information processing, the electrophysiology pioneers Cole, Curtis, Hodgkin, Huxley, and Katz focused their attention on the action potential propagation of the squid giant axon. In 1952, their work culminated in an explicit model of the generation of the action potential. At that same time, the investigation of neuronal electrophysiology in vertebrate CNS exploded due to the invention of intracellular recording and voltage clamp (Marmont, 1949; Cole, 1949; Hodgkin, Huxley and Katz, 1952). Nerve cells were blindly hunted with microcapillary electrodes. Often, however, instead of sought-after neurons, cells that did not generate action potentials were encountered (Phillips, 1956). It was correctly speculated that the encountered cells were glia, however they were incorrectly termed “silent”, “unresponsive”, or “idle”, and therefore uninteresting.

Fifty-seven years after Lugaro’s remarkable paper, Sugaya et al. (1964) noticed that the membrane potential of glial cells actually does change during neuronal excitation. They reported that during seizure discharges and during spreading depression these cells undergo slow depolarization. In 1966, Kuffler and associates established that upon neuronal stimulation glial cells of leech and amphibian depolarize due to the rise of the extracellular K^+ concentration ($[K^+]_{out}$; Orkand et al., 1966; Kuffler and Nicholls, 1966). The glial “ K^+ spatial buffering” hypothesis was born, and the study of glial physiology and role in the CNS was on its way. In the 1970s, glial immunological markers and methods to study glial cells in culture were developed, and once again it was the development of new techniques that pushed the field forward. Since then, advances in ion imaging, microscopy, electrophysiology, and fly and mouse genetics, all led to an astonishing discovery that glial cells express and employ most, if not all, receptors, ion channels, and signaling mechanisms present in neurons. Furthermore, during the last decade, we discovered that glial processes

surrounding synapses not only receive signals from the presynaptic neuron but also respond by releasing feedback signals such as the neurotransmitter glutamate.

When in 1999, Araque, Parpura, Sanzgiri, and Haydon coined the term “tripartite synapse”, establishing glial processes as the third actively participating component of the synapse, the fall of the “neuron doctrine” was official. More than a century after their discovery, it has become clear that only by understanding the bidirectional interaction between glial cells and neurons we can gain an understanding of complex processes such as cognition and behavior. One of the main challenges facing glial biologists in the next few decades is sorting through the enormous catalog of implicated glial functions and interactions to devise a coherent picture of glial physiology and its impact on the organism as a whole. The remaining sections in this chapter are meant to highlight our current knowledge of astrocyte biology, in particular the astrocyte role in extracellular K^+ homeostasis, which was the subject of my dissertation project.

2. TYPES OF GLIA

One of the early anatomists, Del Rio-Hortega (1842), was the first to classify CNS neuroglia into microglia and macroglia, according to their morphology, location, and developmental origin. Macroglia, or astrocytes and oligodendrocytes, were thought to originate from the neuroectoderm, while microglia, Rio-Hortega claimed, were derived from the mesoderm (reviewed in Somjen, 1988). Even though this classification is still used today, a recently emerging theme is that glial lineage is more diverse and more plastic than was previously imagined and novel classification may be required. The glial type that shows most heterogeneity is the “all-capable” astrocyte. Growing evidence indicates that there are several

astrocyte lineages and that astrocytes from different brain regions have distinct properties. Kimelberg (2004) provides an excellent review of the controversy surrounding astrocyte identity and classification. Astrocyte heterogeneity within hippocampus is further discussed in *Physiology of Hippocampal Glia* (page 92). It should also be noted that the peripheral nervous system (PNS) contains glial cells called Schwann cells that are analogous to CNS glia. Like the CNS glia, Schwann cells are either myelinating or non-myelinating, however, their location, morphology, and immunological markers distinguish them from astrocytes and oligodendrocytes.

2.1. MICROGLIA

Microglia have traditionally been regarded as the CNS macrophages and their origin still remains controversial. They represent on average about 10% of the adult CNS cell population (reviewed in Pessac et al., 2001). In mice, microglial progenitors can be detected in neural folds at the early stages of embryogenesis. Murine microglia were shown to originate from the yolk sac at a time in embryogenesis when monocyte and macrophage progenitors of hematopoietic origin are also found (Alliot et al., 1999). Based on this observation, it is now generally accepted that adult microglia originate from monocyte/macrophage precursor cells migrating from the yolk sac into the developing CNS. More recently, however, it has been shown that bone marrow-derived cells can enter the CNS and become cells that phenotypically resemble microglia in the adult mouse (Brazelton et al., 2000; Mezey et al., 2000). Microglia normally exist in a quiescent state in the healthy CNS and are morphologically characterized by a small soma and ramified processes. However, upon "activation" in response to invading viruses and bacteria or CNS injury, microglia undergo

morphological changes including shortening of cellular processes and enlargement of their soma (sometimes referred to as an "amoeboid" phenotype). Activated microglia also up-regulate numerous cell surface antigens and produce innate cytokines and chemokines (reviewed in Town et al., 2005).

2.2. OLIGODENDROCYTES

Oligodendrocytes and astrocytes constitute the second class of neuroglia called macroglia. Macroglia are derived from the neuroepithelial cells that form the neural tube. This early developmental structure contains multipotent stem cells that generate neurons, astrocytes, and oligodendrocytes (reviewed in Rao, 1999). The multipotent stem cells generate intermediate or more restricted precursor cells that undergo progressive maturation to give rise to the postmitotic mature cells. Several distinct astrocyte and oligodendrocyte intermediate precursors have been discovered; however, it also appears that a large population of these cells stems from a common radial glia precursor (Malatesta et al., 2003). Lineage relationships between glial precursors remain a subject of controversy and are further complicated by findings suggestive of neuron-glia developmental interdependence (reviewed in Liu and Rao, 2004; Colognato and French-Constant, 2004).

Oligodendrocytes are the myelinating cells of the CNS. Their membranous processes envelop neuronal axons to reduce the current loss, thereby increasing the velocity of action potential propagation. An oligodendrocyte extends many processes, each of which contacts and repeatedly wraps a stretch of axon with subsequent condensation of this membrane spiral to form a myelin sheath. On the same axon, adjacent myelin segments may belong to different oligodendrocytes. The number of processes that form myelin sheaths projected from

a single oligodendrocyte varies between 1 and 40, depending on the area of the CNS and the species. Newly described myelin functions include clustering of ion channels during axogenesis, participation in development and regulation of axonal caliber, and maintenance of extracellular homeostasis (reviewed in Baumann and Pham-Dinh, 2001). Oligodendrocytes can be distinguished from astrocytes by several criteria. They have smaller nuclei with increased chromatin, denser cytoplasm, and a larger number of microtubules (Glees, 1955). Also, oligodendrocytes lack the intermediate filaments, such as glial fibrillary acidic protein (GFAP), characteristic of most astrocytes (Peters et al., 1976).

2.3. ASTROCYTES

Astrocytes are the most prevalent macroglia in the CNS and make up to 50% of the volume of most brain areas (Kuffler et al., 1984). Over the years it became apparent that astrocytes are a very morphologically and functionally heterogeneous class of cells. Andriezen (1893) initially categorized this broad class of cells into fibrous and protoplasmic glia based on their morphology and localization to white- or gray-matter, respectively. Cajal then named both of these cell populations astrocytes, due to their common star-like shape (1909). When viewed with a light microscope, astrocytes stained by Cajal's gold impregnation method are stellate cells with a cell body of approximately 10 μm and numerous radiating processes. Many astrocyte processes surround blood vessels with enlarged endings called endfeet. Similar astrocytic processes extend to the pial surface of the CNS where they form a continuous sheet called the limiting membrane (Peters et al., 1976). As noted by Cajal (1909), both fibrous and protoplasmic astrocytes contain fibrils and glycogen granules in their cytoplasm. The presence of endfeet, cytoplasmic fibrils, and

glycogen granules are general characteristics of most astrocytes and are useful features for morphological identification of these cells. However, there are several clear distinctions between fibrous and protoplasmic astrocytes.

Fibrous astrocytes are found in the white-matter and display many cylindrical processes that radiate symmetrically away from the soma. These processes extend for long distances, branch infrequently, contain abundant intermediate filaments, and do not appear to be shaped by the surrounding neuropil (Peters et al., 1976). In electron microscopic preparations, fibrous astrocyte cytoplasm is lightly tinted, with scattered glycogen granules and a relatively low density of organelles (reviewed in Levison and Goldman, 1993). Protoplasmic astrocytes have a more complex morphology than fibrous astrocytes. Their processes are highly branched and usually shorter than those of fibrous astrocytes. Compared to fibrous astrocytes, they have fewer intermediate filaments and a greater density of organelles. Furthermore, protoplasmic astrocytes can be subdivided into “smooth” or “velate” (veil-like) cells. Smooth protoplasmic astrocytes have cylindrical processes similar to those of fibrous astrocytes, while the processes of velate protoplasmic astrocytes have membranous extensions that envelop neuronal processes and cell bodies (Levison and Goldman, 1993). When examined in electron micrographs, processes of velate astrocytes seem to fill spaces between neurons, blood vessels, and other glia as if they are formed by their mold cast (Peters et al., 1976). These velate elaborations are especially noteworthy for their intimate associations with synapses (Ventura and Harris, 1999; Lehr and Rusakov, 2002). It is estimated that a single astrocyte in the adult rodent hippocampus envelops approximately 140,000 synapses (Bushong et al., 2002). Generally the membranes of adjacent astrocytes are separated by 10-20 nm gap; however, there are special membrane structures called gap

junctions which interconnect closely apposed astrocytic membranes. Gap junctions are channels assembled from two hemichannels with one hemichannel contributed by each cell. Each hemichannel is composed of six transmembrane subunits called connexins (Cx; reviewed in Dermietzel and Spray, 1993). Astrocytes connected by gap junctions form a syncytium of interconnected cells capable of intercellular communication via ions and small (<1 kDa) signaling molecules that can travel through gap junctions, such as Ca^{2+} and inositol-1,4,5-trisphosphate (IP3). Interestingly, astrocytes are also found to form heterotypic junctions with oligodendrocytes (reviewed in Giaume and Venance, 1995).

Although the classification of astrocytes into fibrous and protoplasmic has merit, it appears to be too simplistic. Histologists also describe cells intermediate in form between oligodendrocytes and astrocytes, white-matter astrocytes with more protoplasmic topology, as well as astrocytes with mixed fibrous and protoplasmic features (Wolff, 1965). During more recent years, observations made in culture and *in vivo* have led to the development of immunocytochemical markers that help identify astrocytes from other glia. Among these are: a) the intermediate filament protein GFAP (Bignami and Dahl, 1974), b) another intermediate filament called vimentin, predominantly expressed by astrocyte precursors (Tapscott et al., 1981), c) S100 β , a Ca^{2+} binding protein named for its solubility in 100% ammonium sulfate and localized to astrocyte cell bodies (Matus and Mughal, 1975; Ghandour et al., 1981), d) an astrocyte-specific enzyme, glutamine synthetase (GS), which converts glutamate into glutamine (Norenberg, 1979), e) and the glutamate-aspartate transporter (GLAST), also specific to astrocytes (Berger and Hediger, 1998; Lehre et al., 1995). There are several problems associated with the use of immunological markers for cell identification (for a good discussion on this subject please refer to Kimelberg, 2004);

however, such cell-specific markers have in recent years largely replaced purely morphological criteria for cell identification. An entire class of astrocytes was established on the basis of expressing one or more of the described astrocyte immunocytochemical markers at some point during their development. These cells were termed specialized astrocytes or intermediate glia and include radial glia, Müller glia, Bergmann glia, tanycytes or ependymal cells, and pituicytes (Privat et al., 1995; McQueen, 1994). As mentioned, these cells express the astrocytic immunomarkers (usually GFAP), but are considered specialized astrocytes because they exhibit a special morphology or are localized in specialized regions of the CNS.

Radial glia have a distinct bipolar shape with a very long fiber that radiates from the ventricular surface to the pial surface of the developing cerebral cortex (reviewed in Rakic, 1995). These elongated fibers guide neurons from areas of rapid division such as ventricular and subventricular zone (VZ, SVZ) to their final destination. In recent years it has also been established that radial glia act as a multipotent stem cell capable of giving rise to neurons, astrocytes, and oligodendrocytes (Doetsch, 2003; reviewed in *Astrocyte Functions*, page 13). In most brain regions they are present only transiently during development; however, there are examples of radial glia that survive into adulthood in the cerebellum (Bergmann glia) and retina (Müller glia). Bergmann glia cell bodies are located in the Purkinje cell layer and their processes radiate through the molecular layer of the cerebellar cortex. Branches of these elongated processes envelop Purkinje cell dendritic trees, and velate processes extending from Bergmann cell bodies envelop Purkinje cell bodies (Wilkin and Levi, 1986). In addition to providing guiding scaffolds for granule cells during cerebellar development, Bergmann glia are involved in signaling cross-talk with Purkinje neurons thereby modulating their activity (Huang and Bordey, 2004; Bellamy and Ogden, 2005). Retinal Müller glia also

extend long processes that transverse the inner and outer limiting membrane of the retina. They are known to siphon K^+ from the extracellular space surrounding the active photoreceptors to the vitreous humor (Karwoski et al., 1989; reviewed in *Potassium Spatial Buffering by Astrocytes*, page 46). These cells also exhibit some functions played by oligodendrocytes and ependymal cells (reviewed in Newman and Reichenbach, 1996). The ependymal cell tanycyte is another type of specialized astrocyte that is often considered a radial glia. In adult mammals, tanycytes line the ventricular system, extending long processes that can reach the pial surface and form endfeet-like structures (Rafols, 1986). They are thought to bridge cerebro-spinal fluid (CSF) to capillaries and endocrine events (reviewed in Rodriguez et al., 2005). Pituicytes are glial cells of the neural lobe that are innervated by hypothalamic neurons. The pituicytes and tanycytes form “synaptoid” contacts with neurons. These contacts include a presynaptic neuronal varicosity with accumulated vesicles and a postsynaptic glial membrane (Wittkowski, 1986). In summary, specialized astrocytes are different in morphology from the classic fibrous or protoplasmic cells but exhibit several astrocytic features such as the presence of astrocyte immunological markers, endfeet structures, and gap junction connectivity.

As pointed out earlier, the characterization of astrocytes is complicated not only by enormous morphological variability of this cell lineage, but also by the existence of cells with clear astrocyte morphology but without expression of characteristic astrocyte markers such as GFAP. One such population of cells in the adult gray-matter was, until recently, referred to as smooth protoplasmic astrocytes because of their characteristic appearance (Levine and Card, 1987). They are now characterized by the expression of a chondroitin sulfate proteoglycan called NG2. It was shown that they are capable of generating

oligodendrocytes in response to demyelination (reviewed in Trotter, 2005). They were, therefore, placed in the oligodendrocyte lineage and referred to as oligodendrocyte precursor cells (OPCs). However, NG2 immunopositive (NG2+) cells have recently been shown to generate interneurons *in vivo* (Belachew et al., 2003; Aguirre and Gallo, 2004) and may represent a highly plastic population of multipotent progenitors that have persisted in adulthood. Furthermore, NG2+ cells appear to receive direct synaptic input from neurons in the adult mouse hippocampus and cerebellum (Bergles et al., 2000; Lin et al., 2005). The ramifications of this finding are not yet understood. Although the inclusion of NG2+ glia in the astrocyte lineage is controversial at the moment, it appears that at least a subpopulation of these cells is derived from the GFAP+ radial glia (Malatesta et al., 2003) and most of them express astrocyte marker S100 β (Hachem et al., 2005).

As evident from the above presented example, the question of astrocyte identity and classification is a complicated one, and it largely depends on our knowledge of astrocyte properties and physiology at a particular time. Early histologists first defined astrocytes as cells that have a characteristic star-like shape. The following generation of glial biologists has modified this definition to include cells that express certain immunocytochemical markers. However, during recent years due to discovery of astrocyte plasticity and multifunctionality it became apparent that both of these definitions are too restrictive. For example, how do we handle changes in marker expression due to development, or changes in morphology due to a disease process; and how do we classify cells that can assume multiple functions (by changing their marker expression) in multiple locations (that require changes of their morphology)? Clearly, there is a lot of work left ahead of us. Since our understanding of glial development and physiology is still in its infancy, the definition of an astrocyte is a

work in progress. Subsequent use of the term glia in the remainder of this document will usually refer to astrocytes.

3. ASTROCYTE FUNCTIONS

Astrocyte functions are incredibly diverse and include almost every aspect of nervous system function, from the birth of cells, cell migrations, and cell interactions that connect and integrate the working elements of the nervous system, to the control of extracellular environment and active participation in synaptic transmission and plasticity. We have come a long way since 1909 when Cajal claimed that no one knows the function of glia (Somjen, 1988). The difficulty we are facing today is not the lack of information, but its overwhelming abundance. Physiological relevance of many described properties and functions of astrocytes remains to be validated *in vivo*, as well as their implicated involvement in CNS pathology. The following section is meant to provide a short review of exciting strides made in elucidating several leading functions assigned to astrocytes.

3.1. CNS DEVELOPMENT

Neuroepithelial stem cells in the embryonic ventricular zone (VZ) generate most of the neurons and glia in the brain. Radial glia are the first cells that can be distinguished from neuroepithelial stem cells, and they appear at the onset of neurogenesis. Cortical neurons are not generated in the cortex itself, but they migrate in a precise inside-out sequence from the proliferative ventricular and subventricular zone (SVZ). By observing morphologies of neurons and glia in the fetal CNS, His (1889) suggested that glia guide developing neurons as they migrate. Decades later, Rakic (1972) confirmed this hypothesis by demonstrating that in

the developing cortex of primates, newborn neurons originating in the VZ follow a radial pathway consisting of single or multiple radial glia fibers which span the expanding cerebral wall. Although postmitotic neurons may need several weeks to reach their final destination, clonally related cells that originate at the same spot in VZ follow the same guide and eventually settle within the same cortical column (Kornack and Rakic, 1995). In addition, recent studies *in vitro* and *in vivo* have provided unequivocal evidence that radial glia represent a population of multipotent stem cells giving rise to neurons, astrocytes, and oligodendrocytes (Malatesta et al., 2000, 2003; Hartfuss et al., 2002; Noctor et al., 2002; reviewed in Gotz et al., 2002). Noctor et al. (2001) demonstrated that radial glia give rise to new neurons which assume a bipolar shape and migrate along the radial fiber of their mother cell that remains attached to the ventricular surface. Thus, the daughter cells are guided by the radial fiber of their mother's cell to the appropriate location in the developing cortical plate (reviewed in Rakic, 2003). At the end of neurogenesis, radial glia transform into protoplasmic and fibrous astrocytes (Schmechel and Rakic, 1979; Voigt, 1989).

Surprisingly, mature astrocytes are themselves capable of neurogenesis in the adult rodent forebrain (Doetsch et al., 1999; Laywell et al., 2000; reviewed in Chanas-Sacre et al., 2000; Gage, 2002). Two sites of adult neurogenesis are the SVZ, along the lateral walls of the lateral ventricle, and the subgranular zone (SGZ), which lies between the granule cell layer and the hilus of the hippocampus. The SVZ gives rise to neurons that migrate into the olfactory bulb, while SGZ gives rise to hippocampal granule neurons. In both of these adult germinal regions, GFAP⁺ protoplasmic astrocytes are the primary *in vivo* precursors (reviewed in Doetsch, 2003). The newborn neurons, during development and in the adult, must be able to find their proper targets and form synaptic connections in order to establish

functional networks. Once again, astrocytes come to the rescue by providing guidance cues for migrating neurons (Nguyen-Ba-Charvet et al., 2004; Ward et al., 2003), influencing their cell fate specification (Blondel et al., 2000), directing dendritic and axonal growth (Shapiro et al., 2005), and promoting synapse formation, functionality, and stability (Ullian et al., 2001; Christopherson et al., 2005; reviewed in Ullian et al., 2004). The molecular mechanisms by which astrocytes influence the above mentioned stages of neuronal development are beginning to unfold and may provide strategies for CNS repair after injury or degenerative neurological diseases.

3.2. ENERGY SUPPLY AND METABOLISM

The human brain consumes about 25% of all glucose and oxygen at rest, even though it represents only 2-3% of the total body mass (reviewed in Pellerin, 2005). These observations emphasize the importance of energy supply to sustain cerebral activity. Golgi was the first to describe the intimate association between astrocyte endfeet and blood vessels. As neurons rarely directly contact blood vessels, he proposed that glia supply neurons with necessary nutrients (Golgi, 1885). The direct functional information about this neuron-glia interaction is only now becoming elucidated. Several *in vitro* studies, have led Pellerin and Magistretti (1994) to propose a model in which glutamate released during synaptic activity and taken up by astrocyte glutamate transporters, stimulates aerobic glycolysis and lactate production in astrocytes. Since lactate appears to be the preferred oxidative substrate for neurons, it is released by astrocytes and taken up into neurons to power subsequent neuronal activity. The drop in glucose concentration in astrocytes then stimulates glucose uptake from the blood vessels via astrocyte perivascular endfeet (Pellerin and Magistretti, 1994). Recent *in situ* and

in vivo evidence has confirmed several parts of this model, while some still remain to be tested. Using two-photon confocal microscopy to monitor fluorescence of a particular glycolysis product, Kasischke et al. (2004), demonstrated that in hippocampal slices astrocytes respond to afferent stimulation of Schaffer collaterals with activation of glycolysis. Furthermore, knockout of astrocyte glutamate transporters, GLAST and glutamate transporter-1 (GLT-1), leads to a large decrease in glucose utilization in response to physiological stimulation of mouse barrel cortex (Voutsinos-Porche et al., 2003a,b). Studies using NMR spectroscopy have provided a clear demonstration that a net lactate transfer occurs between astrocytes and neurons within brain and it increases with increasing levels of neuronal activity (Serres et al., 2003, 2005). In addition, an *in vivo* confirmation for the preferred neuronal use of lactate over glucose was obtained in human subjects by Smith et al. (2003). Several molecular components of the above described astrocyte-to-neuron lactate shuttle, such as glucose and lactate transporters, have been described (reviewed in Pellerin, 2005), but their roles are still poorly understood.

Maintenance of adequate energy supply is also critically linked to the control of cerebral blood flow. The brain's ability to increase blood flow to active regions, termed functional hyperemia, was first described more than a century ago (reviewed in Iadecola, 2004). Interest in hyperemia mechanisms has intensified in recent years because this phenomenon is the basis of functional MRI imaging and is likely involved in brain pathologies such as stroke and Alzheimer disease. Although a variety of mechanisms have been considered, several *in vitro* studies have implicated astrocytes as the primary effectors of the vascular dynamics and cerebral blood flow; although, they yielded conflicting results, with one study indicating that Ca^{2+} signaling in astrocytes induces vasodilation (Zonta et al., 2003) and another study

observing vasoconstriction (Mulligan and MacVicar, 2004). However, Takano et al. (2006) presented an elegant new approach for studying functional hyperemia *in vivo* that may resolve the contradictory findings. They used multiphoton confocal microscopy on transgenic mice in which astrocytes are fluorescently labeled with the calcium-sensitive indicator and perfused the arteries with fluorescent dextran. This allowed for simultaneous monitoring of astrocyte Ca^{2+} signals and vasculature dynamics *in vivo*. The authors report that activation of astrocytic Ca^{2+} signaling, either directly by focal uncaging of caged Ca^{2+} in individual astrocytes or in response to stimulation of neuronal activity, rapidly induces local vasodilation and increased blood flow. Thus, under more physiological conditions astrocytes appear to mediate hyperemia which fits well with their role in the above described coupling of neuronal activity to increased energy consumption.

3.3. BLOOD-BRAIN BARRIER

The blood-brain barrier (BBB) is a selective barrier formed by capillary endothelial cells, surrounded by basal lamina and astrocytic perivascular endfeet. This barrier has a critical role in maintaining homeostasis of the interstitial microenvironment by limiting and regulating molecular exchange of gases, ions, nutrients, hormones, and water between blood and the neural tissue. The BBB acts as a “physical barrier” because complex tight junctions between adjacent endothelial cells force most molecular traffic through the cell, rather than through the cell junctions as in most endothelia (reviewed in Wolburg and Lippoldt, 2002). It is also considered to be a selective “transporter” and “metabolic barrier” due to the presence of transporter and enzyme systems in endothelial cells and astrocytes that regulate facilitated entry and metabolism of required nutrients and exclusion or degradation of potentially

harmful compounds (reviewed in Abbott et al., 2006). The close cell-to-cell association of astrocytes and brain capillaries led to the suggestion that astrocytes mediate the induction of the specific features of the barrier phenotype (Davson and Oldendorf, 1967). There is now strong evidence, particularly from studies in cell culture, that astrocytes control many BBB features, from the development and maintenance of endothelial cell tight junctions (Janzer and Raff, 1987; Dehouck et al., 1990), to the expression and polarized localization of transporters and specialized enzyme systems (Hayashi et al., 1997; Sobue et al., 1999). Several astrocyte-secreted factors, including transforming growth factor- β (TGF β) and glia-derived neurotrophic factor (GDNF), have been shown to induce aspects of the BBB phenotype in endothelial cells of non-CNS origin (reviewed in Haseloff et al., 2005). Defects in BBB function in CNS pathologies that involve glia, such as epilepsy and chronic pain, suggest that continuing astrocyte-mediated trophic support may be necessary for normal endothelial cell function and integrity of the BBB. As already described in *Energy Supply and Metabolism* (page 15), astrocytes may also act to regulate cerebral blood flow based on neuronal energy needs, thereby coordinating the interaction of endothelial cells and pericytes (capillary contractile cells) with neuronal activity (Takano et al., 2006). Furthermore, one of the main BBB functions is to protect the brain from fluctuations in ionic composition that would disturb synaptic and axonal signaling. Astrocytes that form perivascular endfeet at the BBB have a particular role in redistribution of K^+ and water between the interstitial and perivascular space. This astrocyte function is briefly reviewed below and in more detail in *Potassium Spatial Buffering by Astrocytes* (page 46).

3.4. ION AND WATER HOMEOSTASIS

Cells within the CNS are bathed in interstitial fluid that is rich in Na^+ ions (~140 mM) and poor in K^+ ions (~3 mM; Fishman, 1980). The relative concentrations of these cations are reversed inside the cells (mammalian neurons: 5-15 mM Na^+ , ~140 mM K^+ ; Purves et al., 1997), and the resulting chemical gradients across the cell membrane are crucial for many important processes, including neuronal information processing. This process is mediated by fast action potential signaling which depends on the Na^+ influx through voltage-gated Na^+ channels (Na_v) and K^+ efflux through voltage-gated K^+ channels (K_v) resulting in neurotransmitter release and synaptic transmission. As a result of neuronal K^+ release, extracellular K^+ concentration ($[\text{K}^+]_{\text{out}}$) can increase by several mM depending on the stimulation intensity (reviewed in Somjen, 1979; Sykova, 1983). Upon observing that neuronal activity leads to depolarization of the adjacent glial cells and an increase in their $[\text{K}^+]_{\text{in}}$, Orkand et al. (1966) suggested that astrocytes maintain extracellular K^+ homeostasis by distributing K^+ from areas of high $[\text{K}^+]_{\text{out}}$ to areas of low $[\text{K}^+]_{\text{out}}$ through the astrocyte gap junction coupled syncytium (K^+ spatial buffering hypothesis). Such glial K^+ uptake was originally demonstrated in Müller glial cells in the frog retina (Mori et al., 1976) and has been confirmed for astrocytes in a variety of other nervous tissues (Coles and Tsacopoulos, 1979; Schlue and Wuttke, 1983; Ballanyi et al., 1987); however, the mechanisms of K^+ influx and redistribution are not yet completely understood. Several mediators of astrocyte K^+ uptake have been implicated, including Na^+/K^+ -ATPase (Ballanyi et al., 1987; Ransom et al., 2000; D'Ambosio et al., 2002) and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (Walz, 1992), but an inward rectifying K^+ channel, $\text{K}_{\text{ir}}4.1$, has received most attention due to its characteristic channel properties and localization to astrocyte endfeet surrounding synapses and blood vessels

(Higashi et al., 2001). Recent studies in $K_{ir}4.1^{-/-}$ mice have confirmed the necessity of this channel for K^{+} buffering by retinal Müller cells (Kofuji et al., 2000) and endocochlear epithelium (Marcus et al., 2002). Furthermore, both channel- and transporter-mediated K^{+} uptake are accompanied by osmotic water uptake and glial cell swelling (Dietzel et al., 1980, 1989). This process is referred to as regulatory volume decrease (RVD) and has been observed in astrocytes in culture and *in vivo* (Pasantes-Morales et al., 1993, 2002; reviewed in Strange et al., 1996). One of the possible RVD mechanisms is the recently described interplay between $K_{ir}4.1$ and a water channel, aquaporin-4 (AQP4), which colocalize and cluster in the synapse- and vasculature-associated astrocyte processes (Guadagno and Moukhles, 2004; Connors and Kofuji, 2006). These channels appear to mediate the codependent K^{+} and water uptake from the interstitial space and their subsequent release in the perivascular space where they are recycled or extruded (reviewed in Nagelhus et al., 2004). Several lines of evidence suggest that astrocytes may also participate in the regulation of extracellular Na^{+} , Cl^{-} , H^{+} , and bicarbonate ($H_2CO_3^{-}$) homeostasis. These observations, however, have not yet been conclusively substantiated in physiological preparations (reviewed in Simard and Nedergaard, 2004).

3.5. NEUROTRANSMITTER HOMEOSTASIS

Information processing within the CNS is based on intricately coordinated communication between excitatory and inhibitory synaptic units, composed of pre- and postsynaptic nerve endings enwrapped by astrocyte processes. The functional capacity of such synapses depends on biosynthesis, release, receptor interaction, and inactivation of the neurotransmitter in question, thus each of these processes needs to be highly regulated and

controlled. Even before synaptic transmission was understood, an insightful scientist, Lugaro (1907), suggested that glia might terminate the action of substances that transmit signals between neurons, based on his observations that glial processes invest “nervous articulations” (later identified as synapses; Somjen, 1988). Since then it has become evident that astrocytes play a major role in neurotransmitter biosynthesis and termination of excitatory neurotransmission.

Glutamate is the main excitatory neurotransmitter in the CNS and a precursor for γ -aminobutyric acid (GABA), which is responsible for most of inhibitory neurotransmission. Both of these transmitters must be synthesized within the CNS, as they do not cross the BBB. Since neurons lack pyruvate carboxylase, an essential enzyme for *de novo* synthesis of glutamate from glucose, all of the brain’s glutamate is generated by astrocytes and supplied to neurons (reviewed in Hertz and Zielke, 2004). Astrocytes also control recycling of synaptically released glutamate. It has been shown that astrocytes accumulate glutamate from the synaptic cleft and convert it to glutamine by another astrocyte-specific enzyme, glutamine synthetase. Glutamine is then returned to neurons and reconverted to glutamate to power subsequent neurotransmission (Hallermayer et al., 1981; Loo et al., 1995; reviewed in Fonnum, 1984). An almost instantaneous loss of normal vision upon inhibition of the astrocyte-specific metabolic pathways necessary for glutamate production clearly exemplifies the importance of astrocyte involvement in normal function of glutamatergic neurons (Barnett et al., 2000; Pow and Robinson, 1994).

Extensive research has also established that glutamate uptake and termination of excitatory transmission in the CNS is primarily accomplished by astrocyte glutamate transporters. As prolonged exposure of neurons to high levels of glutamate leads to

excitotoxicity marked by neuronal degeneration and death, glutamate concentration in the interstitial space must be tightly controlled (reviewed in Anderson and Swanson, 2000). So far five glutamate transporters (GluTs) have been cloned and named excitatory amino acid transporters 1-5 (EAAT1-5). EAAT1 (or GLAST) and EAAT2 (or GLT-1) account for the majority of the glutamate transport capacity in the CNS and are almost exclusively expressed in astrocytes (Gegelashvili and Schousboe, 1997; Danbolt, 2001). Glutamate transport in neurons is mainly accounted for by EAAT3 (or EAAC1; Kanai and Hediger, 1992). The two transporters, EAAT4 and EAAT5, are expressed in cerebellar Purkinje neurons and retinal Müller cells, respectively (Yamada et al., 1996; Arriza et al., 1997). Uptake of glutamate and H^+ is driven by the coupled transport of $3Na^+$ into the cell and a K^+ out of the cell down their concentration gradients. This creates a net movement of positive charge into the cell which can be monitored using electrophysiological techniques (Bergles and Jahr, 1997; Diamond et al., 1998). Excitatory synaptic activity has been shown to activate rapid GluT currents in astrocytes in the hippocampus (Mennerick and Zorumski, 1994; Bergles and Jahr, 1997; Diamond et al., 1998; Kojima et al., 1999) and cerebellum (Bergles et al., 1997; Clark and Barbour, 1997). Such transporter-associated currents cannot be detected in neurons (Bergles and Jahr, 1998), perhaps due to localization of neuronal GluTs away from the synaptic active zone (Coco et al., 1997; Furuta et al., 1997). Furthermore, selective inhibition of astrocyte GluTs has been reported to increase both the amplitude (Tong and Jahr, 1994) and the duration (Barbour et al., 1994) of glutamate-induced EPSCs. Uptake inhibitors can also enhance the EPSC depression evoked by repetitive stimulation, suggesting a role for glutamate uptake in synaptic plasticity (Turecek and Trussell, 2000). The crucial role of astrocyte transporters *in vivo* is supported by gene knockout and antisense studies. Antisense

knockdown of GLAST and GLT-1, but not the neuronal subtype EAAC1, produces elevated extracellular glutamate levels, excitotoxic neurodegeneration, and paralysis in rats (Rothstein et al., 1996). Mice lacking GLT-1 are prone to lethal spontaneous seizures and display increased susceptibility to loss of hippocampal neurons (Tanaka et al., 1997). Similarly, GLAST-deficient mice display motor discoordination and increased susceptibility to cerebellar injury (Watase et al., 1998). These studies led to increased interest in the role of astrocyte glutamate transport in disease states such as ischemia and epilepsy, further discussed in *Astrocytes in Disease* (page 28).

3.6. TRIPARTITE SYNAPSE

Our view of astrocytes as non-excitabile support cells has radically changed during the past fifteen years due to the astonishing discovery that astrocytes are capable of listening and talking to neuronal elements with chemical messages of their own. Today we view these multi-functional cells as the third essential element of the CNS signal-integration unit, the “tripartite synapse” (Araque et al., 1999). The key findings that helped establish the tripartite nature of most brain synapses are reviewed below, with the focus on studies in acute brain slices (*in situ*) and *in vivo*.

Even though they are not involved in fast electrical signaling, astrocytes are now thought of as excitable cells due to their ability to propagate chemical signals via intracellular Ca^{2+} mobilization. When activated by internal or external signals, astrocytes respond with intracellular Ca^{2+} transients and oscillations, leading to delivery of chemical messages to neighboring cells, an activity that has been termed gliotransmission (Bezzi and Volterra, 2001). A neuron-dependent and spontaneous astrocyte excitation has been documented.

Neuron-dependent excitation has been reported in many brain circuits following nerve fiber stimulation and release of various neurotransmitters and factors including glutamate, GABA, acetylcholine, noradrenaline, dopamine, adenosine triphosphate (ATP), nitric oxide, and brain derived neurotrophic factor (BDNF) (reviewed in Haydon, 2001). Interestingly, astrocytes appear to be able to discriminate and integrate neuronal inputs from different origins (Perea and Araque, 2005). Hippocampal astrocytes in stratum oriens respond with elevations of intracellular Ca^{2+} to stimulation of either Schaffer collaterals (which are glutamatergic) or nerve fibers in the stratum oriens (which are mainly cholinergic). However, when both nerve fibers are stimulated simultaneously, astrocyte responses do not simply correspond to the sum of $[\text{Ca}^{2+}]_{\text{in}}$ transients elicited by the separate stimulation. Instead, they are either smaller or larger, displaying either positive or negative cooperativity, depending on the frequency of the stimulation (Perea and Araque, 2005).

Spontaneous Ca^{2+} transients have been observed in astrocytes in acute brain slices (Parri et al., 2001; Nett et al., 2002; Aguado et al., 2002) and *in vivo* (Hirase et al., 2004; Nimmerjahn et al., 2004). They are generated by Ca^{2+} release from internal stores due to activation of inositol-1,4,5-trisphosphate (IP3) receptors, with possible additional influx of extracellular Ca^{2+} through voltage-gated channels (Parri et al., 2001; Nett et al., 2002; Aguado et al., 2002). Furthermore, they can either remain confined to distal processes that oscillate simultaneously or propagate to variable distances, intracellularly (Nett et al., 2002) or possibly even intercellularly (Parri et al., 2001; Nimmerjahn et al., 2004). Importantly, spontaneous excitation of astrocytes can result in the excitation of nearby neurons, suggesting that astrocytes and neurons may operate in coordinated networks (Parri et al., 2001; Aguado et al., 2002; Hirase et al., 2004; Nimmerjahn et al., 2004). The range of

propagation of astrocyte Ca^{2+} signals under physiological conditions remains undefined and little is known about the influence of environmental factors on astrocyte excitation.

An important response of astrocyte Ca^{2+} -mediated excitation, both by neuronal input and self-generated stimuli, is the release of gliotransmitters or chemicals that act on adjacent neurons, glial cells, and blood vessels. Over the years the number of proposed gliotransmitters has increased and some of their properties and actions have been revealed. They include, but are not limited to glutamate, ATP, adenosine, D-serine, eicosanoids, cytokines, proteins, and peptides, such as acetylcholine-binding protein and atrial natriuretic peptide (reviewed in Volterra and Meldolesi, 2005). In addition, several gliotransmitter release mechanisms have been identified, including a) the Ca^{2+} -dependent release via exocytosis of transmitter containing vesicles (Bezzi et al., 2004; Kreft et al., 2004; Montana et al., 2004; Chen et al., 2005), b) release through Na^{+} -dependent glutamate transporters operating in reverse mode (Rossi et al., 2000), c) release via activation of volume-regulated anion channels (VRACs) due to the cellular volume regulatory response (Kimmelberg et al., 1990; Eggermont et al., 2001), d) release via functional hemmichannels (Ye et al., 2003; Parpura et al., 2004), and e) release via purinergic receptors forming permeant pores (Duan et al., 2003; Fellin et al., 2006). Properties of these release mechanisms in astrocytes, such as their specificity and regulation, are not yet clarified, but what is clear is the remarkable ability of gliotransmission to affect neuronal information processing.

Modulation of neuronal excitability and synaptic transmission by astrocytes was first shown to be mediated by glutamate (Parpura et al., 1994; reviewed in Volterra and Steinhauser, 2004). Recently, modulatory effects mediated by ATP, adenosine, and D-serine, have also emerged (Zhang et al., 2003; Bowser and Khakh, 2004; Yang et al., 2003). The

experimental models that have been most intensely studied are the retinal and hippocampal circuits. Activation of retinal glia, astrocytes and Müller cells, has been shown to modulate the light-evoked spiking activity of ganglion cells that project to the brain, thereby affecting the processing of visual information (Newman and Zahs, 1998). Two opposite effects have been identified so far. The first effect is inhibitory and is mediated by ATP released from Müller cells (Newman, 2003), while the second is stimulatory and mediated by D-serine acting on the N-methyl-D-aspartate (NMDA) receptors (Stevens et al., 2003). D-serine appears to be exclusively synthesized and released by astrocytes and is proven to be an effective coagonist at the glycine-binding site of NMDA glutamate receptors (reviewed in Miller, 2004). In hippocampal slices, astrocyte released glutamate can act on group I mGluRs to produce presynaptic facilitation recorded as an increase in the frequency of spontaneous EPSCs in CA1 pyramidal neurons (Fiacco and McCarthy, 2004). In contrast, astrocyte released ATP mediates synaptic inhibition by suppressing glutamatergic synapses via presynaptic purinergic P2Y receptors (Zhang et al., 2003). Moreover, the observed heterosynaptic suppression of adjacent Schaffer collateral fibers during high frequency activity is mediated by astrocytes which sense the level of activity in the first fiber and tune the activity of the second by releasing ATP (Zhang et al., 2003). Astrocytes can also modulate inhibitory synaptic transmission. The best characterized example of this is the potentiation of hippocampal synapses between the GABA-containing interneurons of the stratum radiatum and CA1 pyramidal neurons (Kang et al., 1998). This effect is not direct but depends on the activation of neighboring astrocytes by GABA. The feedback release of glutamate by astrocytes decreases GABA mediated synaptic failures, possibly through the stimulation of GluR5-containing kainite receptors on the interneurons (Liu et al., 2004).

Furthermore, astrocytes appear to be involved in more global and longer lasting changes of synaptic strength by direct stimulation of postsynaptic excitability, synchronization of neuronal networks, and modulation of synaptic plasticity mechanisms. In hippocampal slices under certain conditions, such as low extracellular Mg^{2+} concentration, CA1 pyramidal neurons show transient slow inward currents (SICs) that are kinetically distinct and larger than synaptic currents. These currents, which are dependent on NMDA receptor activation and are unaffected by the blockade of synaptic transmitter release, depend on spontaneous $[Ca^{2+}]_{in}$ oscillations of astrocytes and are increased in frequency following astrocyte stimulation (Fellin et al., 2004; Perea and Araque, 2005; Angulo et al., 2004). Synchronized SICs could be observed in clusters of neurons suggesting that astrocytes may function as bridging units between circuits that are not directly connected to each other; however, appearance of such currents needs to be verified in more physiologically relevant preparations. Several studies report astrocyte gliotransmission affecting synaptic strength and plasticity in the hippocampus. Astrocyte released D-serine was shown to modulate NMDA receptor-dependent long-term potentiation (LTP) of CA1 pyramidal neurons (Yang et al., 2003). Astrocytes were also shown to control the strength of these synapses by increasing the surface expression of neuronal α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors through the release of tumor necrosis factor- α (TNF α ; Beattie et al., 2002). Pascual et al. (2005) have recently shown that impairment of astrocyte vesicular release machinery alters synaptic transmission and LTP in hippocampal slices by inhibiting ATP release. A glia-induced long-lasting depression of spontaneous synaptic currents of Purkinje neurons has also been shown in cerebellar slices (Brockhaus and Deitmer, 2002).

The above mentioned observations clearly demonstrate that astrocyte release of gliotransmitters can influence synaptic transmission. By synchronizing neuronal activity and affecting long-term synaptic plasticity, astrocytes are able to modulate CNS information processing and memory formation. The new concept of synaptic physiology, the “tripartite synapse”, in which astrocytes play an active role as dynamic regulatory elements of neurotransmission, is now firmly established. However, a considerable amount of work is still needed to fully understand the properties and mechanisms of astrocyte mediated synaptic modulation in different synaptic connections and brain regions, as well as its actual impact on brain function. Furthermore, it should be noted that astrocytes are also involved in bidirectional communication with non-neural cells of the nervous system. For example, astrocytes coordinate spatial positioning of oligodendrocytes during development (Tsai et al., 2002), attract microglia and lymphocytes in response to inflammation or injury (Babcock et al., 2003; Marella and Chabry, 2004), and might even bring reparative stem cells to lesion sites (Imitola et al., 2004). Astrocytes, therefore, appear to be the “all-capable” communication elements that have the capacity to regulate and coordinate different functional components of the CNS.

4. ASTROCYTES IN DISEASE

When one searches the NCBI PubMed Database for articles containing the words “astrocyte” and “disease”, an impressive 2,528 articles and 268 reviews are found, all published within the last three decades. It is almost impossible to name a CNS disorder that has not been associated with glial pathology; however, that is hardly surprising when we consider the abundance and complexity of functional interactions between astrocytes and

neurons, some of which were reviewed in *Astrocyte Functions* (page 13). Astrocytes have a critical role in regulation of synaptic levels of transmitters, buffering of extracellular K^+ , and regulation of volume and pH. They provide metabolic support to active neurons, contribute to coupling between synaptic activity and local blood flow, protect against oxidative stress, and constitute a parallel pathway for propagation and modulation of excitatory signals in the brain.

Injury to the CNS is inevitably accompanied by astrocytic hypertrophy, proliferation, and altered gene expression, a process commonly referred to as reactive astrogliosis (reviewed in Ridet et al., 1997). In addition to altering astrocyte capability to maintain extracellular ion and neurotransmitter levels, such changes in astrocyte physiology lead to changes in their excitation and gliotransmission. The resulting excessive glutamate accumulation in the interstitial space causes enhanced receptor activation that leads to neuronal degeneration and death, a process referred to as excitotoxicity. Excitotoxic cell death mechanisms predominate in the setting of acute, severe energy failure, as occurs with cerebral ischemia, hypoglycemia, head trauma, and status epilepticus (Arundine and Tymianski, 2004; Benarroch, 2005). Glutamate induced injury may trigger massive neuronal apoptosis as seen in amyotrophic lateral sclerosis (Rothstein et al., 1995), Alzheimer's (Sonkusare et al., 2005), Huntington (Shin et al., 2005), and Parkinson disease (Sonsalla et al., 1998). Furthermore, astrocyte malignant activation leads to a family of very aggressive tumors or astrocytomas. The activated cells through their modified glutamate signaling achieve the dual goal of stimulating their own growth and invasiveness while killing surrounding cells (Takano et al., 2001; reviewed in Sontheimer, 2003). Given their critical role in buffering of extracellular K^+ and ion propagation through the syncytium, astrocytes have also been implicated in

epileptogenesis and cortical spreading depression (CSD; Nedergaard et al., 1995; reviewed in D'Ambrosio, 2004). CSD is a spreading wave of nervous tissue depolarization that leads to the inhibition of all cortical activity and may underlie neurologic aura during migraine attacks (Lauritzen, 2001; Sanchez del Rio and Reuter, 2004).

While the above described astrocyte pathology is most likely not the cause but instead a reaction that further exacerbates the disease, astrocyte dysfunction seems to be crucial for pathogenesis of several disease states including chronic pain (reviewed in Wieseler-Frank et al., 2004). A complex interplay between astrocytes and microglia leads to activation of these two cell types in response to immune challenges such as viruses, bacteria, or trauma. Activated astrocytes and microglia release neuroexcitatory proinflammatory cytokines such as TNF α and interleukins, which lead to a feed-forward activation of further production and release of other neuroexcitatory substances, including reactive oxygen species, nitric oxide, ATP, and prostaglandins. Pain enhancement is created by multiple effects of these glia-derived substances on neurons of the pain pathway. For example, they can enhance the release of pain-relevant neurotransmitters from sensory afferent terminals, upregulate the number and/or conductivity of AMPA and NMDA glutamate receptors, and enhance neuronal excitability (Wieseler-Frank et al., 2004). Furthermore, several disorders have been linked to specific mutations in astrocyte associated gene products. Amyotrophic lateral sclerosis (ALS) is a fatal disorder that leads to progressive, selective degeneration of upper and lower motor neurons. In a subset of familial cases ALS is associated with point mutations in the gene that encodes a ubiquitous antioxidant enzyme, Cu/Zn superoxide dismutase (SOD1). Interestingly, in order for motor neuron pathology to appear this enzyme must be mutated in both neurons and astrocytes (Clement et al., 2003). Familial hemiplegic

migraine (FHM) is a rare, severe, autosomal dominant type of migraine (Montagna, 2004). A form of FHM is linked to a missense mutation of the ATP1A2 gene which encodes the $\alpha 2$ subunit of Na^+/K^+ -ATPase that is predominantly expressed by astrocytes in adult tissue (Marconi et al., 2003). Alexander disease is a rare and fatal leukoencephalopathy that most commonly affects infants and young children causing feeding problems, mental and physical retardation, and seizures. The adult forms of this disease resemble multiple sclerosis. The majority of cases of infantile Alexander disease, and at least some cases of the adult form, are due to a heterozygous missense mutation in the GFAP gene (Brenner et al., 2001). Also, seizure susceptibility in mice and humans has been linked to a missense variation in the inward rectifying K^+ channel, $\text{K}_{ir}4.1$ (Buono et al., 2004), which appears to be selectively expressed by astrocytes and oligodendrocytes (Takumi et al., 1995). Involvement of astrocytes in epileptogenesis is multifaceted and is further reviewed in *Astrocyte Dysfunction in Epilepsy* (page 32).

Disturbances of the multifaceted interactions between neurons and astrocytes are increasingly recognized as important pathological mechanisms in a wide range of neurological disorders, some of which are mentioned above. A recurrent question encountered in discussions of astrocyte involvement in CNS pathology is whether glial cells are perpetrators or protectors? Considering the complexity of neuronal and glial signaling mechanisms and their interactions, most likely they are both, possibly even at the same time. Whatever the exact mechanisms, disturbances of neuronal and glial physiology lead to the vicious cycle of reciprocally altered communication in CNS disease states, and we can no longer study one without the other.

5. ASTROCYTE DYSFUNCTION IN EPILEPSY

Epilepsy is one of the most common neurological disorders affecting about 4% of the world population (reviewed in Browne and Holmes, 2001). It is characterized by a repetitive seizure occurrence leading to a disturbance of motor function, cognition, attention, and consciousness. Seizures are induced by hyperexcitability and synchronization of a large population of central neurons, likely caused by imbalance between the excitatory and inhibitory neurotransmission. The convulsant status epilepticus is a life threatening condition in which seizures recur frequently leading to the development of brain edema, changes in blood ion homeostasis and pH, abnormal hormonal regulation, and disturbances in circulatory and respiratory control. Majority of epilepsy research has focused on neuronal mechanisms, but the hypersynchronous firing that is the hallmark of epilepsy could also result from the abnormal function of glial cells by virtue of their critical role in neuronal development, nutrition, homeostasis of the extracellular environment, and modulation of neuronal activity (reviewed in *Astrocyte Functions*, page 13).

5.1. EPILEPTIGENIC MECHANISMS

The processes that render the brain prone to the generation of seizures are often unknown and about 30% of epilepsies remain uncontrolled by present anticonvulsant drugs. Disturbances in neuronal maturation and migration, both of which are dependent on proper functioning of glial cells, are among developmental abnormalities shown to be involved in epileptogenesis (reviewed in Guerrini and Filippi, 2005). Lesion-induced alterations in the central nervous system often also underlie epileptogenesis. These include tumors, trauma, infection, and hypoxia- or ischemia-induced lesions, as well as changes observed after some

forms of status epilepticus (Kapur, 1999). All of these CNS insults are usually associated with the reduction of nerve cell numbers and proliferation of glial cells (Borges et al., 2003). Reactive astrogliosis is a prominent feature of the human epileptic brain, and its induction has been observed in many animal models of epilepsy. Astrogliosis can be a response to the CNS insult that results in epileptogenesis, but it can also be induced and mediated by evoked seizures (Steward et al., 1991). In 1970, Pollen and Trachtenberg hypothesized that by entering a reactive state, glial cells undergo membrane changes that are required for tissue repair but, in doing so, they lose the capability to fully exert homeostatic control over extracellular K^+ and other neuroactive substances (reviewed in D'Ambrosio, 2004).

Fine control and balance of glutamatergic (excitatory) and GABAergic (inhibitory) neurotransmission is imperative for proper functioning of neuronal networks. Decreased inhibitory transmission and/or increased excitatory transmission have been implicated in epileptogenesis. A reduced production of GABA (Meldrum, 1989), diminished excitability of GABAergic cells (Bekenstein et al., 1993), diminished GABA release, alterations in postsynaptic receptors (Kamphuis et al., 1989), and disturbance in the regulation of intracellular Cl^- (Loracher and Lux, 1974) are all conditions that promote the generation of seizures. On the other hand, the main excitatory neurotransmitter glutamate can be released in excess (Meldrum, 1994), the number of excitatory nerve cell terminals can be increased (Sutula et al., 1988), the number, affinity, or gating mechanisms of glutamate receptors can cause increased postsynaptic currents (Walther et al., 1986), and changes in passive and/or active dendritic properties can augment synaptic excitatory coupling (Kuno et al., 1970). Glia may contribute to these disturbances in numerous ways including augmented GABA and

glutamate uptake and consumption. Furthermore, the synaptic content of GABA and glutamate depends on glial metabolic activity (reviewed in *Astrocyte Functions*, page 13).

Changes in ion homeostasis due to increased neuronal activity and impaired glial ion buffering will also contribute to augmented epileptogenesis. Generalized and focal convulsions are characterized by marked changes in the ionic environment. The seizure-like events are accompanied by rises in $[K^+]_{out}$ to about 10 mM in the neocortex and to about 12 mM in hippocampus. $[Na^+]_{out}$ may decrease by up to 15 mM and $[Ca^{2+}]_{out}$ by up to 0.6 mM. Seizures are also associated with a decrease in $[Mg^{2+}]_{out}$ and by acidic pH shifts. Reduction in $[Ca^{2+}]_{out}$ and $[Mg^{2+}]_{out}$ is known to enhance glutamatergic currents, particularly those mediated by NMDA receptors (Kohr and Heinemann, 1988; Heinemann et al., 1992). Additionally, increases in $[K^+]_{out}$ shift the reversal potential of glutamate currents to more depolarized levels, with a subsequent increase in glutamate current amplitude (Heinemann et al., 1990). Augmentation of extracellular ion and transmitter levels is further exaggerated by glial cell swelling which leads to shrinkage of the extracellular space. Each seizure is associated with a decrease in extracellular space size by up to 50% in cats and by about 20% in rats (Gardner-Medwin, 1981). As can be seen, a number of glial dysfunctions are implicated in epileptogenesis. We will focus on the pathological changes of astrocyte ion channels and glutamate receptors shown to affect neuronal excitability.

5.2. INVOLVEMENT OF ASTROCYTE ION CHANNELS

One of the best characterized pro-epileptic agents is increased $[K^+]_{out}$. Seizure activity *in vivo* leads to a significant elevation of $[K^+]_{out}$ (up to 10-12 mM) and high levels of $[K^+]_{out}$ are sufficient to trigger seizure-like events in the hippocampus and several other brain regions

(Traynelis and Dingledine, 1988; reviewed in Somjen, 2002). Potassium buffering has been analyzed in numerous model systems identifying glial Na^+/K^+ -ATPase and K_{ir} channels as important mediators of this astrocyte function (reviewed in *Potassium Spatial Buffering by Astrocytes*, page 46). Interestingly, a reduction of astrocyte K_{ir} current was consistently seen in different epilepsy models *in situ* and in human epileptic tissue (Bordey et al., 2000, 2001; D'Ambrosio et al., 1999; Francke et al., 1997; Schroder et al., 1999). For example, in the pilocarpine seizure model, effects of Ba^{2+} on stimulus induced changes in $[\text{K}^+]_{\text{out}}$ suggested a significant reduction of K_{ir} current in astrocytes in the CA1 hippocampal area of epileptic rats (Gabriel et al., 1998). These findings were in agreement with experiments investigating hippocampal tissue obtained from patients with pharmaco-resistant temporal lobe epilepsy. An impaired regulation of $[\text{K}^+]_{\text{out}}$ was noted in highly sclerotic CA1 region of patients with Ammon's horn sclerosis as compared to control non-sclerotic tissue (Gabriel et al., 1998; Kivi et al., 2000). A diminished astroglial K_{ir} current was later found to be present as well (Jauch et al., 2002; Bordey and Sontheimer, 1998; Hinterkeuser et al., 2000). It has been suggested that post-traumatic intracellular accumulation of Na^+ and Cl^- may decrease the membrane K^+ conductance by blocking K^+ channels (Harvey and Ten Eick, 1989; Bekar and Walz, 2002; Orikabe et al., 2003). Indeed, excess accumulation of intracellular Na^+ can decrease both outwardly rectifying and inwardly rectifying membrane currents in astrocytes (Jabs et al., 1994; Schroder et al., 2002). Such current decrease was observed in post-traumatic CA3 astrocytes *in situ* (D'Ambrosio et al., 1999). In addition, it was suspected that brain injury, by inducing glial cells to divide and move towards the injured site, requires their dedifferentiation which leads to the decrease in K_{ir} conductance. This hypothesis was formulated due to the observation that astrocytes induced to proliferate lose their expression

of inwardly rectifying K^+ currents and gain more outwardly rectifying currents (MacFarlane and Sontheimer, 1997; Bordey et al., 2001). It should also be noted that reduced K_{ir} current was found to accompany mitotic activity in astrocytes in lesion models both *in vitro* and *in situ* (MacFarlane and Sontheimer, 1997; Bordey et al., 2001). The question which of the multiple K_{ir} subunits are affected in sclerosis to reduce inward rectification and K^+ buffering still has to be answered, although preliminary data identified $K_{ir}4.1$ as a potential candidate (Seifert et al., 2002). In support, recent genetic linkage studies have identified an association between missense variations in the gene encoding the $K_{ir}4.1$ potassium channel (KCNJ10) and seizure susceptibility phenotypes in both humans and mice (Lenzen et al., 2005; Buono et al., 2004; Ferraro et al., 2004). Interestingly, the Na^+/K^+ -ATPase α_2 subunit (ATP1A2) gene was also implicated in seizure susceptibility (Ferraro et al., 1999), however a recent study has shown that no association can be found between common variations in the human ATP1A2 gene and idiopathic generalized epilepsy (Luhoff et al., 2005). It remains unclear whether channel dysfunction, increased astrocyte proliferation, or dedifferentiation of formerly mature cells constitutes the basis for the observed loss of astroglial K_{ir} current in epileptic tissue.

Glial Na^+ and Ca^{2+} channels may also play a role in epileptogenesis. Previous work in cell culture and *in situ* suggests that astrocytes and neurons share a common set of voltage-gated Na^+ channels including $Na_v1.1-1.3$, 1.5 and 1.6 (Black et al., 1994, 1998; Oh et al., 1994; Schaller et al., 1995). Although the physiological role of glial Na^+ channels is not well understood, it has been proposed that they might regulate $[Na^+]_{out}$ and thereby control the activity of Na^+ -dependent transporters such as the glutamate transporters and Na^+/K^+ -ATPases (Sontheimer, 2004). Several studies reported changes of Na^+ channel expression in

sclerotic and epileptic CNS. A dramatic upregulation was seen in cultured astrocytes isolated from the seizure focus of human epileptic tissue. The cells possessed depolarized resting membrane potentials and were even capable of generating action potential-like responses upon current injection (O'Connor et al., 1998; Bordey and Sontheimer, 1998a,b). These data suggested that astrocytes overexpressing Na⁺ channels might support spread of seizure activity. Similar overexpression of Na⁺ channels in the reactive astrocytes and the capacity to sustain slow-action potentials was also observed in acute biopsies from human epileptic hippocampus (Bordey and Sontheimer, 1998b), but not in the hippocampal brain slices obtained from human temporal lobe epilepsy patients (Hinterkeuser et al., 2000). Further studies are therefore required to establish a clear role of astrocyte Na⁺ channels in the epileptic tissue.

Voltage-gated Ca²⁺ channels mediate Ca²⁺ influx upon membrane depolarization and regulate various intracellular processes in excitable and non-excitable cells. During seizure activity [Ca²⁺]_{out} decreases at the focus site (Heinemann et al., 1977). In acute hippocampal slices, low [Ca²⁺]_{out} leads to spontaneous seizure-like neuronal discharge patterns implicating a role for Ca²⁺ in the generation of epileptic activity *in vivo* (Haas and Jefferys, 1984; Konnerth et al., 1986). Although the seizure-induced decrease in [Ca²⁺]_{out} is usually attributed to Ca²⁺ influx into neurons, different types of Ca²⁺ channels have been identified in astrocytes and may contribute to [Ca²⁺]_{out} depletion (Verkhratsky and Steinhäuser, 2000). In favor of this hypothesis, immunostaining noted an upregulation of astrocytic L-type Ca²⁺ channels in the kainite model of epilepsy (Westenbroek et al., 1998). In addition, Ca²⁺ influx into astrocytes is likely to add to the synthesis and release of transmitters, cytokines, and

growth factors, thereby indirectly influencing the architecture and activity of neural circuitry (Steinhäuser and Seifert, 2002).

In conclusion, evidence showing reduced astroglial K_{ir} current and increased Na^+ and Ca^{2+} conductance in sclerotic epileptic tissue is accumulating. These alterations in conjunction with the seizure induced shrinkage of the extracellular space may lead to impaired spatial buffering, resulting in strong and prolonged depolarization of glial cells and neurons in response to activity-dependent release of K^+ . Thus, modified properties of astrocytes might directly contribute to or even initiate seizure generation and seizure spread in hippocampal sclerosis (reviewed in Steinhäuser and Seifert, 2002). In addition, the role of oligodendrocytes in the overall extracellular K^+ homeostasis should also not be neglected since recent work has demonstrated that this type of macroglia is suitable for K^+ uptake that may be reduced under certain pathological conditions (Chvatal et al., 1997, 1999; D'Ambrosio et al., 1999).

5.3. INVOLVEMENT OF ASTROCYTE GLUTAMATE RECEPTORS

Various studies have shown involvement of glutamate receptors in seizure development and spread, and increased extracellular levels of glutamate have been found in epileptogenic foci (reviewed in Glass and Dragunow, 1995; Doherty and Dingledine, 2002). While earlier studies usually focused on neurons, recent work has proven that functional metabotropic and ionotropic glutamate receptors are also expressed by astrocytes (Steinhäuser and Gallo, 1996; Porter and McCarthy, 1997) and knowledge about their putative involvement in epilepsy is now gradually emerging (reviewed in Carmant, 2006). Metabotropic glutamate receptor (mGluR) subtypes, mGlu3 (member of group II mGluRs; linked to G_i and inhibition of

adenylate cyclase) and mGlu5 (member of group I mGluRs; linked to G_q , PLC activation, IP3 production, and Ca^{2+} release from internal stores) are the predominant subtypes expressed in glial cells (Schools and Kimelberg, 1999; Winder and Conn, 1996). In animal models of temporal lobe epilepsy, reactive astrocytes of the hippocampus are characterized by a persistent upregulation of mGlu3 and mGlu5 proteins (Aronica et al., 2000; Ferraguti et al., 2001; Ulas et al., 2000). Frequent activation of mGlu5 during seizures enhances IP3 hydrolysis in astrocytes and increases $[Ca^{2+}]_{in}$ (Ong et al., 1999). In addition, mGlu8 (member of group III mGluRs; linked to G_i) appears on astrocyte membrane after pilocarpine-induced epilepsy (Tang et al., 2001). Group I mGluRs are critically involved in the induction of glial Ca^{2+} oscillations (Bezzi et al., 1998; Pasti et al., 1997) and their activation also leads to astrocyte swelling (Hansson, 1994). Moreover, astrocyte Ca^{2+} waves have been shown to travel distances, induce glial glutamate release, and influence neuronal excitability (reviewed in *Astrocyte Functions*, page 13). It is easy to imagine that increased glial activation may lead to increased neuronal activation and hypersynchronization. On the other hand, enhanced activation of mGlu3 in astrocytes may have neuroprotective effects by glutamate stimulated release of TGF β (Bruno et al., 1998). This mechanism might indeed operate in epilepsy since in a rat model of temporal lobe epilepsy, upregulation of mGlu3 was accompanied by enhanced expression of TGF β (Aronica et al., 2000). This growth factor is known to promote neurite sprouting and may contribute to the reorganization of neuronal circuitry typically observed in the epileptic brain (Ishihara et al., 1994). In addition to metabotropic glutamate receptors, glial cells express functional ionotropic glutamate receptors (iGluRs) of the AMPA subtype which are activated by synaptically released glutamate and are Ca^{2+} permeable (Seifert and Steinhäuser, 2001; Jabs et al., 2005; Bergles et

al., 2000; Backus and Berger, 1995; Jabs et al., 1994). In culture, activation of iGluRs induces a rise in $[Ca^{2+}]_{in}$ and triggers Ca^{2+} oscillations that propagated through the astrocyte network (Cornell-Bell et al., 1990; Dani et al., 1992). AMPA receptors in astrocytes *in situ* contribute to the stimulation of glial glutamate release, a process that is Ca^{2+} -dependent (Bezzi et al., 1998). Furthermore, astrocyte glutamate release induced by increases in $[Ca^{2+}]_{in}$ has been shown to influence neuronal spontaneous activity (Fiacco and McCarthy, 2004) and may synchronize neuronal networks (Pascual et al., 2005; Araque et al., 2000). Astrocytes of Ammon's horn sclerotic patients possess prolonged AMPA receptor responses, predicting enhanced depolarization upon activation by neuronally released glutamate (Seifert et al., 2004). These changes might contribute to the increased Ca^{2+} oscillations and Ca^{2+} wave propagation observed in human glial cells cultured from epileptic foci (Lee et al., 1995; Manning and Sontheimer, 1997). Enhanced influx of Ca^{2+} through astroglial mGluRs and AMPA receptors can thus be expected to enhance astrocyte glutamate release and synchronize activity of numerous neurons to generate epileptiform activity. A recent finding by Tian et al. (2005) demonstrates that paroxysmal depolarization shifts that underlie hypersynchronous neuronal firing in epilepsy can be initiated by release of glutamate from extrasynaptic sources or by photolysis of caged Ca^{2+} in hippocampal astrocytes. Furthermore, *in vivo* two-photon imaging showed that several antiepileptic agents reduce the ability of cortical astrocytes to transmit Ca^{2+} signaling (Tian et al., 2005). This study suggests that prolonged episodes of neuronal depolarization evoked by astrocyte glutamate release contribute to epileptogenesis and represents the first clear example of a causative role for astrocytes in epilepsy.

The above presented observations are a small part of an overwhelming wealth of data generated in recent years in support of astrocyte involvement in epilepsy. However, very little work has been done to understand which changes are consequences of chronic seizures and which ones may be causal. It is also important to note that epilepsy is not a single disease, but a neurological disorder that encompasses several syndromes and a broad spectrum of symptoms. Most likely, multiple neuronal and glial signals are needed for seizure initiation and maintenance depending on the location of seizure foci and the particular type of epileptic disorder. Further study of astrocyte involvement in epilepsy may provide novel therapeutic targets and promising treatments without the suppression of normal neuronal transmission associated with current treatment options.

6. POTASSIUM HOMEOSTASIS AND NEURONAL EXCITABILITY

Neuronal excitatory synaptic activity results in opening of transmitter and voltage-gated ion channels leading to action potential propagation which delivers the message to the following synapse and as such mediates information processing within the CNS. Action potential propagation involves synchronized depolarization and hyperpolarization of the neuronal membrane due to Na^+ influx and K^+ efflux, respectively. Even modest neuronal K^+ effluxes elicit considerable changes in $[\text{K}^+]_{\text{out}}$ due to the limited volume of the CNS extracellular space and the low baseline $[\text{K}^+]_{\text{out}}$ (~3 mM; Nicholson and Sykova, 1998; Kume-Kick et al., 2002). These $[\text{K}^+]_{\text{out}}$ changes can impact a wide variety of neuronal processes, such as maintenance of membrane potential, activation and inactivation of voltage-gated channels, synaptic transmission, and electrogenic transport of neurotransmitters (reviewed in Kofuji and Newman, 2004).

6.1. AUGMENTATION OF $[K^+]_{out}$ BY NEURONAL ACTIVITY

The activity-induced increase in extracellular potassium is correlated with the stimulation frequency and number of activated neuronal elements. Measurements reveal that normal physiological stimuli result in $[K^+]_{out}$ increases which rarely exceed 3 mM (Kelly and Van Essen, 1974; Singer and Lux, 1975). For example, in the cat spinal cord, rhythmic flexion/extension of the knee joint causes $[K^+]_{out}$ to increase by about 1.7 mM (Heinemann et al., 1990). Intense stimulation of neuronal pathways causes increases by 5-6 mM above resting level (reviewed in Sykova, 1991). During seizures the accumulation of extracellular potassium is further enhanced, but there seems to be a ceiling level of 12 mM (Lux, 1974; Lothman et al., 1975; Heinemann and Lux, 1977; Somjen, 1979). Only during injury (hypoxia/ischemia, trauma, hypoglycemia) is this ceiling level disrupted and concentrations of up to 25 mM are reached (Hansen, 1985). An extreme case are spreading depression (SD) waves which result in transient elevations of $[K^+]_{out}$ of 30-80 mM in intact nervous tissue (Somjen et al., 1992).

Spreading depression is characterized by a rapid depolarization of a large population of brain cells with massive redistribution of ions between intracellular and extracellular compartments that propagates slowly as a wave in the brain tissue (reviewed in Somjen, 2001). With the aid of K^+ -selective microelectrodes, first Vyskocil et al. (1972) then several others described the astonishing rise of $[K^+]_{out}$ during SD (Lothman and Somjen, 1975; Lothman et al., 1975; Hubschmann and Kornhauser, 1982). The process usually begins with a gradual increase of $[K^+]_{out}$, which remains relatively slow and at a steady velocity until about 10-15 mM is reached. When this level is exceeded, the process becomes explosive, and $[K^+]_{out}$ suddenly rises to a maximum level between 30 and 80 mM, accompanied by a

negative shift of extracellular potential, massive depolarization of glial cells and neurons, and cessation of all neural activity. Interestingly, it has been reported that depolarization of neurons may be delayed sometimes by as much as 10-30 sec after depolarization of glial cells and the negative shift of extracellular potential (Sugaya et al., 1975), implicating glial cells as critical factors in SD propagation. In general, insults such as hypoxia, high $[K^+]_{out}$, and excess glutamate can induce SD or provoke seizure discharge. Whether a seizure is followed by SD is determined by the behavior of $[K^+]_{out}$. As long as $[K^+]_{out}$ does not exceed 8-12 mM SD will not occur, but if the regulation of $[K^+]_{out}$ is overwhelmed SD can occur. Regulation of $[K^+]_{out}$ is one of major astrocyte functions and is reviewed in *Potassium Spatial Buffering by Astrocytes* (page 46). Neurons are thought to initiate the SD response, but glial involvement appears to be needed for its spread (Sugaya et al., 1975; Largo et al., 1996). Migraine, concussion, postictal depression, and hypoxia/ischemia are some of the clinical conditions in which SD is suspected to play a role (reviewed in Somjen, 2001). Interestingly, SD waves have been shown to transform normal astrocytes and microglia into reactive astrocytes and activated microglia (Gehrmann et al., 1993; Kraig et al., 1991).

6.2. INFLUENCE OF ELEVATED $[K^+]_{out}$ ON NEURONAL FUNCTION

Extracellular K^+ concentration affects intracellular and extracellular pH (Pappas and Ransom, 1994; Rose and Deitmer, 1994), cerebral blood flow (Knot et al., 1996), energy metabolism (Salem et al., 1975; Lewis and Schuette, 1975), and ultimately neuronal activity (Xiong and Stinger, 2001; Jensen and Yaari, 1997; Leschinger et al., 1993). Physiological increases in $[K^+]_{out}$ result in small but significant neuronal depolarization which can influence neuronal function in many ways, including modification of spontaneous transmitter release,

threshold for action potential initiation, voltage-sensitive receptor and channel activation, and transmitter release, all leading to the modulation of synaptic transmission. This modulation is biphasic, with small 2-4 mM $[K^+]_{out}$ increases leading to increased neuronal excitability and enhanced synaptic transmission, and higher $[K^+]_{out}$ increases causing synaptic inhibition. Poolos et al. (1987) reported that during repetitive stimulation (2-100 Hz) of Schaffer collaterals in rat CA1 stratum radiatum the $[K^+]_{out}$ increased up to 7 mM. Biphasic changes of the afferent excitability were observed by recording field potentials. Initial responses showed increased conduction velocity and increased amplitudes, however subsequent responses showed progressively decreasing conduction velocity and amplitude tending toward conduction block. This trend was correlated to the rise in $[K^+]_{out}$. Elevated $[K^+]_{out}$ below 6 mM produced increased excitability, most likely due to a small depolarization which brings resting membrane potential closer to the firing threshold. Extracellular K^+ concentration above 6 mM, on the other hand, yielded decreased excitability, presumably from increased inactivation of voltage dependent Na^+ conductance that occurs with increasing depolarization (Poolos et al., 1987). Several studies have reported that 5-7 mM $[K^+]_{out}$ significantly depolarizes resting membrane potential of CA1 pyramidal neurons and induces higher spiking frequencies apparently due to either decreased firing threshold or slowed afterhyperpolarization (Balestrino et al., 1986; Kreisman and Smith, 1993; Voskuyl and Ter Keurs, 1981; Jensen et al., 1994). In contrast, 8.5 mM $[K^+]_{out}$ reduces the incidence of endogenous burst firing in CA3 pyramidal cells, presumably by depolarizing them to a potential at which the burst mechanisms inactivated (Chamberlin et al., 1990).

Extracellular K^+ concentration has also been shown to affect the efficacy of synaptic transmission in several different systems (Balestrino et al., 1986; Hablitz and Lundervold,

1981). Paralleling the excitability studies, small $[K^+]_{out}$ rises cause enhanced synaptic transmission due to neuronal depolarization, increased neuronal spiking, enhanced transmitter release, and augmented responses of postsynaptic NMDA receptors due to their depolarization mediated disinhibition (Chamberlin et al., 1990; Poolos and Kocsis, 1990). However, $[K^+]_{out}$ increases greater than 5 mM reduce both excitatory and inhibitory transmission. In the case of the giant synapse of the squid, evoked EPSCs were found to be decreased in amplitude in high $[K^+]_{out}$, concurrent with the diminished amplitude of the presynaptic action potential (Erulkar and Weight, 1977). The input-output function of transmission through the cuneate nucleus was also depressed, though not by very much, perhaps because depolarization of postsynaptic elements partially compensated for decreased transmitter output (Krnjevic and Morris, 1976). A large increase in $[K^+]_{out}$ in hippocampal slices, also leads to a reduction in the efficacy of synaptic transmission (Rausche et al., 1990). A possible cause may be reduced voltage, Ca^{2+} , and transmitter activated outward currents caused by changes in the ion concentration gradients (Rausche et al., 1989; Ficker and Heinemann, 1992). Interestingly, excitatory and inhibitory transmission display differential sensitivity to elevated $[K^+]_{out}$. In general, synaptic inhibition tends to fail before excitatory synaptic transmission (Jones and Heinemann, 1987; Korn et al., 1987). High $[K^+]_{out}$ has been shown to induce epileptiform activity in the hippocampus by reducing the amplitudes of $GABA_A$ receptor-mediated inhibitory postsynaptic potentials (IPSPs; Korn et al., 1987). However, in a recent study, Meeks and Mennerick (2004) found that in juvenile rat hippocampus $[K^+]_{out}$ accumulation (8-10 mM) depresses EPSCs selectively to IPSCs, attributable to a differential sensitivity of glutamate-releasing versus GABA-releasing fibers to Na^+ channel block and inactivation. Increases in $[K^+]_{out}$ that cause EPSC depression

during high frequency stimulus trains cause an increased probability of action potential conduction failure in single hippocampal axons and depress action potential amplitudes. Authors suggest that selective regulation of evoked glutamate versus GABA signaling by $[K^+]_{out}$ may be an important endogenous mechanism for maintaining relative levels of excitation and inhibition during high-frequency activity (Meeks and Mennerick, 2004).

Changes in synaptic strength such as those observed in elevated $[K^+]_{out}$ are the basis of synaptic plasticity, both short-term such as paired-pulse facilitation (PPF) and post-tetanic potentiation (PTP), and long-term (LTP, LTD). Since these plasticity mechanisms are responsible for higher cerebral functions including learning and memory, maintenance of $[K^+]_{out}$ homeostasis is essential for proper CNS functioning. Importantly, neuronal activity depends not only on the external potassium concentration but also on levels of other ions, as well as transmitter and metabolite levels (Ballanyi, 1995; Ransom, 1992). Homeostasis of all of these molecules is maintained by proper astrocyte functioning as previously reviewed in *Astrocyte Functions* (page 13). For this reason it is impossible to understand CNS information processing without studying glial cells as an integral part of the complex CNS network.

7. POTASSIUM SPATIAL BUFFERING BY ASTROCYTES

Neuronal activity in the central nervous system is accompanied by movement of ions and water across neuronal membranes modifying the composition of the adjacent extracellular space. It is well established that enhanced neuronal activity is accompanied by transient elevations of $[K^+]_{out}$ which can perturb neuronal functioning (reviewed in *Potassium Homeostasis and Neuronal Excitability*, page 41). The ensheathment of neuronal processes

and synapses by astrocyte processes and the characteristic properties of these cells, such as high K^+ permeability and gap junction connectivity, have led to the suggestion that an important role of glial cells is to maintain the homeostasis of the extracellular fluid. Hertz (1965) was the first to postulate that astrocytes are involved in potassium clearance. Hertz suggested that, by distributing K^+ according to some blueprint, glia might regulate the spatial pattern of neuronal excitability (Hertz, 1965; Hertz, 1977). Subsequent measurements of glial membrane potential, $[K^+]_{out}$, and extracellular space volume during physiological or electrical stimulation of neurons have established the idea that astrocytes are responsible for maintaining K^+ homeostasis in the brain. Astrocytes accomplish this function by both passive and active K^+ uptake and K^+ redistribution via gap junction coupled astrocyte syncytium, termed “ K^+ spatial buffering” (Orkand et al., 1966; Newman and Karwoski, 1989; Amedee et al., 1997; Somjen, 2002; Orkand, 1986; Coles, 1989; Walz, 1989). In the case of K^+ uptake, excess K^+ ions are temporarily sequestered into glial cells by transporters and K^+ channels. Eventually, K^+ ions accumulated in glia are released back into the extracellular space and the overall distribution of K^+ across cellular compartments is restored. Furthermore, as first proposed by Orkand et al. (1966), the functionally coupled, highly K^+ permeable glial cells are capable of spatial redistribution of K^+ ions by transferring K^+ from regions of elevated $[K^+]_{out}$ to regions of lower $[K^+]_{out}$. The transfer of K^+ is mediated by current flow through the astrocyte network driven by difference between the syncytium membrane potential (V_m) and the local K^+ equilibrium potential (E_K). The astrocyte syncytium region experiencing increased $[K^+]_{out}$ will have E_K that is more positive than the V_m . This will lead to an inward driving force for K^+ ions which will therefore enter the cell. In regions further removed from the area exposed to high K^+ concentration, the E_K is more negative than the V_m due to the

tendency of the syncytium to remain isopotential. Therefore at these further removed areas there is an outwardly directed driving force for K^+ (reviewed in Kofuji and Newman, 2004; Walz, 2000). In Müller cells of the retina a specialized form of K^+ spatial buffering termed “ K^+ siphoning” has developed. Müller cells stretch through the entire thickness of the retina starting at photoreceptors and ending with specialized endfeet at the inner limiting membrane adjacent to vitreous humor. Due to uneven distribution of K^+ conductance along the Müller cell membrane, K^+ spatial redistribution is accomplished within a single Müller cell. Excess K^+ from the inner plexiform layer of retina, where most retinal synapses are located are siphoned by the Müller cells and released through their endfeet into the vitreous humor (Newman et al., 1984; Newman, 1987; Kofuji and Newman, 2004).

7.1. EVIDENCE FOR POTASSIUM SPATIAL BUFFERING

The first support for glial K^+ uptake and spatial buffering was provided in the classic work by Orkand et al. (1966), who reported that in amphibians, stimulation of the optic nerve leads to slow depolarization and repolarization of the glial cells surrounding the nonmyelinated axons. These K^+ -induced glial depolarizations were later shown to be accompanied by an increase in glial $[K^+]_{in}$. Glial K^+ uptake has since been confirmed in a variety of CNS tissue preparations (Coles and Tsacopoulos, 1979; Schlue and Wuttke, 1983; Ballanyi et al., 1987; Walz, 1992). In an elegant experiment Coles et al. (1986) demonstrated K^+ buffering by Müller glial cells during physiological stimulation of retinal photoreceptors. They recorded neuronal and glial membrane potential, as well as neuronal and glial $[K^+]_{in}$, and demonstrated that light-evoked electrical responses in the drone retina lead to depolarization of both cell types; however, the cell depolarization was accompanied by a

$[K^+]_{in}$ decrease in neurons and $[K^+]_{in}$ increase in the Müller cells (Coles et al., 1986). Subsequently, extensive support for the K^+ spatial buffering hypothesis has come from measurements of extracellular field potentials. The interglial transfer of K^+ ions from areas of elevated $[K^+]_{out}$ to lower $[K^+]_{out}$ generates return current loops in the extracellular space giving rise to extracellular field potentials. These activity-induced slow extracellular field potentials are generated in various CNS regions including the cortex and retina (Gardner-Medwin et al., 1981; Dietzel et al., 1989). In the retina, slow extracellular field potentials are generated upon light stimulation of photoreceptors and current source density analysis indicates these waves originate from K^+ spatial buffering by retinal glial cells (Xu and Karwoski, 1997; Karwoski and Xu, 1999). Measurements of activity-dependent $[K^+]_{out}$ changes in the cortex and cerebellum show that $[K^+]_{out}$ varies with depth and time in a manner consistent with intercellular transfer of K^+ (Gardner-Medwin and Nicholson, 1983). Similar results were obtained in drone retina, where it was estimated that 10 times more K^+ moves as a result of spatial buffering than by simple diffusion through the extracellular space (Coles et al., 1986).

More direct evidence for K^+ spatial buffering has been provided by optical imaging of cat cortical slices and *in vivo* recordings of glial V_m changes and $[K^+]_{out}$ (Holthoff and Witte, 2000; Amzica et al., 2002). Dietzel et al. (1980) hypothesized that during K^+ transfer via glial cells, areas of K^+ influx into glial cells should experience shrinkage of the extracellular space due to concomitant water uptake and glial swelling, while in areas where K^+ and water are released extracellular space should increase. Such changes in extracellular volume following neuronal stimulation were demonstrated by monitoring intrinsic optic signals in brain slices. As predicted, stimulation of cortical areas promoted shrinkage of the

extracellular space followed by swelling in the layers above and below the stimulation. This swelling of extracellular space was associated with local increases in $[K^+]_{out}$ and was dependent on gap junction coupling (Holthoff and Witte, 2000). Amzica et al. (2002) addressed the question of spatial buffering *in vivo* by performing double intragial V_m recordings, together with measurements of the $[K^+]_{out}$ in the cerebral cortex of cats under ketamine and xylazine anesthesia during patterns of slow sleep oscillations and spike-wave seizures. They measured large steady increases of $[K^+]_{out}$ during spike-wave seizures and showed that glial $[K^+]_{in}$ rose before $[K^+]_{out}$ if the glial cells were located at the same distance from the epileptic focus. These findings strongly suggest faster K^+ diffusion through the interglial syncytium than through extracellular space and provide direct evidence for *in vivo* K^+ spatial buffering by astrocytes (Amzica et al., 2002).

7.2. POTASSIUM BUFFERING MECHANISMS

Several mechanisms which aid in the regulation of activity-dependent variations in $[K^+]_{out}$ have been identified. The simplest of these is the diffusion of K^+ through extracellular space. However, as mentioned earlier, due to high K^+ permeability of astrocyte membranes and their direct opposition to neuronal membranes, K^+ appears to move more readily through the glial syncytium than through the extracellular space (Coles et al., 1986; Holthoff and Witte, 2000; Amzica et al., 2002). Furthermore, evidence suggests that K^+ uptake by neurons occurs very slowly, over minutes following tetanic stimulation, primarily by the action of the Na^+/K^+ -ATPase (Heinemann and Lux, 1975; Ballanyi et al., 1984). Thus, active uptake by neurons is not sufficient to buffer rapid $[K^+]_{out}$ increases suggesting a necessity for K^+ uptake by glial cells. During early studies of glial K^+ uptake following neuronal stimulation it was

noticed that increase of glial $[K^+]_{in}$ is mirrored by a decrease of $[Na^+]_{in}$. This phenomenon was originally observed in the drone retina (Coles and Orkand, 1985) and later also in glial cells of mammals (Ballanyi et al., 1987; Ballanyi and Kettenmann, 1990). In the majority of glial cells investigated, the K^+ -evoked changes of $[K^+]_{in}$ and $[Na^+]_{in}$ were also accompanied by an increase in glial $[Cl^-]_{in}$, water influx and swelling (Ballanyi et al., 1987; Kettenmann, 1987; Wuttke, 1990). Further studies revealed that the complexity of ion and water movements across the glial membrane can be explained by an interplay of an active ion transport mechanism, mediated by Na^+/K^+ -ATPase, and passive ion and water fluxes through transporters and channels, mainly the $Na^+/K^+/2Cl^-$ cotransporter, K_{ir} channels, and aquaporins (reviewed in Newman, 1985; Walz, 2000; Kofuji and Newman, 2004).

7.3. ACTIVE POTASSIUM UPTAKE BY Na^+/K^+ -ATPASE

The Na^+/K^+ -ATPase (Na^+ -pump) is a ubiquitous ion-transporting pump found in all animal cells but not in plants and bacteria. It catalyzes active uptake of K^+ and extrusion of Na^+ at the expense of hydrolyzing ATP to ADP and P_i . This uphill transport establishes steep concentration gradients for K^+ and Na^+ ions and is required by all cells. The gradients are then harnessed by other proteins for a variety of essential function, including electrical potential changes mediated by ion channels, the uptake of molecules like neurotransmitters, and the extrusion of Ca^{2+} . The Na^+/K^+ -ATPase has two kinds of subunits: an α subunit containing a binding site for ATP and cardiac glycosides such as ouabain, and a β subunit known to play a role in the biosynthesis and targeting of the enzyme to the plasma membrane. All of the three isozymes of the α subunit are present in neurons, but glia appear to express only the $\alpha 1$ and $\alpha 2$ isozymes. The localization of the β isoforms has not been as

extensively studied, but there is some indication that β_2 , like α_2 , may be more commonly expressed in glia (reviewed in Sweadner, 1995).

The turnover cycle of moving 3Na^+ out of the cell in exchange for moving 2K^+ into the cell generates a continuous outward current which is very slow compared to conductance through ion channels. The cells compensate for the slowness of the pump by expressing a lot of them. In many excitable and non-excitable cells it is one of the most abundant plasma membrane proteins. The importance of Na^+/K^+ -ATPase activity for astrocyte ion homeostasis was demonstrated upon block of this pump by ouabain which caused a large membrane depolarization, loss of $[\text{K}^+]_{\text{in}}$ (-70 mM in 60 min), a gain of $[\text{Na}^+]_{\text{in}}$ (+40 mM in 60 min), and a rise in $[\text{Ca}^{2+}]_{\text{in}}$ (3 fold in 60 min) in cultured astrocytes (Silver and Erecinska, 1997). As reviewed by Sweadner (1995), the pump activity depends on four factors: $[\text{Na}^+]_{\text{in}}$, $[\text{K}^+]_{\text{out}}$, $[\text{ATP}]_{\text{in}}$, and membrane voltage. Upon local increases of $[\text{K}^+]_{\text{out}}$ and subsequent glial depolarization, the Na^+/K^+ -ATPase is expected to transport K^+ ions into the cell in exchange for Na^+ ions. It has been suggested that the Na^+/K^+ -ATPase expressed in glial cells is better suited for $[\text{K}^+]_{\text{out}}$ buffering than the neuronal isoform given observations that the glial isoform has a lower affinity for extracellular K^+ (Grisar et al., 1978, 1979; Franck et al., 1983; Reichenbach et al., 1992). In retina, for example, Müller glia express a Na^+/K^+ -ATPase isoform with a maximal activity at about 10-15 mM $[\text{K}^+]_{\text{out}}$, while the isoform found in rod photoreceptors saturates at $[\text{K}^+]_{\text{out}}$ as low as 3 mM (Reichenbach et al., 1992).

In support of the Na^+/K^+ -ATPase involvement in K^+ uptake by astrocytes, application of the Na^+/K^+ -ATPase inhibitor ouabain was found to decrease astrocyte accumulation of intracellular K^+ and simultaneously deplete intracellular Na^+ following electrical stimulation of guinea-pig olfactory cortex (Ballanyi et al., 1987). Likewise, in hippocampal slices,

Na⁺/K⁺-ATPase blockade increased baseline [K⁺]_{out} and prevented rapid clearance of extracellular K⁺ following neuronal stimulation (D'Ambrosio et al., 2002). In the rat optic nerve, clearance of K⁺ accumulation following axonal stimulation was highly temperature dependent as expected for a carrier-mediated process, and was largely blocked by Na⁺/K⁺-ATPase inhibitors (Ransom et al., 2000). In addition, during spontaneous epileptiform activity in rat dentate gyrus, bath and local application of ouabain increased the baseline [K⁺]_{out}, slowed the rate of [K⁺]_{out} recovery, and induced spreading depression (Xiong and Stringer, 2000).

7.4. PASSIVE POTASSIUM UPTAKE BY K_{IR} CHANNELS

However, a large component of glial K⁺ accumulation is ouabain insensitive. Results by Dietzel et al. (1989) indicate that during electrical stimulation in the cat cortex, the equivalent of 3 to 5 mM [K⁺]_{out} is removed by spatial buffering, 3.4 mM by action of the glial Na⁺/K⁺-ATPase, and 1.4 mM by passive K⁺ uptake into glial cells. Furthermore, it has been shown that astrocyte K⁺ buffering in the rat cortex and cerebellum was not abolished by Na⁺/K⁺-ATPase inhibitors, indicating that it is predominantly mediated by passive K⁺ uptake via channels and transporters (Gardner-Medwin and Nicholson, 1983). The passive K⁺ uptake appears to occur by coupled transport of K⁺ ions, anions, and water, via Ba²⁺ sensitive K⁺ channels, Na⁺/K⁺/2Cl⁻ and K⁺/Cl⁻ cotransporters, and aquaporins (reviewed in Newman, 1985). Walz and Hinks (1986) suggested operation of a transmembrane sodium cycle in glial cells whereby K⁺ is pumped into the cell by the Na⁺/K⁺-ATPase and the drop in intracellular Na⁺ is prevented by a simultaneous stimulation of the electroneutral bumetanide-sensitive Na⁺/K⁺/2Cl⁻ cotransporter. In cultured astrocytes, intracellular K⁺ accumulation following

increased $[K^+]_{out}$ can be partially prevented by blockers of $Na^+/K^+/2Cl^-$ cotransporters, furosemide and bumetanide (Kimelberg and Frangakis, 1985; Walz, 1992; Rose and Ransom, 1996). More recently, it was demonstrated that astrocyte swelling due to $[K^+]_{out}$ increase is reversibly depressed by furosemide and bumetanide in the rat optic nerve preparation. A monoclonal antibody to the NKCC1 form of $Na^+/K^+/2Cl^-$ cotransporter showed that NKCC1 is expressed in optic nerve astrocytes, suggesting the involvement of this particular cotransporter isoform in K^+ buffering (MacVicar et al., 2002).

Even though transporters appear to play a role in K^+ uptake by glia, in most studies the ouabain-insensitive K^+ uptake component is completely abolished by the K^+ channel blocker Ba^{2+} . Ballanyi et al. (1987) reported that in guinea-pig olfactory cortex, Ba^{2+} strongly reduced the stimulus induced rise of glial $[K^+]_{in}$ and ouabain blocked the remaining $[K^+]_{in}$ increase. D'Ambrosio et al. (2002) have established that in the rat CA3 stratum pyramidale Na^+/K^+ -ATPase and K^+ channels, in particular inward rectifying K^+ channels (K_{ir} s), play distinct roles in clearance of extracellular K^+ . Both neuronal and glial Na^+/K^+ -ATPases are involved in setting baseline $[K^+]_{out}$ levels and determining the rate of its recovery. Conversely, glial K_{ir} channels are involved in regulation of baseline levels of $[K^+]_{out}$ and in decreasing the amplitude of the postactivity $[K^+]_{out}$ undershoot (D'Ambrosio et al., 2002). In the mouse retina, K^+ channels appear to mediate most of the K^+ uptake as K^+ siphoning by Müller cells can be completely abolished by blocking K^+ conductance with Ba^{2+} (Kofuji et al., 2000). Similarly, in amphibian retina, light-evoked increases of $[K^+]_{out}$ in vitreous humor are completely blocked by superfusion of retinas with Ba^{2+} (Karwoski et al., 1989; Oakley et al., 1992), and in cat retinas light-evoked $[K^+]_{out}$ increases are three-fold larger following blockade of K^+ channels (Frishman et al., 1992).

Several lines of evidence have confirmed that the astrocyte membrane is selectively permeable to K^+ at rest and passively obeys the Nernst equation over a wide range of $[K^+]_{out}$ (Kettenmann et al., 1983; Kuffler et al., 1966; Lothman and Somjen, 1975; Newman, 1985, 1993). Although many voltage-dependent and -independent K^+ channels have now been found to exist in glia (reviewed in Verkhratsky and Steinhäuser, 2000; Barres et al., 1990; Sontheimer, 1994), properties of K_{ir} channels make them the most likely candidate for K^+ uptake by glial cells. In contrast to other voltage-gated K^+ channels, K_{ir} s are known to exhibit an asymmetrical response to hyperpolarization (high channel conductance), compared to depolarization (low channel conductance) (Katz, 1949); therefore, K^+ enters the cell more readily than it leaves (Doupnik et al., 1995; Stanfield et al., 2002). In addition, K_{ir} channels have high open probability at the normal resting membrane potential of glial cells (-80 to -90 mV) and thus allow uninhibited K^+ flux (Kuffler et al., 1966; Dennis and Gerschenfeld, 1969). Although, as mentioned, astrocytes express additional types of K^+ channels, such as delayed rectifying K^+ channels (K_{dr}) and Ca^{2+} -gated K^+ channels (K_{Ca}), these channel types are mostly inactive at the glial resting membrane potential (Sontheimer, 1994). Another important biophysical property of K_{ir} channels is that their conductance increases with elevations in $[K^+]_{out}$ (Stanfield et al., 2002). This unique property of K_{ir} channels allows increased K^+ conductance into glial cells, and therefore enhanced K^+ clearance rates, when $[K^+]_{out}$ is raised (Newman, 1993). The hypothesis that K_{ir} s participate in K^+ spatial buffering in the CNS has been directly confirmed in the mouse retina (Kofuji et al., 2000) and has led to a considerable interest in their localization, structure, and regulation. Over 20 genes are currently known to encode various K_{ir} channel subunits and they are categorized into seven major subfamilies ($K_{ir}1$ to $K_{ir}7$) based on their regulation mechanisms by intracellular and

extracellular factors (Nichols and Lopatin, 1997; Stanfield et al., 2002). K_{ir} channels are formed by four subunits each with two transmembrane domains separated by a selectivity pore forming re-entry loop (P-loop) and intracellular amino and carboxyl termini. They display pH sensitivity (K_{ir1}), ATP-sensitivity (K_{ir6} , K_{ir4}), and can be regulated by G-proteins (K_{ir3}). It is now established that inward rectification results primarily from voltage-dependent block by intracellular organic cations called polyamines. Of the polyamines, spermine and spermidine are the most potent inducers of rectification although contributions of putrescine and of Mg^{2+} ions are also important (reviewed in Isomoto et al., 1997; Ruppersberg, 2000). This class of ion channels has been shown to play a key role in the physiology of both excitable and non-excitable cells in a variety of tissues. Neuronal K_{ir} channels maintain the resting membrane potential, set a threshold for excitation, and play a major role in cell repolarization following activity (reviewed in Isomoto et al., 1997). In addition, they are involved in cell differentiation and survival of neurons and glia, modify CNS hormone secretion, modulate neurotransmitter release in nigrostriatal system, regulate K^+ homeostasis and K^+ secretion in the kidney, establish endococlear potential necessary for hearing, and regulate cerebral artery dilatation (reviewed in Neusch et al., 2003; Isomoto et al., 1997). It is not surprising then that K_{ir} channelopathies have been implicated in several polygenic CNS diseases such as white matter disease, epilepsy, and Parkinson's disease (Neusch et al., 2003). The direct involvement of glial K_{ir} s in epilepsy was already reviewed in *Astrocyte Dysfunction in Epilepsy* (page 32).

In conclusion, it is important to note that K^+ clearance mechanisms do not act independently and that operation of one mechanism may influence operation of others. The exact role of K^+ uptake and spatial buffering via active Na^+/K^+ -ATPase-mediated uptake and

passive channel- and transporter-mediated ion and water flux in the various parts of the CNS is unresolved. This question is under considerable debate and research has shown that the relative contributions may vary across species and between CNS regions. In the rat optic nerve, for example, $[K^+]_{out}$ buffering seems more dependent on the active uptake mechanism, as post-stimulus recovery of $[K^+]_{out}$ is highly sensitive to Na^+/K^+ -ATPase inhibition but not to glial K^+ channel blockers (Ransom et al., 2000). The authors suggest a model of K^+ buffering which involves post-stimulus K^+ uptake by glial and neuronal Na^+/K^+ -ATPase followed by a slow release of K^+ from glia through Ba^{2+} sensitive K^+ channels. In contrast, in the CA3 region of the rat hippocampus both the Na^+/K^+ -ATPase and glial K^+ -channels seem critical for setting the baseline $[K^+]_{out}$ and the post-stimulus recovery of $[K^+]_{out}$ (D'Ambrosio et al., 2002). Conversely, Jauch et al. (2002) have shown that in the CA1 stratum radiatum of adult rats only Ba^{2+} , but not ouabain or furosemide, affected $[K^+]_{out}$ clearing. Studies in the retina of several species have demonstrated that K^+ siphoning by Müller cells is entirely dependent on functioning of K_{ir} channels (Karwoski et al., 1989; Frishman et al., 1992; Kofuji et al., 2000). One of the challenges for future research is to determine the role that each of these K^+ regulatory mechanisms play in different tissues and during different levels of neuronal activity.

8. GLIAL INWARD RECTIFYING POTASSIUM CHANNEL, $K_{IR4.1}$

Ample evidence points to K_{ir} channels as one of the main mediators of K^+ spatial buffering by astrocytes in the CNS. An ATP- and pH-sensitive, weakly rectifying member of this channel family, $K_{ir4.1}$, has received the most interest due to its broad CNS distribution and a distinct localization pattern. $K_{ir4.1}$ was cloned by three independent laboratories and

was described as the first K_{ir} channel specifically localized to CNS glia (Bond et al., 1994; Bredt et al., 1995; Takumi et al., 1995). Takumi et al. (1995) reported observing $K_{ir}4.1$ mRNA in astrocytes, oligodendrocytes, and Bergman glia in adult rat tissue, as well as in cultured astrocytes, and suggested its involvement in K^+ buffering. Following this discovery several studies established that $K_{ir}4.1$ is expressed by most GFAP+ astrocytes in the brain, retinal Müller cells, and white-matter oligodendrocytes (Poopalasundaram et al., 2000; Higashi et al., 2001; Ishii et al., 2003; Li et al., 2001). Li et al. (2001) reported expression of $K_{ir}4.1$ in cortical neurons in culture; however, this finding has not been substantiated *in situ*. Immunogold electron microscopy study revealed $K_{ir}4.1$ enrichment in astrocyte processes that surround blood vessels and synapses (Higashi et al., 2001). Furthermore, in Müller cells of the mouse retina a water channel, AQP4, was found to co-localize with $K_{ir}4.1$ on Müller cell endfeet facing the vitreous body and blood vessels (Nagelhus et al., 1999). AQP4 and $K_{ir}4.1$ are clustered together by dystrophin-glycoprotein complex that contains PDZ domain protein α -syntrophin, the short dystrophin isoform Dp71, the transmembrane protein β -dystroglycan, and the extracellular matrix receptor α -dystroglycan (Claudepierre et al., 2000; Connors and Kofuji, 2006). As noted earlier, astrocyte K^+ buffering appears to be tightly coupled to water homeostasis (Newman, 1985).

Oligodendrocytes, on the other hand, were found to express $K_{ir}4.1$ mainly in their cell bodies and not their processes, arguing against its role in K^+ uptake by these cells (Poopalasundaram et al., 2000). Several authors even report failing to observe oligodendrocyte $K_{ir}4.1$ expression in the adult rat and mouse brain (Higashi et al., 2001; Hibino et al., 2004). Studies of the embryonic and early postnatal tissue have suggested involvement of $K_{ir}4.1$ in astrocyte and oligodendrocyte development. In embryonic rat brain,

$K_{ir}4.1$ expression appears between E13 and E15 in the neuroepithelium of the cerebral cortex, thalamus, and hypothalamus. By E20 $K_{ir}4.1$ is also found in the cortical plate and hippocampus (Ma et al., 1998). An immunostaining study in the rat optic nerve demonstrated that oligodendrocyte expression of $K_{ir}4.1$ increases between P5 and P10, the major period of oligodendrocyte maturation and the commencement of axon myelination in the optic nerve. Expression in astrocytes, however, develops after P10 and correlates with the development of more negative membrane potential and maturation of K^+ regulatory mechanisms (Kalsi et al., 2004). Generation of $K_{ir}4.1$ knockout ($K_{ir}4.1^{-/-}$) mice confirmed the importance of $K_{ir}4.1$ expression in oligodendrocyte development. $K_{ir}4.1^{-/-}$ mice die prematurely (P8-15) and display severe hypomyelination, white-matter vacuolization, incomplete myelin compaction, and axonal degeneration in the spinal cord. This phenotype was attributed to the improper maturation and myelination of $K_{ir}4.1^{-/-}$ oligodendrocytes, as confirmed by a developmental study of cultured cells (Neusch et al., 2001).

In addition to its indicative localization in astrocyte processes surrounding synapses and blood-vessels, a number of reports provide direct evidence that $K_{ir}4.1$ plays a role in extracellular K^+ homeostasis in several different systems. $K_{ir}4.1$ is expressed in the distal tubular epithelia of the kidney where it is thought to recycle K^+ and maintain the membrane potential of these cells (Ito et al., 1996). It is also found in the inner ear where it appears to be critical in the development and maintenance of the high K^+ concentration and electrical potential of the cochlear endolymph which is essential for hearing (Hibino et al., 1997; Marcus et al., 2002; Rozengurt et al., 2003). Deafness, inner ear degeneration, and lack of endocochlear potential are observed in $K_{ir}4.1^{-/-}$ mice (Marcus et al., 2002; Rozengurt et al., 2003). Furthermore, extracellular K^+ measurements in the brainstem of $K_{ir}4.1^{-/-}$ mice

revealed increased baseline $[K^+]_{out}$ and greater $[K^+]_{out}$ recovery undershoot during strong electrical stimulation, suggesting decreased K^+ permeability of brainstem astrocytes (Neusch et al., 2006). The involvement of $K_{ir}4.1$ in K^+ spatial buffering has been most extensively studied in Müller cells of the retina. Müller cells are involved in a specialized form of K^+ spatial buffering called K^+ siphoning in which the subcellular distribution of K^+ channels directs K^+ clearance through a single cell, from the neuronal photoreceptor layer to the large fluid reservoir called the vitreous humor (reviewed in *Potassium Spatial Buffering by Astrocytes*, page 46). Patch clamp recordings demonstrated a 10-fold increase in input resistance and a large depolarization (from -85 to -13 mV) of $K_{ir}4.1^{-/-}$ Müller cells, as expected if $K_{ir}4.1$ contributes to the large K^+ permeability of these cells. Furthermore, light-evoked electroretinogram showed a complete loss of K^+ fluxes through Müller cells in retinas of $K_{ir}4.1^{-/-}$ mice (Kofuji et al., 2000). Kofuji et al. (2002) went even further in increasing our understanding of K^+ siphoning by discovering the expression of another inward rectifier, $K_{ir}2.1$, in Müller cells. Due to the expression pattern and electrophysiological evidence presented in the previous studies of K^+ siphoning (Newman, 1987, 1993) they proposed a hypothesis that the K^+ influx from neuronal photoreceptors (the K^+ “source”) into Müller cells is mediated by strongly rectifying $K_{ir}2.1$, while the weakly rectifying $K_{ir}4.1$, expressed in Müller cell endfeet, mediates K^+ efflux into the extracellular “sink” of the vitreous humor (Kofuji et al., 2002). This group has also provided a compelling argument for functional coupling of AQP4 and $K_{ir}4.1$, mediated by their clustering via the dystrophin-glycoprotein complex (Connors and Kofuji, 2006). Müller cells lacking a component of this complex, Dp71, fail to cluster $K_{ir}4.1$ in their endfeet (Connors and Kofuji, 2002). Furthermore, coupling of AQP4 and $K_{ir}4.1$ function appears to be essential for extracellular K^+ clearance

by astrocytes in other brain regions. Potassium uptake following neuronal stimulation in hippocampal slices was significantly impaired in α -syntrophin (also a component of the dystrophin-glycoprotein complex) knockout mice which show impaired AQP4 localization (Amiry-Moghaddam et al., 2003). Impaired extracellular K^+ kinetics were also observed in AQP4^{-/-} mice (Binder et al., 2006). Nagelhus and colleagues (2004) have recently proposed a hypothesis that AQP4 works in concert with $K_{ir}4.1$ and the Na^+/HCO_3^- cotransporter to buffer the activity dependent ion, pH, and volume changes of the extracellular space. This hypothesis remains to be tested.

Although significant progress has been made, we still do not have a coherent picture of the coordinated interplay between various mediators of K^+ spatial buffering in the CNS and their involvement in neurological disorders such as epilepsy. In addition to $K_{ir}4.1$, several other molecules such as Na^+/K^+ -ATPase, aquaporins, Na^+/HCO_3^- , K^+/Cl^- , and $Na^+/K^+/2Cl^-$ cotransporters have been implicated in the control of K^+ homeostasis (reviewed in Kofuji and Newman, 2004); however, the above described studies strongly suggest that $K_{ir}4.1$ is critically important for this astrocyte function. Moreover, the $K_{ir}4.1$ gene was recently linked to seizure susceptibility in mice and humans. This gene has been mapped to the distal arm of chromosome 1 in the mouse (Tada et al., 1997). Ferraro et al. (1999) mapped a pentylenetetrazol-induced seizure susceptibility locus to the same region and later identified $K_{ir}4.1$ as a candidate gene (Ferraro et al., 2004). This finding was confirmed by Buono et al. (2004) who reported an association between two missense variations in the $K_{ir}4.1$ gene and seizure susceptibility in humans. Thus, further study of the role of $K_{ir}4.1$ in glial physiology and K^+ spatial buffering may hold promise for novel anticonvulsant therapies.

9. PHYSIOLOGY OF HIPPOCAMPAL ASTROCYTES

Cellular physiology of glial cells was first explored in the 1960s with sharp microelectrode recordings in the invertebrate nervous system (Kuffler et al., 1966; Kuffler and Potter, 1964; Orkand et al., 1966). Glial cell membrane was reported to behave as a “perfect K^+ electrode”, showing no voltage-dependent conductance. Confirming the similarity with invertebrate glia, an almost completely Nernstian relation ($V_m = 59 \cdot \log \frac{[K^+]_{out}}{[K^+]_{in}}$) of glial membrane potential and $[K^+]_{out}$ was also evident in recordings from glial cells in adult mammalian neocortex, optic nerve, and spinal cord (Dennis and Gerschenfeld, 1969; Ransom and Goldring, 1973; Lothman and Somjen, 1975). These and other microelectrode studies did not report any voltage-gated ion conductance in glial cells, supporting the general idea that glia are an electrically silent non-excitabile element of the nervous system. However, development of glial cell culture and improved patch clamp technique brought on a revolution in glial electrophysiology (Suburo and Adler, 1977; McCarthy and De Vellis, 1980). During the following two decades it was clearly demonstrated that glial cells express a similar variety of ion channels and neurotransmitter receptors as neurons, which led to a remarkable shift in understanding the role of glia in the CNS (reviewed in Hertz et al., 1998). Furthermore, the rebirth of *in situ* preparations in more recent years confirmed that diversity of glial channels and receptors is not a culture artifact (reviewed in Verkhratsky and Steinhäuser, 2000). As previously noted in *Astrocyte Functions* (page 13), study of hippocampal and retinal glia has received most attention and has led to the acknowledgment of glial cells as active partners in many, if not all, aspects of CNS development and functioning. Hippocampus and retina are both characterized by a well defined laminar structure and synaptic circuitry making them desirable model systems for

study of neurotransmission. In addition, most hippocampal and retinal synapses are enveloped by astrocyte processes giving us an excellent opportunity to study the bi-directionality of neuron-glia communication. Since the hippocampal slice preparation was used in my study of astrocyte K^+ buffering, I will describe its circuitry and give a historical account of our knowledge of glial physiology in this brain region.

The hippocampus is perhaps the best studied structure in the brain. Together with the adjacent amygdala, it forms the central axis of the limbic system (reviewed in Amaral and Witter, 1989). It is considered critical for spatial learning and awareness, navigation, episodic/event memory, and associational recollection. The hippocampus is formed by two interlocking sheets of cortex and in cross-section has a defined laminar structure with visible layers of pyramidal neurons. Connections within the hippocampus follow this laminar format and are uni-directional, forming a closed loop that mainly originates and ends in the adjacent entorhinal cortex (**Fig. 1**). The principal cell layers are the CA1-3 pyramidal cell regions and the dentate gyrus (DG). The perforant path is the major input to the hippocampus. Axons of the perforant path arise in the entorhinal cortex and project to the granule cells of the dentate gyrus and pyramidal cells of the CA3 region. It was in this pathway that LTP was first discovered (Bliss and Lomo, 1970, 1973). Axons of DG granule cells, called mossy fibers (MF), extend to CA3 pyramidal cells, forming their major input. Information flow continues via the Schaffer collateral (SC) pathway formed by axons of CA3 cells which project to the CA1 region. These axons either come from CA3 neurons in the same hippocampus (ipsilateral) or from the contralateral hippocampus in the opposite hemisphere (commissural fibers). The pathway from CA1 back to the entorhinal cortex forms the principal output from the hippocampus. These CA1 projection fibers course through the outermost layer of the

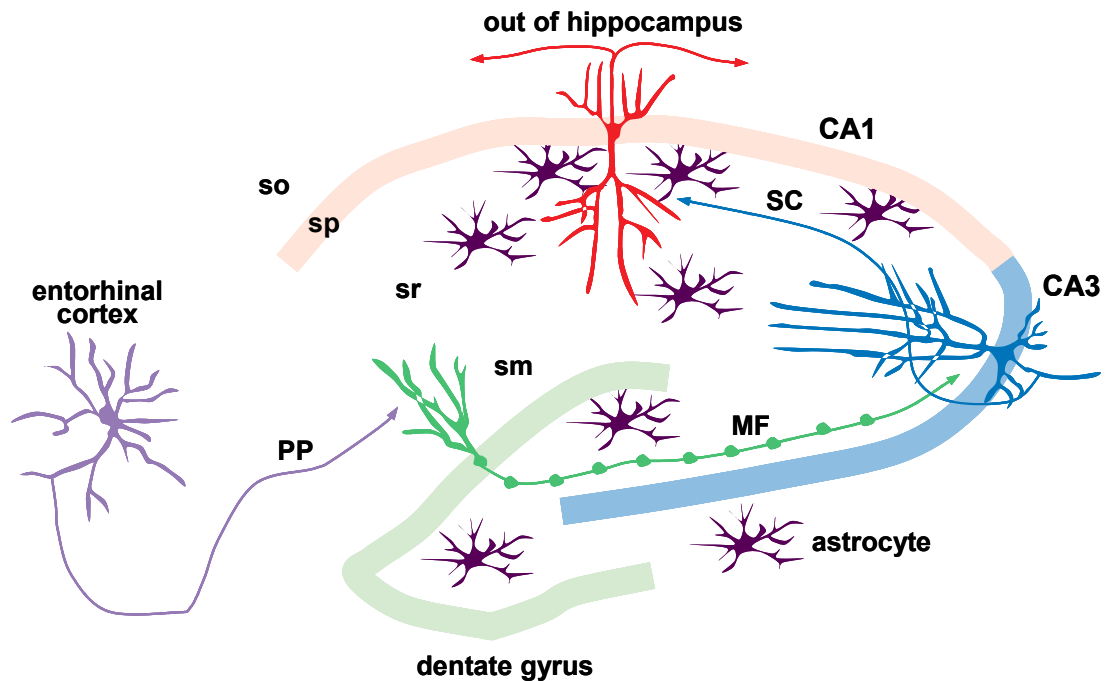


Figure 1. Tri-synaptic circuit of the hippocampus. The hippocampus forms a uni-directional synaptic network with input from entorhinal cortex (EC) that makes connections with the dentate gyrus (DG) and CA3 pyramidal neurons via the perforant path (PP). CA3 neurons also receive input from the DG via the Mossy Fibers (MF) and send axons to CA1 pyramidal cells via Schaffer collateral pathway (SC). CA1 neurons in turn send the main hippocampal output back to the EC, forming a loop. Hippocampal strata include stratum oriens (so), pyramidale (sp), radiatum (sr), and moleculare (sm). Most hippocampal synapses are enveloped by astrocyte processes with a single astrocyte overseeing an estimated 140,000 synapses (Bushong et al., 2002). Figure adapted from Christie and Cameron (2006).

hippocampus called the alveus. Just below the alveus is the stratum oriens (so) which borders the pyramidal cell layer and contains nonpyramidal neurons. The principal pyramidal cell layer (CA1-3) is also known as the stratum pyramidale (sp). Below stratum pyramidale is the stratum radiatum (sr) where CA3 axons or Schaffer collaterals synapse onto CA1 dendrites. Immediately underneath the stratum radiatum is the stratum moleculare (sm) which contains the perforant pathway fibers (reviewed in Amaral and Witter, 1989, 1995).

Steinhäuser et al. (1992) were the first to patch clamp glial cells in acutely isolated mouse (postnatal day, P10-12) hippocampal slices. They observed a broad spectrum of current patterns and grouped the cells into four subpopulations, three of which displayed a varying degree of voltage- and time-dependent currents (39% of patched cells), and one displaying symmetrical inward and outward currents with no apparent voltage dependence or current decay (61%). To confirm the glial-identity of patch clamped cells, they were filled with a tracer dye during patching and later inspected with the electron microscope. All of the patch-filled cells were identified as astrocyte-like glia by their size, chromatin distribution, and lack of synaptic membrane specializations (Steinhäuser et al., 1992). The following year, Sontheimer and Waxman (1993) went on to determine the current properties of glial cells in rat hippocampal slices (P5-24). They, too, filled the patched cells with a tracer dye and later identified them by presence (astrocytes) or absence (oligodendrocytes) of GFAP immunostaining. Astrocytes were shown to express three types of K^+ currents, singly or in combination: delayed rectifying currents ($I_{K_{dr}}$) sensitive to tetraethylammonium (TEA), transient "A"-type currents (I_{K_a}) sensitive to 4-aminopyridine (4-AP), and inward rectifying currents ($I_{K_{ir}}$) sensitive to Ba^{2+} . In addition, voltage-activated Na^+ currents (I_{Na_v}) sensitive to tetrodotoxin (TTX) were observed in ~10% of GFAP+ cells. Oligodendrocytes, identified by

their morphology and lack of GFAP staining, had either time- and voltage-independent currents or a distinct K^+ current characterized by inward rectification (Sontheimer and Waxman, 1993). Steinhäuser et al. (1994) focused their continued studies on non-myelinating hippocampal glia presumed to be astrocytes, and classified these cells by their current pattern into “complex” glia, expressing voltage-gated channels, and “passive” glia, lacking voltage-dependent currents and displaying a linear current-voltage (IV) relationship, similar to what was previously observed in the adult invertebrate and mammalian CNS (Kuffler et al., 1966; Dennis and Gerschenfeld, 1969; Ransom and Goldring, 1973; Lothman and Somjen, 1975). Furthermore, they demonstrated that most of the passive cells are GFAP+, suggesting that they are mature protoplasmic astrocytes, while none of the complex cells stained for GFAP and may represent an immature astrocyte population. Both cell types were shown to express functional $GABA_A$ receptors and iGluRs, most likely of kainate type. Passive cells also displayed functional GluT currents (Steinhäuser et al., 1994). These findings represent the first demonstration of neurotransmitter receptor expression on glial cells in intact tissue. It was later shown that both passive and complex cells also have mGluRs, as well as adrenergic, purinergic, serotonergic, muscarinic, and peptidergic receptors linked to activation of phospholipase C or adenylate cyclase (Cai and Kimelberg, 1997; Pasti et al., 1997; reviewed in Porter and McCarthy, 1997).

Prompted by the reports of developmentally regulated channel expression in neurons and oligodendrocytes (Spitzer, 1991), Kressin et al. (1995) looked at the electrophysiological development of complex glia within the mouse CA1 stratum radiatum (P5-35). In the early postnatal days, the current pattern of these cells appears to be dominated by two types of K^+ outward currents, the IK_{dr} and IK_a . At this stage, all complex cells also express significant

TTX-sensitive I_{Na_v} currents. During maturation, the contribution of $I_{K_{dr}}$ and I_{K_a} significantly decreases and almost all cells after P20 lack TTX-sensitive currents. This downregulation of inward Na^+ and outward K^+ currents is accompanied by a substantial increase in the $I_{K_{ir}}$ and a passive voltage-independent conductance which is selectively K^+ permeable and insensitive to TEA and 4-AP. These two conductances appear to stabilize the cells' membrane potential close to E_K . Increasing evidence of electrical coupling was also found with maturation, but still the majority of complex cells failed to couple (Kressin et al., 1995; Bordey and Sontheimer, 1997). This reported replacement of voltage-gated Na^+ and K^+ outward currents by passive and inward rectifier K^+ currents also occurs during development of oligodendrocytes from their precursors in corpus callosum slices (Berger et al., 1991). Furthermore, Kressin et al. (1995) substantiated the idea that complex cells are immature astrocytes by showing that in older animals they usually immunostain for GFAP (also shown by Chvatal et al., 1995) and they always fail to stain for oligodendrocyte precursor markers, Olig1 and Olig4. The first observation is in agreement with reports made by Nixdorf-Bergweiler et al. (1994) who demonstrated a large increase in the number of GFAP+ cells in the mouse CA1 stratum radiatum between P8 and P24. Several other findings implicated that complex glia are part of the astrocyte lineage, including their expression of an astrocyte-specific enzyme, glutamine synthetase (Akopian et al., 1996), and reports showing that both passive and complex astrocytes injected with a tracer dye have the spongiform morphology consistent with that of protoplasmic astrocytes (Jabs et al., 1997). Interestingly, some investigators such as Bordey and Sontheimer (1997, 1998) reported only finding complex cell type in their hippocampal preparations and showed that they are consistently GFAP+. They attributed the discrepancy between their observations and those made in other

laboratories to technical issues such as poor cell access that may lead to a transient masking of voltage-activated currents and passive appearance of otherwise complex cells (Bordey and Sontheimer, 1998). Due to such reports, the following few years were marked by a debate over the existence of purely passive glia and the identity of complex glia which was questioned when the NG2⁺ cells (thought of as oligodendrocyte precursors) were found to display the “complex” electrophysiological phenotype (Bergles et al., 2000). As reviewed in *Types of Glia* (page 4), the NG2⁺ cells were originally referred to as smooth protoplasmic astrocytes due to their astrocyte-like morphology (Levine and Card, 1987). However, more recently it was shown that they constitute the principal dividing cell population of adult brain, accounting for 75% of all cortical cells that are pulse labeled with BrdU (Horner et al., 2000; Levine et al., 2001; Dawson et al. 2003). They appear to preferentially differentiate into oligodendrocytes in mature damaged brain (Ong and Levine, 1999; Watanabe et al., 2002; Wilson et al., 2003), but they may also give rise to GABAergic neurons in the hippocampus (Belachew et al., 2003). In transgenic reporter mice, NG2⁺ glia express green-fluorescent protein (GFP) under the control of oligodendrocyte-associated promoters, such as proteolipid protein (PLP; Mallon et al., 2002) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) promoter (Belachew et al., 2001), as well as under the control of astrocyte-associated promoters, such as S100 β (Lin and Bergles, 2002; Hachem et al., 2005) and GFAP promoter (Mathias et al, 2003). They usually do not immunostain for GFAP, but start expressing GFAP or vimentin after lesion to the adult brain (Alonso, 2005). Furthermore, NG2⁺ glia engage in rapid signaling with glutamatergic and GABAergic neurons through direct neuron-glia synapses in the cerebellum and hippocampus (Bergles et

al., 2000; Lin et al., 2005). This glial population, therefore, represents an enigmatic multipotent cell type in need of further analysis.

More recently, the Steinhäuser laboratory employed electrophysiology in conjunction with single-cell RT-PCR and immunostaining to better define properties of passive and complex glia within hippocampus (Matthias et al., 2003; Wallraff et al., 2004; Jabs et al., 2005). They used GFAP-eGFP mice expressing enhanced-GFP (eGFP) under the control of human GFAP promoter (*gfa2*), which was previously shown to drive astrocyte-specific eGFP expression (Brenner and Messing, 1996; Nolte et al., 2001). In hippocampal slices of P6-20 GFAP-eGFP mice, passive glia are brightly fluorescent and distinguished by functional GluT expression and high levels of GLT-1 and GLAST mRNA. They are therefore termed GluT cells. Complex glia, on the other hand, are faintly fluorescent, display functional AMPA receptor currents and contain mRNA for AMPA receptor subunits, GluR1-4. Overlap of GluT and iGluR currents is never observed and passive glia always lack iGluRs on the transcriptional level; however, ~80% of complex or GluR cells express GLT-1 and GLAST mRNA. The transcripts for neuronal transporter ECCA1 are also found in ~45% of complex cells (Matthias et al., 2003). Furthermore, the immunostaining study at P14 revealed that ~95% of complex (GluR) glia express S100 β , whereas GFAP is less frequently expressed (~40%). One-third of GluR cells are also NG2 immunopositive and in 21% of those, NG2 is coexpressed with GFAP. Conversely, passive or GluT glia are never NG2+ and always GFAP+ (Matthias et al., 2003). In P30 hippocampal slices GluT cells are extensively coupled while GluR cells lack gap junction coupling and Cx43 transcripts (Wallraff et al., 2004). As was previously shown for NG2+ cells (Bergles et al., 2000), the S100 β + GluR cells also receive GABAergic and glutamatergic input from surrounding neurons, as well as direct

input from Schaffer collaterals upon stimulation (Jabs et al., 2005). Interestingly, it is not yet known whether GluR cells are capable of releasing glutamate, but GluT cells have been shown to release glutamate via regulated exocytosis (Bezzi et al., 2004) and modulate neuronal activity (Fiacco and McCarthy, 2004; Liu et al., 2004; Volterra and Steinhäuser, 2004). The above summarized morphological, immunocytochemical, and functional characteristics of passive or GluT glia suggest that they are the classical protoplasmic astrocytes. It is harder to define the complex (GluR) glia since they appear to be segregated into at least two subpopulations. One of those is the NG2+ multi-potent precursor population, while the other probably represents immature astrocytes which will later upregulate GluT and passive K^+ currents (Kressin et al., 1995; Matthias et al., 2003).

A very recent extensive developmental study of glial cells in the hippocampus summarizes and further corroborates the previously described findings. Zhou et al. (2006) performed *in situ* whole-cell recordings from morphologically identified glia in the rat hippocampal CA1 region (P1-106) and post-stained the recorded cells with GLAST and NG2 antibodies. The main findings of this study are summarized in **Figure 2**. Patched cells were classified into three different types according to their whole-cell current pattern: passive glia (PG), and outward rectifying- (ORG) and variably rectifying glia (VRG) (previously grouped into complex glia by other investigators). Outward rectifying glia display mostly voltage-gated outward K^+ currents ($I_{K_{dr}}$ and I_{K_a}) and inward Na^+ currents (I_{Na_v}) until P22 (**Fig. 2A**). Between P1-3 they are equally split into GLAST+ and NG2+ populations, but they are mainly NG2+ (80%) in juvenile animals (P4-21), and they cannot be found in adult hippocampus (**Fig. 2B**). Variably rectifying glia displaying voltage-gated outward ($I_{K_{dr}}$ and I_{K_a}) and inward ($I_{K_{ir}}$) K^+ currents (**Fig. 2A**) are also present at P1, peak at P5 and decline to a

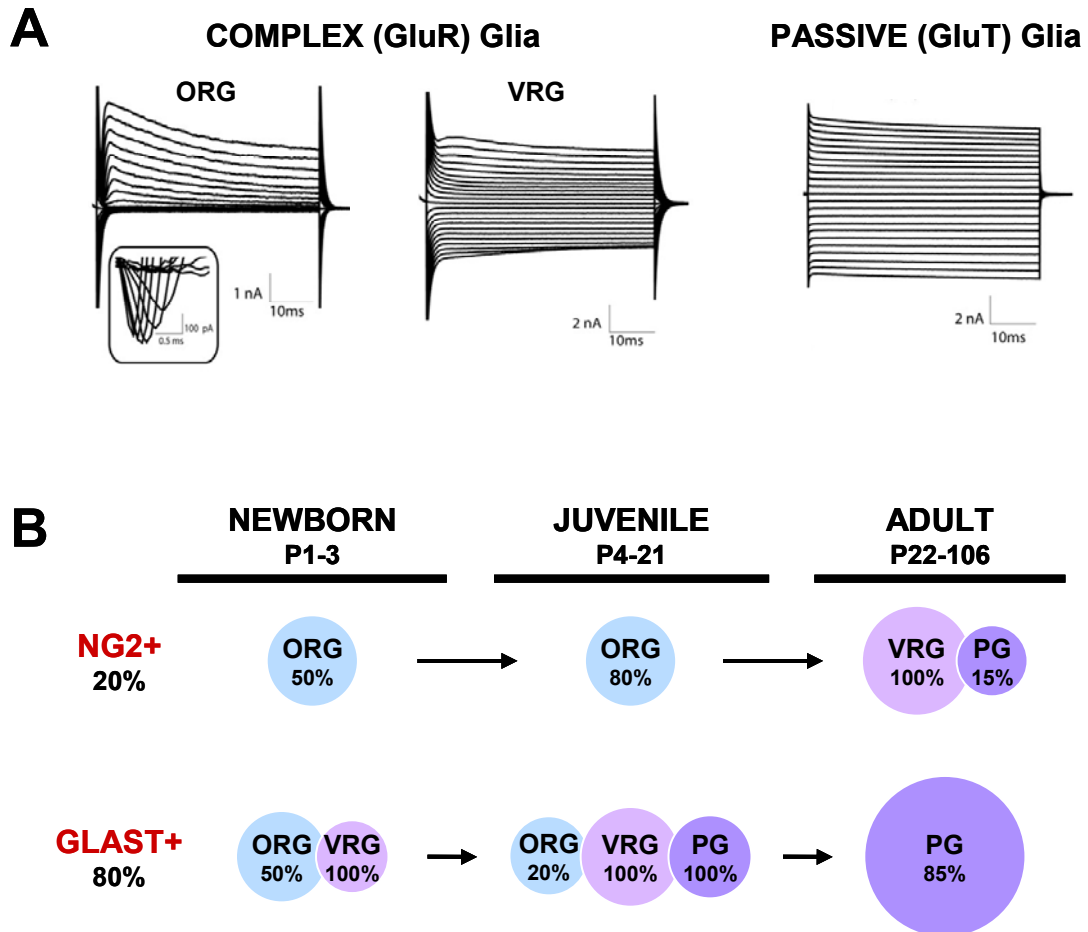


Figure 2. Heterogeneity of hippocampal glia. (A) Representative whole-cell current recordings from hippocampal glia. Glial cells displaying voltage- and time-dependent currents are termed complex or GluR glia. They are subdivided into *outwardly rectifying glia* (ORG), characterized by outward K^+ conductance and inward Na^+ conductance (shown in inset), and *variably rectifying glia* (VRG) characterized by outward and inward K^+ conductance. In contrast to complex glia, passive or GluT glia (PG) are distinguished by predominant time- and voltage-independent K^+ currents. (B) Possible relationships between GLAST and NG2 lineage glia and their electrophysiological phenotypes in rat hippocampus. (%) Percent of NG2 vs. GLAST immunopositive cells of the noted phenotype at a given age. Figure adapted from Zhou et al. (2006).

stationary level of ~10% in adult. They are GLAST+ from newborn to juvenile, but only NG2+ in the adult (**Fig. 2B**). Electrophysiologically passive glia show up at P4 and increase to 90% in the adult, where 85% of these cells are GLAST+ and coupled and 15% appear to be NG2+ and not coupled. Importantly, GLAST and NG2 immunostaining were never found to overlap. Overall these findings suggest that a) two separate glial lineages marked by GLAST vs. NG2 expression are present in the hippocampus since birth, b) the GLAST lineage is always the predominant type with the NG2 lineage making about 20% of the population, c) during brain maturation, GLAST+ glia progress from outward rectifying and variably rectifying to passive electrophysiological phenotype in the adult, while NG2+ glia convert from outward rectifying to variably rectifying (Zhou et al., 2006).

The above described study, therefore, brings us full circle to the original electrophysiology studies in the intact CNS demonstrating that membrane of mature astrocytes acts as a passive “ohmic” resistor (Kuffler et al., 1996; Dennis and Gerschenfeld, 1969; Ransom and Goldring, 1973; Lothman and Somjen, 1975). The heterogeneity of electrophysiological properties of glia in *in situ* preparations from young developing tissue can be attributed to the coexistence of glial cells from several lineages at different developmental stages. Glia in the cerebral cortex, spinal cord (Bordey and Sontheimer, 2000), caudal medulla (Grass et al., 2004) and dentate gyrus (Isokawa and McKhann, 2005) all appear to share similar physiological properties with cells in the hippocampal stratum radiatum and can also be subdivided into passive and complex glia. Passive glia represent the mature astrocyte population of highly connected cells which envelop blood vessels and synapses, possess an intrinsic Ca^{2+} -mediated excitability, and are involved in glutamate uptake and release. A large percentage of complex glia in the developing CNS are most

likely the immature astrocyte population which will become the passive glia, while in the adult they are the poorly understood NG2+ progenitor cells possibly capable of direct synaptic communication with neurons.

CHAPTER II. INITIAL CHARACTERIZATION OF THE $K_{IR}4.1$ CONDITIONAL KNOCKOUT PHENOTYPE

1. INTRODUCTION

Epilepsy is one of the most common neurological disorders affecting about 4% of the population; however, its cellular basis is largely unknown and about 30% of all epilepsies remain uncontrolled (Browne and Holmes, 2001). Numerous studies implicate that neuronal hyperexcitability, which is a hallmark of this disorder, may result from abnormal astrocyte function and impairment of their critical role in neuronal development, homeostasis of the extracellular environment, and modulation of neuronal activity (reviewed in *Astrocyte Dysfunction in Epilepsy*, page 32). Elevated $[K^+]_{out}$ is one of the best characterized pro-epileptic agents (Traynelis and Dingledine, 1988; Jensen and Yaari, 1997; Leschinger et al., 1993), and impaired regulation of $[K^+]_{out}$ has been consistently observed in epileptic tissue (Gabriel et al., 1998; Kivi et al., 2000). Furthermore, observations made from surgical specimens of patients with epilepsy suggest that diminished astrocyte K_{IR} currents underlie the seizure-associated impairment of $[K^+]_{out}$ homeostasis (Ballanyi et al., 1987; Bordey and Sontheimer, 1998; Hinterkenser et al., 2000; Schroder et al., 2000).

Information processing within the CNS depends on fast action potential signaling which delivers the message from one synaptically coupled neuronal cell to another. Action potential propagation involves synchronized depolarization and hyperpolarization of the neuronal membrane, mediated by Na^+ influx and K^+ efflux through voltage-gated channels. Due to the

small volume of interstitial space and the low baseline $[K^+]_{out}$, action potential mediated K^+ effluxes elicit substantial changes in $[K^+]_{out}$ (reviewed in *Augmentation of $[K^+]_{out}$ by Neuronal Activity*, page 41). During spontaneous neuronal activity in the cerebellum $[K^+]_{out}$ increases 1-3 mM around the soma and dendrites of Purkinje cells (Hounsgaard and Nicholson, 1983). Moreover, seizure-like activity *in vivo* is characterized by up to 10-12 mM increases in $[K^+]_{out}$ (Fisher et al., 1976; Lothman and Somjen, 1976), while CNS injury, such as hypoxia/ischemia and concussion, may lead to transient elevations of $[K^+]_{out}$ up to 80 mM (Somjen et al., 1992). Since such $[K^+]_{out}$ changes modulate neuronal excitability and synaptic transmission both directly (by affecting the neuronal membrane potential, voltage-gated channels, and neurotransmitter dynamics) and indirectly (by affecting pH homeostasis, cerebral blood flow, and energy metabolism), $[K^+]_{out}$ must be tightly and effectively regulated (reviewed in *Influence of Elevated $[K^+]_{out}$ on Neuronal Function*, page 43). Numerous studies examined the relationship between $[K^+]_{out}$ and neuronal excitability and demonstrated that small local rises of $[K^+]_{out}$ (as low as 0.5 mM) trigger epileptiform activity and enhanced synaptic transmission, while rises greater than 6 mM eventually elicit complete synaptic inhibition (Heinemann et al., 1986; Yaari et al., 1986; Leschinger et al., 1993; Poolos et al., 1987; Chamberlin et al., 1990). While neurons themselves are involved in regulation of $[K^+]_{out}$, it is well accepted that astrocytes have the predominant role in maintaining K^+ homeostasis (reviewed in *Potassium Spatial Buffering by Astrocytes*, page 46).

The pioneering work of Kuffler's group demonstrated that nerve impulses cause slow depolarization of glial cells due to K^+ influx across their membrane (Orkand et al., 1966; Kuffler, 1967). Based on this observation they proposed the "K⁺ spatial buffering hypothesis" which states that astrocytes take up excess extracellular potassium ions,

distribute them via the gap junction-coupled cell syncytium, and extrude the ions at sites where $[K^+]_{out}$ is low. Subsequently this phenomenon was confirmed in a variety of nervous tissue preparations (Mori et al., 1967; Kettenmann et al., 1983; Coles et al., 1986; Holthoff and Witte, 2000; reviewed in *Evidence for Potassium Spatial Buffering*, page 48). By simultaneously recording astrocyte membrane potential and $[K^+]_{out}$, Amzica et al. (2002) directly demonstrated that astrocytes take up K^+ during neuronal activity *in vivo* and that K^+ moves faster through the astrocyte syncytium than through the interstitial space. Although several possible mediators of astrocyte K^+ uptake have been proposed, including Na^+/K^+ -ATPase and $Na^+/K^+/2Cl^-$ cotransporters, pharmacological studies suggest that K_{ir} channels dominantly participate in K^+ buffering (reviewed in *Passive Potassium Uptake by K_{ir} Channels*, page 53). Glial K^+ uptake induced by repetitive stimulation of the olfactory tract is strongly inhibited by the K_{ir} channel blocker Ba^{2+} , although a small part of the uptake is ouabain-sensitive indicating Na^+/K^+ -ATPase involvement (Ballanyi et al., 1987). D'Ambrosio and coworkers demonstrated that blocking glial K_{ir} s significantly raises $[K^+]_{out}$ during low frequency neuronal stimulation (2002). In the retina of several different species, K^+ buffering by Müller glia is completely abolished upon K_{ir} blockade (Karwoski et al., 1989; Oakley et al., 1992; Frishman et al., 1992). In addition, as mentioned above, evidence is accumulating that glial K_{ir} channels play an important role in epileptogenesis.

In contrast to other voltage-gated K^+ channels, K_{ir} channels have a high open probability at the normal resting membrane potential of glial cells (-80 to -90 mV), and their conductance is augmented by elevations in $[K^+]_{out}$ (Katz, 1949; Doupnik et al., 1995; Stanfield et al., 2002). These unique electrophysiological properties make them well suited for K^+ uptake. Appearance and upregulation of astrocyte K_{ir} conductance (Kressin et al.,

1995; Bordey and Sontheimer, 1997; Zhou et al., 2006) is concurrent with the appearance of $[K^+]_{out}$ maintenance mechanisms during the early postnatal development of the brain (Kalsi et al., 2004). The first identified glia-associated K_{ir} was the weakly rectifying $K_{ir}4.1$ (Takumi et al., 1995), found in astrocyte processes surrounding synapses and blood-vessels and oligodendrocyte cell bodies (Higashi et al., 2001; Poopalasundaram et al., 2000; Ishii et al., 2003; reviewed in *Glial Inward Rectifying Potassium Channel, $K_{ir}4.1$* , page 57). $K_{ir}4.1$ localization and intimate association with AQP4 in perivascular astrocyte processes implicated its involvement in K^+ buffering (Negelhus et al., 1999). Subsequently, generation and study of $K_{ir}4.1^{-/-}$ mice confirmed the importance of $K_{ir}4.1$ in K^+ buffering by several cell types, including Müller glia and cochlear epithelium (Kofuji et al., 2000; Marcus et al., 2002; Rozengurt et al., 2003). In support of these findings, a recent quantitative trait loci mapping identified the $K_{ir}4.1$ gene (KCNJ10) as a putative seizure susceptibility gene in mice (Ferraro et al., 2004). Moreover, a missense (R271C) variation in the $K_{ir}4.1$ gene was linked to general seizure susceptibility in humans (Buono et al., 2004).

Due to its apparent involvement in oligodendrocyte development and K^+ homeostasis in the kidney, general knockout of $K_{ir}4.1$ leads to severe pathology, including pronounced white-matter vacuolization, and to early postnatal lethality (P7-14; Kofuji et al., 2000; Neusch et al., 2001). Our laboratory set out to generate a conditional knockout of $K_{ir}4.1$ restricted to astrocytes to directly study its role in astrocyte K^+ buffering and hopefully circumvent the pathology associated with systemic loss of this channel. We employed the well described Cre/loxP system which takes advantage of the bacteriophage enzyme Cre-recombinase (Cre) capable of excising a DNA fragment surrounded by its recognition sequences, termed loxP sites (Orban et al., 1992; reviewed in Le and Sauer, 2000). Two lines

of mice were made: a recombinant $K_{ir}4.1$ floxed ($K_{ir}4.1^{f/f}$) line and a transgenic B6-Tg(GFAP-Cre)1McCarthy (*gfa2-Cre*) line. The targeting constructs used for mouse generation are depicted in **Figure 3A** and **3B**. We utilized a 2.2 kB fragment of the human GFAP promoter (termed *gfa2*; generously provided by Dr. Albee Messing) to drive Cre-recombinase expression selectively in astrocytes. The intermediate filament GFAP is a well accepted astrocyte marker, and *gfa2* promoter was previously shown to drive astrocyte-specific expression of reporter genes *in vitro* and *in vivo* (Brenner et al., 1994; Brenner and Messing, 1996; Nolte et al., 2001). Detailed description of methods employed for the construct and mouse generation can be found in *Materials and Methods* (page 166). Removal of the neo/tk selection cassette which was surrounded by FRT sites, was accomplished by first breeding $K_{ir}4.1^{f/f}$ mice to FLPeR mice (courtesy of Dr. Susan M. Dymecki). FLPeR mice express Flp-recombinase under the control of β -actin promoter. This transgene was integrated into the ROSA26 locus which has been shown to drive transgene expression in most cells, including germline (Farley et al., 2000). Mouse generation and the initial characterization of transgene expression were done by Kristi Casper with help from the UNC Animal Models Core Facility. **Figure 3C** demonstrates that most if not all GFAP immunopositive cells (green fluorescence) in CA1 stratum radiatum of an adult *gfa2-Cre* animal express Cre-recombinase (red fluorescence). For simplicity purposes $K_{ir}4.1^{f/f}/gfa2-Cre$ mice will be referred to as $K_{ir}4.1$ cKO and $K_{ir}4.1^{f/+}/gfa2-Cre$ mice as $K_{ir}4.1^{f/-}$.

The initial characterization of the $K_{ir}4.1$ cKO mice is described below. It involved a study of their behavioral, cellular, morphological, and electrophysiological phenotype. The *gfa2* promoter-driven Cre recombination of the floxed $K_{ir}4.1$ gene was found to lead to the CNS restricted loss of $K_{ir}4.1$, a severe depolarization of all glial types studied, and a pronounced

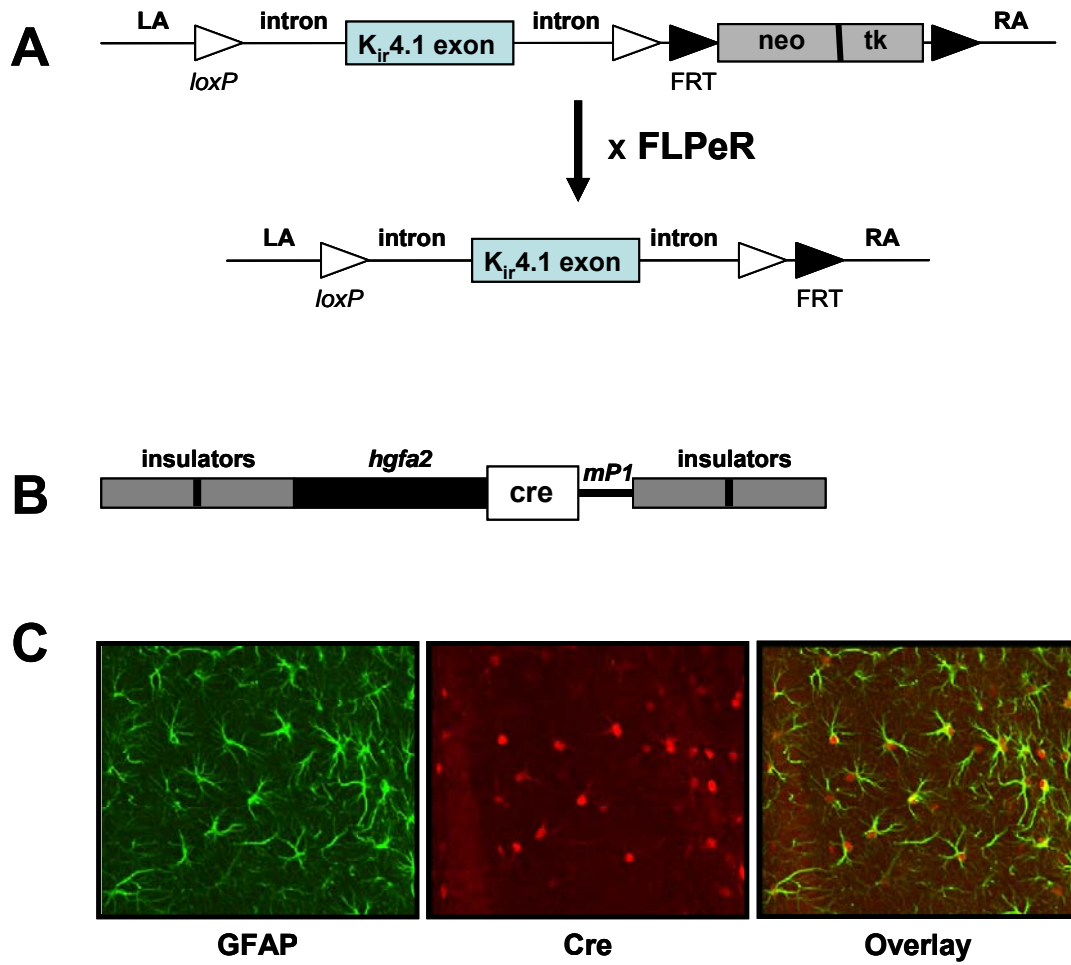


Figure 3. Generation of the $K_{ir}4.1$ cKO mice. (A) Targeting construct for the recombinant $K_{ir}4.1^{f/f}$ mouse line. Neo/tk selection cassette was removed by crossing $K_{ir}4.1^{f/f}$ and FLPeR mice. (B) Targeting construct for the transgenic *gfa2*-Cre line. (C) Fluorescent immunostaining for GFAP and Cre in the *gfa2*-Cre CA1 stratum radiatum.

behavioral phenotype, including ataxia and seizures. These findings are suggestive of $K_{ir}4.1$ involvement in maintenance of the glial membrane potential and CNS K^+ homeostasis.

2. RESULTS

2.1. $K_{ir}4.1$ CKO PHENOTYPE INCLUDES ATAXIA, SEIZURES, AND EARLY LETHALITY

The first step in our study of $K_{ir}4.1$ cKO animals was to characterize their behavioral phenotype. $K_{ir}4.1$ cKO mice were born in expected Mendelian ratio. They were indistinguishable from their WT littermates until P12-15 when they could be recognized by runted appearance (**Fig. 4A**) and wobbly movements. With time they developed pronounced body tremor, lethargy, and ataxia with frequent falls to the side. Once overturned cKO mice had a hard time regaining their upright position. Hind leg splaying and paralysis was often observed (**Fig. 4B**), as well as visual placing deficiency due to either complete or partial eye closure, probably attributable to involvement of $K_{ir}4.1$ in Müller cell homeostasis (Kofuji et al., 2000). $K_{ir}4.1$ cKO mice stopped gaining weight around P15 after reaching the weight of 5-6 g (**Fig. 4D**) and most died between P20 and P25 (**Fig. 4E**). When stimulated by sudden movements, cKO mice displayed grand mal seizures with hyperextension of the back and limb rigidity (**Fig. 4C**). Seizures of clonic type, characterized by rhythmic jerking movements of front and hind limbs, as well as tonic type, characterized by stiffening of the body and limbs, have been observed. Seizure recovery usually occurred within 30 sec after which mice resumed movement. Seizures of any type have never been seen in WT, $K_{ir}4.1^{f/f}$ or $K_{ir}4.1^{+/-}$ mice. Seizure occurrence in $K_{ir}4.1$ cKO mice suggests $K_{ir}4.1$ involvement in K^+ spatial buffering and control of neuronal excitability. Further behavioral testing was not done

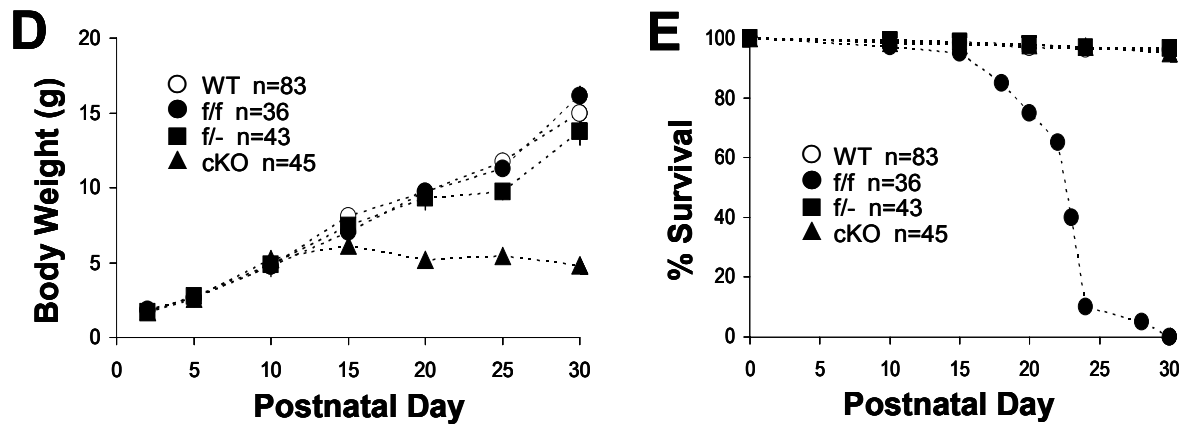


Figure 4. $K_{ir}4.1$ cKO phenotype. $K_{ir}4.1$ cKO mice exhibit growth retardation (A, D), ataxia and hind-leg paralysis (B), stress-induced seizures (C), and premature lethality (E).

since $K_{ir}4.1$ cKO mice cannot climb or suspend themselves from the cage bars and would not be able to perform in standard behavioral tests.

2.2. *GFA2* PROMOTER DRIVES COMPLETE REMOVAL OF $K_{IR}4.1$ FROM THE CNS

To establish that *gfa2* promoter-driven $K_{ir}4.1$ cKO leads to loss of $K_{ir}4.1$ from astrocytes but not other cells in which this protein is abundant, such as kidney tubular epithelial cells (Ito et al., 1996), we employed western blotting and fluorescent immunostaining. Detailed protocols for these and other later described techniques can be found in *Materials and Methods* (page 166). Brain and kidney homogenates from littermate WT, $K_{ir}4.1^{fl/fl}$, $K_{ir}4.1^{+/-}$, and $K_{ir}4.1$ cKO animals of several different ages (P15-25) were prepared and processed using a standard western blotting protocol. Complete absence of $K_{ir}4.1$ protein was observed in the brain tissue of $K_{ir}4.1$ cKO mice, while no reduction in $K_{ir}4.1$ level was seen in the cKO kidney (**Fig. 5A**). Levels of $K_{ir}4.1$ protein from all examined tissues of $K_{ir}4.1^{fl/fl}$ and $K_{ir}4.1^{+/-}$ mice were comparable to WT controls. Blotting for β -actin served as the protein loading control in all experiments.

Fluorescent immunostaining of frozen brain and spinal cord sections was used to look at the loss of $K_{ir}4.1$ from different cell populations and to examine cellular modifications that could explain $K_{ir}4.1$ cKO behavioral phenotype. Briefly, brains and spinal cords of P10-30 mice were drop-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and frozen in optimal cutting temperature media prior to sectioning on a cryostat. Tissue sections (14 μ m) were blocked to minimize non-specific staining, and antibodies applied at room temperature for varying lengths of time. Antibodies against GFAP and astrocyte-specific glutamate transporter, GLAST, were used for astrocyte identification. Antibodies against 2,3-cyclic-

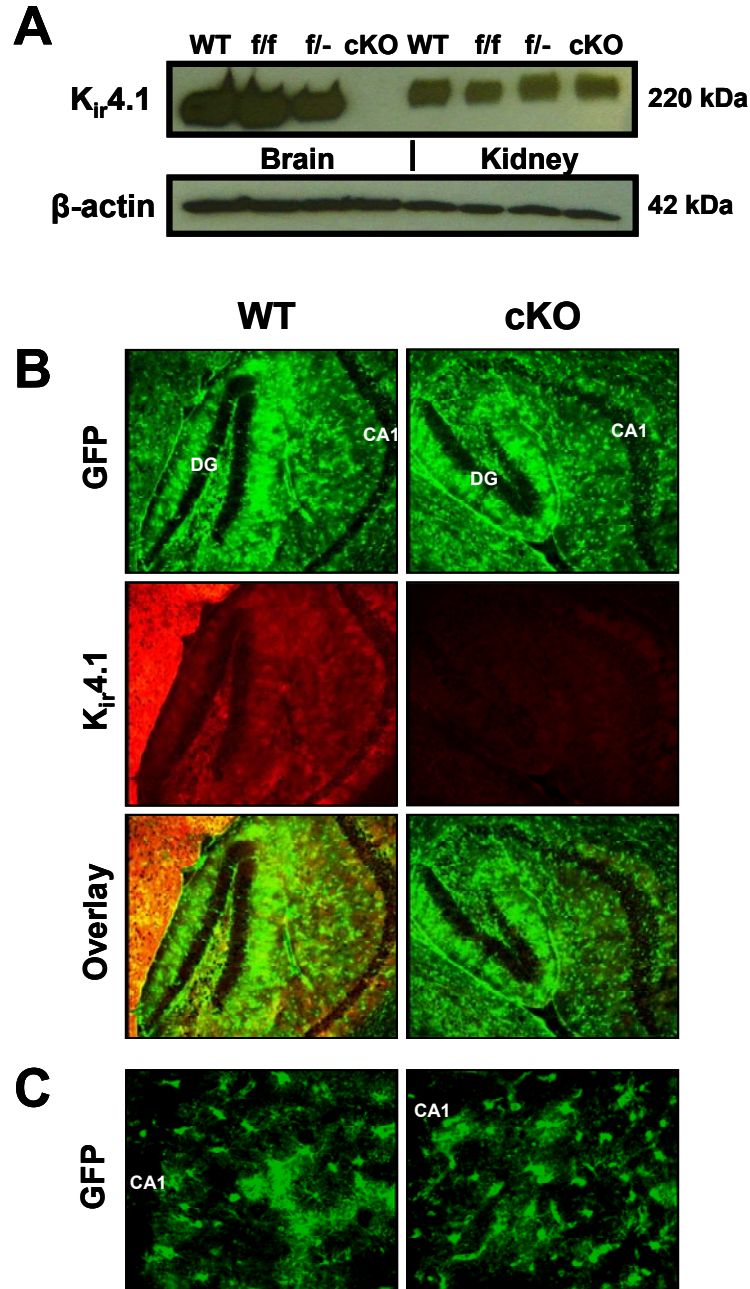


Figure 5. Loss and localization of $K_{ir}4.1$. (A) $K_{ir}4.1$ western blot from littermate WT, $K_{ir}4.1^{f/f}$, $K_{ir}4.1^{f/-}$, and $K_{ir}4.1$ cKO brain and kidney tissue (P20). Blotting for β -actin was used as a loading control. (B) Fluorescent GFP and $K_{ir}4.1$ immunostaining of WT/S100 β -eGFP and $K_{ir}4.1$ cKO/S100 β -eGFP hippocampus (P25); (DG) Dentate gyrus; (CA1) CA1 pyramidal cell layer. (C) GFP immunostaining in CA1 stratum radiatum of WT/S100 β -eGFP and $K_{ir}4.1$ cKO/S100 β -eGFP mice (P25); (CA1) CA1 pyramidal cell layer.

nucleotide-3-phospho-diesterase (CNP), proteolipid protein (PLP), and myelin-basic protein (MBP) were used to stain oligodendrocytes and myelin. NeuN, calbindin, and neurofilament antibodies identified neurons and their processes. In addition to the immunological marker study, $K_{ir}4.1^{f/f}$ and *gfa2*-Cre animals were crossed to two different reporter lines expressing eGFP in astrocytes: GFAP-eGFP (Nolte et al., 2001) and S100B-eGFP (Vives et al., 2003). Adult GFAP-eGFP animals display astrocyte specific eGFP expression that reliably overlaps with GFAP antibody labeling throughout CNS (Nolte et al., 2001). In adult S100B-eGFP mice, the highest level of eGFP expression is found in ependymocytes and astrocytes, however, several neuronal populations in the cerebellum, forebrain, and medulla, as well as NG2+ glia are also eGFP labeled (Vives et al., 2003). Upon obtaining the proper crosses we were able to compare morphology, localization, and number of eGFP expressing cells between WT and $K_{ir}4.1$ cKO mice. We concentrated our attention on the hippocampus since this region was the focus of our electrophysiological study. As can be seen in **Figure 5B**, most eGFP expressing cells within WT/S100B-eGFP hippocampus were immunopositive for $K_{ir}4.1$ (red fluorescence). Neuronal cell bodies in the pyramidal and dentate gyrus cell layers were devoid of $K_{ir}4.1$ staining and eGFP. Very robust $K_{ir}4.1$ staining was observed in thalamus, brain stem, cerebellar molecular layer, and spinal cord gray matter (**Fig. 6A**) in agreement with other published studies (Poopalasundaram et al., 2000; Kalsi et al., 2004). This staining is attributed to astrocyte and oligodendrocyte expression of $K_{ir}4.1$; however, due to its diffuse and punctuate nature we found it hard to definitively exclude its localization on neuronal processes. Even though $K_{ir}4.1$ cKO hippocampi consistently appeared smaller than those of WT littermates, the distribution and morphology of cells expressing eGFP in littermate WT/S100-eGFP and $K_{ir}4.1$ cKO/S100-eGFP stratum radiatum appeared unaltered

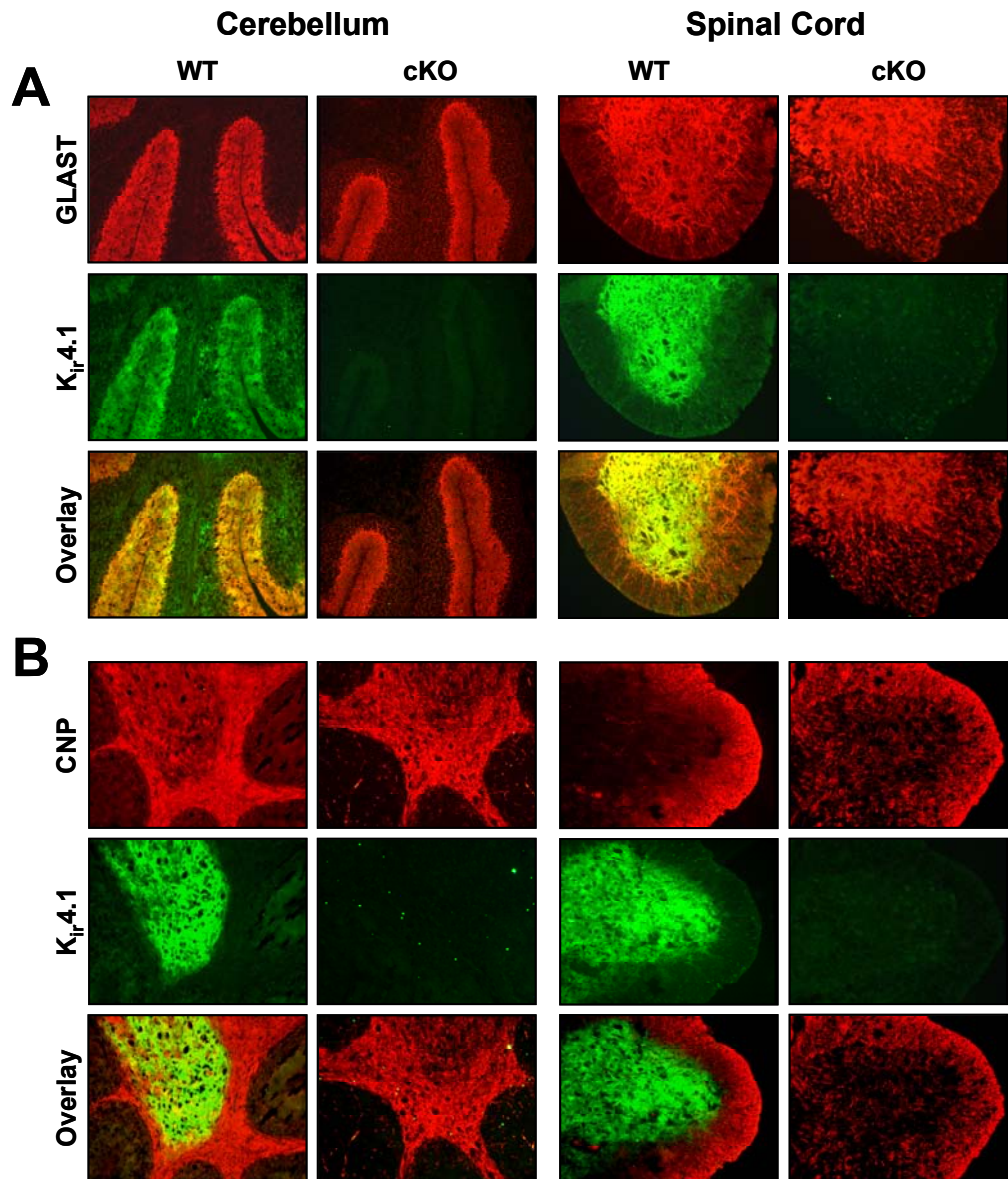


Figure 6. Loss of K_{ir}4.1 from astrocytes and oligodendrocytes. Fluorescent immunostaining for K_{ir}4.1, astrocyte marker GLAST (A), and oligodendrocyte marker CNP (B) in the WT and K_{ir}4.1 cKO cerebellum and spinal cord (P21).

(**Fig. 5C**). The same was observed in other brain regions of the $K_{ir}4.1$ cKO/S100-eGFP and in $K_{ir}4.1$ cKO/GFAP-eGFP mice (data not shown).

As expected, $K_{ir}4.1$ cKO hippocampus displayed complete loss of $K_{ir}4.1$ (**Fig. 5B**), but to our surprise $K_{ir}4.1$ staining could not be seen in any other area of the cKO brain. Since $K_{ir}4.1$ is expressed by astrocytes and oligodendrocytes, this finding was suggestive of *gfa2* driven Cre recombination in both of these cell types. Further studies in our and other laboratories confirmed *gfa2* activity in GFAP+ progenitor cells that give rise to astrocytes, oligodendrocytes, and neurons (Malatesta et al., 2003; Casper and McCarthy, 2006). Double immunostaining with $K_{ir}4.1$ antibody and the above outlined cellular markers confirmed the loss of $K_{ir}4.1$ from GLAST expressing astrocytes (**Fig. 6A**) and CNP expressing oligodendrocytes (**Fig. 6B**) throughout brain and spinal cord of $K_{ir}4.1$ cKO mice. Reduction of staining for myelin proteins (CNP, MBP, and PLP) was observed, indicating loss of myelin (**Fig. 6B**, CNP shown). GFAP and neurofilament staining in the $K_{ir}4.1$ cKO revealed disorganized and fragmented processes of cerebellar Bergman glia, spinal cord white-matter astrocytes and motor neurons (data not shown). $K_{ir}4.1^{f/f}$ and $K_{ir}4.1^{+/-}$ brains and spinal cords were comparable to the WT controls, confirming that neither floxing nor the removal of one copy of the $K_{ir}4.1$ gene influences $K_{ir}4.1$ distribution and function.

2.3. $K_{IR}4.1$ LOSS LEADS TO CNS MORPHOLOGICAL CHANGES AND WHITE-MATTER VACUOLIZATION

To further examine cellular basis of the observed $K_{ir}4.1$ cKO behavioral phenotype, brains and spinal cords of WT, $K_{ir}4.1^{f/f}$, $K_{ir}4.1^{+/-}$, and cKO mice (P10-25) were paraffin embedded, serially cut into coronal or sagittal 4 μ m sections and stained with histological stains hemotoxylin (purple nuclear stain), eosin (pink cytoplasm stain), and solochrome (blue

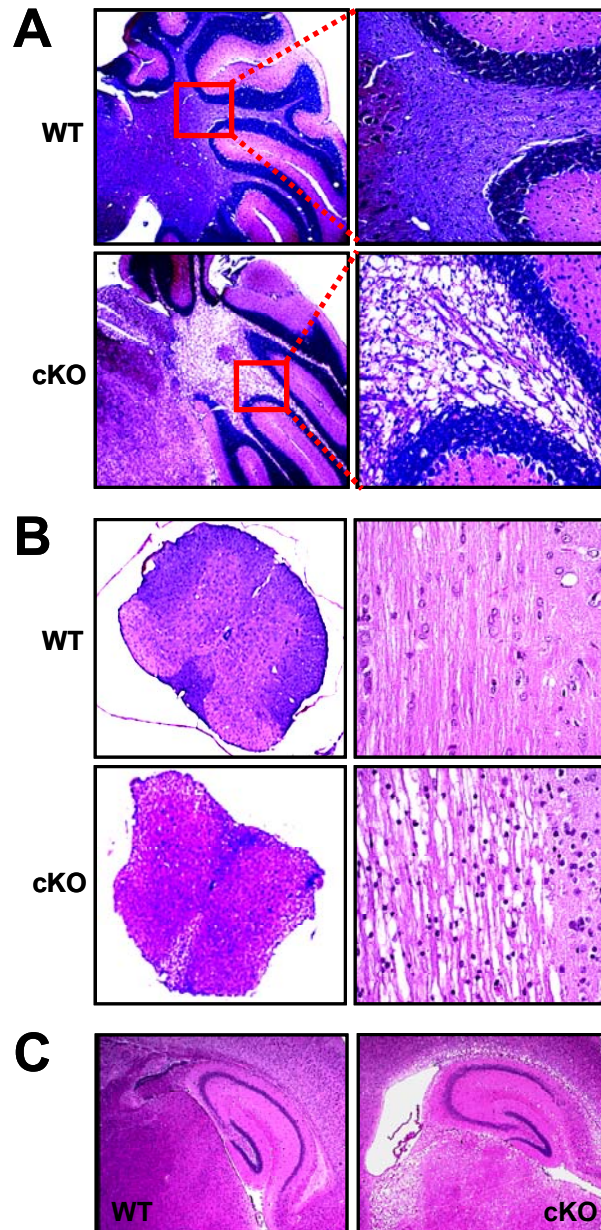


Figure 7. CNS morphological changes and white-matter vacuolization in the Kir4.1 cKO. (A) Paraffin embedded saggital sections of the WT and Kir4.1 cKO cerebellum stained with solochrome and eosin (P25). (B) Left: Transverse thoracic spinal cord sections stained with solochrome and eosin; Right: Longitudinal thoracic spinal cord sections stained with hemotoxylin and eosin. (C) Saggital sections of the hippocampus stained with hemotoxylin and eosin.

myelin stain). **Figure 7** shows cerebellum (**A**), spinal cord (**B**), and hippocampus (**C**) of littermate WT and cKO animals (P25). Extensive white-matter vacuolization is evident throughout the brain and spinal cord of the $K_{ir4.1}$ cKO mouse, most notable being the depicted vacuolization of the cerebellar internal capsule, corpus callosum, thalamus, and spinal cord white-matter. Identical dysmyelinating phenotype was observed in $K_{ir4.1}^{-/-}$ mice. Electron microscopy study of their spinal cords revealed numerous vacuoles, aberrant uncompact or unattached myelin sheaths and axonal degeneration (Neusch et al., 2001). On a gross anatomical level $K_{ir4.1}$ cKO brains were smaller, with thinner cortex, smaller hippocampus, and enlarged lateral ventricles (**Fig. 7C**). Neuronal layering of cortex, hippocampus, and cerebellum appeared normal. Closer examination identified increased number of small round nuclei suggestive of gliosis (most evident in cKO spinal cord transverse section, **Fig. 7B** bottom right). Not surprisingly, demyelinating and dysmyelinating disorders are usually accompanied by gliosis, attributed to upregulation of OPCs in an attempt of remyelination (Fancy et al., 2004). Floxed and heterozygous mice did not show any histological pathology (data not shown). Our overall findings confirm the importance of $K_{ir4.1}$ in oligodendrocyte development and myelination and provide some understanding of the observed ataxia and paralysis in the $K_{ir4.1}$ cKO mice.

2.4. $K_{ir4.1}$ cKO HIPPOCAMPAL GLIA ARE SEVERELY DEPOLARIZED

Because of its laminar anatomy (**Fig.1**) and proposed involvement in learning and memory hippocampus is one of the most extensively studied structures in the brain. Electrophysiological properties of hippocampal glia (reviewed in *Physiology of Hippocampal Glia*, page 62) and neurons are well described and documented. Furthermore, numerous

studies of neuronal hyperexcitability and epileptiform activity have been conducted in this brain region. We therefore examined the effect of $K_{ir}4.1$ removal on glial development and electrophysiological properties in acutely isolated hippocampal slices. The first step in investigating the role of this channel in glial physiology was to examine changes in basal membrane properties, including resting membrane potential (V_m), membrane resistance (R_m), and whole-cell currents, caused by $K_{ir}4.1$ removal.

All of the subsequently described experiments in this and following two chapters involved preparation of either sagittal (astrocyte study) or coronal (oligodendrocyte study) hippocampal slices from brains of WT, $K_{ir}4.1^{f/f}$, $K_{ir}4.1^{+/-}$, and $K_{ir}4.1$ cKO mice (P5-30). Slices were continuously perfused with oxygenated artificial cerebrospinal fluid (ACSF) at room temperature. Cells were patched with glass pipettes (7-8 $M\Omega$ resistance) filled with standard intracellular solution containing 145 mM K-gluconate, 2 mM $MgCl_2$, 10 mM HEPES, 4 mM Mg-ATP, 14 mM phosphocreatine and 0.25 mM EGTA. Cell tracer dye Alexa 488 hydrazide (astrocyte study) or Alexa 568 hydrazide (oligodendrocyte study) was included in the patch pipette to examine cell morphology and gap junction coupling. The membrane potential was stepped from -90 mV (WT, $K_{ir}4.1^{f/f}$, $K_{ir}4.1^{f/-}$ cells) or -40 mV ($K_{ir}4.1$ cKO cells) in 20 mV steps, (between -180 and +80 mV) to measure whole-cell currents. Data constraints were set ($R_m > 1 M\Omega$; access resistance, $R_a < 50 M\Omega$; membrane capacitance, $C_m > 20$ pF) and all of the data not meeting this criteria was eliminated from the analysis. Data are reported as mean \pm standard error of the mean (SEM) and results considered significant (*) if $p < 0.05$ in a two-sample t-test assuming unequal variance.

As described in the *Physiology of Hippocampal Glia* (page 62) hippocampus contains two distinct subpopulations of astrocyte-like glia named complex (GluR) and passive (GluT)

cells due to the nature of their whole-cell current pattern (**Fig. 2**). These two populations of cells differ not only in their electrophysiological properties but in cellular marker expression, gap junction coupling, and expression of glutamate transporters versus receptors. Passive glia display large time- and voltage-independent leak currents, are consistently GFAP and GLAST immunopositive, are extensively coupled via gap junctions, and express glutamate transporters, but not ionotropic glutamate receptors. Conversely, complex cells display time- and voltage-dependent K^+ and Na^+ currents, are mostly S100 β + with about 20% of cells also being NG2+, and have functional ionotropic glutamate receptors of the AMPA subtype. As stressed earlier, numerous questions concerning astrocyte identity and nomenclature still remain to be answered; however, observations from our and other laboratories indicate that passive glia are mature protoplasmic astrocytes and will be referred to as such from here on (Matthias et al., 2003; Wallraff et al., 2004; Zhou et al., 2006). Complex glia, on the other hand, appear to be a mixed population of immature astrocytes and NG2+ multi-potent precursors (Zhou et al., 2006). In order to provide further insight into their physiology and possibly identity, we decided to study passive astrocytes and complex glia within the hippocampus, as well as mature myelinating oligodendrocytes in the corpus callosum.

Passive astrocytes (**Table I; Fig. 8A,B**) in WT CA1 stratum radiatum have a very low R_m of $4.53 \pm 0.33 \text{ M}\Omega$ which explains the large size of their whole-cell currents. Their V_m of $-82.62 \pm 0.74 \text{ mV}$ ($n=67$) is close to the potassium equilibrium potential (E_K) suggesting high resting K^+ conductance. Loss of $K_{ir}4.1$ in the cKO passive cells led to a 49 mV depolarization ($-33.43 \pm 1.64 \text{ mV}$, $n=50$) and a significant 2.4 fold increase in their R_m ($10.78 \pm 1.76 \text{ M}\Omega$, $n=50$). Floxing of the $K_{ir}4.1$ exon did not lead to significant changes in astrocyte membrane properties. Heterozygous cells ($K_{ir}4.1^{f/+}$) were slightly depolarization ($\Delta V_m = 4.41 \text{ mV}$)

without a significant change in R_m (**Fig. 8B**). Surprisingly, no change in the whole-cell currents, cell morphology, or gap junction coupling was found among the four genotypes (**Fig. 8A**, WT and cKO cells shown).

Table I	Passive Astrocyte Membrane Properties			
	WT	$K_{ir4.1}^{ff}$	$K_{ir4.1}^{+/-}$	$K_{ir4.1}$ cKO
V_m (mV)	-82.62 ± 0.74	-84.13 ± 0.86	-78.21 ± 1.62	-33.43 ± 1.64
R_m (MΩ)	4.53 ± 0.33	4.69 ± 0.57	4.55 ± 0.38	10.78 ± 1.76
n	67	22	28	50

Table II	Oligodendrocyte Membrane Properties			
	WT	$K_{ir4.1}^{ff}$	$K_{ir4.1}^{+/-}$	$K_{ir4.1}$ cKO
V_m (mV)	-72.03 ± 3.10	-68.40 ± 2.40	-70.60 ± 2.51	-25.14 ± 1.40
R_m (MΩ)	25.95 ± 4.50	25.87 ± 3.40	20.23 ± 3.10	15.83 ± 4.71
n	18	16	20	16

Oligodendrocyte study (**Table II**; **Fig. 8C,D**) was limited to corpus callosum cells that had myelinating morphology and whole-cell decaying currents typical of mature oligodendrocytes (Gipson and Bordey, 2002; Berger et al., 1991). WT oligodendrocytes displayed R_m of 25.95 ± 4.50 M Ω and V_m of -72.03 ± 3.10 mV (n=18), in agreement with the literature reports (Berger et al., 1991). Comparable to passive astrocyte data, there was no significant difference in V_m and R_m between WT and $K_{ir4.1}^{ff}$ or $K_{ir4.1}^{+/-}$ oligodendrocytes (**Fig. 8D**), but $K_{ir4.1}$ cKO cells were depolarized 47 mV (-25.14 ± 1.40 mV, n=16). In contrast to $K_{ir4.1}$ cKO passive astrocytes, we observed a significant decrease in cKO oligodendrocyte R_m (15.83 ± 4.71 M Ω , n=16), which can possibly be explained by the loss and/or leakiness of their membrane due to the myelin damage that was observed by histology. Upon tracer dye (Alexa 568 hydrazide) filling of $K_{ir4.1}$ cKO oligodendrocytes, we

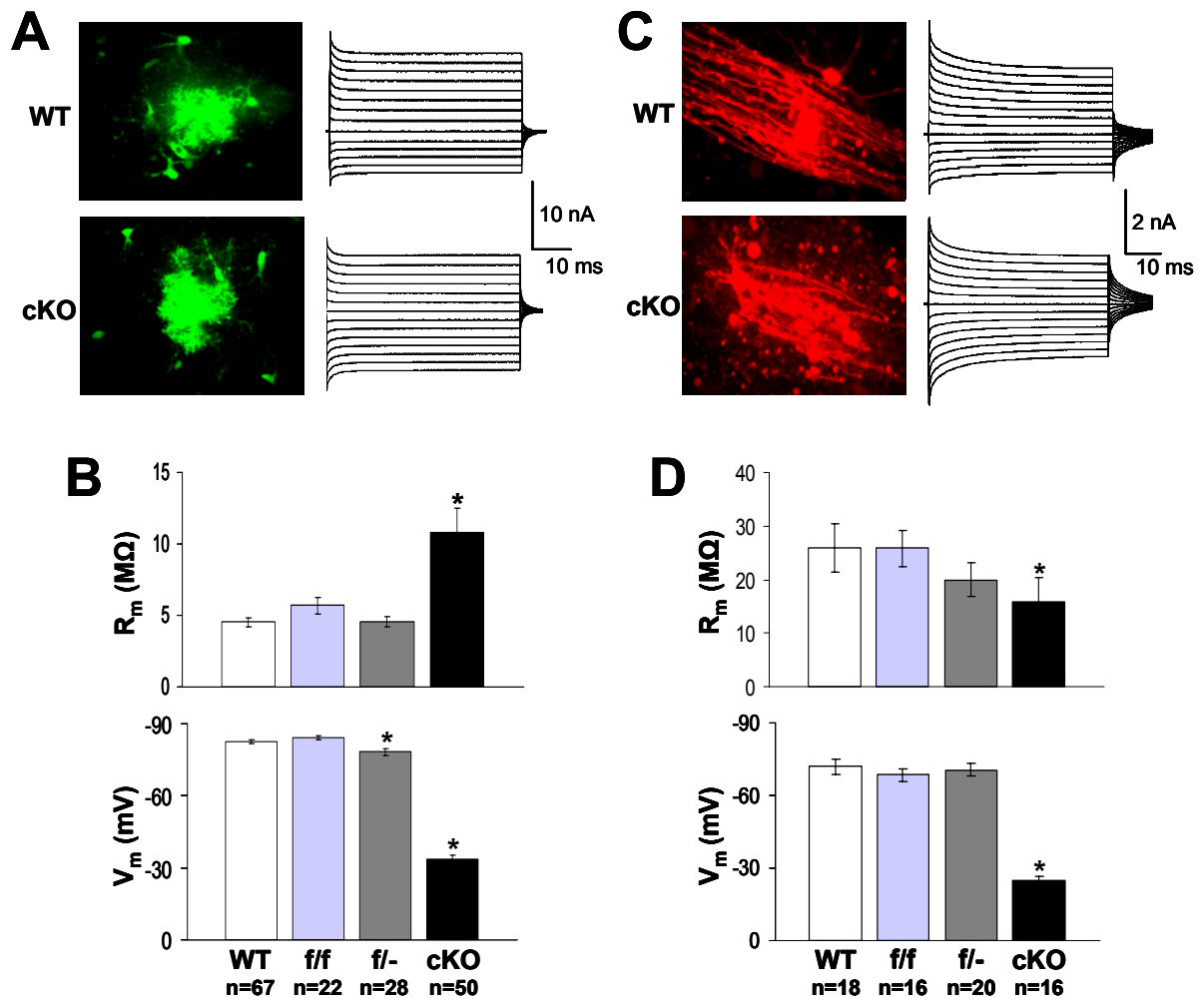


Figure 8. Morphology and membrane properties of passive astrocytes and myelinating oligodendrocytes. (A) Left: Astrocyte gap junction coupling assessed by diffusion of the Alexa 488 hydrazide tracer dye. Right: Representative whole-cell currents of WT and $K_{ir}4.1$ cKO passive astrocytes. (B) Membrane resistance (R_m) and resting membrane potential (V_m) of passive astrocytes in the CA1 stratum radiatum. Mean \pm SEM, * p <0.05 (C) Left: Morphology of mature myelinating oligodendrocytes in WT and $K_{ir}4.1$ cKO corpus callosum. Right: Representative whole-cell currents of WT and cKO oligodendrocytes. (D) Membrane resistance (R_m) and resting potential (V_m) of oligodendrocytes in the corpus callosum. Mean \pm SEM, * p <0.05

saw a reduction in the number of myelinated parallel fibers and appearance of large round swellings along the fibers (**Fig. 8C**).

Complex glia (**Table III**; **Fig. 9**) in WT (V_m : -76.16 ± 2.17 mV, R_m : 132.53 ± 38.78 M Ω , $n=23$), $K_{ir4.1}^{f/f}$ and $K_{ir4.1}^{f/-}$ CA1 stratum radiatum displayed R_m and V_m similar to values reported in literature (Bordey and Sontheimer, 2000; Matthias et al., 2003). Surprisingly, complex cells could not be found during our initial study in the hippocampus of P10-30 $K_{ir4.1}$ cKO mice. As mentioned earlier, during hippocampal development the number of complex cells decreases with increasing age of the animal (Kressin et al., 1995; Bordey and Sontheimer, 2007; Zhou et al., 2006). We therefore examined hippocampi of younger animals (P5-10) that usually contain >70% of complex cells. Three different $K_{ir4.1}$ cKO mice were examined and 4 out of 57 (7%) patched cells were of the complex type. Substantial reduction of inward currents was observed in all of the cells patched (**Fig. 9A**). Furthermore, complex glia in the $K_{ir4.1}$ cKO hippocampus were markedly depolarized ($\Delta V_m = 37.66$ mV, -38.50 ± 6.58 mV, $n=4$) and exhibited a 4.1 fold increase in R_m (536.63 ± 149.15 M Ω , $n=4$; **Fig. 9B**). These changes were similar to those observed in $K_{ir4.1}$ cKO passive cells.

Table III	Complex Glia Membrane Properties			
	WT	$K_{ir4.1}^{f/f}$	$K_{ir4.1}^{+/-}$	$K_{ir4.1}$ cKO
V_m (mV)	-76.16 ± 2.17	-80.50 ± 3.89	-79.56 ± 0.90	-38.50 ± 6.58
R_m (MΩ)	132.53 ± 38.78	181.03 ± 62.25	109.06 ± 25.75	536.63 ± 149.15
n	23	14	12	4

Due to the observed apparent loss of complex glia in $K_{ir4.1}$ cKO animals, we decided to quantify relative number of complex cells encountered in the WT versus cKO hippocampus (**Fig. 9C**). Since no difference was found between WT, $K_{ir4.1}^{f/f}$ and $K_{ir4.1}^{f/-}$ hippocampi those data were pooled and termed WT*. There was an 11 fold decrease in % of complex

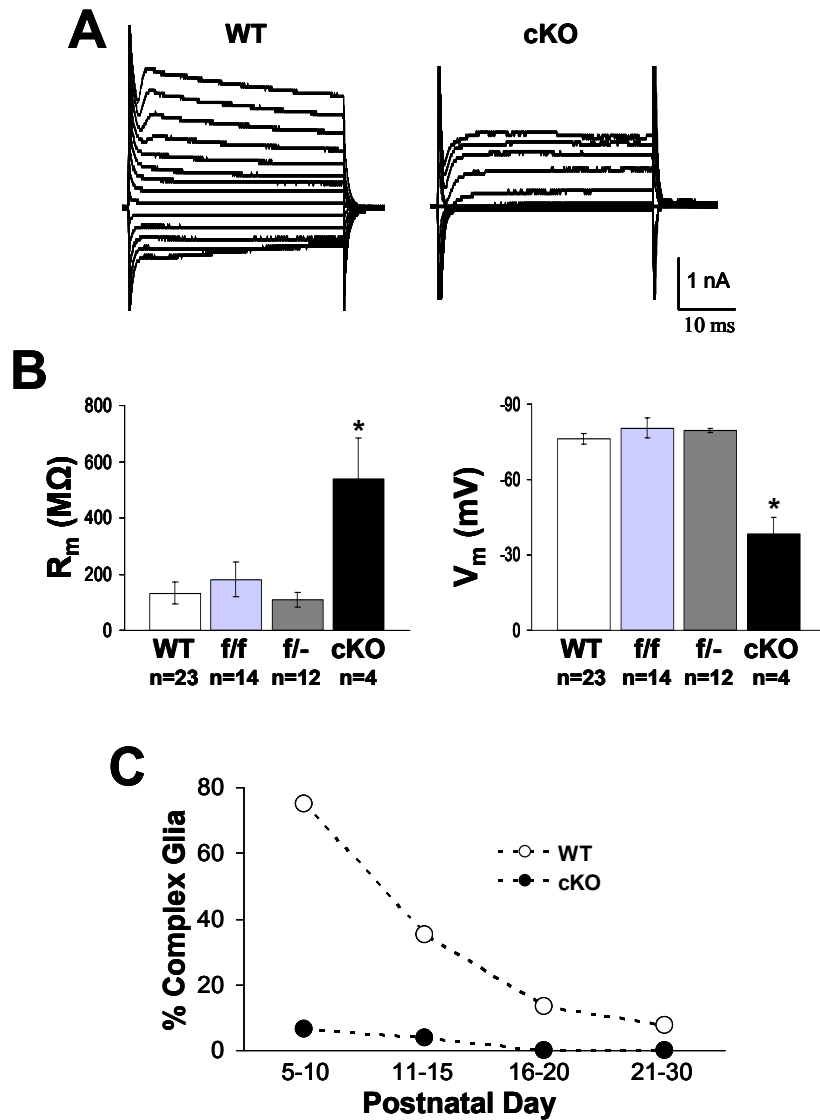


Figure 9. Membrane properties and distribution of complex glia. (A) Representative whole-cell currents of WT and $K_{ir}4.1$ cKO complex glia in CA1 stratum radiatum. (B) Membrane resistance (R_m) and resting membrane potential (V_m) of complex cells. Mean \pm SEM, * $p < 0.05$ (C) Relative percent (%) of patched complex cells within WT and $K_{ir}4.1$ cKO hippocampus.

cells in P5-10 cKO hippocampus from 75% (35/47) in WT* to 7% (4/57) in $K_{ir}4.1$ cKO, and a 9 fold decrease in P11-15 cKO from 36% (39/95) in WT* to 4% (1/26) in $K_{ir}4.1$ cKO. Complex cells were never encountered in P16-20 or P21-30 cKO slices, while % of complex cells in WT* hippocampus in those age groups was 13% (16/121) and 8% (4/49), respectively.

In summary, we observed several parallels between the three studied glial populations in changes of electrophysiological properties caused by removal of $K_{ir}4.1$, the main one being their striking membrane depolarization. This finding implicates that $K_{ir}4.1$ sets the membrane potential of passive and complex glia, as well as mature oligodendrocytes. In addition, decrease in the proportion of complex cells encountered within the CA1 stratum radiatum is suggestive of $K_{ir}4.1$ playing a role in their development.

3. DISCUSSION

K_{ir} channels are expressed by a wide variety of excitable and non-excitable cells and play an important role in the maintenance of their resting membrane potential and proper K^+ homeostasis (reviewed in Reimann and Ashcroft, 1999; Neusch et al., 2003). $K_{ir}4.1$ was the first cloned K_{ir} channel expressed predominantly in glial cells, namely astrocytes and oligodendrocytes (Takumi et al., 1995). Its channel properties and localization along astrocyte processes surrounding neuronal elements and blood vessels make it an excellent candidate for mediating the K^+ uptake by these cells (Higashi et al., 2001). We generated a conditional knockout of $K_{ir}4.1$ driven by the *gfa2* promoter in order to study its role in astrocyte physiology and K^+ buffering. This promoter consists of 2.2 kilobase of 5'-flanking DNA of the human GFAP gene and has been found to drive astrocyte-specific expression in

cultured cells and *gfa2-lacZ* reporter mouse line (Besnard et al., 1991; Brenner et al., 1994). However, during the initial characterization of the transgenic *gfa2-Cre* line created in our laboratory it became evident that *gfa2* promoter is active in GFAP+ progenitor cells that give rise not only to astrocytes, but also to a large population of oligodendrocytes and neurons throughout brain (Casper and McCarthy, 2006). Numerous studies have since confirmed our findings. GFAP-expressing neuronal precursors have been found in subventricular zone, dentate gyrus, and cerebellum (Doetsch et al., 1999; Laywell et al., 2000; Fukuda et al., 2003; reviewed in Doetsch, 2003). Malatesta et al. (2003), using Cre/loxP *in vivo* fate-mapping, established that GFAP+ radial glia produce most of the cortical projection neurons, astrocytes, and oligodendrocytes, as well as a subpopulation of NG2+ glia. Through appropriate breeding of $K_{ir}4.1^{f/f}$ and *gfa2-Cre* mouse lines we obtained $K_{ir}4.1$ cKO mice and began their characterization by establishing the extent and localization of Cre-mediated $K_{ir}4.1$ excision. Western blot and immunostaining analysis demonstrated loss of $K_{ir}4.1$ from the brain and spinal cord of cKO animals, while $K_{ir}4.1$ protein levels were unchanged in all other tissues examined (kidney and heart). The complete lack of $K_{ir}4.1$ expression in the cKO nervous tissue suggests that Cre-dependent excision of the floxed $K_{ir}4.1$ gene occurs prior to the differentiation of the GFAP+ multi-potent precursors. In fact, the first appearance of $K_{ir}4.1$ mRNA in the brain (E13-15; Ma et al., 1998) actually coincides with the appearance of the *gfa2* promoter-driven Cre expression in radial glia (E12-14) that later generate most CNS neurons and macroglia (Malatesta et al., 2003). To generate a true astrocyte-specific knockout we would therefore need to control the onset of Cre expression and avoid astrocyte development. Such temporal control of transgene expression is possible with the use of

inducible conditional knockout technology (reviewed in Bockamp et al., 2002) and our laboratory is currently in the process of screening several inducible systems.

The behavioral phenotype of our $K_{ir}4.1$ cKO mice is very similar to that of the previously described $K_{ir}4.1^{-/-}$ mice (Neusch et al., 2001) and includes severe defects in control of voluntary movements, posture and balance, ataxia, hind leg paralysis, body tremor, and premature death. The $K_{ir}4.1^{-/-}$ mice, however, die on average two weeks earlier than the $K_{ir}4.1$ cKO animals, probably due to the kidney associated pathology since $K_{ir}4.1$ appears to play a major role in K^{+} recycling by kidney tubular epithelium (Ito et al., 1996; Fujita et al., 2002). Surprisingly, no reports of seizures have been made in $K_{ir}4.1^{-/-}$ animals, while we observe clonic and tonic seizures in response to stressful stimulation in all examined $K_{ir}4.1$ cKO mice. A possible explanation for this discrepancy is the early lethality of $K_{ir}4.1^{-/-}$ animals. The seizures in $K_{ir}4.1$ cKO mice are much more pronounced after the second postnatal week by which time most of $K_{ir}4.1^{-/-}$ animals have already deceased. Seizure occurrence may reflect neuronal hyperexcitability due to the impairment of K^{+} buffering by astrocytes lacking K_{ir} conductance. The causative role of decreased astrocyte K_{ir} conductance in epileptogenesis has been substantiated by several *in situ* and *in vivo* studies (Gabriel et al., 1998; D'Ambrosio et al., 1999; Schroder et al., 1999; Bordey et al., 2001; reviewed in *Astrocyte Dysfunction in Epilepsy*, page 32). However, we cannot exclude the influence of the observed oligodendrocyte pathology in the $K_{ir}4.1$ cKO hyperexcitability and motor impairment. Severe white-matter vacuolization, accompanied by a decrease in the level of myelin proteins, CNP, MBP, and PLP, is evident throughout the brain and spinal cord of $K_{ir}4.1$ cKO animals. Neusch et al. (2001) reported similar pathology in the spinal cord of $K_{ir}4.1^{-/-}$ mice and ascribed it to the failure of myelin production and compaction by $K_{ir}4.1^{-/-}$

oligodendrocytes. Their electron microscopy study revealed that spinal cord hypo/dysmyelination is accompanied by axonal degeneration and glial apoptosis, both of which are probably present in the brain as well. Such hypo/dysmyelination and consequent axonal degeneration were previously shown to occur following genetic ablation of proteins directly involved in the structure or metabolism of myelin, such as in PLP/DM20^{-/-} mice (Griffiths et al., 1998; Kondo et al., 1995; Sanchez et al., 1996; King et al., 1997; Brady et al., 1999). Thus, the white-matter pathology caused by the loss of K_{ir}4.1 represents the first report of an ion channel playing a role in myelination, apparently through its role in oligodendrocyte development and maturation (Neusch et al., 2001). The diverse roles that K_{ir} channels play in cell development and ontogenesis are beginning to emerge from several transgenic animal studies (reviewed in Neusch et al., 2003). For example, studies of the “weaver mouse”, which contains a spontaneous point mutation in the pore region of the K_{ir}3.2 subunit, leading to the loss of its K⁺ selectivity (Kofuji et al., 1996; Slesinger et al., 1996), have provided strong evidence that K_{ir} channels play a role in neurogenesis. Defective differentiation of cerebellar and substantia nigra neurons is observed, as well as cell-cycle abnormalities, failed axogenesis and migration of cerebellar granule neurons, all eventually leading to granule cell apoptosis and cerebellar atrophy (Maricich et al., 1997; Oo et al., 1996; Harrison and Roffler-Tarlov, 1998; Migheli et al., 1999). Interestingly, homozygous weaver mice display a very similar behavioral phenotype as the K_{ir}4.1 cKO animals, including ataxia, tremor, and tonic-clonic seizures (Eisenberg and Messer, 1989).

In addition to their role in ontogenesis, K_{ir} channels have classically been thought of as responsible for maintaining the neuronal resting membrane potential and thereby setting a threshold for their excitation (Hille, 1992). However, it has become apparent that glial

membrane potential also depends on K_{ir} expression. In oligodendrocyte and astrocyte precursor cells, upregulation of K_{ir} channels is associated with the development of their highly negative membrane potential (Berger et al., 1991; Knutson et al., 1997; Kressin et al., 1995; Bordey and Sontheimer, 1997). The development of hippocampal GLAST+/GFAP+ astrocytes progresses through a series of electrophysiological phenotypes, from outwardly rectifying to variably rectifying to passive (**Fig. 2**; reviewed in *Physiology of Hippocampal Glia*, page 62). The K_{dr} , K_a , and Na_v conductances observed in early postnatal days (P1-15) are slowly downregulated and replaced by increasing K_{ir} and passive voltage-independent conductances (Kressin et al., 1995; Zhou et al., 2006). Functional expression of K_{ir} s and their importance in membrane potential maintenance have been demonstrated in astrocytes and oligodendrocytes by pharmacological studies *in vitro* and *in situ* (Amedee et al., 1997; Kettenmann and Ransom, 1995; Bordey and Sontheimer, 1997). $K_{ir4.1}$ in particular appears to be necessary for the membrane potential maintenance of Müller glia and cultured oligodendrocytes, as these cells undergo severe depolarization upon its loss in $K_{ir4.1}^{-/-}$ mice (Kofuji et al., 2000; Neusch et al., 2001). However, presence and functionality of K_{ir} channels in passive glia is questionable since their whole-cell currents do not display any voltage dependence. Furthermore, studies of passive current properties are very limited as they were largely ignored by glial electrophysiologists due to their enigmatic leak properties and apparent insensitivity to K^+ channel blockers (Kressin et al., 1995). Interestingly, our data demonstrate that $K_{ir4.1}$ appears to be critical for the membrane potential development and/or maintenance of both glial types in the hippocampus, as well as mature myelinating oligodendrocytes in the corpus callosum. We find that passive astrocytes, complex glia, and oligodendrocytes lacking $K_{ir4.1}$ are all markedly depolarized (40-50 mV) as compared to

wild-type cells. However, even though an expected increase in membrane resistance, usually associated with channel loss, was seen in both passive (2.5 fold increase) and complex (4 fold increase) $K_{ir}4.1$ cKO glia, only in complex cells did we see the appropriate loss of inward current suggestive of $K_{ir}4.1$ functional expression in this cell type. Oligodendrocyte membrane resistance was actually decreased in the cKO animals, possibly due to the observed myelin damage. Furthermore, the large leak currents of passive astrocytes and oligodendrocytes remained mostly unaffected following $K_{ir}4.1$ deletion. It should be noted that membrane resistance of passive cells was only ~ 10 M Ω even after a 2.5 fold increase in the $K_{ir}4.1$ cKO, which is 50 fold less than R_m of $K_{ir}4.1$ cKO complex glia. After observing these somewhat contradicting findings of the severe cell depolarization accompanied by seemingly unchanged current properties in $K_{ir}4.1$ cKO passive glia and mature oligodendrocytes, we thought of two possible explanations. The first one considers our ability to record K_{ir} conductance in the presence of large passive currents, and the second one considers the influence of the knockout-induced developmental changes on the cell physiology and membrane properties.

Several studies implicate that $K_{ir}4.1$ localization and clustering are critical for its function (Connors and Kofuji, 2000; Amiry-Moghaddam et al., 2003; Saadoun et al., 2003). In Müller glia $K_{ir}4.1$ appears to be functionally coupled to AQP4 and localized to the cells' endfeet by the dystrophin-glycoprotein complex (Connors and Kofuji, 2006). Even though the existence of such $K_{ir}4.1$ /AQP4 clustering mechanisms has not yet been directly demonstrated in hippocampal astrocyte processes, an electron microscopy study did confirm $K_{ir}4.1$ presence in astrocyte perisynaptic and perivascular processes (Higashi et al., 2001). Furthermore, mice lacking a component of the dystrophin-glycoprotein complex, α -syntrophin, show impaired

K⁺ uptake in the hippocampus (Amiry-Moghaddam et al., 2003). Considering the complexity and volume of the protoplasmic astrocyte process network (beautifully demonstrated by Bushong et al., 2002), the space clamp issues in such process bearing cells, and the size of their leak conductance, it is easy to imagine that currents generated by K_{ir} channels located in distal perisynaptic and perivascular processes may be impossible to record. Understanding the nature and characteristics of leak currents may enable their inhibition and subsequent unmasking of the K_{ir} and/or other voltage-gated conductances in passive astrocytes. This issue was addressed and will be described in the following chapter.

As already discussed, the *gfa2*-driven Cre recombination of the floxed K_{ir}4.1 locus occurs early in development, prior to differentiation of the GFAP⁺ progenitors into different glial types. This implies that astrocytes and oligodendrocytes in our K_{ir}4.1 cKO mice lack K_{ir}4.1 from their birth. Demonstrating the importance of K_{ir} expression in cellular development, block and genetic inactivation of K_{ir} channels have been shown to inhibit or alter cell-cycle progression and proper development of astrocytes, oligodendrocytes, and neurons (MacFarlane and Sontheimer, 1997; Sontheimer et al., 1989; Neusch et al., 2001; Marichich et al., 1997). In the rat optic nerve, oligodendrocyte expression of K_{ir}4.1 substantially increases during the major period of oligodendrocyte maturation and commencement of myelination (P5-10; Kalsi et al., 2004). Consistent with this observation, lack of K_{ir}4.1 in cultured oligodendrocytes appears to slow differentiation, maintaining the cells in an immature state incapable of proper myelination (Neusch et al., 2001). Optic nerve astrocytes, on the other hand, upregulate K_{ir}4.1 expression after P10, which correlates with the development of their highly negative membrane potential (Kalsi et al., 2004). Similarly, during hippocampal development K_{ir} conductance of complex glia progressively increases as

these cells mature into passive astrocytes displaying membrane potential close to E_K (Kressin et al., 1995; Bordey and Sontheimer, 1997; Zhou et al., 2006). The proportion of $I_{K_{ir}}$ expressing cells, termed variably rectifying glia, in the rat hippocampus peaks at P5 (~60%) when all of them are GLAST+, and then slowly declines to ~10% in the adult where all of these cells are NG2+ (Zhou et al., 2006). In agreement with this study, we find that 75% of the patched glia in mouse P5-10 hippocampal slices are of complex phenotype, either outward- or variably-rectifying. Their number declines to 8% in P21-30 slices when only variably rectifying glia are found. Interestingly, we observe a striking loss of complex glia in $K_{ir}4.1$ cKO hippocampus. Between P5-10 only 7% of the patched cells were of complex type, and following P15 we never encountered complex glia in the $K_{ir}4.1$ cKO CA1 stratum radiatum. These observations suggest that $K_{ir}4.1$ loss influences development of both GLAST+ and NG2+ glia. To look at possible changes in glial number, distribution, and/or morphology we crossed $K_{ir}4.1$ cKO mice to two reporter lines, GFAP-eGFP and S100 β -eGFP. In the adult GFAP-eGFP mice eGFP is expressed by GFAP+/GLAST+ passive astrocytes (Nolte et al., 2001), while in the S100 β -eGFP mouse line eGFP is found in both GFAP+ and NG2+ glia (Vives et al., 2003; Hachem et al., 2005). Surprisingly, no apparent changes in the eGFP staining pattern were observed between wild-type and $K_{ir}4.1$ cKO hippocampi. We did notice marked disorganization of astrocyte processes in $K_{ir}4.1$ cKO cerebellar and spinal cord white-matter which is probably a consequence of the severe white-matter vacuolization. A detailed study involving electron microscopy and glial lineage tracing would be necessary to unequivocally rule out influence of $K_{ir}4.1$ loss on astrocyte morphology and differentiation; however, the lack of apparent alterations in astrocyte distribution and morphology in the $K_{ir}4.1$ cKO mice crossed with transgenic eGFP reporter

lines and during patch filling of cKO astrocytes with a fluorescent tracer dye, suggest that loss of $K_{ir}4.1$ expression mainly influences development of their electrophysiological properties. $K_{ir}4.1$ cKO astrocytes appear to be unable to follow the natural pattern of their electrophysiological maturation from the outward rectifying to variably rectifying to passive, and progress directly from outward rectifying to passive phenotype. Post-staining of patched cells would be required to determine the fate of the NG2+ glia. Most likely the few complex glia we patched in P5-10 $K_{ir}4.1$ cKO hippocampus were of this type, but by the adulthood they too appear to become passive.

Complex cells within hippocampus have been shown to express other K_{ir} s, including $K_{ir}2.1-2.3$ (Schroder et al., 2002). Why these channels do not compensate for $K_{ir}4.1$ loss could possibly be explained by the late onset of their expression. $K_{ir}4.1$ expression appears early in the embryonic development. $K_{ir}4.1$ transcripts are already evident in the rat cerebral cortex, thalamus, and hypothalamus at E13-15, and by E17-20 they are also found in the hippocampus (Ma et al., 1998). $K_{ir}2.1-2.3$ mRNAs appear in the peripheral nervous system during the embryonic development (E13), but in the hippocampus they are observed only after P10 (Karschin and Karschin, 1997). The early expression of $K_{ir}4.1$ and its influence on the cell membrane potential and K^+ homeostasis may be necessary to put into motion the cell-cycle mechanisms responsible for development of the cells' final channel, transporter, and receptor complement. Immature glia lacking $K_{ir}4.1$ may, therefore, fail to express a required set of channels and transporters necessary for maintenance of ion gradients across their membrane resulting in the severely depolarized passive glia we find in the $K_{ir}4.1$ cKO hippocampus. Maintenance of astrocyte membrane potential close to E_K is one of the prerequisites for their ability to take up extracellular K^+ . Furthermore, steep Na^+ and K^+

gradients across astrocyte membrane are necessary for their volume and pH regulation, neurotransmitter uptake, and Ca^{2+} signaling, all of which are extremely important for proper CNS functioning (reviewed in *Astrocyte Functions*, page 13). Studies of the $\text{K}_{\text{ir}}4.1$ involvement in the maintenance of astrocyte membrane potential and the influence that $\text{K}_{\text{ir}}4.1$ loss exerts on astrocyte and neuronal functioning are described in the following two chapters.

CHAPTER III. ROLE OF $K_{IR}4.1$ IN ASTROCYTE MEMBRANE POTENTIAL MAINTENANCE

1. INTRODUCTION

The classic studies of Kuffler et al. (1966) and Orkand et al. (1966) first described electrophysiological properties of vertebrate glia *in situ*. Sharp microelectrode impalements of glia in the frog and mud puppy optic nerve revealed that glial cells had low membrane potential between -70 and -90 mV, failed to display voltage-dependent conductances, were electrically coupled to neighboring glial cells, and responded to alterations in $[K^+]_{out}$ with E_m changes that closely followed the Nernst relation for a K^+ -selective electrode. The first electrophysiological recordings from mammalian glia *in situ* (Tasaki and Chang, 1958) revealed that like glia in amphibian optic nerve, “silent cells” presumed to be astrocytes had low resting membrane potential and responded to cortical stimulation with slow depolarizations. Subsequent studies in the mammalian spinal cord, cortex, and optic nerve all confirmed almost exclusive K^+ permeability of the glial membrane and lack of voltage-dependent currents (Lothman and Somjen, 1975; Futamachi and Pedley, 1976; Picker and Goldring, 1982; Ransom and Goldring, 1973). However, with the advent of improved electrophysiological techniques and glial cell culture, it was later shown that astrocytes express a variety of voltage-gated channels (reviewed in Verkhratsky and Steinhäuser, 2000).

The GFAP/GLAST-immunopositive astrocytes within mammalian hippocampus undergo a developmental progression from so called complex phenotype, displaying multiple voltage-

gated conductances including $I_{K_{dr}}$, I_{K_a} , $I_{K_{ir}}$, and I_{Na_v} , to passive glia which resemble the originally described “silent cells” (Kressin et al., 2005; Zhou et al., 2006; reviewed in *Physiology of Hippocampal Glia*, page62). In the early postnatal days, the current pattern of complex cells is dominated by large outward K^+ currents generated by TEA-sensitive K_{dr} and 4-AP-sensitive K_a channels. Some TTX-sensitive I_{Na_v} is also seen, usually prior to P15-20. During maturation the contribution of these conductances significantly decreases and is accompanied by a substantial increase in the $I_{K_{ir}}$ and the passive voltage-independent conductance, until the adulthood when all that can be seen are the passive currents. These developmental changes are further accompanied by the hyperpolarization of the astrocytes membrane (Kressin et al., 1995; Bordey and Sontheimer; 1997), increase in their glutamate and K^+ uptake capability (Kalsi et al., 2004; Matthias et al., 2003), and enhanced gap junction coupling (Kressin et al., 1995; Wallraf et al., 2004). Due to their increased conductance at negative potentials, K_{ir} channels are thought to underlie the developmental hyperpolarization of the astrocyte membrane potential and to maintain it close to the E_K (Kressin et al., 1995; Bordey and Sontheimer; 1997). However, one caveat in all of the previous studies of K_{ir} function in astrocytes was the assumption that these cells comprise a homogenous cell population. Due to perplexing and unknown nature of passive currents, passive astrocytes were generally ignored and complex cells were primarily used in studies of astrocyte electrophysiology. Reports describing electrophysiological properties of passive astrocytes are therefore extremely limited. This is in spite of the fact that passive astrocytes are the predominant type of glia encountered in the adult CNS gray matter (Lothman and Somjen, 1975; Futamachi and Pedley, 1976; Picker and Goldring, 1982; Zhou et al., 2006).

As the first identified K_{ir} channel expressed predominantly in astrocytes and oligodendrocytes, $K_{ir4.1}$ was speculated to be the membrane potential setting channel of these cells (Takumi et al., 1995). Our data indeed demonstrate large depolarization of astrocytes and oligodendrocytes in the hippocampus of $K_{ir4.1}$ cKO animals, strongly supporting this hypothesis. However, as discussed in the previous chapter, the somewhat contradicting finding that despite the severe depolarization of mature $K_{ir4.1}$ cKO astrocytes and oligodendrocytes their whole-cells currents remain seemingly unchanged, begs further clarification of $K_{ir4.1}$ role in astrocyte physiology. In the below described study we examined two possibilities that may hold the answer to these conflicting observations.

First, the $K_{ir4.1}$ cKO-induced glial depolarization may reflect a developmental phenomenon, whereby $K_{ir4.1}$ expression is needed for the development of alternate mechanisms for membrane potential maintenance in mature cells. Early appearance of $K_{ir4.1}$ in the CNS (Ma et al., 1998), decrease in the proportion of the complex glia encountered within the $K_{ir4.1}$ cKO hippocampus, and maturation impairments of the $K_{ir4.1}^{-/-}$ cultured oligodendrocytes (Neusch et al., 2001), all point to the $K_{ir4.1}$ involvement in glial development. Interestingly, an early study of ion channel properties of cultured astrocytes identified Na^+/K^+ -ATPase as the major effector of astrocyte membrane potential (Sontheimer et al., 1994). The Na^+/K^+ -ATPase is a plasma membrane ion-pump found in all animal cells. It catalyzes the active uptake of K^+ and extrusion of Na^+ , thereby establishing ion concentration gradients between the inside and outside of the cell (reviewed in Sweadner, 1995). Block of Na^+/K^+ -ATPase in neurons causes a reversible <10 mV depolarization since their membrane potential is largely set by K_{ir} and leak channels (Tavalin et al., 1997; Senatorov and Hu, 1997). In contrast, block of Na^+/K^+ -ATPase in cultured astrocytes leads

to >20 mV depolarization and subsequent cell death, suggesting that this ion-pump plays a significant role in the maintenance of ion gradients across astrocyte membrane (Sontheimer et al., 1994). As reviewed earlier in *Active Potassium Uptake by Na⁺/K⁺-ATPase* (page 51), it has been suggested that the astrocyte-expressed isoforms of Na⁺/K⁺-ATPase are significantly different from those expressed by neurons and are better suited for K⁺ uptake (Grisar et al., 1978; Franck et al., 1983; Reichenbach et al., 1992). Block of astrocyte Na⁺/K⁺-ATPase leads to increased neuronal excitability and seizures (Walts and Wuttke, 1999; D'ambrosio et al., 2000; Walz, 2000). Loss of K_{ir}4.1 may impair development of astrocyte membrane potential by influencing expression and/or activity of the Na⁺/K⁺-ATPase or other channels and transporters involved in the maintenance of their cellular ion homeostasis. Such Na⁺/K⁺-ATPase impairment would ultimately lead to neuronal hyperexcitability and seizures such as those observed in the K_{ir}4.1 cKO.

Second, technical limitations may preclude the study of passive astrocyte voltage-dependent currents, such as I_{K_{ir}}, due to presence of overwhelming voltage-independent leak conductances and extremely low membrane input-resistance. Specifically, our ability to voltage-clamp passive astrocytes in order to activate voltage-gated K_{ir} channels, located primarily on their fine processes, is extremely limited due to the space clamp issues. Imposing a uniform membrane voltage on low-resistance, morphologically complex cells linked in an electrotonic syncytium is beyond the capabilities of our current technology. We therefore may not be able to see K_{ir} channel activity, or lack there of in the K_{ir}4.1 cKO passive cells. Several investigators speculated that low membrane resistance and large leak currents of passive astrocytes arise from uninhibited intercellular current flow (Kressin et al., 2005; Bordey and Sontheimer, 2000; Walz, 2000). It is well established that astrocytes form

an interconnected cell syncytium via gap junction coupling. Filling of a single hippocampal astrocyte with a small tracer dye leads to filling of hundreds of surrounding cells (Konietzko and Müller, 1994; Frisch et al., 2003; Wallraff et al., 2004). This phenomenon is thought to facilitate important astrocyte functions such as K^+ buffering and neurotransmitter uptake (Nakase and Naus, 2004; Theis et al., 2003). Presence of large membrane pores or hemichannels capable of current conductance has also been noted in astrocytes, and they appear to be involved in the release of gliotransmitters that mediate neuron-glia crosstalk (Arcuino et al., 2002; Ye et al., 2003). Another candidate that may be responsible for the passive astrocyte currents are the newly cloned two-pore K^+ channels (K_{2P}) also termed background or leak channels (reviewed in Kim, 2005). These channels are structurally unique as compared to other K^+ channels since each subunit possesses two pore-forming domains and four transmembrane segments. In mammals, sixteen K_{2P} channel genes have been identified and divided into six subfamilies based on amino acid homology (TREK, TALK, TWIK, TRESK, THIK and TASK). They are gated by pH, stretch, heat, coupling to G-proteins and anesthetics, and although their physiological functions are largely unknown, K_{2P} channels appear to play a crucial role in setting the resting membrane potential and regulating cell excitability (Aller et al., 2005; Kang and Kim, 2006). Characterization and possible identification of channels responsible for passive astrocyte currents would not only enable their inhibition in order to increase astrocyte input-resistance and improve voltage clamp, but would also provide better understanding of astrocyte physiology and function.

A pharmacological approach was taken to further characterize passive astrocyte currents and investigate $K_{ir}4.1$ involvement in setting the astrocyte membrane potential. Our study indicates that wild-type passive astrocytes and oligodendrocytes express minimal K_{ir}

conductance, while K_{ir} channels appear to be critical for the membrane potential maintenance of complex glia. Furthermore, loss of $K_{ir}4.1$ does not change ion-selectivity properties of the passive astrocyte membrane, but it possibly affects Na^+/K^+ -ATPase function thereby indirectly leading to the membrane depolarization. We also demonstrate that passive astrocyte currents do not arise from gap junction coupling or hemichannel expression, and even though they appear to be K^+ selective, they cannot be blocked by the well-established K^+ -channel blockers. Since K_{2P} channels also display substantial resistance to K^+ -channel blockers (O'Connell et al., 2005; reviewed in Kim, 2005), they may be responsible for the passive astrocyte currents.

2. RESULTS

2.1. WILD-TYPE PASSIVE ASTROCYTES AND OLIGODENDROCYTES DISPLAY MINIMAL RESTING K_{IR} CONDUCTANCE

We first wanted to establish whether $K_{ir}4.1$ directly sets the membrane potential of astrocytes and oligodendrocytes, or is involved in its development. If $K_{ir}4.1$ directly maintains the membrane potential of glial cells within hippocampus, pharmacological block of K_{ir} channels should mimic the glial depolarization observed in $K_{ir}4.1$ cKO. Ba^{2+} ions at μM concentrations are selective blockers of K_{ir} channels and have been used as such in numerous studies (Ransom and Sontheimer, 1995; D'Ambrosio, 2002; Akopian et al., 1997). Changes in whole-cell currents, V_m , and R_m , due to the bath application of Ba^{2+} were recorded in passive astrocytes, complex glia, and oligodendrocytes in hippocampal slices. Acutely isolated 300 μm hippocampal slices were prepared from P15-25 WT and $K_{ir}4.1$ cKO mice. Glial cells were patched with standard pipette solution and their whole-cell currents in response to step voltage protocol and membrane properties were recorded in control ACSF.

Perfusion was then switched to Ba²⁺ supplemented ACSF and run for 10 min while monitoring V_m changes in current clamp. This time was sufficient for V_m to reach the steady state. At the end of the 10 min Ba²⁺ perfusion, whole-cell currents and membrane properties were recorded again. Perfusion was switched back to ACSF to look at the reversibility of the Ba²⁺ effect. All of the recordings in which R_a changed more than 10% within the 10 min recording period were taken out of the analysis, as well as those where starting R_a > 50 MΩ.

Ba²⁺ block (100 μM) led to a large 7.5 fold increase in R_m (control: 161.06 ± 64.06 MΩ, n=5; Ba²⁺: 1202.55 ± 221.34 MΩ, n=5) of WT complex glia, complete loss of inward currents and 58 mV depolarization (control: -73.66 ± 4.28 mV, n=5; Ba²⁺: -18.50 ± 5.44 mV, n=5) (**Fig. 10A,B; Table IV**). These data are consistent with data seen in the literature and confirm that K_{ir} channels directly set the resting membrane potential of complex cells (Ransom and Sontheimer, 1995; Akopian et al., 1997).

In contrast, Ba²⁺ exerted a much smaller effect on passive astrocytes and oligodendrocytes. Wild-type passive astrocytes depolarized approximately 6 mV during 100 μM Ba²⁺ application (control: -82.04 ± 1.16 mV, n=11; Ba²⁺: -76.28 ± 1.40 mV, n=6). Ba²⁺ concentration was therefore raised to 1 mM; however, even with this Ba²⁺ concentration passive astrocyte membrane depolarized only 8.9 mV on average (control: -81.24 ± 0.96 mV, n=26; Ba²⁺: -72.34 ± 2.55 mV, n=10) with a 1.8 fold increase in R_m (control: 4.30 ± 0.45 MΩ, n=26; Ba²⁺: 7.60 ± 1.58 MΩ, n=10) (**Fig. 10D; Table IV**). Similarly, 1 mM Ba²⁺ caused a relatively small depolarization of oligodendrocytes (ΔV_m= 14.7 mV), but did not significantly increase their R_m (**Fig. 10F; Table IV**). There was nevertheless a trend towards a small decrease in both outward and inward whole-cell current due to Ba²⁺ block in passive astrocytes and oligodendrocytes (**Fig. 10C,E**). A small but insignificant depolarization was

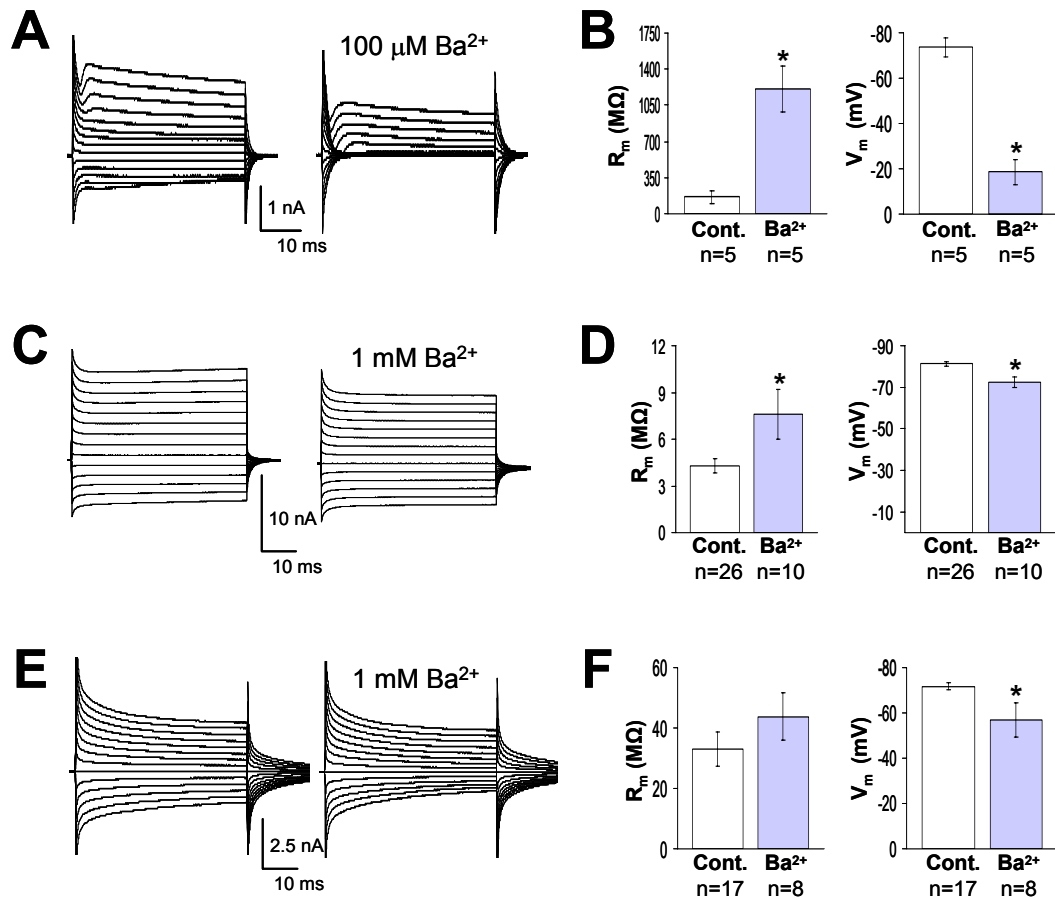


Figure 10. Influence of K_{ir} channel block on wild-type glia. Representative whole-cell current traces in control ACSF (left) and Ba^{2+} supplemented ACSF (right) from WT complex glia (A), passive astrocyte (C), and corpus callosum oligodendrocyte (E). Membrane resistance (R_m) and resting membrane potential (V_m) of complex glia (B), passive astrocytes (D), and oligodendrocytes (F) in WT hippocampus and corpus callosum, before and after Ba^{2+} application. Mean \pm SEM, * $p < 0.05$

also seen in passive $K_{ir}4.1$ cKO cells ($\Delta V_m = 5.75$ mV) without a change in their R_m (Table IV). The inability of Ba^{2+} to recapitulate passive astrocyte and oligodendrocyte depolarization caused by the loss of $K_{ir}4.1$ disputes a direct role of this channel in setting their membrane potential. Since very little is known about the nature of passive currents and the mechanism of membrane potential maintenance in passive astrocytes we proceeded by investigating those questions in following experiments.

Table IV	WT Complex Glia		WT Passive Astrocytes		cKO Passive Astrocytes		WT Oligodendrocytes	
	Control	100 μ M Ba^{2+}	Control	1 mM Ba^{2+}	Control	1 mM Ba^{2+}	Control	1 mM Ba^{2+}
V_m (mV)	-73.66 \pm 4.28	-18.50 \pm 5.44	-81.24 \pm 0.96	-72.34 \pm 2.55	-36.40 \pm 5.28	-30.65 \pm 3.35	-71.67 \pm 1.51	-56.90 \pm 7.51
R_m (M Ω)	161.06 \pm 64.06	1202.55 \pm 221.34	4.30 \pm 0.45	7.60 \pm 1.58	6.95 \pm 1.83	7.45 \pm 1.75	33.00 \pm 5.51	43.73 \pm 7.81
n	5	5	26	10	18	10	17	8

2.2. CLASSIC K^+ AND Ca^{2+} CHANNEL BLOCKERS DO NOT INFLUENCE PASSIVE ASTROCYTE CURRENTS

Passive astrocytes have a reported high K^+ permeability which is evident from their highly negative resting membrane potential close to E_K (Ransom and Goldring, 1973; Lotham and Somjen, 1975; Kressin et al., 1995; Bordey and Sontheimer, 2000). A pharmacological approach was taken to look at expression of voltage-gated K^+ and Ca^{2+} channels in these cells. Known classic K^+ channel blockers TEA, 4-AP, and Tris were used. TEA blocks delayed rectifying channels (K_{dr}) and 4-AP blocks transient A-type channels (K_a), while intracellular Tris non-selectively blocks outward K^+ conductance (Hille, 1992; Bordey and Sontheimer, 1999; Molitor and Manis, 1999; Smitherman and Sontheimer, 2001). To block Ca^{2+} channels we used Cd^{2+} ions (Hille, 1992; Smitherman and Sontheimer, 2001). Polyamine spermine, which is responsible for inward rectifying nature of K_{ir} channels

by blocking outward K^+ current (Biedermann et al., 1998; Skatchkov et al., 2000; Stanfield and Sutcliffe, 2003), was used to confirm the lack of significant K_{ir} conductance in passive cells. Another goal of this study was to gain an insight into the possible mechanism of depolarization seen in $K_{ir}4.1$ cKO glia. If any of the above listed blockers could recapitulate the cKO passive astrocyte depolarization it would suggest that $K_{ir}4.1$ deletion affects expression or function of alternate K^+ and/or Ca^{2+} channels.

Intracellular block of K^+ channels was achieved by replacing the standard K-gluconate pipette solution with a Tris pipette solution containing 90 mM Tris- PO_4 , 108 mM Tris base, 20 mM TEA, 11 mM EGTA and 10 mM sucrose. Spermine also acts from the intracellular side and was therefore added at 500 μ M to the standard pipette solution in a separate set of experiments. For extracellular block, ACSF was modified by replacing 25 mM NaCl with 20 mM TEA and 5 mM 4-AP and adding 0.5 mM $CdCl_2$ (block-ACSF). To evaluate the effect of the intracellular block, CA1 stratum radiatum WT passive astrocytes (P15-25) were patched with either standard, spermine supplemented, or Tris pipette solution. Membrane properties and currents were recorded following patching and 15 min post-patching. In the extracellular block experiments cells were patched with standard pipette solution in control ACSF. Five min after patching perfusion was switched to block-ACSF and 15 min later cell properties were recorded. To test the reliability of our methods, we did a limited number of experiments in complex cells and observed results consistent with those reported (Smitherman and Sontheimer, 2001; Bordey and Sontheimer, 1999).

Intracellular spermine block and extracellular TEA, 4-AP and Cd^{2+} block did not lead to any apparent changes of the passive astrocyte membrane properties or currents (**Fig. 11**). Mean V_m and R_m values in control and experimental conditions can be seen in **Table V**. This

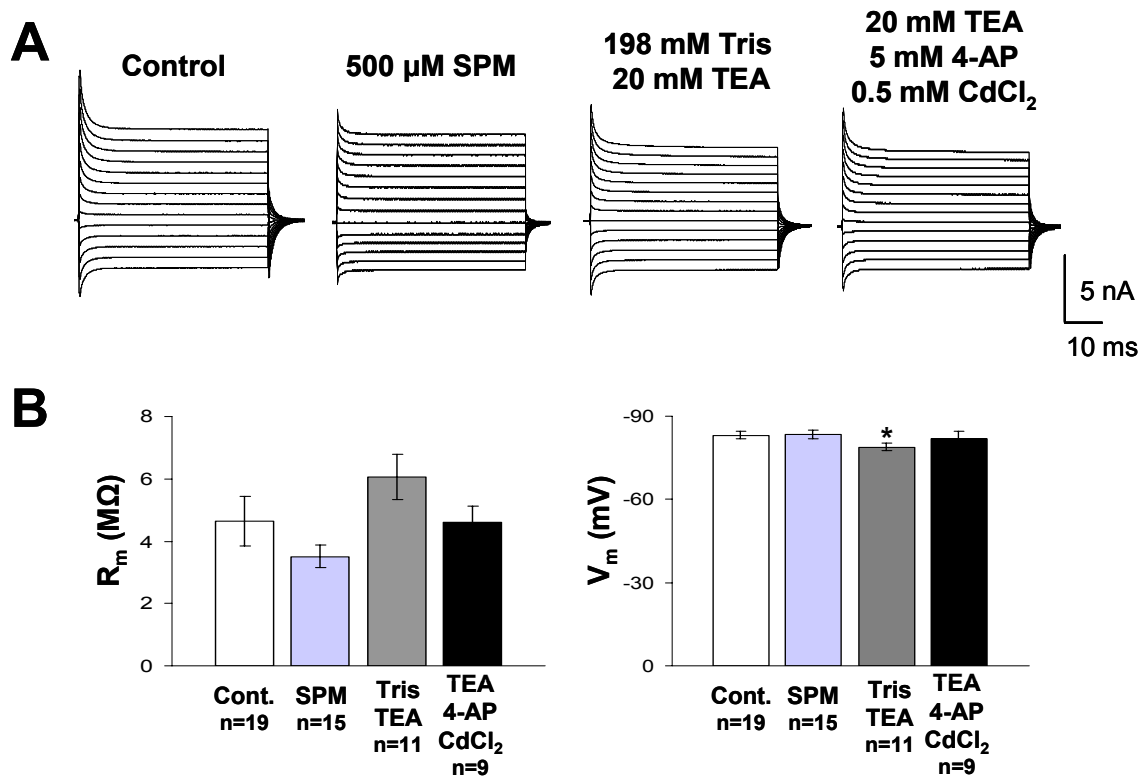


Figure 11. Influence of K^+ and Ca^{2+} channel blockers on passive currents of wild-type astrocytes. (A) Representative whole-cell currents of hippocampal astrocytes under control conditions and following K^+ and Ca^{2+} channel block with indicated blockers. (B) Membrane resistance (R_m) and resting membrane potential (V_m) of passive astrocytes in control or blocker-supplemented conditions. Mean \pm SEM, * $p < 0.05$

was not surprising as passive currents do not show any voltage- or time-dependence. However, Tris and TEA intracellular block did lead to a significant ($p=0.05$) but relatively small cell depolarization ($\Delta V_m = 4.27$ mV; -78.83 ± 1.37 mV, $n=9$), without significantly affecting their R_m (although there was a trend towards higher R_m values, 6.05 ± 0.73 M Ω , $n=9$; **Table V**, **Fig. 11B**). We suspect that the observed cell depolarization is due to the partial block of passive or leak channels by Tris-base.

Thus far presented data suggests that passive astrocytes do not have significant K_{dr} , K_a , or K_{ir} mediated resting conductance. Upon thorough review of our findings and the available literature two possible explanations for the nature of passive currents arose: presence of recently cloned two-pore K^+ leak channels (K_{2p}) and/or current dissipation due to high gap junction and hemichannel expression. We proceeded with the study of gap junction/hemichannel involvement. Study of K_{2p} is beyond the scope of this document and will not be described further.

Table V	Control	500 μM spermine	20 mM TEA 5 mM 4-AP 0.5 mM Cd²⁺	198 mM Tris 20 mM TEA
V_m (mV)	-83.1 ± 1.37	-83.54 ± 1.52	-82.04 ± 2.47	-78.83 ± 1.37
R_m (MΩ)	4.64 ± 0.81	3.51 ± 0.37	4.59 ± 0.54	6.05 ± 0.73
n	19	15	11	9

2.3. GAP JUNCTION AND HEMICHANNEL BLOCK DOES NOT ABOLISH PASSIVE ASTROCYTE CURRENTS

Due to the observed high degree of gap junction coupling and hemichannel expression in passive astrocytes, it was hypothesised that low R_m and large leak currents of these cells arise from uninhibited intercellular current flow (Walz, 2000). We tested this hypothesis by

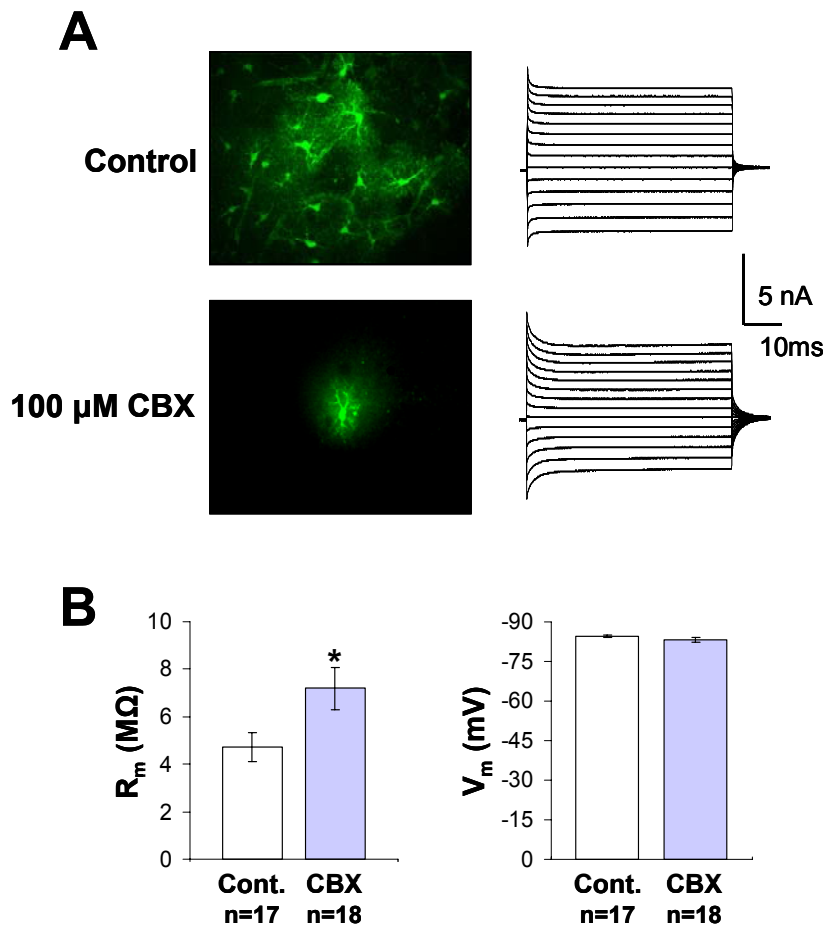


Figure 12. Influence of gap junction/hemichannel blocker carbenoxolone on passive astrocyte currents. (A) Left: Gap junction coupling of hippocampal passive astrocytes assessed by diffusion of neurobiotin before and after CBX application. Right: Representative whole-cell currents of passive astrocytes in control and CBX supplemented ACSF. (B) Membrane resistance (R_m) and resting membrane potential (V_m) of passive astrocytes before and after CBX application. Mean \pm SEM, * $p < 0.05$

examining the effect of gap junction block on passive astrocyte whole-cell currents. Astrocytes in WT (P15-25) CA1 stratum radiatum were patched in control ACSF or in the carbenoxolone (CBX) supplemented ACSF. CBX is a well characterized gap junction and hemichannel blocker and was used at a 100 μ M concentration (Peters et al., 2003; Sanchez-Alvarez, 2004). Slices were perfused with CBX supplemented ACSF for 20 min prior to patching in order to insure complete gap junction block. Tracer dyes Alexa 488 Hydrazide (500 μ M) and neurobiotin (0.5%) were included in the pipette solution to assess gap junction coupling and CBX effect. Astrocytes were patched for 10 min to allow dye diffusion. Cell membrane properties and currents were recorded in control and CBX supplemented ACSF. Slices were fixed and stained with the appropriate fluorescent antibody to visualize neurobiotin diffusion.

Carbenoxolone caused a complete block of gap junction coupling of passive hippocampal astrocytes as assessed by the lack of tracer dye diffusion in carbenoxolone treated slices (**Fig. 12A**). It also induced a significant 1.5 fold increase in astrocyte R_m (control: 4.71 ± 0.60 M Ω , n=17; CBX: 7.18 ± 0.88 M Ω , n=18), slightly decreasing the passive current size (**Fig. 12A,B**). It did not however cause any change in the cell's resting membrane potential (control: -84.55 ± 1.36 mV, n=17; CBX: -83.24 ± 2.07 mV, n=18; **Fig. 12B**). Since blocking gap junctions caused only a marginal R_m increase in passive astrocytes (R_m in CBX is still below 10 M Ω while in non-coupled cells such as complex astrocytes it is >100 M Ω) it appears that gap junction coupling and/or hemichannels do not mediate passive currents in astrocytes.

2.4. HIGH POTASSIUM SELECTIVITY OF THE PASSIVE ASTROCYTE MEMBRANE IS MAINTAINED IN THE $K_{ir4.1}$ cKO

The perplexing finding that WT passive astrocytes exhibit minimal K_{ir} conductance while deletion of $K_{ir4.1}$ causes severe depolarization of their membrane, initiated our exploration of other possible mechanisms of cKO cell depolarization. Interestingly, whole-cell currents of $K_{ir4.1}$ cKO passive astrocytes did not qualitatively change as compared to WT cell currents, suggesting that the channel complement of these cells has not changed. However, a large depolarization of the membrane potential such as that in $K_{ir4.1}$ cKO astrocytes may arise from a change in channel selectivity which would allow increased Na^+ and/or Cl^- permeability. To examine the possible change in membrane ion selectivity induced by $K_{ir4.1}$ loss, K^+ , Na^+ , and Cl^- ion substitution experiments were done by changing the extracellular concentration of these ions and observing the resulting changes of whole-cell currents, V_m , and R_m of passive cells. Ion concentrations were changed by equimolarly replacing NaCl with choline-Cl, Na-gluconate, or KCl. Control ACSF contained 2.5 mM K^+ , 153 mM Na^+ and 135 mM Cl^- . Low Na^+ ACSF contained 28 mM Na^+ , while low Cl^- ACSF contained 8 mM Cl^- . Minimal concentrations of Na^+ and Cl^- were maintained to insure proper ACSF pH buffering (by Na_2HCO_3) and working of the AgCl electrode, respectively. We employed two different experimental designs since switch from control to low $[Cl^-]_o$ ACSF caused voltage artifacts due to the nature of AgCl electrode. Whole-cell currents, V_m , and R_m were recorded in control ACSF 5 min post-patching and then again 15 min after switching to perfusion with experimental solutions. In the second set of experiments membrane properties and currents were recorded from cells patched 15 min after switching to experimental ACSF. Results were identical in the two experimental designs in Na^+ and K^+ substitution experiments and data

was therefore pooled. Only data from the second set of experiments was analyzed in the Cl⁻ substitution study.

We first used four different extracellular K⁺ concentrations ([K⁺]_{out} = 2.5, 10, 25 and 50 mM) and plotted the resulting V_m versus log [K⁺]_{out}/[K⁺]_{in} for WT and K_{ir}4.1 cKO cells. Graded increase of [K⁺]_{out} from 2.5 to 50 mM caused increasing depolarization of the passive astrocyte membrane in WT and K_{ir}4.1 cKO passive astrocytes (**Table VI, Fig. 13A**). As stated by the Nernst equation $E_K = 58.17 \log [K^+]_{out}/[K^+]_{in}$ (at 20°C). For a cell membrane selectively permeable to K⁺ ions, V_m would equal E_K and slope of V_m vs. log [K⁺]_{out}/[K⁺]_{in} would be 58.17. Reduction in the slope indicates reduction in K⁺ selectivity of the membrane. When we plot the resulting wild-type V_m values vs. log [K⁺]_{out}/[K⁺]_{in}, we obtain a slope of 50.10 confirming the high selectivity of passive astrocyte membrane for K⁺ ions. A large decrease in K⁺ selectivity of K_{ir}4.1 cKO passive astrocyte membrane was suggested by the resulting slope of 16.63 (**Fig. 13A**).

Table VI		2.5 mM [K⁺]_o	10 mM [K⁺]_o	25 mM [K⁺]_o	50 mM [K⁺]_o
V_m (mV)	WT	-82.31 ± 0.98 n=62	-48.61 ± 1.48 n=19	-28.90 ± 0.91 n=15	-17.98 ± 1.03 n=16
	cKO	-39.11 ± 2.42 n=46	-25.51 ± 1.75 n=8	-21.76 ± 0.71 n=6	-17.05 ± 1.07 n=8

We therefore examined Na⁺ and Cl⁻ permeability of WT versus cKO passive cell membrane by reducing the concentration of these ions in ACSF and observing the resulting V_m, R_m, and current changes as described above. A large reduction of [Na⁺]_{out} (from 153 to 28 mM) is expected to hyperpolarize a membrane permeable to Na⁺. Conversely, reduction of [Cl⁻]_{out} (from 135 to 8 mM) should lead to a depolarization of Cl⁻ permeable membrane. To our surprise, membrane properties of K_{ir}4.1 cKO passive astrocytes (R_m: 6.87 ± 0.78 MΩ,

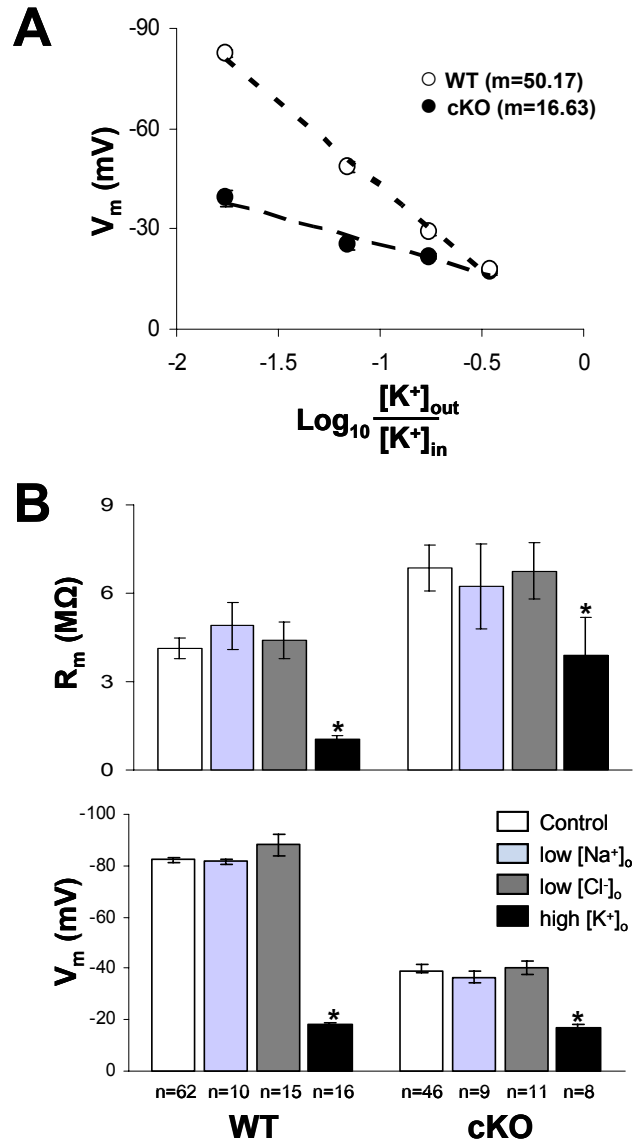


Figure 13. Ion substitution effects on passive astrocyte membrane properties. (A) Effect of increasing $[K^+]_{out}$ on the passive astrocyte membrane potential in WT and $K_{ir}4.1$ cKO; (m) slope. (B) Membrane resistance (R_m) and resting membrane potential (V_m) of passive astrocytes in control, low $[Cl^-]_{out}$, low $[Na^+]_{out}$, and high $[K^+]_{out}$ -ACSF. Mean \pm SEM, * $p < 0.05$

V_m : -39.11 ± 2.42 mV, $n=46$) were not affected by low Na^+ or low Cl^- concentration (**Table VII, Fig. 13B**), indicating that increased Na^+ and/or Cl^- permeability is not the cause of their depolarization. As expected, WT passive cells (R_m : 4.13 ± 0.35 M Ω , V_m : -82.31 ± 0.98 mV, $n=62$) also did not display significant Na^+ and Cl^- resting permeability (**Table VII, Fig. 13B**). In contrast, as already shown in **Figure 13A**, an increase in extracellular K^+ (50 mM) was able to depolarize WT (-17.98 ± 1.03 mV, $n=16$) and cKO (-17.05 ± 1.07 mV, $n=8$) cells and significantly decrease their R_m (WT: 1.04 ± 0.14 M Ω , $n=16$; cKO: 3.89 ± 1.28 M Ω , $n=8$; **Fig. 13B, Table VII**) due to increased K^+ conductance. Data was further verified with several different Na^+ and Cl^- concentrations including complete removal of extracellular Cl^- accompanied with a use of a salt bridge and complete removal of intracellular Na^+ (data not shown). Overall, our findings suggest that selectivity of the passive astrocyte currents is not affected by the removal of $\text{K}_{ir}4.1$.

Table VII		Control ACSF 2.5 mM K^+ 153 mM Na^+ 135 mM Cl^-	low Na^+ ACSF 2.5 mM K^+ 28 mM Na^+ 135 mM Cl^-	low Cl^- ACSF 2.5 mM K^+ 153 mM Na^+ 8 mM Cl^-	high K^+ ACSF 50 mM K^+ 105 mM Na^+ 135 mM Cl^-
V_m (mV)	WT	-82.31 ± 0.98 $n=62$	-81.70 ± 0.92 $n=10$	-87.99 ± 4.50 $n=15$	-17.98 ± 1.03 $n=16$
	cKO	-39.11 ± 2.42 $n=46$	-36.66 ± 2.37 $n=9$	-40.24 ± 2.32 $n=11$	-17.05 ± 1.07 $n=8$
R_m (MΩ)	WT	4.13 ± 0.35 $n=62$	4.90 ± 0.80 $n=10$	4.41 ± 0.63 $n=15$	1.04 ± 0.14 $n=16$
	cKO	6.87 ± 0.78 $n=46$	6.23 ± 1.44 $n=9$	6.75 ± 0.95 $n=11$	3.89 ± 1.28 $n=8$

2.5. INHIBITION OF Na^+/K^+ -ATPASE LEADS TO $\text{K}_{ir}4.1$ cKO-LIKE DEPOLARIZATION OF WILD-TYPE PASSIVE ASTROCYTES

Faced with contradicting observations that $\text{K}_{ir}4.1$ cKO causes severe passive astrocyte depolarization yet passive cells do not appear to have significant resting K_{ir} conductance, nor

is the membrane ion selectivity of $K_{ir}4.1$ cKO cells changed, we examined another mechanism known to regulate membrane potential. In most mammalian cells, a powerful Na^+/K^+ -ATPase (Na^+ -pump) maintains Na^+ and K^+ gradients across the membrane. As such, it has an important role in regulating cell volume, intracellular pH and Ca^{2+} levels, and in driving a variety of other Na^+ -gradient dependent processes such as glucose and amino acid transport (reviewed in Therien and Blostein, 2000). The pump is electrogenic in nature because it extrudes 3 Na^+ for every 2 K^+ entering the cell, generating a continuous outward current flow and a negative membrane potential.

A well characterized Na^+/K^+ -ATPase blocker, cardiac glycoside ouabain (OUA), was used to look at the pump activity in WT vs. $K_{ir}4.1$ cKO cells by measuring the ouabain induced changes in membrane potential (**Fig. 14**) and holding current (data not shown) of WT and cKO hippocampal passive astrocytes. Since ouabain causes neuronal depolarization and therefore increased excitability, 1 μ M TTX was included in all experiments to prevent neuronal firing. Astrocytes were first patched in TTX supplemented ACSF without addition of ouabain to confirm that TTX does not influence their properties. Ouabain (1 mM) application led to a severe depolarization of WT passive astrocytes as can be seen from the voltage trace in **Figure 14A**. Cells depolarized at the rate of approximately 3 mV/min and their V_m reached a steady-state in approximately 15 min. The ouabain effect was not reversible and with time led to the complete run-down of astrocyte membrane potential. Interestingly, upon ouabain application WT complex astrocytes responded similarly to neurons with a small reversible depolarization (data not shown). In contrast to WT passive astrocytes, $K_{ir}4.1$ cKO passive cells responded to ouabain with a small depolarization followed by a hyperpolarization and a complete recovery of their initial membrane potential

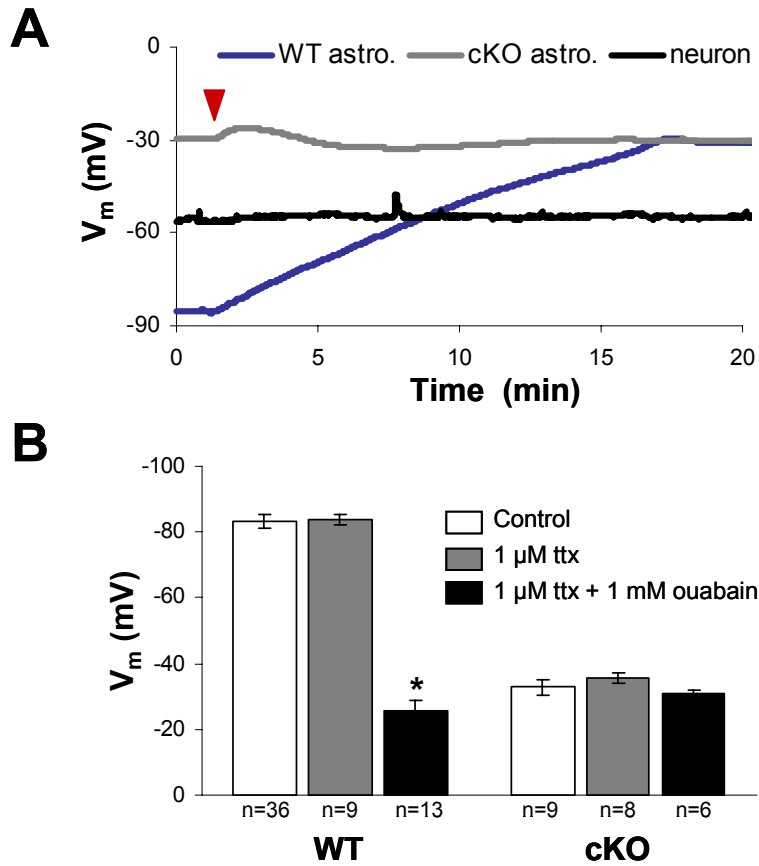


Figure 14. Influence of Na^+/K^+ -ATPase block on passive astrocyte membrane potential. (A) Membrane potential (V_m) traces during Na^+/K^+ -ATPase block with ouabain. Red arrowhead marks start of ouabain application. (B) Resting membrane potential (V_m) of WT and $\text{K}_{\text{ir}}4.1$ cKO passive astrocytes in control and ouabain-ACSF. Mean \pm SEM, * $p < 0.05$

(**Fig. 14A**). The accompanying neuronal trace illustrates that TTX inhibits ouabain induced depolarization of neurons, ruling out the possibility that astrocyte depolarization is indirectly caused by neuronal depolarization and subsequent release of ions and neurotransmitters (**Fig. 14A**). Several different ouabain concentrations were used (5 μ M to 1 mM) all exerting similar depolarizing effect on WT cells, with lower concentrations requiring longer time to steady state V_m (data not shown). Ouabain (1mM) caused a 58 mV depolarization of WT passive astrocytes (control: -83.30 ± 2.24 mV, n=36; TTX: -83.67 ± 1.67 mV, n=9; OUA+TTX: -25.76 ± 3.12 mV, n=11), bringing their V_m very close to the V_m of $K_{ir}4.1$ cKO cells (-32.86 ± 2.25 mV, n=9). Ouabain also increased the R_m of WT cells (control: 4.16 ± 0.61 M Ω , n=36; TTX: 4.77 ± 0.91 M Ω , n=9; OUA+TTX: 9.07 ± 1.49 M Ω , n=11) to the similar extent as the cKO of $K_{ir}4.1$ (7.74 ± 1.56 M Ω , n=9; **Table VIII, Fig. 14B**).

Table VIII	WT Passive Astrocytes			cKO Passive Astrocytes		
	Control	1 μ M TTX	1 mM OUA 1 μ M TTX	Control	1 μ M TTX	1 mM OUA 1 μ M TTX
V_m (mV)	-83.30 ± 2.24	-83.67 ± 1.67	-25.76 ± 3.12	-32.86 ± 2.25	-35.38 ± 1.54	-30.73 ± 1.36
R_m (M Ω)	4.16 ± 0.61	4.77 ± 0.91	9.07 ± 1.49	7.74 ± 1.56	8.41 ± 0.91	6.03 ± 1.11
n	36	9	11	9	8	6

In addition, we recorded ouabain induced change in holding current (also called pump current; Lancaster et al., 2000; Despa et al., 2003) in voltage-clamp mode while the cell membrane was held at either -80 mV (close to WT V_m) or -30 mV (close to cKO V_m) to eliminate the effect of the membrane potential difference between the two cell populations. As expected, ouabain induced current change mimicked the observed V_m change. A continuous slow inward current reflective of depolarization was seen in WT astrocytes. In $K_{ir}4.1$ cKO cells inward current was also observed; however, it was completely reversed by a

slower outward current suggestive of compensation for the ouabain Na^+ -pump block. The ability of $\text{K}_{\text{ir}}4.1$ cKO astrocytes to reverse the ouabain induced depolarization (presumably caused by Na^+ and Ca^{2+} influx and K^+ efflux) and maintain their V_m appeared to be due to activation of compensatory Cl^- influx as it was inhibited by removal of extracellular Cl^- (data not shown). Activation of Cl^- conductance in astrocytes has been observed upon cellular stress due to hypoxia, starvation, pH and volume change (Parkerson and Sontheimer, 2004). In the absence of extracellular Cl^- , ouabain depolarized cKO astrocytes up to 15 mV, but it never led to a complete run down of their membrane potential as was seen in WT cells (data not shown). The above described results strongly suggest that $\text{K}_{\text{ir}}4.1$ cKO causes a change in Na^+/K^+ -ATPase activity and/or regulation which in turn leads to cell depolarization.

We further employed several different approaches to look at the Na^+ -pump activity and compensatory Cl^- conductance in $\text{K}_{\text{ir}}4.1$ cKO cells, including ATPase activity assay and extensive study of effects of Cl^- channel and transporter blockers on WT and cKO cells. Unfortunately, we were unable to obtain conclusive results. It became apparent that the complexity of the channel/transporter interplay requires systematic dissection of their expression and functionality which is beyond the scope of this study. Interestingly, Cl^- channel blockers (DIDS, NPPB, niflumic acid) caused a slow hyperpolarization of $\text{K}_{\text{ir}}4.1$ cKO passive astrocytes suggestive of resting Cl^- conductance that could explain their depolarization. However, the finding that the complete removal of intracellular and extracellular Cl^- does not influence their membrane properties argues against this possibility. Clearly the complexity of our system and the lack of literature on passive astrocyte channel and transport properties make it hard to interpret these results.

3. DISCUSSION

Pharmacological blockade of K_{ir} channels in passive astrocytes, complex glia, and mature oligodendrocytes suggests that $K_{ir4.1}$ plays both a direct and indirect role in glial membrane potential maintenance, depending on the glial type and consistent with its developmentally regulated expression pattern. The Ba^{2+} -induced severe depolarization (~ 60 mV) of wild-type complex glia, complete loss of their inward currents, and a large 7.5-fold increase in their input-resistance, all implicate that K_{ir} channels comprise a major component of the resting ion conductance of complex cells, thereby setting their membrane potential. These findings are therefore in agreement with the previously noted role of K_{ir} channels in the development of the deep negative membrane potential of complex glia during their maturation (Kressin et al., 2005; Bordey and Sontheimer, 1997; Zhou et al., 2006). Membrane properties of the few remaining complex glia in the $K_{ir4.1}$ cKO hippocampus, were almost identical to those observed in wild-type cells exposed to K_{ir} blocker Ba^{2+} , including ~ 40 mV depolarization, lack of inward currents, and a 4-fold increase in membrane resistance. Following the cloning of $K_{ir4.1}$ and discovery of its glial localization it was shown that complex glia also express other K_{ir} channels, such as $K_{ir2.1-2.3}$, $K_{ir5.1}$, and $K_{ir6.1}$ (Schroder et al., 2002; Hibino et al., 2004). Presence of alternate K_{ir} subtypes probably accounts for the larger R_m and V_m effect seen upon Ba^{2+} block in wild-type cells than in the $K_{ir4.1}$ cKO. We also cannot exclude the knockout-induced developmental changes and cells' attempt at membrane potential compensation. Nevertheless, our findings implicate that $K_{ir4.1}$ is primarily responsible for setting the membrane potential of complex astrocytes. However, the inability of μM Ba^{2+} to mimic passive astrocyte and oligodendrocyte depolarization seen in the $K_{ir4.1}$ cKO, in the light of its ability to do so in complex glia, leads us to conclude that K_{ir} channels do not

directly set membrane potential of passive astrocytes and mature oligodendrocytes. While $K_{ir}4.1$ cKO passive astrocytes display ~ 50 mV depolarization, a 10-fold smaller depolarization was observed due to the Ba^{2+} (100 μ M) block of K_{irs} in wild-type cells. Furthermore, similar depolarization could also be observed upon Ba^{2+} application in $K_{ir}4.1$ cKO cells, suggesting that Ba^{2+} -induced depolarization in wild-type passive astrocytes may not be related to the $K_{ir}4.1$ block. Increased Ba^{2+} concentration (1 mM) led to a larger astrocyte and oligodendrocyte depolarization (~ 10 mV); nevertheless, given that 1 mM Ba^{2+} can block other voltage-gated K^+ channels (K_a , K_{dr}), as well as 2-pore K^+ channels (K_{2p}), this effect cannot be solely attributed to the block of K_{ir} channels. These data suggest that mature astrocytes and oligodendrocytes display minimal K_{ir} conductance, indicating that alternate channels and/or transporters are responsible for setting their membrane potential. Furthermore, as passive astrocytes and oligodendrocytes develop from precursors displaying complex electrophysiological phenotype and pronounced K_{ir} conductance, their severe depolarization in the $K_{ir}4.1$ cKO suggests that $K_{ir}4.1$ loss and subsequent depolarization of the precursor cells impair development of the mechanisms responsible for membrane potential maintenance in the mature cell.

During the subsequent study of passive currents which seemed to be the most likely effectors of the astrocyte membrane potential, we noticed an apparent decrease in the K^+ -selectivity of the $K_{ir}4.1$ cKO astrocyte membrane. Incremental increases in $[K^+]_{out}$ generated smaller shifts of the $K_{ir}4.1$ cKO astrocyte membrane potential, as compared to wild-type cells. This resulted in a significantly shallower slope ($m=17$) of the $\log[K^+]_{out}$ vs. V_m plot than is predicted for a K^+ -selective membrane by the Nernst equation ($m=59$). However, the ion substitution studies disproved presence of significant Na^+ or Cl^- conductance in the wild-

type and knockout cells alike. Therefore, loss of $K_{ir}4.1$ does not appear to change the ion-selectivity of the passive astrocyte membrane, leaving us with a perplexing observation that the large K^+ -selective conductance is not capable of maintaining the membrane potential of $K_{ir}4.1$ cKO cells close to the E_K . The low input-resistance of passive astrocytes suggests that K^+ -channels of these cells are mostly in their open state and allow free diffusion of K^+ in and out of the cell. Such channels may be ideal for K^+ buffering as passive cells could simply act as an extension of extracellular space. Channel regulation would control the diffusion space and therefore time of clearance and $[K^+]_{out}$ to fine tune neuronal excitability. However, such system would depend on a mechanism capable of actively maintaining the astrocyte membrane potential close to E_K to allow for the directional changes of the K^+ flux (influx vs. efflux) depending on $[K^+]_{out}$. Our data provide an insight into regulation of passive astrocyte membrane potential by Na^+/K^+ -ATPase. We show that Na^+/K^+ -ATPase selective blocker, ouabain, causes severe depolarization of passive astrocytes strongly implicating its importance in astrocyte membrane potential maintenance. Since ouabain depolarizes wild-type cells to the extent of their $K_{ir}4.1$ cKO counterparts, which are largely ouabain insensitive, $K_{ir}4.1$ absence during passive astrocyte development possibly impairs Na^+/K^+ -ATPase expression, localization, and/or functionality leading to cell depolarization. Chronic exposure to high $[K^+]_{out}$, which may be present in $K_{ir}4.1$ cKO tissue, has been shown to reduce Na^+/K^+ -ATPase activity (Sweadner, 1995). As previously noted, $K_{ir}4.1$ channels are clustered and functionally coupled to AQP4 by the dystrophin-glycoprotein complex (Nagelhus et al., 1999; Claudepierre et al., 2000; Amiry-Moghaddam et al., 2003; Connors and Kofuji, 2006). A recent study has introduced Na^+/K^+ -ATPase as a possible member of this protein complex. Sakuraba et al. (2005) show that AQP4 and Na^+/K^+ -ATPase can be co-

immunoprecipitated from the rat cerebellum, and that they couple K^+ transport and water permeability *in vitro*. AQP4, therefore, may act as a bridging molecule between the $K_{ir}4.1$ and Na^+/K^+ -ATPase, bringing them together in a functional complex responsible for extracellular K^+ homeostasis. As reviewed in *Potassium Spatial Buffering by Astrocytes* (page 46), numerous studies implicate that K^+ buffering within the CNS is accomplished by an interplay between the active Na^+/K^+ -ATPase-mediated K^+ uptake and passive channel- and transporter-mediated ion and water flux. The role that each of these mechanisms play in different tissues and during different levels of neuronal activity is still to be determined. Interestingly, a decrease in the Na^+/K^+ -ATPase activity has been reported in animal models of epilepsy and in hippocampal tissue from epileptic patients (Brines et al, 1995; Fernandes et al, 1996), both of which also display decreased K_{ir} conductance (Ballanyi et al., 1987; Bordey and Sontheimer, 1998; Schroder et al., 2000). It is conceivable then, that by removing the $K_{ir}4.1$ in our cKO animals we have caused a disturbance of the putative $K_{ir}4.1/AQP4/Na^+/K^+$ -ATPase complex responsible not only for the astrocyte membrane potential homeostasis, but for the ion and water homeostasis of the extracellular space, as well. The study of astrocyte K^+ uptake in the $K_{ir}4.1$ cKO, and the influence that its impairment plays in neuronal excitability and synaptic transmission, will be described in the following chapter.

In an attempt to better understand passive astrocyte currents and devise a strategy for their inhibition to establish better voltage clamp conditions during our astrocyte recordings, we provided some novel insights about their properties, as well as confirmed some of the previously published observations. As established for glia in cortex, spinal cord, and optic nerve by the early microelectrode studies (Lothman and Somjen, 1975; Futamachi and

Pedley, 1976; Picker and Goldring, 1982; Ransom and Goldring, 1973), we demonstrate that the passive astrocytes membrane in the hippocampus also behaves as an almost perfect K^+ -selective electrode ($\log[K^+]_{out}$ vs. V_m slope of 51 compared to the Nernst relation slope of 59). A few studies have proposed Cl^- contribution to the passive currents (Lascola and Kraig, 1996; Nobile et al., 2000; Makara et al., 2003); however, our ion substitution studies indicate that passive astrocytes lack significant resting Cl^- and Na^+ conductances. Astrocyte chloride conductance appears to be activated upon cellular stress such as hypoxia or osmotic changes (Parkerson and Sontheimer, 2004; Ernest et al., 2005), which may explain our finding of the depolarization-induced Cl^- conductance in $K_{ir}4.1$ cKO astrocytes exposed to ouabain. Seeing that astrocyte membrane is highly K^+ -selective, we used several well-established K^+ -channel blockers such as TEA (blocks K_{dr}), 4-AP (blocks K_a), spermine (endogenous K_{ir} blocker), and intercellular Tris (non-selective K^+ channel blocker) to explore their effects on the passive astrocyte whole-cell currents and membrane properties and possibly reveal presence of K^+ voltage-gated channels in these cells. In addition, to look at the presence of Ca^{2+} channels which have been shown in astrocytes *in vitro* (D'Ascenzo et al., 2004; Barres et al., 1989; Corvalan et al., 1990), we used Ca^{2+} channel blocker Cd^{2+} . None of these reagents notably affected wild-type passive astrocyte membrane properties or currents, suggestive of minimal if any functional expression of K^+ and Ca^{2+} voltage-gated channels in passive cells. Recent report by Wallraff et al. (2004) showing minimal effects of TEA, 4-AP, Cs^{2+} , Ba^{2+} (K^+ channel blockers), and Co^{2+} (Ca^{2+} channel blocker) on passive astrocyte membrane properties supports our findings. Two other earlier studies also reported that passive currents could not be blocked by TEA and 4-AP (Kressin et al., 1995) or Cs^{2+} (D'Ambrosio et al., 1998). To test whether passive currents arise from current dissipation via gap junctions or

hemichannels these channels were blocked with carbenoxolone (Ye et al., 2003; Peters et al., 2003; Ozog et al., 2002; Sanchez-Alvarez et al., 2004). No effect on the cells' membrane potential and only a small reduction of their whole-cell currents were observed upon carbenoxolone application, which reliably resulted in the complete loss of tracer dye spreading between cells. Furthermore, since we routinely patch uncoupled passive astrocytes displaying whole-cell currents and membrane properties comparable to those of the coupled cells, we do not believe that cell coupling is responsible for astrocyte passive currents. Our conclusion is additionally supported by two other studies in which alternate well-characterized gap junction blockers (halothane and octanol) were used and both produced only minor effects on passive currents (Kressin et al., 1995; Wallraff et al., 2004). A possibility remains that carbenoxolone and other gap junction blockers can block passage of tracer dyes but fail to block intercellular ion flow, explaining why we and others are not seeing an effect of these substances on passive currents. However, this possibility is not very likely since Ceelen et al., (2001), by using dual patch clamp, demonstrated that octanol blocks current flow between coupled astrocytes in the rat retina. The above presented results argue against the presence of voltage-gated K^+ or Ca^{2+} channels in passive astrocytes and the proposed hypothesis that their low membrane potential and large passive currents arise from extensive gap junction coupling and hemichannel expression. Passive astrocyte current properties, such as voltage- and time-independence and resistance to K_V channel blockers, are most similar to the properties of the fairly new class of K^+ channels, the K_{2P} channels. A number of different K_{2P} channels, including TASK-1, TASK-3, and TREK-2, were found in astrocytes in culture (Gnatenco et al., 2002), while immunostaining studies demonstrated TASK-1 expression by hippocampal astrocytes (Kindler et al., 2000) and TASK-1,2

expression by Müller glia (Eaton et al., 2004). Furthermore, Skatchkov et al. (2006) recently confirmed functional expression of TASK-like channels in Müller cells. Confirmation of K_{2P} expression and exploration of their functional roles in protoplasmic passive astrocytes will provide further understanding of astrocyte physiology that may clarify mechanisms of their membrane potential and ion homeostasis maintenance.

CHAPTER IV. INFLUENCE OF $K_{IR}4.1$ cKO ON ASTROCYTE FUNCTION AND NEURONAL ACTIVITY

1. INTRODUCTION

When one considers the abundance and complexity of reported neuron-glia interactions, it becomes evident that CNS functioning and therefore functioning of the organism as a whole depends on the multidirectional communication between these cell types. Over the years, astrocytes have emerged as the most heterogeneous and multi-functional glial population (reviewed in *Astrocyte Functions*, page 13). Astrocytes play a critical role in CNS development by participating in neurogenesis, guidance of neuronal migration, and synaptogenesis. They provide metabolic support to active neurons, regulate volume, pH and ion homeostasis of the interstitial space, and safeguard the brain from toxic substances through their involvement in the induction and maintenance of the blood-brain barrier. However, astrocyte functions receiving most interest are their regulation of synaptic levels of neurotransmitters, in particular glutamate, buffering of extracellular K^+ , and release of neuroactive substances or gliotransmitters, all of which have been shown to directly modulate neuronal excitability and transmission.

Functional capacity of the CNS excitatory synapses depends on the ability of astrocytes to synthesize and recycle glutamate (reviewed in *Neurotransmitter Homeostasis*, page 20). Two astrocyte-specific enzymes, pyruvate carboxylase and glutamine synthetase, are involved in *de novo* synthesis of glutamate from glucose and the return of synaptically

released glutamate to the presynaptic cell, respectively (reviewed in Pellerin, 2005). Furthermore, it has been established that termination of excitatory transmission depends on the activity of astrocyte glutamate transporters (GluTs). Two of the five cloned GluTs, GLAST and GLT-1, are almost exclusively expressed by astrocytes and account for the majority of the glutamate transport capacity in the CNS (Gegelashvili and Schousboe, 1997; Danbolt, 2001; Diamond and Jahr, 2000). These transporters couple transport of glutamate and H⁺ to the transport of 3Na⁺ and K⁺ down their concentration gradients, thereby creating a net current flow into the cell (Vandenberg, 1998). Excitatory synaptic activity has been shown to activate rapid GluT currents in astrocytes in the hippocampus, cerebellum, and olfactory bulb (Mennerick and Zorumski, 1994; Bergles and Jahr, 1997; Kojima et al., 1999; Clark and Barbour, 1997; De Saint Jan and Westbrook, 2005). Selective inhibition of astrocyte GluTs can increase amplitude and duration of neuronal excitatory postsynaptic currents (EPSCs; Tong and Jahr, 1994; Barbour et al., 1994), as well as modulate the mGluR mediated-excitation of inhibitory neurons (Huang et al., 2004). Antisense knockdown of GLAST and GLT-1 in rats causes elevation of extracellular glutamate, excitatory neurodegeneration, and ultimately paralysis (Rothstein et al., 1996). Furthermore, mice lacking GLT-1 are prone to lethal spontaneous seizures (Tanaka et al., 1997), while those lacking GLAST display motor discoordination (Watase et al., 1998). Glutamate-induced excitotoxic neuronal death has been implicated in several disorders including cerebral ischemia, hypoglycemia, head trauma, epilepsy, and amyotrophic lateral sclerosis, as well as in Alzheimer, Huntington, and Parkinson disease (Arundine and Tymianski, 2004; Benarroch, 2005; Rothstein et al., 1995; Sonkusare et al., 2005; Shin et al., 2005; Blum et al.,

2001), further emphasizing the importance of glutamate homeostasis maintenance, which appears to be one of main astrocyte functions.

As reviewed in earlier chapters, the ability of astrocytes to maintain extracellular K^+ homeostasis is also well established. There is now considerable evidence that $[K^+]_{out}$ fluctuates from its normal level of about 3.5 mM up to 12 mM in conditions of high neuronal activity (reviewed in *Potassium Homeostasis and Neuronal Excitability*, page 41). Moderate elevation of $[K^+]_{out}$ (1-4 mM) leads to neuronal hyperexcitability and seizure-like activity, while more severe $[K^+]_{out}$ increase (6 mM and above) leads to the phenomenon called spreading depression which completely abolishes neuronal firing (reviewed in Laming et al., 2000). Neuronal depolarization induced by elevated $[K^+]_{out}$ has been shown to influence threshold for action potential initiation, activation of voltage-sensitive receptors and channels, and transmitter release (Poolos et al., 1987; Balestrino et al., 1986; Kreisman and Smith, 1993; Voskuyil and Ter Keurs, 1981; Jensen et al., 1994; Chamberlin et al., 1990; Poolos and Kocsis, 1990), all leading to the modulation of synaptic transmission. Both excitatory and inhibitory transmission are prone to $[K^+]_{out}$ modulation (Balestrino et al., 1986; Hablitz and Lundervolt, 1981; Rausche et al., 1990; Chamberlin et al., 1990; Poolos and Kocsis, 1990). Interestingly, they display different sensitivity to elevated $[K^+]_{out}$, and in general, synaptic inhibition tends to fail before excitatory synaptic transmission (Jones et al., 1987; Korn et al., 1987). Active uptake of K^+ by neurons, mainly mediated by their Na^+/K^+ -ATPase, is a relatively slow process and appears insufficient for buffering of rapid activity-induced $[K^+]_{out}$ increases (Heinemann and Lux, 1975; Ballanyi et al., 1984). Most neuronally extruded K^+ is therefore taken up by astrocytes, distributed through their syncytium, and released in areas of low $[K^+]_{out}$, a process known as K^+ spatial buffering (reviewed on page

46). Furthermore, it has been shown that astrocyte K^+ uptake is accompanied by Cl^- , Na^+ , and water fluxes (Coles and Orkand, 1985; Ballanyi et al., 1987; Ballanyi and Kettenmann, 1990; Kettenmann, 1987; Wuttke, 1990), and the exact mechanism of this complex process is not understood. In addition to glial K_{ir} channels and Na^+/K^+ -ATPase, involvement of aquaporins and several transporters, mainly K^+/Cl^- , $Na^+/K^+/2Cl^-$ and Na^+/HCO_3^- cotransporters, has been implicated (Simard and Nedergaard, 2004). Disturbance of astrocyte K^+ buffering capacity has been observed in epileptogenesis (Steinhäuser and Seifert, 2002; D'Ambrosio, 2004) and spreading depression which may underlie migraine attacks (Somjen, 2001; Martins-Ferreira et al., 2001; Sanchez-del-Rio and Reuter, 2004; Bolay and Moskowitz, 2005).

In addition to the above noted ability of astrocytes to modulate neurotransmission by maintaining glutamate and K^+ homeostasis, astrocytes have recently been shown to directly participate in synaptic transmission through release of gliotransmitters, including glutamate, ATP, and D-serine (reviewed in *Tripartite Synapse*, page 23). For example, glutamate released by astrocytes in the hippocampus causes facilitation of the spontaneous neuronal activity (Fiacco and McCarthy, 2004), while astrocyte released ATP mediates synaptic inhibition (Zhang et al., 2003). Such changes of synaptic strength are the basis of synaptic plasticity, short-term and long-term potentiation (LTP) and depression (LTD). LTP and LTD are prime candidates for mediating learning and memory, as well as many other forms of experience-dependent plasticity. Changes in synaptic strength can occur by presynaptic mechanisms, such as altered neurotransmitter release, and postsynaptic mechanisms, such as altered activity and/or abundance of postsynaptic AMPA receptors (reviewed in Malenka and Bear, 2004). The induction of LTP and LTD, as characterized in the CA1 region of the hippocampus, occurs in the postsynaptic neuron and requires calcium influx through NMDA

receptors. Level and mode of the postsynaptic calcium elevations seem to determine whether synaptic potentiation or depression will occur (reviewed in Sheng and Kim, 2002). Two forms of short-term potentiation (STP, lasting seconds to minutes), paired-pulse potentiation (PPF) and post-tetanic potentiation (PTP), have been shown to be presynaptic in origin and involve an increase in the number of transmitter quanta released due to the effects of the residual elevation in presynaptic $[Ca^{2+}]_{in}$. Short-term depression (STD) is usually attributed to depletion of the readily releasable neurotransmitter vesicles (reviewed in Zucker and Regehr, 2002). Potential astrocyte involvement in short- and long-term plasticity has been observed at several synapses. In hippocampal cell culture, stimulation of astrocytes can depress the strength of synaptic contacts between neurons (Araque et al., 1998). This is the result of astrocytic Ca^{2+} -evoked glutamate release that inhibits glutamate release from neurons by activating presynaptic mGluRs (Araque et al., 1998; Araque et al., 2000). An *in situ* study demonstrated astrocyte involvement in the enhancement of transmission between interneurons and pyramidal cells in hippocampal slices (Kang et al., 1998). During repetitive stimulation, inhibitory neurons activate GABA_B receptors on astrocytes, which leads to $[Ca^{2+}]_{in}$ increase and feeds back through an unknown mechanism to the presynaptic terminal to enhance transmission. Astrocytes released D-serine (Yang et al., 2003) and ATP (Pascual et al., 2005) were also shown to control the strength of hippocampal synapses and modulate NMDA-dependent LTP. Furthermore, Janigro et al. (1997) demonstrated that block of glial K_{ir} channels and concomitant rise of $[K^+]_{out}$ influence long-term synaptic plasticity by inhibiting LTD maintenance. They hypothesized that because of the impaired glial K^+ uptake stimulation paradigms normally used to elicit LTD may actually result in potentiation of synaptic activity due to K^+ -mediated neuronal depolarization (Janigro et al., 1997). It has also

been demonstrated that astrocytes can potentiate neurotransmission by releasing TNF α which increases surface expression of neuronal AMPA receptors (Beattie et al., 2002). Collectively, these studies suggest that astrocytes contribute to multiple forms of synaptic plasticity. Elucidation of possible mechanisms and physiological relevance is in its early stages and promises to remain an exciting area of research in coming years.

In addition to direct impairment of K⁺ buffering caused by the loss of K_{ir}4.1, we hypothesized that K_{ir}4.1 cKO astrocyte depolarization and possible impairment of their Na⁺/K⁺-ATPase may lead to the loss of membrane ion gradients, which will subsequently cause impaired glutamate uptake. We therefore examined K⁺ and glutamate uptake by K_{ir}4.1 cKO astrocytes and found them both to be greatly reduced. Furthermore, we observed decreased spontaneous activity of CA1 pyramidal neurons and enhanced synaptic potentiation within the K_{ir}4.1 cKO hippocampus. These findings provide further support that astrocyte K⁺ buffering and glutamate uptake modulate activity of pre- and postsynaptic neuronal elements and thereby influence synaptic strength and plasticity.

2. RESULTS

2.1. POTASSIUM AND GLUTAMATE UPTAKE BY K_{IR}4.1 CKO PASSIVE ASTROCYTES ARE GREATLY IMPAIRED

Axons of CA3 pyramidal neurons (Schaffer collaterals) synapse on dendrites of CA1 cells in the hippocampal CA1 stratum radiatum. The synapses are enveloped by astrocyte processes which take up released K⁺ and glutamate during neuronal activity, thereby insuring proper neuronal excitability and neurotransmission (Ventura and Harris, 1999; Seil, 2001; Bushong et al., 2002). To test the ability of K_{ir}4.1 cKO passive astrocytes to buffer K⁺ and take up synaptically released glutamate, we examined astrocyte whole-cell current generated

upon stimulation of the Schaffer collateral pathway. Passive astrocytes within CA1 stratum radiatum of WT and $K_{ir}4.1$ cKO (P15-20) hippocampal slices were patched and their membrane properties recorded. Stimulating glass electrode filled with ACSF was positioned 50 μm away from the patched astrocyte. Great care was taken to insure that the positioning of the recording and stimulating electrode was consistent between experiments. Schaffer collaterals were stimulated with five 100 μs / 200 μA pulses at 50 Hz. Astrocyte whole-cell current responses to the Schaffer collateral stimulation were recorded during the control condition (ACSF perfusion), after 2 min perfusion with 100 μM Ba^{2+} supplemented ACSF, and finally after 2 min perfusion with 100 μM Ba^{2+} and threo- β -benzyloxyaspartate (TBOA) supplemented ACSF. Stimulation was induced in triplicate with 15 sec delay between each run and the obtained responses averaged for each condition. 100 μM Ba^{2+} (K_{ir} channel blocker) and TBOA (non-selective GluT blocker) have previously been shown to completely inhibit astrocyte K^+ and glutamate uptake, respectively (Lüscher et al., 1998; De Saint Jan and Westbrook, 2005). Ba^{2+} -sensitive current or K^+ uptake current was obtained by subtracting the current trace obtained during Ba^{2+} block (Ba^{2+} -trace) from the control trace and recording the peak amplitude of the resulting inward current. TBOA-sensitive current or GluT current was obtained by subtracting the Ba^{2+} /TBOA-trace from the Ba^{2+} -trace and again recording the peak inward current amplitude. This analysis was done with Clampfit 9.0 software. Only recordings where starting R_a was less than 50 $\text{M}\Omega$ and varied less than $\pm 20\%$ during the experiment were included in the analysis. Data were analyzed using two sample t-test assuming unequal variance, reported as mean \pm SEM, and assumed significant (*) if $p < 0.05$.

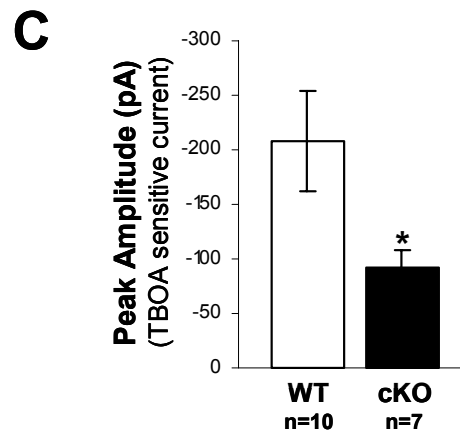
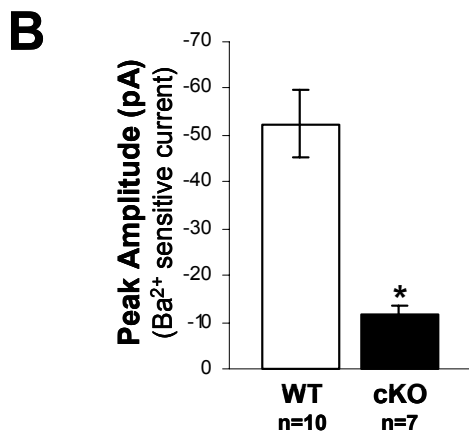
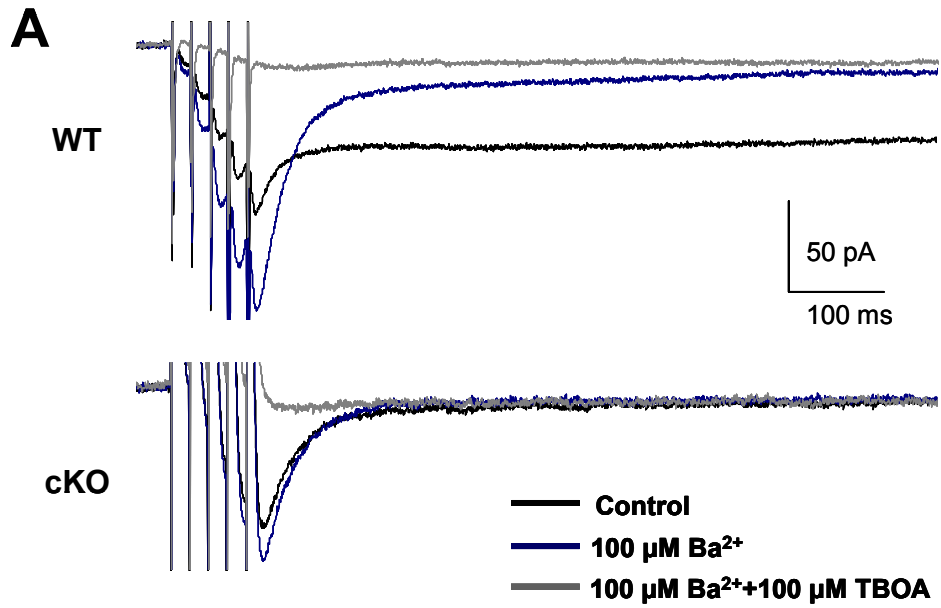


Figure 15. Passive astrocyte potassium and glutamate uptake during Schaffer collateral stimulation. (A) Whole-cell current traces from WT and $K_{ir}4.1$ cKO passive astrocytes induced by Schaffer collateral stimulation in the control condition (black trace), after Ba^{2+} block (blue trace), and after Ba^{2+} /TBOA block (gray trace). (B) Peak amplitude of K^+ uptake (Ba^{2+} -sensitive) current. (C) Peak amplitude of GluT (TBOA-sensitive) current. Mean \pm SEM, * $p < 0.05$

Figure 15A displays averaged astrocyte responses to Schaffer collateral stimulation during control condition (black trace), Ba^{2+} block (blue trace), and Ba^{2+} /TBOA block (gray trace). Wild-type responses were obtained from 10 astrocytes in 4 different animals, while $K_{ir}4.1$ cKO responses were gathered from 7 cells in 3 animals. In WT cells Ba^{2+} blocked the slow component of the current which persisted 10-15 s after stimulation, while TBOA blocked the faster GluT mediated component. In contrast, $K_{ir}4.1$ cKO astrocytes mostly lacked Ba^{2+} -sensitive current. In addition, Ba^{2+} caused a large increase of the GluT mediated fast current in WT but not in cKO astrocytes. Upon quantification of the collected data we obtained values for the peak amplitude of Ba^{2+} (**Fig. 15B**) and TBOA (**Fig. 15C**) sensitive current in two cell populations. Wild-type astrocyte peak K^+ uptake (Ba^{2+} -sensitive) current (-52.43 ± 7.20 pA, $n=10$) was 4.5 fold greater than the K^+ uptake current of $K_{ir}4.1$ cKO cells (-11.80 ± 1.71 pA, $n=7$). GluT generated (TBOA-sensitive) peak current in $K_{ir}4.1$ cKO astrocytes (-91.38 ± 16.65 pA, $n=7$) was also decreased as compared to that of WT cells (-207.73 ± 45.76 pA, $n=10$) in the presence of Ba^{2+} . In the control condition, prior to applying Ba^{2+} , the fast component of the recorded uptake current was approximately 20% smaller in cKO cells as compared to WT (**Fig. 15A**). These observations suggest that both K^+ and glutamate uptake are largely impaired in $K_{ir}4.1$ cKO passive astrocytes, most likely due to the loss of $K_{ir}4.1$ and GluT inhibition by membrane depolarization. However, two caveats need to be kept in mind when interpreting this data. First, due to the design of our experiment we cannot exclude the possibility that decreased uptake current in $K_{ir}4.1$ cKO astrocytes is attributed to the change in neuronal activity and synaptic transmission, therefore change in K^+ extrusion and glutamate release. Differences in WT versus $K_{ir}4.1$ cKO neuronal spontaneous and induced activity, as well as synaptic transmission, were addressed and will

be described in sections 2.2 and 2.3 of this chapter. Second, because of the severe depolarization of $K_{ir}4.1$ cKO astrocytes and the dependence of K^+ uptake via K_{ir} channels on the membrane potential, inward K^+ flow (i.e. K^+ uptake) may only be possible when $[K^+]_{out}$ is extremely high. For K^+ to move into the cell E_K has to be more depolarized than V_m . In $K_{ir}4.1$ cKO cells resting at -35 mV this should only happen when $[K^+]_{out} > 35$ mM, if we assume that $[K^+]_{in}$ is set at 145 mM by our patching solution. Our stimulating conditions may not generate enough K^+ extrusion for cKO cells to sense it. We therefore tested the ability of $K_{ir}4.1$ cKO cells to take up K^+ while directly increasing $[K^+]_{out}$ (5-35 mM) and the ability of Ba^{2+} to block the induced current flow.

ACSF potassium concentration was increased by equimolarly replacing NaCl with KCl. Three different potassium concentrations were used: 5, 10, and 35 mM, while control ACSF contained 2.5 mM K^+ . Since we have previously shown that $[Na^+]_{out}$ changes do not affect passive astrocyte properties (**Fig. 15B**), we were not concerned about decreasing the Na^+ concentration. TTX (1 μ M) was included in all solutions to control neuronal activity and eliminate its effect on the astrocyte uptake current. Wild-type and $K_{ir}4.1$ cKO passive astrocytes were patched and their whole-cell current recorded while cells were voltage clamped at their resting membrane potential or at -90 mV to exclude data variability due to the membrane potential difference. During the recording we first bath applied high K^+ ACSF for 1 min, allowed membrane current to recover to baseline, then pretreated the slice with 100 μ M Ba^{2+} for 2 min, followed by a second 1 min high K^+ perfusion (**Fig. 16**). This Ba^{2+} concentration was chosen because it was previously shown to reliably abolish all K_{ir} current in complex glia (**Fig. 10A**). In wild-type passive astrocytes 5 mM $[K^+]_{out}$ application generated a large transient inward current (>1 nA) that was partially blocked by 100 μ M Ba^{2+}

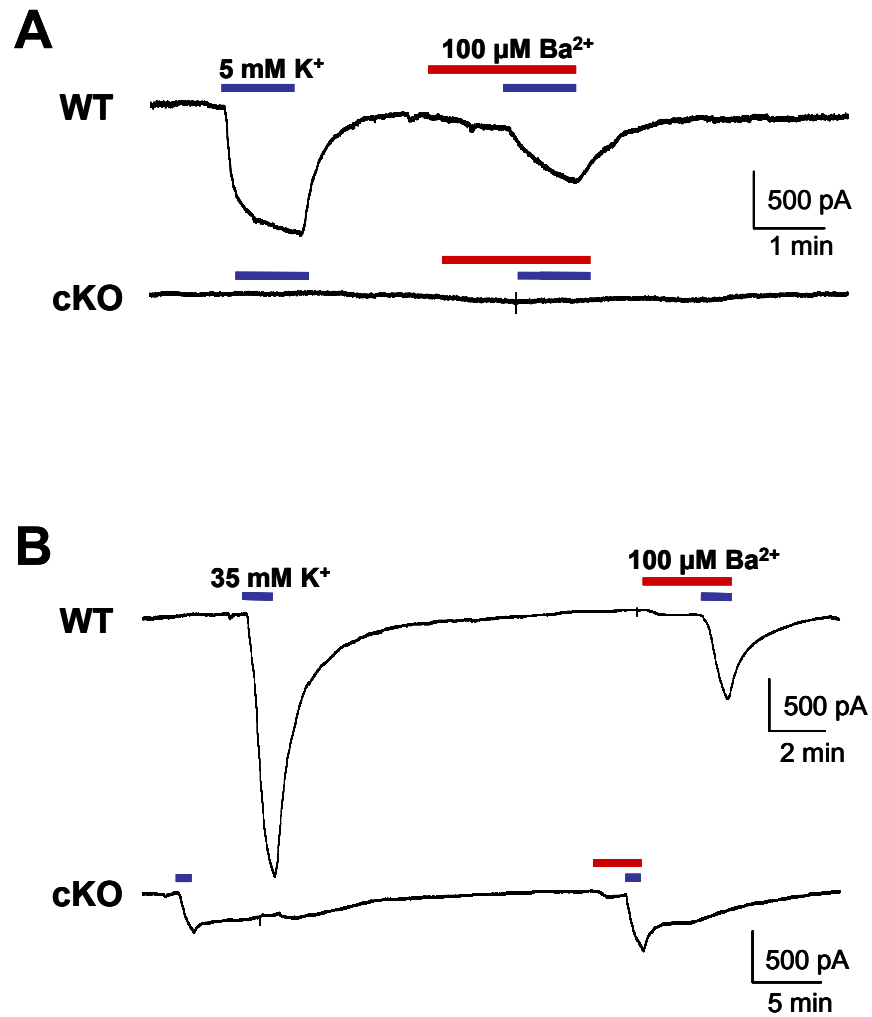


Figure 16. Potassium uptake by wild-type and Kir4.1 cKO passive astrocytes. Whole-cell current traces from WT and Kir4.1 cKO astrocytes induced by the bath application of 5 mM K⁺ (A) and 35 mM K⁺ (B), without and with a 2 min 100 μM Ba²⁺ pretreatment.

(**Fig. 16A**). As expected, 10 mM $[K^+]_{out}$ (data not shown) and 35 mM $[K^+]_{out}$ generated a larger uptake current with faster transients (**Fig. 16B**). Ba^{2+} consistently blocked approximately 60% of the K^+ current suggesting either that a Ba^{2+} -insensitive mechanism also contributes to K^+ uptake by astrocytes or that our block was incomplete and may require longer and/or higher Ba^{2+} application. In contrast, upon 5 mM $[K^+]_{out}$ application no inward current was seen in passive $K_{ir}4.1$ cKO astrocytes (**Fig. 16A**). However, 10 mM $[K^+]_{out}$ was detected by cKO cells and it caused small current deflections (<200 pA) that were Ba^{2+} -insensitive (data not shown). The same trend was observed during 35 mM $[K^+]_{out}$ application with the K^+ uptake current being slightly larger (**Fig. 16B**). These experiments clearly demonstrate that $K_{ir}4.1$ cKO cells have reduced K^+ buffering capability. They are however capable of detecting large extracellular K^+ increases (>10 mM) and utilize a Ba^{2+} -insensitive mechanism for K^+ uptake.

2.2. SPONTANEOUS ACTIVITY OF CA1 PYRAMIDAL NEURONS IS DECREASED IN THE $K_{ir}4.1$ cKO

Small rises in $[K^+]_{out}$ lead to neuronal depolarization, reduced action potential threshold, and increase in frequency of spontaneous excitatory postsynaptic currents (sEPSCs; Laming et al., 2000). However, prolonged exposure to high extracellular K^+ can lead to a phenomenon called spreading depression marked by a decrease in neuronal excitability (reviewed in Somjen, 2001). We therefore examined basic electrophysiological properties of CA1 pyramidal neurons in $K_{ir}4.1$ cKO and WT mice (P15-20), as well as their spontaneous and evoked activity. This was accomplished by patching neurons in hippocampal slices and recording their membrane properties (V_m , R_m) and whole-cell currents in response to a voltage step protocol. After the initial whole-cell current recording, we recorded spontaneous

synaptic activity (sEPSCs) for 10 min while voltage clamping the cells at -70 mV, and then proceeded to examine action potential properties. Action potentials were induced by 500 ms current injections of increasing amplitude (20-300 pA) in 20 pA increments to examine action potential frequency, threshold, and waveform properties (such as rise and decay slope and undershoot amplitude). Action potential and sEPSC properties were analyzed using Clampfit 9.0 analysis software and compared between the two genotypes. Recordings during which R_a changed more than $\pm 20\%$ were not included in the analysis.

As can be seen in **Figure 17**, neuronal whole-cell currents (**Fig. 17A**) and membrane properties (**Fig. 17B**) in $K_{ir}4.1$ cKO hippocampus were comparable to those of WT cells (WT: $V_m = -57.51 \pm 1.20$ mV, $R_m = 201.42 \pm 14.84$ M Ω , n=23; cKO: $V_m = -58.88 \pm 1.19$ mV, $R_m = 206.02 \pm 15.49$ M Ω , n=17). These data suggest that basic neuronal properties during resting unstimulated conditions may not be affected in the $K_{ir}4.1$ cKO. Action potential induction further revealed that $K_{ir}4.1$ cKO CA1 pyramidal neurons do not appear hyperexcitable as the frequency of their firing due to the increasing stimulation intensity was not significantly different from that seen in WT cells (**Fig. 17D**). We did however observe a trend towards decreased action potential frequency during high stimulation (200-300 pA) in $K_{ir}4.1$ cKO cells, as well as a small increase in the hyperpolarization undershoot after the action potential train (**Fig. 17C**).

During the study of neuronal spontaneous activity we noticed an apparent decrease in frequency and size of sEPSCs in $K_{ir}4.1$ cKO neurons. Wild-type and $K_{ir}4.1$ cKO representative current recordings and individual averaged sEPSCs are shown in **Figure 18A** and **18B**, respectively. Both sEPSC frequency (WT: 0.41 ± 0.07 Hz, n=23; cKO: 0.23 ± 0.06 Hz, n=19) and peak amplitude (WT: -12.59 ± 0.79 pA, n=23; cKO: -10.34 ± 0.78 , n=19)

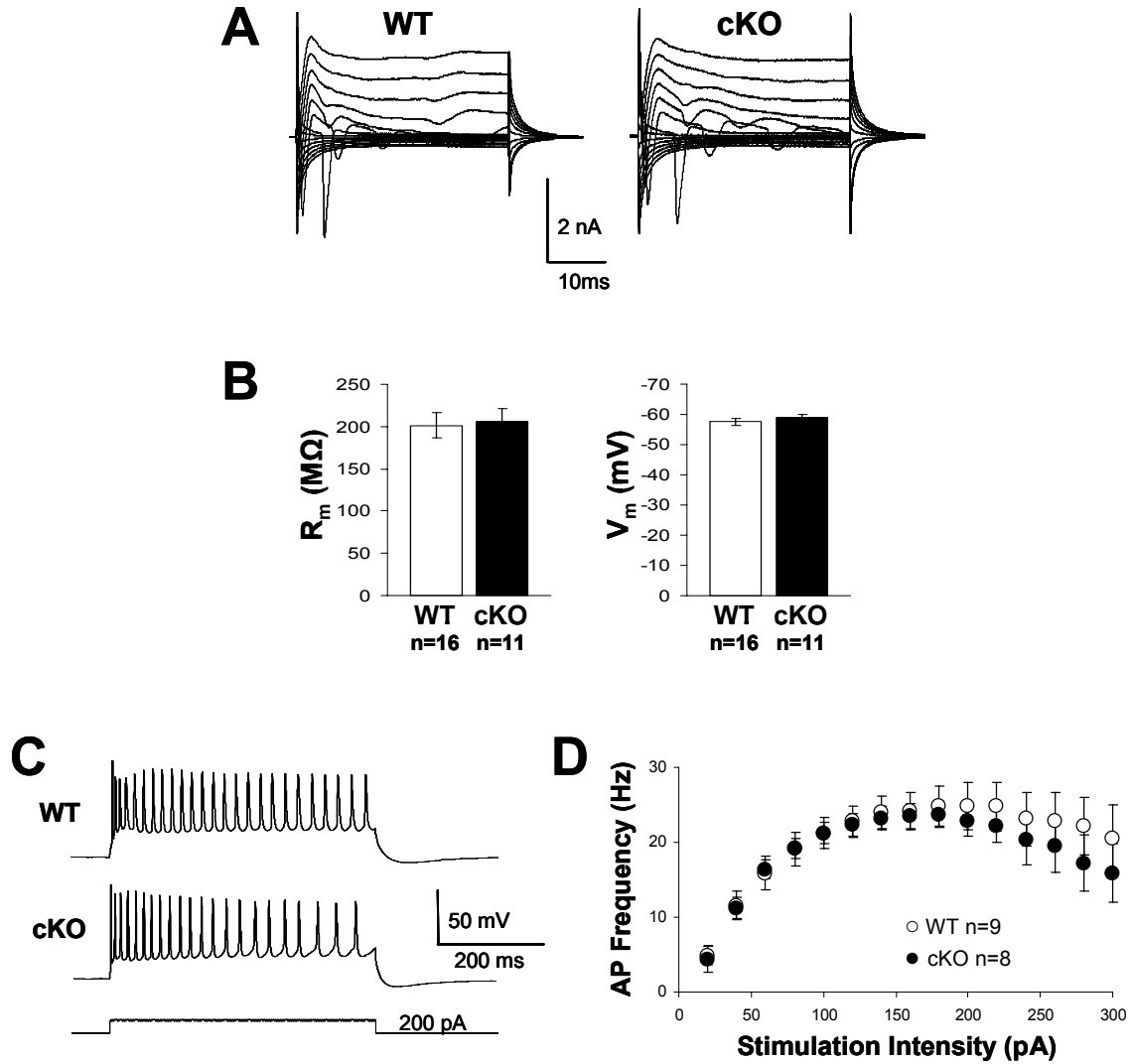


Figure 17. Membrane and action potential properties of wild-type and $K_{ir}4.1$ cKO pyramidal neurons. (A) Representative whole-cell currents of WT and $K_{ir}4.1$ cKO CA1 pyramidal neurons. (B) Membrane resistance (R_m) and resting membrane potential (V_m) of WT and $K_{ir}4.1$ cKO neurons; Mean \pm SEM. (C) Representative action potential recordings generated by 200 pA / 500 ms current injection (D) Influence of stimulation intensity on the action potential frequency of WT and $K_{ir}4.1$ cKO neurons; Mean \pm SEM.

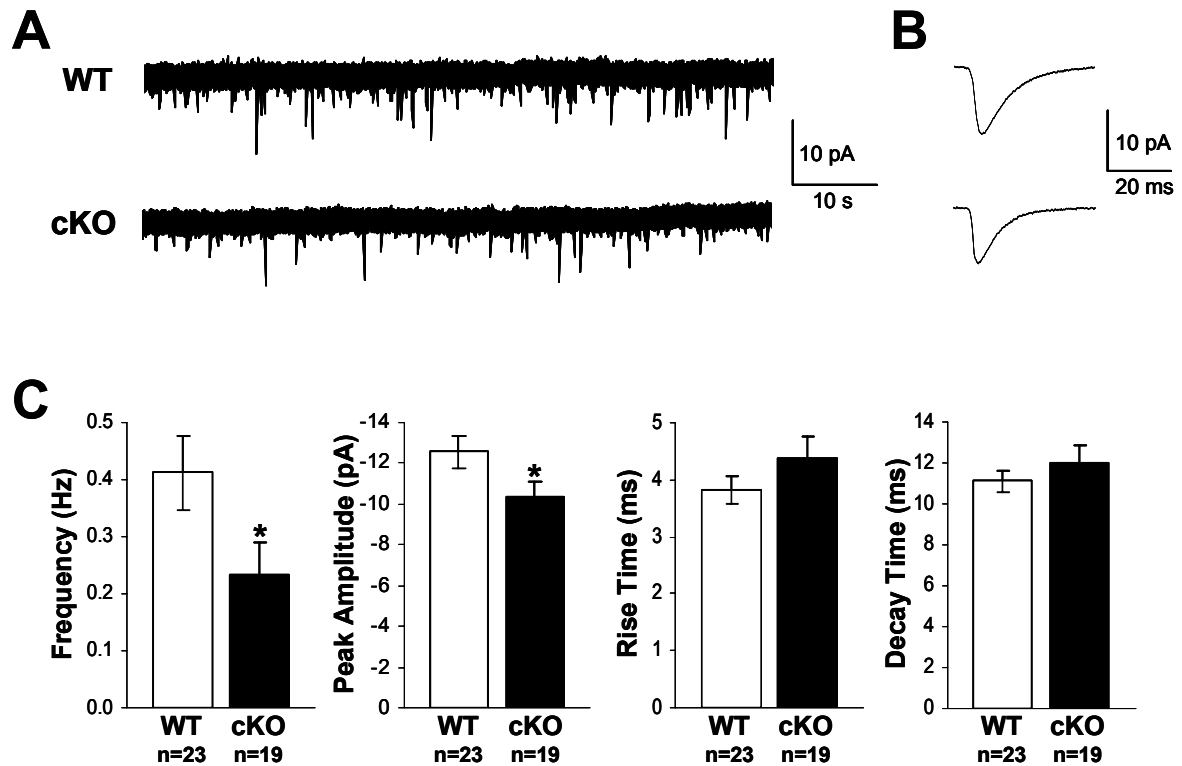


Figure 18. Spontaneous activity of wild-type and $K_{ir}4.1$ cKO neurons. (A) Representative current recordings from WT and $K_{ir}4.1$ cKO CA1 pyramidal neurons. (B) Representative averaged sEPSC traces from WT and $K_{ir}4.1$ cKO neurons. (C) sEPSC frequency, peak amplitude, rise and decay time; Mean \pm SEM, * $p < 0.05$.

were significantly smaller in cKO neurons (**Fig. 18C**). Mean sEPSC rise and decay time were comparable between the two populations (**Fig. 18C, Table IX**) and are similar to the values reported in literature for the AMPA receptor mediated synaptic currents (Saviane et al., 2002; Losonczy et al., 2003; Zhang et al., 2005). We eliminated inhibitory postsynaptic currents by voltage clamping the cells at -70 mV which is the reversal potential for Cl⁻ under our conditions, and NMDA receptor mediated EPSCs were inhibited due to presence of Mg²⁺ in our extracellular solution. The above reported findings suggest that K_{ir}4.1 deletion, which leads to astrocyte depolarization and impairment of their ability to buffer K⁺ and glutamate, causes a decrease of the neuronal spontaneous activity, but surprisingly does not cause changes of the neuronal membrane and action potential properties.

Table IX	sEPSC Properties				
	Frequency (Hz)	Peak Amplitude (pA)	Rise Time (ms)	Decay Time (ms)	n
WT	0.41 ± 0.07	-12.59 ± 0.79	3.82 ± 0.24	11.12 ± 0.52	23
cKO	0.23 ± 0.06	-10.34 ± 0.78	4.38 ± 0.38	11.97 ± 0.85	19

2.3. SYNAPTIC POTENTIATION IN THE K_{ir}4.1 cKO HIPPOCAMPUS IS ENHANCED

Following the study of neuronal single-cell properties, we proceeded by looking at the role of K_{ir}4.1 mediated K⁺ buffering in synaptic transmission and plasticity. One of the best studied synaptic fields in the brain is the CA1 stratum radiatum where Schaffer collateral fibers (CA3 pyramidal cell axons) synapse onto dendrites of CA1 cells. Stimulation of the Schaffer collateral pathway and recording of the generated field excitatory postsynaptic potentials (fEPSPs) allows for assessment of changes in basal synaptic transmission and synaptic plasticity (STP, LTP) caused by loss of K_{ir}4.1. We would like to thank Dr. Ben Philpot for his generous help with this study.

The fEPSPs were recorded from sagittal hippocampal slices (WT and $K_{ir}4.1$ cKO, P20-25) in a submersion recording chamber perfused with oxygenated ACSF maintained at 30°C. Stimulating electrode was positioned in CA1 stratum radiatum below the pyramidal cell layer. Recording electrode was positioned parallel to the stimulating electrode 300-400 μ m away. Input/output curve was generated by stepping the stimulation amplitude from 0 to 80 μ A. Stimulation amplitude that elicited half the maximum response and stimulation rate of 0.033 Hz (1 pulse/30 sec) were used throughout the experiment unless otherwise stated. After 5 min of stable baseline recording a paired-pulse facilitation (PPF) experiment was run. PPF protocol included 10 two-pulse pairs with increasing inter-pulse interval from 25 to 250 ms. Percent facilitation was calculated by dividing fEPSP slope elicited by the 2nd pulse with fEPSP slope elicited by the 1st pulse. Long-term potentiation (LTP) was induced with a single 1-sec 100-Hz train after 15 min of stable baseline recording (<5% drift). LTP was sampled for 45 min following induction and potentiation was calculated by dividing the average slope of 30-45 min post-induction responses with the average slope of 0-15 min pre-induction baseline responses. We also compared the average slope of 0-15 min post-induction responses with the average slope of 0-15 min baseline responses and designated this potentiation as short-term (STP). Post-tetanic potentiation (PTP) was measured as the average slope of 0-2 min post-induction responses compared to the average slope of 13-15 min baseline responses. Single PPF and LTP experiment was done per slice. Slices that showed <105% LTP were not included in the PTP, STP and LTP analysis (2 WT and 2 cKO slices).

Wild-type and $K_{ir}4.1$ cKO fEPSP traces, obtained by averaging responses from twelve 15 min baseline recordings for each genotype, are shown in **Figure 19A**. The first fast biphasic

wave is the stimulus artifact. Next small negative-going wave is the presynaptic fiber volley which is followed by the EPSP. As can be seen from the averaged traces (**Fig. 19A**) and the analysis of EPSP properties (**Table X**), $K_{ir}4.1$ cKO fEPSPs are comparable to those recorded in the WT hippocampus. To compare basal properties of synaptic transmission we generated two different input/output curves (**Fig. 19B,C**). **Figure 19B** shows that there is no significant difference between responses elicited by graded increase of stimulation amplitude (0-80 μ A) in $K_{ir}4.1$ cKO as compared to WT slices. Plotting the slope of EPSP (measure of postsynaptic response) as the function of fiber volley amplitude (thought to correlate with the number of presynaptic axons stimulated) confirms that $K_{ir}4.1$ cKO does not have a significant effect on the basal properties of single-pulse evoked neurotransmission (**Fig. 19C**).

Table X	fEPSP Properties				
	Rise Time (ms)	Rise Slope (μ V/ms)	Decay Time (ms)	Decay Slope (μ V/ms)	n
WT	1.43 \pm 0.18	-569.10 \pm 101.83	8.76 \pm 0.41	79.30 \pm 8.24	12
cKO	1.57 \pm 0.12	-442.46 \pm 46.34	8.13 \pm 0.42	76.60 \pm 6.56	12

Study of $K_{ir}4.1$ cKO influence on synaptic plasticity was initiated by examining paired-pulse facilitation (PPF). PPF is a presynaptic form of short-term plasticity in which the second of two pulses, delivered within 25-250 ms, elicits a larger synaptic response than the first due to residual increase in $[Ca^{2+}]_{in}$ in the presynaptic terminal (representative trace displayed in **Fig. 19D**). Once more, no difference was seen between $K_{ir}4.1$ cKO and WT slices (**Fig. 19D**). However, upon completing the LTP study we observed that $K_{ir}4.1$ cKO slices exhibited a significantly greater degree of potentiation than WT slices in the first 20 min following tetanus and nonsignificantly greater thereafter (**Fig. 20A**). $K_{ir}4.1$ cKO PTP (first 2 min following tetanus) was 30% greater (WT: 137.0 \pm 3.9%, n=10; cKO: 166.8 \pm

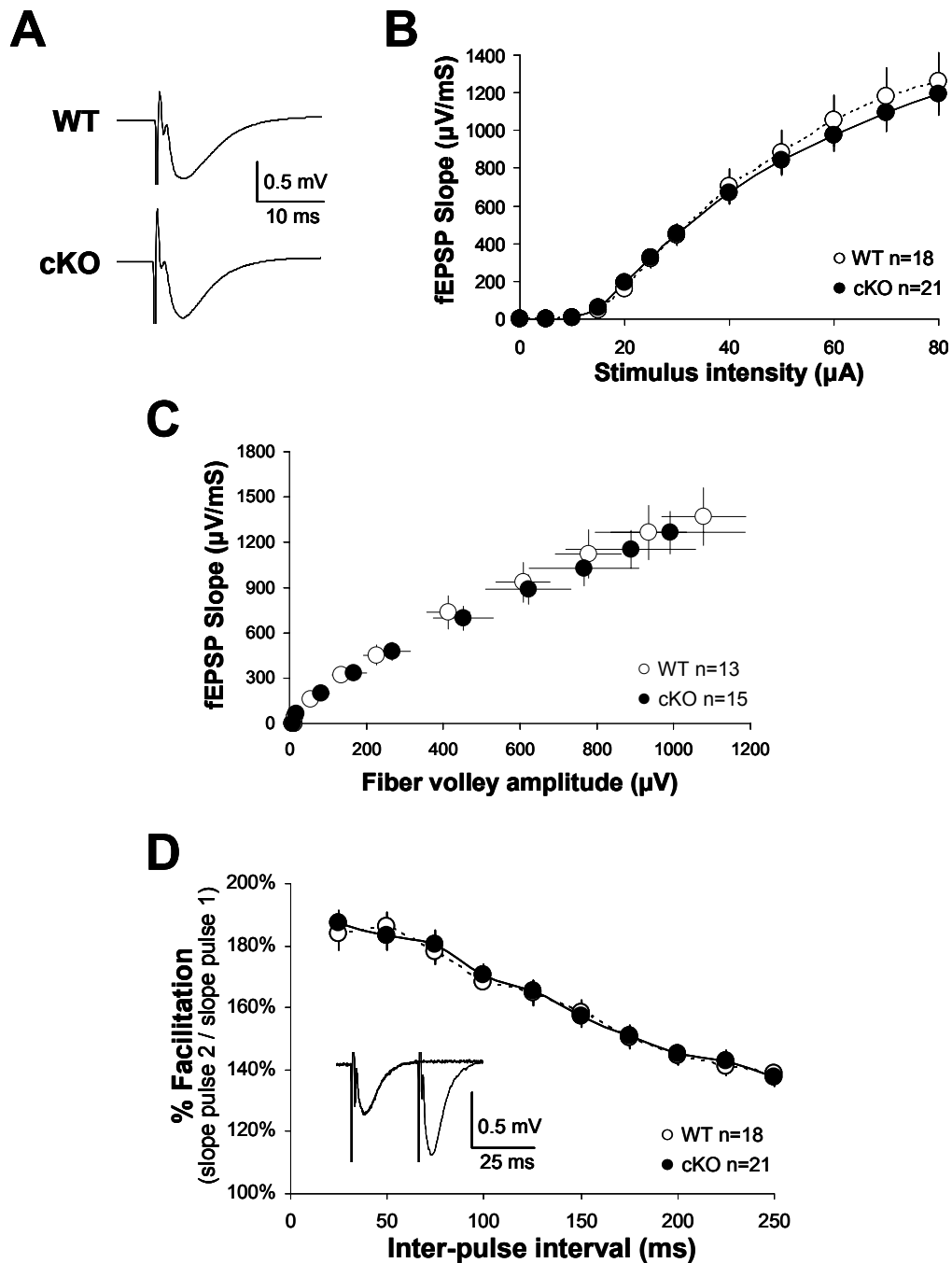


Figure 19. Synaptic transmission in wild-type and $K_{ir}4.1$ cKO hippocampus. (A) Representative averaged WT and $K_{ir}4.1$ cKO fEPSP traces. **(B)** Influence of stimulation intensity on the fEPSP slope. **(C)** Correlation between fiber volley amplitude (presynaptic activity) and fEPSP slope (postsynaptic activity). **(D)** Paired-pulse facilitation (representative WT current trace shown as inset).

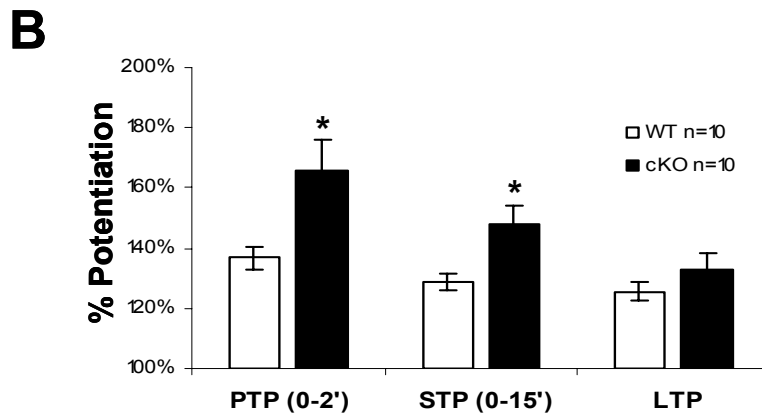
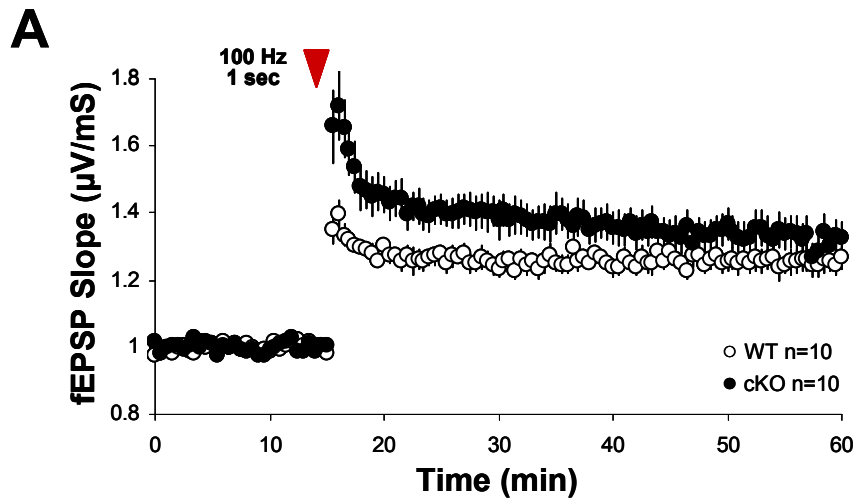


Figure 20. Synaptic potentiation in wild-type and $K_{ir}4.1$ cKO hippocampus. (A) Long-term potentiation (LTP). **(B)** Percent potentiation: post-tetanic potentiation (PTP), short-term potentiation (STP), and LTP; Mean \pm SEM, * $p < 0.05$.

10.2%, n=10) and STP (first 15 min following tetanus) was 19% greater (WT: $128.8 \pm 2.5\%$, n=10; cKO: $147.8 \pm 6.3\%$, n=10) than in WT. LTP (30-45 min following tetanus) was $126.0 \pm 3.2\%$ in the WT (n=10) and $133.1 \pm 5.1\%$ in the cKO (n=10) (**Fig. 20B**). These data suggest that $K_{ir}4.1$ mediated K^+ buffering functions during the early stages of LTP, a period when short-term plasticity still contributes to LTP induction. Since a change in synaptic potentiation was observed without the change in basal synaptic transmission, it appears that $K_{ir}4.1$ is primarily needed for clearing of large K^+ elevations following high synaptic activity but not during basal neurotransmission.

3. DISCUSSION

In the previous two chapters we reported and discussed several changes in astrocyte physiology induced by the loss of $K_{ir}4.1$, the main one being their depolarization. Such membrane depolarization could arise from the run down of membrane ion gradients. The experiment in which a selective Na^+/K^+ -ATPase blocker, ouabain, causes wild-type astrocyte depolarization comparable to that seen in the $K_{ir}4.1$ cKO, suggests that the cKO cell depolarization may indeed arise from the impaired ion distribution brought on by the loss of Na^+/K^+ -ATPase function. As mentioned earlier, Na^+/K^+ -ATPase and AQP4 can be co-immunoprecipitated from the mouse cerebellum (Sakuraba et al., 2005). This important pump may therefore be localized on astrocyte perisynaptic and perivascular processes together with AQP4 and $K_{ir}4.1$, forming a molecular complex responsible for the interstitial ion and water homeostasis. Addition of ouabain causes intercellular loss of K^+ , gain of Na^+ and Ca^{2+} , and small alkalization in cultured astrocytes (Erecinska et al., 1991; Harold and walz, 1992, Sontheimer et al., 1994). Silver and Erecinska (1997) demonstrated that during a

60 min Na^+/K^+ -ATPase inhibition, ions move down their concentration gradients with $[\text{Na}^+]_{\text{in}}$ increasing ~ 40 mM, $[\text{K}^+]_{\text{in}}$ decreasing ~ 70 mM, and $[\text{Ca}^{2+}]_{\text{in}}$ increasing ~ 3 -fold. Such impairment of ion gradients and accompanied astrocyte membrane depolarization are expected to decrease the activity of all ion gradient-dependent transporters and voltage-gated channels, including glutamate transporters and K_{ir} s. Glutamate transporters are voltage- as well as ion-gradient-dependent, and disturbances in ion gradients like those occurring in ischemia have been shown to reduce the effectiveness of the glutamate uptake (Kimelberg et al., 1993). Hippocampal passive astrocytes express high levels of two GluT subtypes, GLAST and GLT-1 (Matthias et al., 2003), which appear to mediate most of the synaptic glutamate uptake (Gegelashvili and Schousboe, 1997; Diamond and Jahr, 2000; Danbolt, 2001). Cell depolarization and increase in $[\text{Na}^+]_{\text{in}}$ has been shown to inhibit their function and at times cause transporter reversal and glutamate release (Bordey and Sontheimer, 2003; D'Ambrosio, 2004). We therefore directly measured passive astrocyte K^+ and glutamate uptake current induced by the electrical stimulation of Schaffer collateral fibers. Our experiments demonstrate $>70\%$ reduction of the K^+ uptake by $\text{K}_{\text{ir}4.1}$ cKO astrocytes and $>50\%$ reduction of their glutamate uptake, as determined from the peak amplitude of the Ba^{2+} -sensitive and TBOA-sensitive uptake current, respectively. Interestingly, Ba^{2+} application led to an almost 100% increase of the fast GluT-mediated component of the uptake current in wild-type astrocytes, likely due to the combination of direct Ba^{2+} -induced neuronal depolarization and indirect depolarization caused by elevated K^+ in response to glial K_{ir} block. In $\text{K}_{\text{ir}4.1}$ cKO cells only a small ($<20\%$) increase was seen implicating that their glutamate uptake capability has reached its limit and/or that stimulated presynaptic terminals in the $\text{K}_{\text{ir}4.1}$ cKO stratum radiatum release less glutamate upon Ba^{2+} depolarization than in

the wild-type. Since CA3 to CA1 synapses in the $K_{ir}4.1$ cKO hippocampus display normal synaptic transmission upon low levels of stimulation, the impaired GluT activity is most likely to blame for the decreased augmentation of glutamate uptake current by Ba^{2+} in the $K_{ir}4.1$ cKO.

In the rat CA1 stratum radiatum 100 pulse/100 Hz stimulation of Schaffer collaterals raises $[K^+]_{out}$ up to 7 mM (Poolos et al., 1987). Our stimulation protocol of 5 pulses at 50 Hz probably only generates 1-2 mM $[K^+]_{out}$ increase. As previously discussed, K^+ influx in $K_{ir}4.1$ cKO astrocytes resting at -35 mV would occur only when $[K^+]_{out}$ increases more than 35 mM, if we assume that our patching solution dictates 145 mM $[K^+]_{in}$. Due to the poor space clamp of passive astrocytes we are unable to hyperpolarize $K_{ir}4.1$ cKO cell membrane to the normal resting potential of the wild-type astrocytes to look at K^+ uptake under such conditions. We therefore bath applied 5-35 mM K^+ and measured the induced inward current before and after Ba^{2+} block of K_{ir} channels. In wild-type astrocytes 2 min pretreatment with 100 μ M Ba^{2+} completely abolished K^+ uptake current due to Schaffer collateral stimulation, implicating that it was indeed mediated by K_{ir} channels. We employed the same blocking protocol during the bath application of high K^+ and demonstrated that Ba^{2+} blocks >60% of K^+ uptake current in wild-type astrocytes under these conditions, while in the $K_{ir}4.1$ cKO cells none of the uptake current was blocked by Ba^{2+} . Collectively, our results implicate that astrocyte K^+ uptake in the hippocampus is mainly mediated by Ba^{2+} -sensitive $K_{ir}4.1$ channels, although during higher $[K^+]_{out}$ elevations (>2 mM), a Ba^{2+} -insensitive mechanism, possibly uptake by K_{2P} channels or Na^+/K^+ -ATPase, appears to be involved as well. Further exploration of the astrocyte channel, transporter, and receptor properties and the development of methods for their selective study in an intact tissue, in the presence of neurons with those

same membrane components, will be needed to resolve the exact roles of K_{2P} channels, Na^+/K^+ -ATPase, and K_{ir} s in astrocyte K^+ buffering. Neusch et al. (2006) have recently tackled these questions by examining the $[K^+]_{out}$ dynamics using K^+ -selective microelectrodes in the brainstem of $K_{ir4.1}^{-/-}$ mice. Their data demonstrate that $[K^+]_{out}$ recovery after neuronal stimulation is almost twice as long in the $K_{ir4.1}^{-/-}$ mice than in the wild-type animals. Furthermore, $K_{ir4.1}^{-/-}$ brainstem slices showed a clear undershoot of $[K^+]_{out}$ baseline following the recovery from stimulation-induced $[K^+]_{out}$ increase, suggestive of $K_{ir4.1}$ role in the K^+ return to the extracellular space. These findings agree with the hypothesis proposed by D'Ambrosio et al. (2002) that K_{ir} channels and Na^+/K^+ -ATPase cooperate in the K^+ uptake, however, while Na^+/K^+ -ATPase uptake persists after cessation of neuronal activity causing the $[K^+]_{out}$ undershoot, K_{ir} channels switch to K^+ extrusion in order to balance this undershoot. Direct observations of the astrocyte uptake current following neuronal stimulation in our experiments implicate dominant role of $K_{ir4.1}$ in K^+ uptake by astrocytes. Barium was reliably able to block all of the TBOA-insensitive uptake in wild-type astrocytes, while in $K_{ir4.1}$ cKO cells it mostly failed to produce any effect on the uptake current. Interestingly, recovery of the high K^+ induced inward current in $K_{ir4.1}$ cKO astrocytes was substantially prolonged, suggestive of $K_{ir4.1}$ role in K^+ return to the extracellular space.

Extracellular K^+ has been shown to influence neuronal excitability and the efficacy of synaptic transmission in several different systems (Balestrino et al., 1986; Korn et al., 1987; Chamberlin et al., 1990; Meeks and Mennerick, 2004). Inhibition of astrocyte glutamate transporters directly affects excitatory neurotransmission (Tong and Jahr, 1994; Barbour et al., 1994; Turecek and Trussell, 2000), as well as animal behavior (Rothstein et al., 1996;

Tanaka et al., 1997; Watase et al., 1998). Reduction of astrocyte K^+ and glutamate uptake observed in $K_{ir}4.1$ cKO hippocampus would therefore be expected to impair neuronal functioning. Furthermore, as reviewed in *Tripartite Synapse* (page 23), astrocyte excitability is based on the spontaneous and stimulated intracellular Ca^{2+} mobilization which activates gliotransmitter release. Astrocyte released neuro-active substances have been shown to affect neuronal excitability (Zhang et al., 2003; Tian et al., 2005), excitatory and inhibitory synaptic transmission and plasticity (Fiacco and McCarthy, 2004; Kang et al., 1998; Yang et al., 2003; Beattie et al., 2002; Pascual et al., 2005), as well as synaptogenesis and neuronal wiring (Fasen et al., 2003; Elmariah et al., 2005; reviewed in Collazos-Castro and Nieto-Sampedro, 2001; Ullian, 2004). Changes of $[Ca^{2+}]_{in}$ such as those induced by Na^+/K^+ -ATPase inhibition may therefore impair astrocyte Ca^{2+} signaling and lead to changes in gliotransmitter release, thus affecting neuronal activity in a variety of ways. Surprisingly, basic membrane properties, V_m , R_m , and whole-cell currents, as well as excitability of the CA1 pyramidal neurons, assessed by the induction of action potential firing with increasing stimulation intensity, all appeared unaffected in the $K_{ir}4.1$ cKO. Small but insignificant decrease in action potential frequency during high stimulation and enhanced after-hyperpolarization were seen in $K_{ir}4.1$ cKO neurons, reminiscent of the accommodation changes observed following prolonged neuronal activity (Mo et al., 2002; Savic et al., 2001; Madison and Nicoll, 1984; Madison and Nicoll, 1982). However, study of neuronal spontaneous activity in the $K_{ir}4.1$ cKO demonstrated marked decrease in frequency and amplitude of sEPSCs. $K_{ir}4.1$ cKO sEPSC frequency was ~45% reduced as compared to the wild-type, while sEPSC amplitude showed ~20% reduction. Changes in the EPSC frequency are classically attributed to changes in the presynaptic function, such as alterations in the

probability of transmitter release, while changes in EPSC amplitude appear to involve modification of the postsynaptic terminal responsiveness (reviewed in Stevens, 2004; Sheng and Jong Kim, 2002), both of which can be modulated by extracellular ion and transmitter levels, as well as by gliotransmitters. For example, small $[K^+]_{out}$ rises cause enhancement of the presynaptic transmitter release usually due to increased neuronal spiking, and augmented responses of postsynaptic NMDA receptors due to their depolarization mediated disinhibition (Chamberlin et al., 1990; Poolos and Kocsis, 1990). Larger $[K^+]_{out}$ increases (>5 mM), on the other hand, reduce neuronal firing presumably by the depolarization induced inactivation of Na^+ channels (Poolos et al., 1987). Astrocyte released ATP has been shown to fine tune synaptic activity by binding to the presynaptic purinergic P2Y receptors and inhibiting glutamate release (Zhang et al., 2003). Through their release of $TNF\alpha$, astrocytes also influence postsynaptic effectiveness by modulating the expression of AMPA receptors (Beattie et al., 2002). Which of these mechanisms are responsible for the observed decrease of neuronal spontaneous activity in the $K_{ir}4.1$ cKO hippocampus will be explored in our future studies. Such inhibition of spontaneous activity may have developed in response to the increased basal interstitial levels of glutamate and K^+ in order to reduce neuronal hyperexcitability and epileptogenesis. Measurements of $[K^+]_{out}$ levels during resting and stimulated conditions *in vivo* are underway in the laboratory of our collaborator Dr. Florin Amzica at the Université Laval in Québec, Canada. Preliminary results indicate increased basal $[K^+]_{out}$ in the cortex of $K_{ir}4.1$ cKO mice.

Janigro et al. (1997) demonstrated that blocking glial K_{ir} channels and thereby impairing K^+ buffering prevents LTD maintenance in the hippocampus. They attributed this observation to the K^+ -mediated depolarization of neuronal synaptic elements which results in

potentiation of synaptic activity (Janigro et al., 1997). Our experiments extend these findings by demonstrating that $K_{ir}4.1$ cKO leads to marked enhancement of synaptic potentiation in the hippocampal CA1 stratum radiatum. Synaptic potentiation in $K_{ir}4.1$ cKO hippocampus was significantly elevated up to 20 min after tetanic stimulation, with PTP (2 min post-stimulus) displaying 30% and STP (15 min post-stimulus) 20% enhancement compared to wild-type. PTP is thought to last 30 sec to several minutes and be presynaptic in origin, involving an increase in the number of transmitter quanta released without a change in quantal size or postsynaptic effectiveness (reviewed in Zucker and Ragehr, 2002). LTP, lasting hours to days, has a presynaptic and a postsynaptic component as its induction and expression require activation of postsynaptic NMDA receptors and increasing the number of postsynaptic AMPA receptors, respectively (reviewed in Malenka and Bear, 2004). Since the enhancement of synaptic potentiation we observe in $K_{ir}4.1$ cKO mice lasts about 20 min it may involve both pre- and postsynaptic mechanisms. As mentioned earlier, elevated $[K^+]_{out}$ causes neuronal depolarization that can lead to enhanced glutamate release from the presynaptic cell and enhanced NMDA receptor activation in the postsynaptic cell (Chamberlin et al., 1990; Poolos and Kocsis, 1990). The observed slowed $[K^+]_{out}$ clearance in $K_{ir}4.1^{-/-}$ brainstem (Neusch et al., 2006) implicates that strong neuronal stimulation may cause substantial interstitial accumulation of K^+ that will subsequently lead to synaptic augmentation. In addition, impaired glutamate uptake and Na^+/K^+ -ATPase function may also contribute to the enhanced potentiation of the $K_{ir}4.1$ cKO synapses. Surprisingly, synaptic transmission induced by a single pulse stimulation, as well as PPF, were unaffected in the $K_{ir}4.1$ cKO. Neuronal mechanisms, such as increased Na^+/K^+ -ATPase mediated K^+ uptake and/or reduced neurotransmitter release, may be capable of compensating for the reduction of

K^+ and glutamate uptake by $K_{ir}4.1$ cKO astrocytes during low activity levels. However, glial GluT and K_{ir} activity appear to be necessary for uptake of large glutamate and $[K^+]_{out}$ elevations generated by increased neuronal activity. Presence of stress-induced rather than spontaneous seizures in $K_{ir}4.1$ cKO animals supports this hypothesis.

In summary, our data demonstrate that impairment of astrocyte K^+ and glutamate uptake, induced by the loss of $K_{ir}4.1$, affects neuronal functioning by decreasing neuronal spontaneous activity and enhancing synaptic potentiation. This is yet another example of the possible astrocyte involvement in the CNS information processing, and its further exploration promises to provide novel understanding of the emerging neuron-glia functional co-dependence.

CHAPTER V. GENERAL DISCUSSION AND FUTURE DIRECTIONS

Buffering of extracellular K^+ is one of the most studied functions of astrocytes. Nevertheless, little information is available about the differential role of implicated K^+ -buffering mechanisms and their influence on extracellular K^+ homeostasis, neuronal activity, and animal behavior. In particular, little is known about the specific role of glial K_{ir} channels in intact tissue preparations and *in vivo*. $K_{ir}4.1$ is one of the most abundant K_{ir} channels in the CNS found almost exclusively in astrocytes and oligodendrocytes (Poopalasunduram et al., 2000; Higashi et al., 2001; Ishii et al., 2003). Its involvement in K^+ buffering by astrocytes has been implicated by immunohistochemical, electrophysiological, and genetic linkage studies (Higashi et al., 2001, Kofuji et al., 2001; Buono et al., 2004; Neusch et al., 2006). In this manuscript, we describe generation and characterization of the conditional knockout of $K_{ir}4.1$ directed to glia by the human GFAP promoter, *gfa2*. Contrary to previously published reports of astrocyte-restricted *gfa2* activity (Brenner et al., 1994; Brenner and Messing, 1996), our immunohistochemical studies demonstrated loss of $K_{ir}4.1$ from astrocytes and oligodendrocytes in the $K_{ir}4.1$ cKO brain and spinal cord, suggestive of *gfa2*-driven Cre recombination in glial precursors. Our laboratory and several others have since confirmed that progenitor cells displaying *gfa2* promoter activity give rise to astrocytes, oligodendrocytes, and neurons (Malatesta et al., 2000, 2003; Namba et al., 2005; Casper and McCarthy, 2006). $K_{ir}4.1$ cKO mice die prematurely, display severe white-matter vacuolization, ataxia, and stress-induced seizures. These observations provide further support

for the previously demonstrated $K_{ir}4.1$ involvement in myelination (Neusch et al., 2001) and seizure susceptibility (Ferraro et al., 2004; Buono et al., 2004).

Highly selective membrane permeability to K^+ and a strongly negative resting membrane potential are considered fundamental properties of mature astrocytes (Kuffler et al., 1966; Orkand et al., 1966; Ransom and Goldring, 1973; Lotham and Somjen, 1975). Astrocytes express a wide range of K^+ channels, but K_{ir} channels are thought to be responsible for the high K^+ permeability and maintaining the resting membrane potential close to E_K (reviewed in Verkhratsky and Steinhauser, 2000). Electrophysiological studies have demonstrated that K_{ir} conductance increases during astrocyte development, concurrent with the development of their K^+ uptake ability (Sykova et al., 1992; Kressin et al., 1995; Bordey and Sontheimer, 1997). Astrocytes express message for $K_{ir}2.1$, $K_{ir}2.2$, and $K_{ir}2.3$ (Schroder et al., 2000; Schroder et al., 2002), and can be immunolabeled for these K_{ir} subunits, as well as for $K_{ir}4.1$, $K_{ir}5.1$, $K_{ir}6.1$, and $K_{ir}6.2$, in a region specific manner (Takumi et al., 1995; Poopalasundaram et al., 2000; Higashi et al., 2001; Ishii et al., 2003; Hibino et al., 2004; Zhou et al., 2002; Thomzig et al., 2001). Despite presence of multiple K_{ir} subunits, our data demonstrate dominant role of $K_{ir}4.1$ in membrane potential maintenance of hippocampal astrocytes and corpus-callosum oligodendrocytes. In the $K_{ir}4.1$ cKO both of these cell populations are severely (>30 mV) depolarized. Furthermore, our data indicate that $K_{ir}4.1$ is directly responsible for setting the membrane potential of the hippocampal glia displaying time- and voltage-dependent currents. These cells are termed complex glia, and most are immature astrocytes that give rise to mature protoplasmic cells displaying passive voltage-independent currents. We also observe a large decrease in the relative number of complex glia in the $K_{ir}4.1$ cKO hippocampus without an apparent decrease in the total number of GFAP- and

S100 β -immunopositive cells, implicating that K_{ir}4.1 removal possibly leads to premature functional expression of passive currents. These observations are consistent with and extend previous reports based on studies of retinal Müller cells (Kofuji et al., 2000) and cultured spinal cord oligodendrocytes (Neusch et al., 2001), which showed that lack of K_{ir}4.1 depolarized their resting membrane potential and impaired oligodendrocyte development. However, our study of mature/passive wild-type astrocytes and myelinating oligodendrocytes indicates that they express minimal resting K_{ir} conductance, which influences but is not critical for their membrane potential maintenance. Furthermore, we provide a novel insight into the possible mechanism of passive astrocyte membrane potential maintenance by demonstrating that inhibition of their Na⁺/K⁺-ATPase leads to a severe membrane depolarization, comparable to that seen in K_{ir}4.1 cKO cells. Collectively, these data implicate K_{ir}4.1 importance in the development of the deeply negative astrocyte membrane potential. By directly setting the membrane potential of astrocyte precursors, K_{ir}4.1 appears to mediate proper development of the mature cell channel and transporter complement, including functional expression of their Na⁺/K⁺-ATPase. However, this hypothesis needs to be further explored and substantiated. To study astrocyte development and provide better understanding of the mature/passive astrocyte membrane physiology which is still largely a mystery, we will employ single-cell RT-PCR in combination with electrophysiological and lineage characterization *in situ*. One of the focuses of this study will be the recently isolated family of K_{2P} channels, particularly TASK and TREK channels. Since we have demonstrated that passive astrocyte currents are largely unaffected by the gap junction/hemichannel blocker carbenoxolone and display K_{2P}-like resistance to K⁺ channel blockers, including Ba²⁺, TEA, and 4-AP (Lesage, 2003), K_{2P} channels appear to be the most likely candidates mediating

mature astrocyte currents. These channels are responsible for background or leak K^+ conductance in excitable tissues and as such modulate their membrane potential (reviewed in Bayliss et al., 2003; Lesage, 2003). Furthermore, blockade of TASK channels depolarizes Müller cell membrane by about 50% (Skatchkov et al., 2005), and recent studies have shown that Müller glia express TREK-1 and TREK-2 (Eaton et al., 2004). TASK-1, TASK-3 and TREK-2 mRNA has been demonstrated by RT-PCR in cultured astrocytes (Gnatenco et al., 2002; Ferroni et al., 2003), and astrocytes *in vivo* have been shown to be immunopositive for TASK-1, TASK-2 and TASK-3 channels (Rusznak et al., 2004). These channels appear to be involved in homeostasis of glial membrane potential and cell volume, and may have a protective role during metabolic disturbances and ischemia (Ferroni et al., 2003; Skatchkov et al., 2005).

As mentioned above, glia express a variety of K_{ir} subunits, including strongly rectifying $K_{ir}2.1-2.3$ (Schroder et al., 2000; Schroder et al., 2002), weakly rectifying ATP-dependant $K_{ir}4.1$ which can form strongly rectifying heteromeric channels with $K_{ir}5.1$ (Ishii et al., 2003; Hibino et al., 2004), and ATP-sensitive $K_{ir}6.1$ and $K_{ir}6.2$ (Zhou et al., 2002; Thomzig et al., 2001). Comparison of immunolabelling for $K_{ir}2.0$, $K_{ir}4.1$, and $K_{ir}5.1$ implicates co-assembly of these subunits in Bergmann glia, Müller glia, astrocytes, and oligodendrocytes (Leonoudakis et al., 2001; Kofuji et al., 2002; Ishii et al., 2003; Hibino et al., 2004). The differences in conductance and sensitivity to intracellular and extracellular factors, including pH, ATP, G-proteins, neurotransmitters, and hormones, suggests that different K_{ir} channels expressed by glial cells may have multiple and distinct functional roles. Most notably, K_{ir} channels are implicated in astrocyte K^+ buffering, but the identity and differential roles of the K_{ir} subunits involved are not yet resolved. Study of $K_{ir}4.1^{-/-}$ mice has shown that ablation of

this channel results in reduced capacity for K^+ buffering in Müller glia (Kofuji et al., 2001), endocochlear epithelium (Marcus et al., 2002), and brainstem astrocytes (Neusch et al., 2006). Moreover, the subcellular localization of $K_{ir}4.1$ channels is consistent with a specific role in the astrocyte transport of K^+ between neurons and blood vessels (Higashi et al., 2001). Astrocyte perisynaptic and perivascular processes are also enriched with $K_{ir}5.1$ (Hibino et al., 2004) and AQP4 (Guadagno and Moukhles, 2004). In retinal Müller glia the processes surrounding neurons express $K_{ir}4.1$, $K_{ir}5.1$ and $K_{ir}2.1$, while endfeet facing the vitreous humor and processes surrounding blood vessels are enriched with $K_{ir}4.1$ and AQP4 (Nagelhus et al., 1999; Kofuji et al., 2002; Ishii et al., 2003). Observation of such subcellular localization of K_{ir} channels led to the proposed K^+ buffering model in which K^+ released during neuronal activity is taken up by glia via strongly rectifying heteromeric $K_{ir}4.1/K_{ir}5.1$ and $K_{ir}4.1/K_{ir}2.1$ channels, and possibly homomeric $K_{ir}2.1$ channels, and K^+ is then extruded from glia into extracellular “sinks” via weakly rectifying $K_{ir}4.1$ homomeric channels (Kofuji et al., 2002; Kofuji and Newman, 2004; Butt and Kalsi et al., 2006). However, while in the retina K_{ir} channels appear to dominate K^+ uptake (Oakly et al., 1992; Newman, 1993; Linn et al., 1998; Kofuji et al., 2001), in other systems additional K^+ uptake mechanisms have been implicated. Comprehensive studies of $[K^+]_{out}$ dynamics in the rat optic nerve and CA3 stratum radiatum brought forth two additional models of the $[K^+]_{out}$ homeostasis maintenance (Ransom et al., 2000; D’Ambrosio et al., 2002). The first model states that baseline $[K^+]_{out}$ and the magnitude of $[K^+]_{out}$ elevation induced by neural activity are mainly determined by glial Na^+/K^+ -ATPase (~80%) and less by K_{ir} channels. Neuronal K^+ uptake carried out by Na^+/K^+ -ATPase, compared to glial K^+ uptake, builds slowly and independently of $[K^+]_{out}$ but instead depends on $[Na^+]_{in}$ increases. The time course of $[K^+]_{out}$ decline following stimulation

is determined by: a) cessation of axonal K^+ efflux due to axon depolarization, b) glial K^+ uptake, the rate of which will fall rapidly as $[K^+]_{out}$ falls, c) axonal K^+ uptake, which has a slow buildup and a slow decline, and d) the slow release of K^+ from glia for reuptake by axons. Axonal pump rate determines the sustained, slow fall of $[K^+]_{out}$ and causes the undershoot in $[K^+]_{out}$ (Ransom et al., 2000). The second model developed by D'Ambrosio et al. (2002) suggests cooperation of K_{ir} channels and Na^+/K^+ -ATPase, whereby glial and neuronal Na^+/K^+ -ATPase are involved in setting the baseline $[K^+]_{out}$, determining the rate of its recovery and the level of its postactivity undershoot. Glial K_{ir} channels, on the other hand, are involved in the regulation of baseline $[K^+]_{out}$ and in decreasing the amplitude of the postactivity $[K^+]_{out}$ undershoot, but do not affect the rate of K^+ clearance. In agreement with the proposed models, recent measurements of stimulation induced $[K^+]_{out}$ changes in brainstem slices of juvenile (P6-10) $K_{ir4.1}^{-/-}$ mice demonstrate that $K_{ir4.1}$ is not involved in determining the level of stimulus-induced $[K^+]_{out}$ elevation, but does regulate level of the postactivity $[K^+]_{out}$ undershoot (Neusch et al., 2006). However, in contrast to the proposed models, $K_{ir4.1}$ removal also influences the rate of $[K^+]_{out}$ recovery by slowing it down almost two-fold. Possible explanations for this discrepancy may involve differences in age and brain region studied, as well as the knockout-induced changes in astrocyte physiology and alternate K^+ buffering mechanisms. For example, as described above, loss of $K_{ir4.1}$ leads to astrocyte depolarization which will reduce the electrochemical driving force for K^+ influx. By directly measuring the K^+ uptake current in $K_{ir4.1}$ cKO hippocampal astrocytes induced by the Schaffer collateral stimulation, we demonstrated an almost complete loss of K^+ uptake by astrocytes lacking $K_{ir4.1}$, while bath application of >10 mM $[K^+]_{out}$ resulted in small Ba^{2+} -insensitive K^+ uptake. Our results therefore suggest that most of the Ba^{2+} -sensitive (K_{ir}

mediated) K^+ uptake by hippocampal astrocytes is carried out via $K_{ir}4.1$ channels, and as implicated by the Neusch et al. (2006) findings, they likely control the rate of extracellular K^+ clearance and the postactivity return of K^+ to the extracellular space. Whether these roles are accomplished by subcellular colocalization and heterologous assembly of $K_{ir}4.1$ with other K_{ir} subunits, such as seen in Müller glia (Nagelhus et al., 1999; Kofuji et al., 2002; Ishii et al., 2003), still needs to be determined. Furthermore, astrocyte membrane potential and K_{ir} expression are physiologically dynamic and are regulated by diverse extracellular and intracellular factors including extracellular glutamate, intracellular cyclic AMP levels, extracellular and intracellular ATP and pH, and the extent of cell coupling via gap junctions (McKhann et al., 1997; Isomoto et al., 1997; D'ambrosio et al., 1998; Ruppersberg, 2000), thereby providing mechanisms for coupling of glial function with neuronal activity. How these K_{ir} regulating mechanisms influence K^+ buffering and neuronal excitability is also not known.

The classical view of neuromodulation has recently been extended and elaborated in a variety of directions. Recent work has prompted a re-evaluation of the part that astrocytes play in brain development and signaling. As we have reviewed throughout this manuscript, astrocytes express ion channels, transporters, and neurotransmitter receptors that sense local synaptic activity and in response modulate extracellular ion and transmitter homeostasis by their coordinated uptake and/or release. Our findings shed new light on the role of glial $K_{ir}4.1$ channels in the modulation of neuronal activity and synaptic plasticity. In addition to the almost complete abolition of the astrocyte K^+ uptake, we demonstrate that loss of $K_{ir}4.1$ also leads to impaired glutamate uptake. These $K_{ir}4.1$ cKO-induced changes in astrocyte function affect neuronal activity in two main ways. First, we observe reduced spontaneous activity of

the $K_{ir}4.1$ cKO CA1 pyramidal neurons exemplified by the lower frequency and amplitude of their sEPSCs, and second, we demonstrate enhanced synaptic potentiation in the $K_{ir}4.1$ cKO hippocampus. The exact mechanisms of the observed changes in synaptic strength in $K_{ir}4.1$ cKO will be the focus of our future studies. Changes in the frequency of sEPSCs are generally thought to reflect a presynaptic change in release probability (Bouron et al., 2001), while dynamic changes of postsynaptic receptor complement appear to primarily modulate sEPSC amplitudes (Liao et al., 1995; Oliet et al., 1996). Spontaneous neurotransmitter release contributes to the maintenance of dendritic structure and may modulate information processing in the CNS (McKinney et al., 1999). Electrical activity of the nerve endings, neurotransmitters, hypotonic solution, polycations, neurotrophic factors, and immunoglobulins can all modify the rate of spontaneous release (reviewed in Bouron et al., 2001). $K_{ir}4.1$ loss may result in tonic elevation of basal $[K^+]_{out}$ due to impaired K^+ uptake, while the observed $K_{ir}4.1$ cKO astrocyte depolarization may reflect a run-down of intracellular ion gradients leading to impaired transmitter uptake and/or release, all contributing to the inhibition of CA1 pyramidal cell spontaneous activity in the $K_{ir}4.1$ cKO hippocampus. For example, prolonged low-level electrical activity caused by slight $[K^+]_{out}$ elevation may cause spontaneous activity accommodation such is seen after LTD induction protocols which reduce both sEPSC frequency and amplitude (Goda and Stevens, 1996; Carroll et al., 1999). These effects are mediated by direct presynaptic modulatory action that reduces the basal release by an unknown mechanism, and by a reduction in the number of postsynaptic AMPA receptors (Carroll et al., 1999). Activation of neuronal presynaptic mGluRs has also been shown to depress spontaneous transmitter release (Gereau and Conn, 1995; Morishita et al., 1997; Kamiya and Ozawa, 1999; Bushell et al., 1999). It is therefore

conceivable that elevated extrasynaptic glutamate, in response to impaired glutamate uptake by $K_{ir}4.1$ cKO astrocytes, modulates transmitter release by activating presynaptic mGluRs. In addition, adenosine is a potent neuromodulator shown to inhibit spontaneous release by activating A1-type adenosine receptors (Scholz and Miller, 1992; Scanziani et al., 1992; Bouron and Reuter, 1997). In the hippocampus, astrocyte released ATP is converted to adenosine and has been shown to negatively influence excitatory synaptic strength and plasticity (Pacsual et al., 2005), while positively modulating inhibitory transmission (Kawaqamura et al., 2004). Since ATP release by astrocytes appears to be mediated by a Ca^{2+} -dependant vesicular release, we will examine stimulated and spontaneous Ca^{2+} dynamics in $K_{ir}4.1$ cKO astrocytes, as well as employ a pharmacological approach to demonstrate possible involvement of neuronal adenosine receptors in the observed changes of synaptic strength. As noted, in addition to affecting spontaneous neuronal activity, loss of glial $K_{ir}4.1$ significantly affected synaptic plasticity, specifically by augmenting synaptic potentiation up to 20 min poststimulus (100 Hz, 1 s). Synaptic plasticity is regarded as a physiological process of crucial importance because short- and long-lasting changes in synaptic strength are thought to provide the molecular basis of learning and memory (Elgersma and Silva, 1999). Short-term plasticity lasting several sec or minutes is considered to be presynaptic in origin (reviewed in Zucker and Regehr, 2002). Several important presynaptic factors control synaptic strength including, but not limited to, the size of synaptic vesicles and their neurotransmitter content, the number of release sites, and the probability of quantal release at each site. In the rat hippocampus, Janigro et al. (1997) documented direct modulation of synaptic plasticity by impaired K^+ buffering by glia. They demonstrated that Cs^+ by interfering with normal K^+ uptake into astrocytes (by directly blocking inward rectifier K^+

channels and by causing cell depolarization, thus collapsing the gradient for K^+ entry into the cell) inhibits maintenance of LTD. This observation was attributed to increased neuronal excitability in the presence of elevated $[K^+]_{out}$ which causes increased efficacy of synaptic potential generation and increased probability of transmitter release, thereby masking or overlapping with the induced LTD. Given the slowed clearance of $[K^+]_{out}$ in the $K_{ir}4.1^{-/-}$ brainstem (Neusch et al., 2006), the tetanic stimulation used to elicit LTP in the $K_{ir}4.1$ cKO hippocampal slices most likely leads to accumulation of extracellular K^+ , which potentiates synaptic transmission through the depolarization-induced increase of the presynaptic cell activity and by possibly relieving the Mg^{2+} block of the postsynaptic NMDA receptors. *In vivo* measurements of neuronal and glial activity, as well as $[K^+]_{out}$ dynamics in the $K_{ir}4.1$ cKO cortex are currently underway in the laboratory of Dr. Florin Amzica who has extensive expertise in this area of research (Amzica et al., 2002; Amzica and Massimini, 2002). Furthermore, astrocytes can modulate synaptic strength and plasticity by release of gliotransmitters, such as glutamate (Fiacco and McCarthy, 2004), ATP (Pascual et al., 2005), and D-serine (Yang et al., 2003). Specific interference with astrocyte vesicular release of transmitters in the mouse hippocampus causes reduction of extracellular ATP levels, enhanced synaptic potentiation, but decreased LTP and heterosynaptic depression (Pascual et al., 2005). These observations suggest that by suppressing excitatory transmission astrocytes regulate the degree of synaptic plasticity and heterosynaptic activity. A pharmacological approach will be taken to explore involvement of gliotransmitters in the observed enhancement of synaptic potentiation in the $K_{ir}4.1$ cKO hippocampus. Future studies of K^+ buffering and gliotransmission in the $K_{ir}4.1$ cKO will add to our understanding of ways in

which astrocytes modulate activity of neuronal synaptic elements, and by fine tuning their strength influence synaptic transmission and plasticity.

Surprisingly, CA1 pyramidal cell excitability, assessed from the frequency of action potential firing induced by single-cell current injections, and the basal synaptic transmission, induced by a single field stimulation, were not significantly altered in the $K_{ir}4.1$ cKO. Comparable results were obtained in the $K_{ir}4.1^{-/-}$ brainstem where rhythmic respiratory network activity was undisturbed by the impairment of K^+ buffering (Neusch et al., 2006). These results implicate that alternate mechanisms such as neuronal and glial Na^+/K^+ -ATPase may be adequate for K^+ clearance during low levels of neuronal activity. Casullo and Krnjevic (1987) have previously demonstrated that during low-frequency asynchronous neuronal activity, potassium buildup is buffered independently from glial depolarizations. Rises in bulk $[K^+]_{out}$ activity during such conditions range from 0.1 to 1.5 mM, depending on the brain region (Somjen, 2002). Several studies demonstrate that striking changes of synaptic function are usually found when $[K^+]_{out}$ is raised above 5 mM (reviewed in Somjen, 1979). Below that level, the sensitivity of synaptic transmission to $[K^+]_{out}$ appears to be functionally negligible, which may represent a neuron-intrinsic mechanism of synaptic strength control to maintain fidelity of transmission during $[K^+]_{out}$ alterations. In summary, our results implicate that while $K_{ir}4.1$ -mediated K^+ buffering by astrocytes does not modulate synaptic function during low levels of neuronal activity, it does, however, play a role in modulation of synaptic strength and plasticity during elevated neuronal activity. The exact mechanisms of this modulation are to be determined in our future studies.

Impressive progress in understanding of astrocyte function has been made in the past few years, mainly because of the combination of molecular and electrophysiological approaches

together with improved imaging methods. Glial biologists have provided us with a large list of glial properties and putative functions, but we still have only a rudimentary understanding of when they are used and how they manifest themselves in neurophysiology, behavior, and disease processes. It is our belief that by developing animal models that provide selective perturbation of glial gene expression while monitoring its effects on neurophysiology and animal behavior, we will come closer to understanding their role in the CNS information processing. Our laboratory is at the forefront of generating such animal models for study of astrocyte physiology and function in particular (Pascual et al., 2005; Casper and McCarthy, 2006). Since *gfa2* promoter activity was confirmed in neural and glial progenitor cells, we developed several lines of inducible Cre mice in an attempt to developmentally regulate and limit Cre recombination of floxed genes to astrocytes. One of these lines, named *gfa2*-CreERT13, contains *gfa2*-driven mutated estrogen receptor hormone-binding domain (ERT) fused to Cre-recombinase. In cells expressing CreERT addition of tamoxifen causes its translocation to the nucleus where recombination of the floxed gene can occur. Fidelity of this system was tested with two different reporter lines and has shown promising results of tamoxifen mediated Cre recombination. We have already crossed our $K_{ir}4.1^{fl/fl}$ line with the *gfa2*-CreERT13 line and are in the process of determining induction conditions that produce most reliable and complete gene recombination. This will enable direct study of $K_{ir}4.1$ role in astrocyte physiology and K^+ buffering at different stages of CNS development and further expand our understanding of this neuron-glia functional interaction. The ways in which neurons and glia cooperate to insure proper CNS functioning are most likely far from exhausted, and the pace of their discovery will only increase in coming years. As proclaimed by Volterra and Meldolesi (2005): “The astrocyte revolution is bound to continue,” as we

discover more ways in which these cells contribute to the health and disease of the nervous system.

CHAPTER VI. MATERIALS AND METHODS

1. GENERATION OF THE $K_{ir}4.1$ CONDITIONAL KNOCKOUT MICE

To generate astrocyte-specific conditional knockout (cKO) of $K_{ir}4.1$, two lines of mice were made: a recombinant $K_{ir}4.1$ floxed ($K_{ir}4.1^{f/f}$) line and a transgenic *gfa2*-Cre line. For the targeting construct of $K_{ir}4.1^{f/f}$ line, a p1 clone containing the $K_{ir}4.1$ gene was first isolated and mapped. *loxP* sites were integrated upstream and downstream of the exon containing the entire open reading frame encoding the $K_{ir}4.1$ protein. The targeting construct was electroporated into ES cells, which then underwent antibiotic resistance selection. Colonies surviving selection were tested for homologous recombination and incorporation of the *loxP* sites by PCR and Southern blot. Two clones were identified and injected into C57Bl/6J blastocysts. Chimeric mice were bred to C57Bl/6J mice to identify germline transmission of the targeted $K_{ir}4.1$ allele. In order to remove the neo/tk selection cassette which was surrounded with FRT sites, $K_{ir}4.1^{f/f}$ mice were first bred to FLPeR mice (courtesy of Dr. Susan M. Dymecki). FLPeR mice express Flp-recombinase under the control of β -actin promoter. This transgene was integrated into the ROSA26 locus which has been shown to drive transgene expression in most cells, including germline (Farley et al., 2000).

The transgenic *gfa2*-Cre line was generated by replacing the lacZ coding sequence in the pgfa2lac1 vector (courtesy of Dr. Michael Brenner) with the coding sequence of Cre-recombinase from the pBS185 vector. pgfa2lac1 contains the 2.2 kB human GFAP promoter (*gfa2*) and a polyA signal from the mouse protamine1 gene (Brenner et al., 1994). This

cassette was placed between four copies of genomic insulator sequence. The construct was linearized and injected into C3H/C57 hybrid embryos. Founder mice were identified for transgene incorporation by PCR and Southern blot. *gfa2*-Cre mice were crossed to two different reporter lines in order to establish expression of the transgene and fidelity of the Cre mediated recombination.

2. WESTERN BLOTTING

Mice (P15-25) were anesthetized by isoflurane inhalation. Brains were rapidly removed following decapitation and homogenized with a rotor-stator homogenizer in 1.5 ml of ice-cold phosphate-buffered saline (PBS) supplemented with Complete phosphatase inhibitor cocktail (Roche). Homogenates were centrifuged (1000xg, 10 min at 4°C), supernatant transferred to a fresh tube and centrifuged again (12000xg, 20 min at 4°C). Pellets were resuspended in 500 µl of 0.5% Triton X-100/PBS. Sample aliquots were flash-frozen and kept at -80°C until use. Protein concentration was determined with a Bio-Rad DC protein assay (Lowry method). Samples were diluted with 5xSample buffer prior to loading (50 µg of protein/lane) onto 10% Tris-Glycine gel and run at 125 V for 1 h. Gel was transferred to nitrocellulose membrane (100 V for 90 min). Membrane was blocked with 5% milk/PBST (PBS with 0.1% Tween-20) 2 h at room temperature (RT) and incubated with primary antibodies (1:1000 r α $K_{ir}4.1$, Alomone Labs; 1:10000 m α β-actin, Sigma) overnight at 4°C in 5% milk/PBST. Following 3x10 min washes in PBST, membrane was incubated with HRP-conjugated secondary antibodies (2 h at RT), washed again and processed for ECL detection. Incubation of $K_{ir}4.1$ antibody with the antigen peptide yielded a blank blot, confirming the specificity of this antibody.

3. HISTOLOGICAL ANALYSIS AND IMMUNOSTAINING

Mice were anesthetized by intraperitoneal injection of 20% urethane prior to perfusion with 4% paraformaldehyde (PFA). Brains and spinal cords were removed and postfixed overnight at 4°C in 4% PFA. Tissue was then dehydrated, embedded in paraffin, sectioned at 6 µm thickness with a Leica microtome, and placed on slides. Sections were stained with hematoxylin and eosin or solochrome and eosin and imaged using an Olympus light microscope. For immunostaining, perfused brains and spinal cords were cryoprotected in 30% sucrose overnight at 4°C prior to freezing in OCT. Frozen tissue was sectioned at 14 µm thickness. Sections were blocked with blocking solution (20% normal goat serum, 0.2% Triton X-100, 2% BSA) for 4 h at RT and further incubated with primary antibody (1:500 r α Kir4.1, Alomone Labs; 1:5000 gp α GFAP, Advanced ImmunoChemical; 1:500 m α CNP, Sigma; m α MBP, Sigma; m α PLP, Serotec; 1:1000 m α neurofilament, Sternberger Monoclonals, Inc.; 1:1000 gp α GLAST, Chemicon; 1:1000 m α NeuN, Chemicon; 1:500 m α calbindin, Sigma) overnight at 4°C. Fluorescent secondary antibody was applied for 3 h at RT. Images were captured on the Zeis Axioskop fluorescent microscope.

4. WHOLE-CELL ELECTROPHYSIOLOGY

Mice (P5-30) were anesthetized by isoflurane inhalation. Brains were rapidly removed following decapitation and submerged into 4°C slicing buffer (125 mM NaCl, 10 mM glucose, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 2.5 mM KCl, 3.8 mM MgCl₂, 100 µM kynurenic acid) bubbled with 95% O₂, 5% CO₂. Sagittal or coronal sections (300µm) were cut on a Leica VT1000S vibrotome and hippocampus dissected. Hippocampal slices were then incubated in oxygenated 35°C ACSF for 45 min (125 mM NaCl, 10 mM glucose, 1.25

mM NaH₂PO₄, 26 mM NaHCO₃, 2.5 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgCl₂). After cooling down to RT slices were transferred to a recording chamber in an upright fixed-stage Olympus LSM-GB200 argon/krypton confocal microscope and perfused with oxygenated RT ACSF. Borosilicate glass pipettes were pulled on a Narishige PP-83 vertical pipette puller and were not fire-polished. Pipettes had a resistance of 7-9 MΩ and contained 145 mM K-gluconate, 2 mM MgCl₂, 10 mM HEPES, 4 mM Mg-ATP, 14 mM phosphocreatine, 0.25 mM EGTA, and 500 μM Alexa 488 or 568 hydrazide, pH 7.3. Cells were visualized and patched using DIC optics. Whole cell-patch clamp recordings were performed using the Axopatch 200B amplifier and PClamp 9.0 software (Axon Instruments). The membrane potential was stepped from V_m (unless otherwise stated) to -180 mV and up to 80 mV in 20 mV increments to measure whole-cell currents. A test pulse of -5 mV was included after each step to monitor changes in access resistance. The signal was low-pass filtered at 5 kHz and digitized at 100 kHz using a Digidata 1200 computer interface (Axon Instruments). Perfusion solutions were switched using a Warner Instruments valve controller. Exchange of the solution in the recording chamber was completed in about 20 sec.

5. EXTRACELLULAR ELECTROPHYSIOLOGY

Mice (P20-25) were anesthetized with a lethal dose of barbiturates and rapidly decapitated. Brains were bisected in oxygenated ice-cold dissection buffer (75 mM sucrose, 87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 10 mM glucose, 7 mM MgCl₂, 0.5 mM CaCl₂, 1.3 mM ascorbic acid) and sagittal 400 μm sections were cut using a Leica VT1000S vibrotome. Hippocampus was dissected and slices were left to recover for 45 min in an incubation chamber filled with warmed (30°C) oxygenated ACSF (124 mM NaCl,

3 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 20 mM glucose, 2 mM CaCl₂, 1 mM MgCl₂, 0.75 mM ascorbic acid). After 15 min at room temperature slices were transferred to a submersion recording chamber maintained at 30°C and perfused with ACSF (without ascorbic acid). Concentric bipolar stimulating electrode was positioned in CA1 sr ~250 μm below the pyramidal cell layer. Glass recording electrode (~1 MΩ) filled with ACSF was positioned parallel to the stimulating electrode 300-400 μm away. An attempt was made to maintain similar orientation of the electrodes relative to the pyramidal cell layer and dentate gyrus in order to minimize changes in fEPSP properties due to electrode positioning. Input/output curve was generated by stepping the stimulation amplitude from 0 to 80 μA. Stimulation amplitude that elicited half the maximum response and stimulation rate of 0.033 Hz (1 pulse every 30 sec) were used throughout the experiment unless otherwise stated. PPF protocol included 10 two-pulse pairs with increasing inter-pulse interval from 25 to 250 ms. Long-term potentiation (LTP) was induced with a single 1-sec 100-Hz train after 15 min of stable baseline recording (<5% drift). LTP was sampled for 45 min following induction.

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