Expression and Function of $K_{ir}7.1$ in the Murine Central Nervous System

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Abstract

Glia express a variety of ion channels, but the precise subtypes expressed by astrocytes and oligodendrocytes has not been fully elucidated. The Kir7.1 subtype of inwardly rectifying potassium channels (Kir) is highly expressed in retinal pigment epithelium and has been demonstrated in Purkinje neurons of the adult rat cerebellum and pyramidal neurons of the hippocampus, but it has not previously been identified in glia. Using quantitative real time PCR, an ion channel profile for the developing mouse optic nerve was constructed and Kir7.1 was identified as one of the major ion channels present. Immunostaining revealed widespread expression of Kir7.1 in glia and neurons in the mouse brain with the highest expression found in optic nerve oligodendrocytes. A major function of Kir is to maintain the membrane potential of glia in the face of large ionic shifts associated with normal neuronal function and pathology. Oligodendrocytes are particularly susceptible to ischemia so the role of Kir7.1 in maintaining oligodendrocyte integrity during oxygen and glucose deprivation (ODG) in the isolated intact mouse optic nerve was examined, using the Kir7.1 channel blocker VU590. Blockade of Kir7.1 resulted in increased cell death of optic nerve oligodendrocytes in normoxic conditions by activating caspase-dependent apoptotic pathways and significantly augmented cell death induced by ODG. Moreover, intracellular calcium fluctuations dependent on store operated calcium entry in optic nerve glia were identified as a potential mechanism for the cellular stress induced by Kir7.1 inhibition. The results presented within this thesis demonstrate functional expression of Kir7.1 in glial cells, and indicate they are important in maintaining oligodendrocytic integrity in both physiological and pathological conditions.
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Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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Dissemination

List of Poster Presentations

Sept 2014  SoNG, Southampton UK
July 2013  IUPS, Birmingham, UK
July 2013  Euroglia, Berlin, Germany
Oct 2012  Society for Neuroscience, New Orleans, USA
July 2012  Physiology 2012, Edinburgh, UK
Sept 2011  10thEuroglia European Meeting, Prague, Czech Republic
July 2011  8thIBRO World Congress of Neuroscience – Florence, Italy
July 2011  Young Physiologists Symposium, Oxford, U.K.
July 2010  Anatomical Society summer meeting, Portsmouth, UK

List of Oral Presentations

July 2012  Young Physiologists Symposium, Edinburgh, UK
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>$[\text{Ca}^{2+}]_i$</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>$[\text{K}^+]_i$</td>
<td>Intracellular potassium concentration</td>
</tr>
<tr>
<td>$[\text{Na}^+]_i$</td>
<td>Intracellular sodium concentration</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial Cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ASIC</td>
<td>Acid Sensing Ion Channel</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine -5'-triphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>Barium</td>
</tr>
<tr>
<td>Cav$^+$</td>
<td>Voltage Gated Ca$^{2+}$ Channel</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium Induced Calcium Release</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CRAC</td>
<td>Ca$^{2+}$-release activated Ca$^{2+}$ channel</td>
</tr>
<tr>
<td>DIV</td>
<td>Days <em>in vitro</em></td>
</tr>
<tr>
<td>dsRED</td>
<td>Discosoma sp. red fluorescent protein</td>
</tr>
<tr>
<td>ECS</td>
<td>Extracellular Space</td>
</tr>
<tr>
<td>eGFP</td>
<td>(Enhanced) Green fluorescence protein</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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</table>
IP₃R  Inositol triphosphate Receptor
Kᵢᵣ  Inward rectifier potassium ion channel
Kₖᵃ  Calcium activated K⁺ Channel
KO  Knockout
Kᵥ  Voltage Gated K⁺ Channel
Naᵥ  Voltage Gated Na⁺ Channel
NCX  Sodium (Na⁺) – Calcium (Ca²⁺) Exchanger
NG2  Neuron-glial Antigen 2
NMDA  N-methyl-D-aspartate
OGD  Oxygen and Glucose Deprivation
OGN  Oxygen and Glucose Normal
OPC  Oligodendrocyte Progenitor Cell
PDZ  Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PLP1  Proteolipid protein
PMCA  Plasma Membrane Ca²⁺ ATPase
qRT-PCR  Quantitative Real Time Polymerase Chain reaction
RMP  Resting membrane potential
RPE  Retinal Pigment Epithelium
rtPCR  Reverse Transcription Polymerase Chain reaction
SERCA  Sarco- Endoplasmic Reticulum
SOCE  Store Operated Calcium Entry
STIM  Stromal Interaction Molecule
TRP  Transient Receptor Potential channels
Chapter 1 - General Introduction
1.1. **Cells of the central nervous system**

The two main cell types of the central nervous system (CNS) are neurones and glia. Neurones are electrically excitable cells and are responsible for transmitting electrical and chemical signals, thus receiving, processing and conveying information throughout the nervous system (Levitan et al., 2001). Glia are electrically inexcitable and are made up of astrocytes, myelinating oligodendrocytes, oligodendrocyte progenitor cells (OPCs) and microglial cells. Glial cells were previously considered to be the connective tissue of the brain, but it is now known that their functions are vital to the normal physiology and function of the nervous system (Verkhratsky and Butt, 2013). Neurones and glia are neural cells that originate from a common multipotent neural progenitor cell of the neuroepithelium, under the control of multiple signals. In contrast, microglial cells are mesodermal cells derived from foetal macrophages that enter the CNS during embryonic development to give rise to microglia (Kessaris et al., 2001; Colognato and Ffrench-Constant, 2004). It was also believed that neuronal precursor cells disappear around birth so that there would be no development of new neurones in the mature brain. This notion changed as evidence started accumulating on the presence of populations of neural stem cells in the subventricular zone (SVZ) and the dentate gyrus of the hippocampus, that give rise to neurones throughout life (Kernie and Parent, 2010).

1.1.1. **Astrocytes**

Astrocytes are multi-functional glial cells and are the main homeostatic element of the CNS. A defining characteristic of astrocytes is the expression of the glial fibrillary
Acidic protein (GFAP; Figure 1.1), an intermediate filament that forms the astrocytic cytoskeleton and is used as a marker for mature astrocytes (Kimelberg, 2004). Astrocytes have a very heterogeneous morphological phenotype in the various brain regions which include protoplasmic astrocytes of grey matter of the brain and the spinal cord, which have a number of thick processes that are often found to be in contact with blood vessels and they are associated with neuronal cell somata and synapses, fibrous astrocytes localized in the white matter which usually display the “traditional” stellate morphology with longer and thinner processes and are associated with the nodes of Ranvier, classical radial glia that act as pluripotent neural precursor cells during development, radial Müller glia of the retina, cerebellar Bergmann glia and velate astrocytes, tanycytes that connect ventricular walls with parts of hypothalamus and spinal cord, pituicytes in the neuro-hypophysis, and perivascular and marginal astrocytes. In addition, astroglia cover several types of specialized cells such as ependymocytes, choroid plexus cells and retinal pigment epithelial cells (Parpura and Verkhratsky, 2012).

Astrocytes support neuronal functions in many ways:

- **they provide structural support by organization of the CNS in domains;** the distribution of astrocytes throughout the adult CNS is highly organized and parcellates the gray matter of the entire brain and spinal cord into small astrocyte-delimited domains that form the basis for a neuro-vascular unit, which is central for neurovascular coupling and the metabolic support of neurons. The parcelling process starts during the later stages of embryogenesis and has been termed
"tiling" (Nedergaard et al., 2003; Verkhratsky and Butt, 2013). The astrocytic domains are very well-defined and the elements within each domain, i.e. neurons, synaptic terminals and blood vessels are integrated and surrounded by protoplasmic astrocyte processes that express all receptors necessary for synaptic support and local signalling, while distal signalling and coupling of neighbouring astrocytes occurs through the most distal astrocytic processes which are rich in gap junctions (Bushong et al., 2002; Verkhratsky and Butt, 2013).

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**they contribute to the formation of the blood-brain barrier;** The physical substrate of the BBB are the endothelial cells of the brain capillaries which are sealed by tight junctions that inhibit the paracellular transport of molecules even as small as ions. The barrier function is not fixed, but can be modulated and regulated, both in health and in pathology and the role of astrocytes is to completely ensheath the CNS capillaries in order to regulate and maintain the properties of the BBB. In vitro studies have provided ample evidence that astrocytes can regulate the BBB by adjusting the physical barrier (inducing tighter tight junctions), controlling the transport barrier (via the expression and polarized localisation of transporters on the astrocytic endfeet), and regulating the metabolic barrier thus ensuring an adequate supply of essential water-soluble nutrients and metabolites required by nervous tissue, and for which the BBB has low passive permeability (Dupport et al., 1998; Hasseloff et al., 2005; Abbott et al., 2006; Abbott et al., 2010; Verkhratsky and Butt, 2013). More recently, pericytes have been also shown to contribute to BBB induction (Dohgu et al., 2005).
• **they are responsible for the formation of glial scar tissue (reactive gliosis) in case of brain injury:** Astroglialosis is triggered by signals (represented by neurotransmitters such as ATP and glutamate, cytokines, growth factors and blood factors) derived from damaged cells at the core of any insult regardless of aetiology. Generally, astrocytic transformation into reactive astrocytes begins with the upregulation of GFAP and vimentin, and hypertrophy of the astrocytic cell body and processes. Two morphological types of reactive astrogliosis have been identified to date: i) isomorphic gliosis in which the normal architecture of CNS tissue is not altered, astroglial microdomain organisation is preserved and the remodelling of neuronal networks is facilitated, and ii) anisomorphic gliosis during which the reactive astrocytes proliferate and lose their domain organisation. In anisomorphic gliosis, astrocytes also produce keratin and chondroitin thus inhibiting axonal regeneration and resulting in the formation of a permanent glial scar (Sofroniew, 2009; Robel et al., 2011; Verkhratsky and Butt, 2013).

• **they provide metabolic support through the “astrocyte-neurone lactate shuttle”;**

The brain has very high energy requirements mainly due to the need for maintenance and restoration of ion gradients following signalling processes such as postsynaptic and action potentials, as well as uptake and recycling of neurotransmitters (Attwell and Laughlin, 2001; Alle et al., 2009). It is now generally accepted that the metabolic needs of active neural tissue are met, at least partially, by non-oxidative glucose metabolism (i.e., glycolysis) (Figley and Stroman, 2011; Bélanger et al., 2011). Experimental evidence has demonstrated that the amount of glucose taken up by astrocytes is disproportionately high
compared to their energy requirements (Barros et al., 2009; Chuquet et al., 2010) and the reason for this is explained by the astrocyte-neuron lactate shuttle (ANLS) model proposed by Pellerin and Magistretti (Pellerin and Magistretti, 1994). Perivascular astroglial processes possess GLUT1 transporters via which they uptake glucose and through the process of aerobic glycolysis they convert it into pyruvate and subsequently into lactate by lactate dehydrogenase type 5 (LDH5), an enzyme exclusive to astrocytes. Lactate is released into the ECS and is taken up by neurons via the monocarboxylase transporters (MCT-1 and 2), where it is converted back into pyruvate by LDH1 and is utilised to produce energy. Glutamate released during synaptic transmission and uptaken by astrocytes acts as a signal for astrocytes to increase their delivery of energy supply to active neurones. This coupling of high synaptic activity and energy delivery from astrocytes to neurons is critical for higher cognitive brain functions (Suzuki et al., 2011; Verkhratsky and Butt, 2013).

- **they provide developmental support with neurone migration and the formation of new synapses;** Following the generation of neural stem cells from neuroectodermal cells, the first cells to develop are radial glia, an important function of which is to provide a scaffold along which the neural precursor cell can migrate (Kriegstein and Alvarez-Buylla, 2009). This is particularly important where neurones will be organised in layers e.g. cerebral cortex and cerebellum (Rakic, 2003 a and b). When neurones have reached their final sites, they will start extending axons and forming synapses. The biggest wave of synaptogenesis starts shortly after birth in the mammalian brain and lasts for several weeks, while
synapses continuously strengthen, weaken, die or are formed throughout life (Kelsch et al., 2010). Experimentally, increased synaptogenesis has been observed when neurons are cultured together with astrocytes due to the production of cholesterol by astrocytes, as this lipid is essential for new membranes that are formed during synaptogenesis (Mauch et al., 2001). Moreover, glia produce signals by which they can influence the expression of proteins that are essential for synapse formation. Following the formation of new synapses, astrocytes control their maturation through several signals such as TNFa (tumour necrosis factor alpha) or ADNF (activity-dependent neurotrophic factor) which induce the insertion of glutamate receptors and NMDA receptors unto post-synaptic membranes respectively (Eroglu and Barres, 2010). Finally, astrocytes can also limit the number of synapses or be involved in the elimination of synapses in the CNS (Verkhratsky and Butt, 2013).

- they contribute to CNS microenvironment regulation by maintaining the extracellular space (ECS) water and pH homeostasis and preventing neurotoxicity by neurotransmitter uptake; Astrocytes express water channels termed aquaporins on their perivascular and subpial endfeet, through which they regulate water exchange between the blood supply and the CNS ECS in response to changes in the extracellular osmolarity (Kimelberg, 1992) or in cases of high synaptic activity, during which the ECS surrounding active synapses is restricted thus increasing the efficacy of synaptic transmission (Nagelhus et al., 2004; Haj-Yasein et al., 2012). Moreover, astrocytes are involved in the maintenance of extracellular pH in the CNS by releasing protons and lactate via the MCT-1.
transporter or by removing protons via glutamate transporters. These mechanisms are controlled by transmembrane ion gradients and can operate in forward or reverse modes (Verkhratsky and Butt, 2013). Importantly, astrocytes are responsible for the uptake of neurotransmitters following synaptic activity, thus preventing accumulation of these molecules in the synaptic cleft. More specifically, they possess glutamate transporters via which they remove glutamate from the synaptic cleft, convert it into glutamine with the enzyme glutamine synthetase and finally transport the glutamine back into neurons via the glutamate-glutamine shuttle, where it is converted back into glutamate and released during the next synaptic potential (Danbolt, 2001; Eulenburg and Gomeza, 2010). In the case of inhibitory neurons, astrocytes uptake GABA through glutamate transporters GAT-3 and GAT-1. Importantly, GABA is synthesised from glutamate in neuronal terminals which also arrives from the glutamate-glutamine shuttle, which in this case is termed GABA-glutamine shuttle (Ortinski et al., 2010). Finally, removal of ATP is also undertaken by astrocytes via specifically expressed transporters (Boison et al., 2010).

- **they help regulate the ECS ion homeostasis through the various ion channels and transporters they express;** The maintenance of the extracellular ion composition by astrocytes will be discussed in detail throughout this thesis.

Even within a region, astrocytes differ in their morphology as well as the expression of ion channels as they must adapt to the functional requirements of
different brain regions (Allen and Barres, 2009; Matyash and Kettenmann, 2010; Verkhratsky and Butt, 2013).

1.1.2. Oligodendrocytes

The exclusive function of oligodendrocytes (Figure 1.2) is the production and preservation of large sheets of compacted myelin (myelin sheaths) that provide insulation to neuronal axons within the CNS and form the nodes of Ranvier which facilitate the saltatory transmission of axonal signals. A single oligodendrocyte can myelinate multiple (as many as 50) axons; failure to form myelin (hypomyelination), or failure to maintain the myelin sheath (demyelination), impairs the conduction of signals in the affected nerves and can lead to neurological disease such as Multiple Sclerosis (MS) and Cerebral Palsy.

There are several oligodendroglial specific markers, used to distinguish between OPCs and mature oligodendrocytes. Among the most commonly used is Proteolipid Protein (PLP), a myelin protein produced by mature oligodendrocytes (Mela and
Goldman, 2009; Azim et al., 2012) and the NG2 chondroitin sulfate proteoglycan used to distinguish OPCs (Nishiyama et al., 1996).

Figure 1. 2: Oligodendrocyte in the mouse striatum. Myelinating processes are stained for PLP and are ensheathing multiple axons (arrows). The nuclei were stained with Hoechst Blue (Papanikolaou, unpublished)

1.1.3. Oligodendrocyte progenitor cells (OPCs)

During development, OPCs give rise to oligodendrocytes and then persist in the adult CNS as a pool of OPCs throughout the brain parenchyma (Figure 1.3). In the adult CNS, 90% of OPCs are not mitotically active, but following injury or demyelination they become mitotically active and regenerate oligodendrocytes, astrocytes and possibly neurones (Leoni et al., 2009; Zhao et al., 2009). OPCs have been shown to form functional synapses with neurones (Kukley et al., 2008; Bergles et al., 2010).
1.1.4. Microglia

Microglial cells (Figure 1.4) comprise 10-15% of the glial population and they are usually referred to as “the resident macrophages of the CNS” (Kettenmann et al., 2011). They remain in a resting state in physiological conditions but when a pathogenic factor is identified, they are activated and act rapidly by attacking the pathogen and subsequently removing the remains of the damage by phagocytosis (Verkhratsky and Butt, 2013; Amor et al., 2010).
1.1.5. Ependymal cells

Ependymal cells line the ventricles and the central canal of the spinal cord. The majority of ependymal cells are ciliated and responsible for the constant flow of the cerebrospinal fluid (CSF). There is a special group of ependymal cells within the ventricles; the choroid plexus, that are responsible for CSF production and forming the blood-CSF barrier (Chojnacki et al., 2009).
1.2. Excitability of Neural Cells

Neuronal excitability is well described in textbooks. In brief, activation of postsynaptic neurotransmitter receptors triggers a shift in the membrane potential, either an excitatory or inhibitory post synaptic potential (EPSP, IPSP), which is transmitted to the cell soma and thence to the axon hillock, or initial segment, the site of action potential initiation. The action potential is a rapid change in the membrane potential that depends on the membrane being depolarized sufficiently to a threshold. Whether the membrane is depolarized to threshold to generate the all-or-nothing action potential depends on the sum of the EPSP and IPSP electrical signals transmitted from the dendrites. Even though glial cells are not electrically excitable, i.e. they cannot generate action potentials like neurones, they display what have been termed ‘calcium excitability’ and ‘sodium excitability’, involving a range of plasmalemmal and intracellular calcium (Ca\(^{2+}\)) channels and sodium (Na\(^{+}\)) channels (Nedergaard et al., 2010; Kirischuk et al., 2012). The main route for glial responses to chemical stimulation is via Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores such as the Endoplasmic Reticulum (ER) and the mitochondria, and by triggering Ca\(^{2+}\) influx through plasmalemmal Ca\(^{2+}\) channels. Sodium signalling has also been described in astrocytes and is thought to represent an additional mechanism for neurone-glial signalling (Hamilton et al., 2008; Verkhratsky et al., 2012; Verkhratsky and Butt, 2013).

All of these events in neural cell excitability are dependent on the expression and site-specific localisation of families of voltage-gated ion channels, which mediate Ca\(^ {2+}\)- and Na\(^ {+}\)-signalling in glia and synaptic signalling and electrical activity in neurones. The main differences between neurons and glia is the expression of high densities of
voltage-gated Na⁺ and Ca²⁺ channels in neurones, which mediate the action potential and synaptic signaling, respectively as well as different ion distributions across their cell membranes (Figure 1.5). In addition, glia and neurons differ in their intracellular ion concentrations, in particular a higher concentration of Na⁺ and Cl⁻, due to the activity of Na⁺-Cl⁻ co-transporters (Verkhratsky and Butt, 2013). A key feature of glia compared to neurons is their much higher K⁺ selective permeability of their plasmalemma, which is due primarily to high expression of inwardly rectifying potassium channels (Kir), and which maintains the characteristic strongly negative glial resting membrane potential (RMP), close to the equilibrium potential for potassium (E_K). Kir channels are also a feature of neurones, with important roles in stabilizing the neuronal membrane potential and repolarisation of axons during the action potential (Armstrong and Hille, 1998).

Figure 1.5: Cytoplasmic ion concentrations in neurones and astrocytes. (Verkhratsky and Butt, 2013).
1.2.1. Sodium (Na$^+$)

Na$^+$ channels can be found in both electrically excitable (neurones) and electrically silent cells (glia), where they display the same molecular structure and biophysical properties. The main difference is the density of channel expression on the cell membrane; on neuronal cell membranes their density can reach up to 10000 channels per µm$^2$ whereas glial cells express about one channel per 10µm$^2$. Glial progenitor cells show much denser Na$^+$ channel expression than mature glia which is thought to suggest their involvement in glial proliferation, differentiation and migration, whereas their functions in mature astrocytes and oligodendrocytes have yet to be elucidated. The main route for Na$^+$ influx in mature glia is through ionotopic glutamate and purinoceptors, which produce substantial Na$^+$ fluxes upon activation and possibly via Transient Receptor Potential (TRP) channels (Parpura and Verkhratsky, 2012, Verkhratsky and Butt, 2013). Na$^+$ signals are responsible for moderating important astroglial functions such as metabolic support for neurones, K$^+$ buffering ([Na$^+$]$_i$ levels regulate the function of the Na$^+$/K$^+$-ATPase and the Na$^+$/K$^+$/Cl$^-$ co-transporter NKCC1), pH homeostasis, and even Ca$^{2+}$ signalling by moderating the activity of the Na$^+$/Ca$^{2+}$ exchanger (NCX) (see details in subsection 1.2.5) (Kirischuk et al., 1997; Suzuki et al., 2011; Kirischuk et al., 2012)

1.2.2. Calcium (Ca$^{2+}$)

Ca$^{2+}$-permeable ion channels that allow Ca$^{2+}$ influx into the cell are represented by several families, including the highly Ca$^{2+}$ selective voltage-gated Ca$^{2+}$ channels (Ca$_v$), cationic channels with various degrees of Ca$^{2+}$ permeability, such as ionotopic neurotransmitter receptors (e.g. glutamatergic, purinoreceptors), and the family of
TRP channels, as well as the plasmalemmal Ca\textsuperscript{2+}-release activated Ca\textsuperscript{2+} channels (CRAC). Intracellular Ca\textsuperscript{2+} channels include the inositol 1,4,5-trisphosphate receptors (IP\textsubscript{3}R) and ryanodine receptors (RyR), which cause an increase in [Ca\textsuperscript{2+}]\textsubscript{i}, by releasing Ca\textsuperscript{2+} from the ER stores into the cytoplasm. The maintenance of [Ca\textsuperscript{2+}]\textsubscript{i} levels is brought about by sequestration of Ca\textsuperscript{2+} into the ER lumen by transporters such as the Sarco-endoplasmic reticulum ATPases (SERCA or Sarco-endoplasmic reticulum Ca\textsuperscript{2+} pumps) or by extrusion of Ca\textsuperscript{2+} into the ECS by plasmalemmal Ca\textsuperscript{2+} ATPases (PMCA) and ion exchangers, such as NCX (Solovyova and Verkhratsky, 2003; Verkhratsky and Butt, 2013). The mitochondria, which are able to accumulate Ca\textsuperscript{2+} through the Ca\textsuperscript{2+} uniporter and to release Ca\textsuperscript{2+} through the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger are also involved in regulating cellular Ca\textsuperscript{2+} homeostasis (Nicholls, 2005; Parpura and Verkhratsky, 2012).

As with Na\textsuperscript{+} channels, Ca\textsubscript{v} channels are more highly expressed in glial progenitors than mature glia and are thought to regulate early developmental processes in oligodendrocytes, including expression of myelin genes. Their expression persists in mature oligodendrocytes, in which they are suggested to modulate Ca\textsuperscript{2+} entry in processes and controlling myelination, but there is little evidence for functional expression of Ca\textsubscript{v} channels in mature astrocytes (Verkhratsky and Butt, 2013). However, there are indications of their upregulation in astrocytes in pathology, for example in reactive astrogliosis and epilepsy (Parpura et al., 2011).
The IP₃R is a cation-selective channel gated by IP₃ and located on the ER membrane. There are 3 mammalian subtypes of IP₃R; IP₃R1, IP₃R2 and IP₃R3 (Bezprozvanny, 2005; Stutzmann and Mattson, 2011). IP₃R2 is the main subtype expressed in astrocytes and IP₃-mediated Ca²⁺ signalling is also present in OPCs, oligodendrocytes and microglia even though IP₃R expression in OPCs and oligodendrocytes has not been resolved (Zhao et al., 2000; Fiacco and McCarthy, 2006; Butt, 2006; Kettenmann et al., 2011; Kanno and Nishizaki, 2012). The IP₃Rs are simultaneously controlled by IP₃ and Ca²⁺ ions and therefore local increase in [Ca²⁺], facilitates receptor opening and promotes Ca²⁺-induced Ca²⁺ release (CICR) through neighbouring IP₃R. In addition to intracellular and luminal Ca²⁺, IP₃R can be modulated by ATP and pharmacological modulators (Alexander et al., 2009). All the aforementioned modes of IP₃R activation underlie the propagation of Ca²⁺ waves which are important in many aspects of glial physiology, such as long range signalling via the astrocytic syncytium, the transfer of signal from distal astrocytic processes to the soma and neurotransmitter release. These Ca²⁺ waves can be completely stopped by blockade of SERCA pumps (e.g. by thapsigargin) or inhibition of phospholipase C (PLC), which catalyses the production of IP₃ by hydrolysing phosphatidylinositol 4,5-bisphosphate (PIP₂) (Porter and McCarthy, 1995; Kirischuk et al., 1995; Kirischuk et al., 1996; Kirischuk et al., 1999). Moreover, blockade of IP₃R using the membrane-permeable antagonist diphenylboric acid 2-aminoethyl ester (2-APB) is also sufficient to inhibit Ca²⁺ waves in astrocytes and Ca²⁺-dependent neurotransmitter release (Hua et al., 2004; Fellin et al., 2004; Shigetomi et al., 2008). Previous studies have linked IP₃R with some neurodegenerative diseases. In ischaemic stroke, Ca²⁺ release through IP₃R
is enhanced (Stutzmann and Mattson, 2011). In AD, presenilin (PS) 1 activates IP₃R to release Ca²⁺ and increase [Ca²⁺]ᵢ (Berridge, 2010).

1.2.3. Ca²⁺ and neural cell death

Ca²⁺ is an important intracellular messenger that mediates many of the physiological responses to chemical and electrical stimulation. However, it can also activate caspases, so excessive influx may lead to the activation of apoptotic pathways and cell death (Mattson, 2007). Intracellular Ca²⁺ ([Ca²⁺]ᵢ) overload has been implicated in several neurodegenerative diseases, such as ischaemic stroke, amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) (Mattson, 2007; Stutzmann and Mattson, 2011). In stroke for example, compromised blood flow (ischaemia) results in a massive release of glutamate which can activate glial and neuronal N-Methyl-D-aspartate (NMDA) receptors and result in Ca²⁺ influx (Salter and Fern, 2005). In addition, membrane associated oxidative stress can also impair the function of glutamate transporters, promote membrane depolarization and result in further Ca²⁺ influx (Mattson, 2007). However, the [Ca²⁺]ᵢ increase is not solely due to Ca²⁺ influx, but also due to release from intracellular Ca²⁺ stores. As soon as Ca²⁺ enters the cell, it binds with receptors present on the ER and can activate CICR and more Ca²⁺ to be released into the cytosol via IP₃R and RyR (Berridge, 2010).
1.2.4. Store Operated Calcium Entry (SOCE)

Store-operated Ca\(^{2+}\) entry (SOCE) is present in most electrically excitable and non-excitable cells and it refers to the opening of plasmalemmal Ca\(^{2+}\) channels and subsequent Ca\(^{2+}\) influx in response to low Ca\(^{2+}\) concentration in the ER lumen ([Ca\(^{2+}\)]\(_L\)) (Parekh and Putney, 2005). SOCE activation provides Ca\(^{2+}\) for replenishment of the ER store, but it is also important for producing the sustained (“plateau”) phase of the Ca\(^{2+}\) signal that often lasts even after the agonist-evoked signal has ended. There are several parts comprising the molecular machinery of SOCE. Some cells express store-operated plasmalemmal channels characterised by extremely high Ca\(^{2+}\) selectivity and very low single channel conductance (I\(_{\text{CRAC}}\)). For the activation of these channels, the stromal interaction molecule (STIM) proteins that are located on the ER membrane and detect [Ca\(^{2+}\)]\(_L\) are translocated near the cell membrane and interact with Orai proteins that form the plasmalemmal channel (Feske et al., 2006; Putney, 2007; Verkhratsky and Butt, 2013). Astrocytes show functional expression of SOCE with Stim1/Orai1 overexpression or silencing resulting in the modulation of thrombin-induced intracellular Ca\(^{2+}\) dynamics (Pivneva et al., 2008; Akita and Okada, 2011; Moreno et al., 2012; Motiani et al., 2013).

In recent years, it has been shown that SOCE may also involve activation of TRP channels. The superfamily of TRP channels are non-selective cation channels that are expressed in many cell types and can be activated by various intra- and extracellular signals (Venkatachalam and Montell, 2007). TRP allow both Na\(^+\) and Ca\(^{2+}\) ions to enter the cell in both excitatable and non-excitable tissues (Ramsey et al., 2006). TRP channels

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constitute at least seven subfamilies, TRPA (TRP-ankyrin), TRPC (TRP-canonical), TRPM (TRP-melastatin), TRPV (TRP-vanilloid), TRPP (TRP-polycystin), TRPML (TRP-mucolipin) and TRPN (TRP-NOMPC) which is not found in mammals. More specifically the TRPC (canonical) subfamily; Trpc1 mRNA and protein have been detected in astrocytes and these channels have been shown to modulate astroglial Ca²⁺ signals while involvement of Trpc4 and Trpc5 as auxiliary subunits has also been postulated (Grimaldi et al., 2003; Golovina, 2005; Smyth et al., 2006; Malarkey et al., 2008).

1.2.5. Pumps and transporters

Plasmalemmal Na⁺/K⁺-ATPase is a member of the P-type family of active cation transport proteins, which is ubiquitous in animal cells, that catalyses the efflux of three Na⁺ and influx of two K⁺ per ATP hydrolysed, resulting in a net loss of positive charge within the cell (Jorgensen et al., 2003). The glial Na⁺/K⁺-ATPases are activated following an increase in intracellular Na⁺ concentration ([Na⁺]ᵢ) and hence every transient [Na⁺]ᵢ elevation promotes Na⁺ efflux in exchange for K⁺ influx. In addition, their saturation is much higher than the neuronal pumps (up to 15mM compared to 3mM respectively) which is suitable mechanism for the removal of extracellular K⁺ ([K⁺]ₒ) during neuronal action potential propagation (Parpura and Verkhratsky, 2012; Verkhratsky and Butt, 2013).

Na⁺/Ca²⁺ exchanger (NCX) is expressed in the plasma membrane of virtually all animal cells and it is arguably the most physiologically important ion exchanger in glia. It can operate either in the forward mode (removal of one Ca²⁺ in exchange for the import of three Na⁺ ions) or in the reverse mode (extrusion of Na⁺ and influx of Ca²⁺
ions). The mode of operation depends on the membrane potential and the transmembrane concentration gradients for Na\(^+\) and Ca\(^{2+}\). As the resting [Na\(^+\)]\(_i\) of astrocytes is quite high (15-20mM), the reversal potential of the NCX is close to the RMP (-80mV) which results in the NCX dynamically fluctuating between forward/reverse mode, thus mediating both Ca\(^{2+}\) and Na\(^+\) fluctuations. Even mild depolarisation of the glial membrane or Na\(^+\) influx through either ionotropic receptors or neurotransmitter transporters can trigger NCX reversal. In mammals, there are three genes encoding for the NCX: NCX1, NCX2, and NCX3 and they can all be found in glia (Blaustein and Lederer, 1999; Parpura and Verkhratsky, 2012; Verkhratsky and Butt, 2013). The NCLX is the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger and it is also involved in regulating glial Na\(^+\) (Nicholls, 2005; Palty et al., 2010).

**Na\(^+\)/Ca\(^{2+}\) -K\(^+\) exchanger (NCKX)** allows the influx of four Na\(^+\) ions in exchange for one Ca\(^{2+}\) and one K\(^+\) ion. The genes encoding for NCKX are NCKX1 and NCKX2 (Kraev et al., 2001).

### 1.2.6. Potassium (K\(^+\))

Potassium channels fall into four major classes, namely; i) Voltage Gated, ii) Calcium activated, iii) Inwardly Rectifying and iv) Tandem Pore Domain (Gutman et al., 2005).

Various members from all of the different classes have been observed in glial cells where they display similar biophysical characteristics to those that are found in neurones or muscle cells. K\(^+\) channels are the most abundant detected in glia and the glial resting membrane conductance is dominated by passive K\(^+\) currents (Verkhratsky
and Butt, 2013). Generally, the expression of outwardly rectifying K+ channels has been implicated in regulation of their proliferation, as they show a down-regulation trend with glial differentiation. However, various subtypes of voltage gated K+ channels (Kv), Ca2+ -activated K+ channels (KCa) and rapidly inactivating A-type K+ currents (KA) have been detected in mature, non-proliferating glia although their functional relevance is still unclear (Verkhratsky and Steinhäuser, 2000; Verkhratsky and Butt, 2013). Glia also express voltage independent K+ channels which are represented by the two pore forming domain channels (K2P) and are hypothesized to affect glial membrane potential; however, direct experimental evidence for this is lacking (Bay, 2013; Verkhratsky and Butt, 2013).
1.3. **Kir channels**

Undoubtedly the most physiologically relevant $K^+$ channels (and by far the most studied) in CNS glia are the inwardly rectifying $K^+$ channels ($Kir$). These are predominantly responsible for maintaining the glial resting membrane potential, which is close to the $E_K$. $Kir$ channel expression and more specifically the $Kir2.x$ and $Kir3.x$ subtypes have also been found in neurones, where they have been implicated with the regulation of postsynaptic excitability (Hibino et al., 2010).

Since 1993, genes encoding several $Kir$ channel proteins have been identified and can be categorised into 7 subfamilies ($Kir1.x$ to $Kir7.x$) based on sequence homology. These $Kir$ subfamilies can be further grouped (Figure 1.6) based on their functional attributes including their degree of rectification, single channel conductance and sensitivities to chemical blockers such as barium ($Ba^{2+}$) or caesium ($Cs^+$)(Hibino et al, 2010).

![Figure 1.6: Kir channel subunits can be categorized into four functional groups. (Figure modified from Hibino et al, 2010)](image)
The characteristic inward rectification of K<sub>ir</sub> channels results primarily from a voltage-dependent block by intracellular organic cations such as polyamines or Mg<sup>2+</sup> ions, which physically block the conduction pathway during depolarization, thereby impeding the outward flow of K<sup>+</sup>. The polyamines spermine and spermidine are the most potent inducers of rectification although contributions of putrescine and Mg<sup>2+</sup> are also important (Osawa et al., 2009). The degree of rectification varies greatly among members of the K<sub>ir</sub> superfamily and is critical to their respective functional roles. K<sub>ir2.x</sub> and K<sub>ir3.x</sub> subunits form classical "strong" inward rectifier channels, while other members encode channels with "intermediate" (K<sub>ir4.x</sub>) or "weak" (K<sub>ir1.1</sub>, K<sub>ir6.x</sub> and K<sub>ir7.x</sub>) rectification. Structurally, each K<sub>ir</sub> subunit consists of two transmembrane domains separating a pore forming loop (Figure 1.7).

![Figure 1.7: Overall architecture of K<sub>ir</sub> channels.](image)

**Figure 1.7:** Overall architecture of K<sub>ir</sub> channels. a. Schematic drawing of a K<sub>ir</sub> channel subunit. Each subunit comprises two transmembrane helices (M1 and M2), a pore-forming region containing the pore-helix (P), and a cytoplasmic domain formed by the amino (N) and carboxy (C) termini. b. Side view of a K<sub>ir</sub> channel showing the transmembrane domain of two subunits (green and blue) and the C-terminal domains of their neighbouring subunits (red and yellow). White spheres represent K<sup>+</sup> ions in the selectivity filter.
Functional Kir channels are assembled as tetramers of subunits surrounding a single transmembrane aqueous pore (Bichet et al., 2003). Kir channels can be assembled as homotetramers (i.e. four identical protein subunits), however the simple architecture and strong homology between the basic subunits allows for heterotetrameric combinations to form functional Kir channels.

Kir channels have variable functions in multiple cell types including macrophages, cardiac myocytes, endothelial cells and CNS cells. For example, in cardiac myocytes, Kir2.x, Kir3.x and Kir6.x subfamilies are important for the maintenance of prolonged action potential by slowing membrane repolarisation (Anumonwo and Lopatin, 2010), whereas in kidney Kir1.x, Kir2.x, Kir4.x, Kir5.x and Kir7.x subfamilies regulate Na⁺, K⁺ and Mg²⁺ transport through epithelial cells (Wang et al., 2010). In endothelial cells, vascular smooth muscle (VSM) cells and pericytes, Kir2.x and Kir6.x subfamilies are involved in dilation, induced by elevated extracellular K⁺ (Jackson, 2005), and in pancreatic β cells Kir6.2 are involved in regulation of insulin release induced by elevated glucose levels and in response to an increase in ATP concentration (Flechtner et al., 2006).

1.3.1. Glial Kir channels

The research evidence that has been produced so far, confirms that CNS glia can express most of the Kir channel subfamilies. The Kir4.1 subtype is highly specific, widespread and strongly expressed in the CNS and has been demonstrated in astrocytes and oligodendrocytes of the grey and white matter (Poopalasundaram et al., 2000; Li et al., 2001; Kalsi et al., 2004), in Bergmann glia and Muller glia (Takumi et al., 1995; Ishii et al., 1997; Schröder et al., 2000; Higashi et al., 2001). There is evidence
demonstrating strong expression of Kir5.1 in astroglial endfeet, in Muller glia as well as in oligodendrocytes in vitro (Kalsi and Butt, 2004; Brasko, 2013). In addition, the strongly rectifying Kir2.1 and Kir2.2 have also been identified in retinal Muller glia (Kofuji et al., 2002; Ishii et al., 2003; Raap et al., 2002) and hippocampal astrocytes (Schröder et al., 2002). Moreover, immunolabelling for Kir2.2 has been demonstrated in Bergmann glia and astrocytes of the cerebellum (Leonoudakis et al., 2001), while astrocytes and oligodendrocytes of the forebrain and hindbrain displayed heterogeneous labeling for Kir2.1, Kir2.2 and Kir2.3. Cultured astrocytes also seem to express Kir2.1 (Perillán et al., 2000). The expression of the Kir6.x subtypes has been shown in Muller glia (Eaton et al., 2002) and astrocytes of the dorsal vagal nucleus, as well as in astrocytes and oligodendrocytes of the corpus callosum and cerebellar white matter (Zhou et al., 2002). However, in previous studies no expression had been found for Kir6.2 mRNA in hippocampal, cortical and cerebellar astrocytes, oligodendrocytes (Dunn-Meynell et al., 1998) or Bergmann glia (Thomzig et al., 2001). The data on glial expression of Kir3.x is sparse but there is evidence of immunopositivity of astrocytes in vitro and mRNA expression in Muller cells of the Guinea pig retina.

The main functions of Kir channels in glia are:

- To maintain the highly negative RMP, which is around -85 mV, and its high permeability for K⁺ ions.
- To undertake repolarisation of the glial membrane after the influx of Na⁺ or Ca²⁺ during neuronal action potentials.
- Potassium buffering by means of the glial syncytium, which facilitates the movement of K⁺ ions from areas of the brain with increased [K⁺]o to distant areas with lower [K⁺]o.
• They are also involved in ion and water transport by being colocalised with water channels (aquaporins) (Saadoun et al., 2003; Goodyear et al., 2009).

Some of the most important functions of astrocytes and oligodendrocytes are dependent on the strongly negative RMP and K⁺ permeability and it is safe to say that these functions depend on their expression of Kir channels.

1.3.2. Astrocytes and K⁺ buffering

A major function of astrocytes is the maintenance of extracellular [K⁺]. During their repolarisation after the transmission of an action potential, neurones release K⁺ into the ECS. The K⁺ concentration in the ECS is maintained at 3 – 5 mM (Jurkat-Rott et al., 2009), and an excessive increase has pathological effects on the membrane potentials of neurones and glia, on intra- and extracellular pH, further synaptic transmissions, as well as on cerebral blood flow and energy metabolism. Therefore, it is of immense importance for the overflow of K⁺ to be removed (Ransom et al., 2000). The process of redistribution of the excess potassium ions within the ECS was named “spatial buffering” by Orkand et al (Orkand et al., 1966) and in 1983 it was recognized that it is glial cells that carry out this function (Coles and Orkand, 1983) (Figure 1.8).

The dominant role of Kir channels for K⁺ buffering was originally demonstrated by Newman in retinal Müller cells from a frog (Newman, 1986) and more recently in murine Müller cells (Kofuji et al., 2000). A few years later the role of Kir channels in spatial buffering and the restoration and maintenance of the membrane potential was shown also in hippocampal astrocytes in Kir4.1 knockout mice (Djukic et al., 2007) and in astrocytes of the spinal cord (Olsen et al., 2006). In Kir4.1 knock-out mice, ablation of
Kir4.1 results in a dramatic reduction in Kir currents, depolarization and reduced capacity for K⁺ buffering in glia.

Kir channels have also been connected to astrocytic differentiation. It has been discovered that while mature astrocytes show high activity of Kir channels, the process of astrocytic de-differentiation during reactive gliosis results in Kir down-regulation (Kalsi et al., 2004).

Figure 1.8: The astrocytic syncytium (Verkhratsky and Butt, 2013).
1.3.3. Oligodendrocytes and Myelination

The importance of Kir4.1 expression in oligodendrocytes was documented when Neusch et al (2001) observed significantly decreased motor functionality in mice with a null mutation the Kir4.1 gene. The main cause for this motor impairment was hypomyelination of the spinal cord followed by neuronal degeneration and the scientists found that cultured oligodendrocytes from these mice were depolarized and had underdeveloped morphological characteristics. It became obvious that Kir4.1 in oligodendrocytes are responsible for retaining the RMP thus managing important cell functions such as signalling, differentiation and myelination as well as regulating the extracellular [K⁺] within the spinal cord.
1.4. **Kir7.1 - the newest member of the Kir family**

1.4.1. Structure and physiology

The gene encoding for Kir7.1 (KCNJ13) was first cloned in 1998, by three independent research groups (Döring et al., 1998; Partiseti et al., 1998; Krapivinsky et al., 1998). Its amino acid sequence and biophysical characteristics are quite distinct from other Kir channels and it is only 38% identical to its closest relative Kir4.2. As a result, Kir7.1 channels are relatively insensitive to Ba\(^{2+}\) and Cs\(^{+}\), classical blockers of Kir channels, with IC\(_{50}\) values of >1mM and >10mM respectively. Moreover, the permeability of Kir7.1 is independent of [K\(^{+}\)]\(_o\) and it displays an exceptionally small single channel conductance. The residue responsible for these unusual characteristics of the Kir7.1 channel pore (Figure 1.9) is methionine125 (M125), which is located in the same position as arginine148 (R) in all other Kir subtypes. Replacement of this residue with the conserved R dramatically increased the single channel conductance of the channel by ~20 fold, Ba\(^{2+}\) sensitivity by ~10 fold and allowed the channel to exhibit the rectification profile of other Kir channels (Sabirov et al., 1997; Döring et al., 1998; Krapivinsky et al., 1998).

**Figure 1.9: Comparison of Kir channel pore regions.** The conserved leucine (L), arginine (R) and glutamic acid (E) are replaced by serine (S), methionine (M) and glycine (G) in Kir7.1 (Krapivinsky et al., 1998).
The low unitary conductance in conjunction with the lack of specific pharmacological blockers has made the electrophysiological study of native \( \text{Kir}7.1 \) currents very challenging. However, recently a novel specific blocker for ROMK and \( \text{Kir}7.1 \) channels was discovered. The new compound, termed VU590, blocks \( \text{Kir}1.1 \) and \( \text{Kir}7.1 \) channels at low micromolar concentrations without inhibiting \( \text{Kir}4.1 \) or \( \text{Kir}2.1 \) channel activity (Lewis et al., 2009; Bhave et al., 2010). Despite the relative simplicity and similarity of \( \text{Kir} \) channels, structurally different compounds display such specificity in their blockade. For example, tricyclic antidepressants such as nortriptyline block \( \text{Kir}4.1 \) channels, whereas the antimalarial agent chloroquine inhibits \( \text{Kir}2.1 \) and it is noteworthy that they exhibit no effect on \( \text{Kir}1.1 \) (Rodríguez-Menchaca et al., 2008; Furutani et al., 2009). It has been postulated that VU590 at high micromolar concentrations may partially inhibit endogenous HEK-293 currents consisting mainly of \( \text{Kv} \). However, this blockade was as low as 10% (Bhave et al., 2011).

At present, evidence for heteromeric assembly of \( \text{Kir}7.1 \) with other \( \text{Kir} \) subunits is lacking, although this does not negate the possibility of the existence of heteromers. Furthermore, Partiseti et al. (1998) reported that \( \text{Kir}7.1 \) channels, unlike the related \( \text{Kir}6 \) subfamily members, do not associate with SUR1 accessory subunits when the two are co-transfected in HEK293 cells.

1.4.2. Distribution of \( \text{Kir}7.1 \) in human and animal tissues

\( \text{Kir}7.1 \) protein and/or mRNA has been detected in various organs such as epithelial cells in the kidney, thyroid and retina, in CNS neurones, ependymal cells, choroid plexus epithelium and spinal cord, myometrial smooth muscle, small intestine, neural
regions of the gastric mucosa as well as gastric parietal cells, and also in the lung, prostate, liver, pancreas, cochlear nucleus, testis and ovaries (Table 1.1).

Table 1.1: Kir7.1 expression in various tissues.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Species</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Human, Rat, Guiney Pig</td>
<td>rtPCR; Northern Blot; Immunohistochemistry; Western Blot</td>
<td>Krapivinsky et al., 1998; Partiseti et al., 1998; Döring et al., 1998; Ookata et al., 2000; Derst et al., 2001; Suzuki et al., 2003; Pondugula et al., 2006; Yang et al., 2008</td>
</tr>
<tr>
<td>Brain</td>
<td>Human, Rat, Guiney Pig, Rabbit, Cichlid</td>
<td>rtPCR; Northern Blot; In situ hybridisation; Immunofluorescence; Western Blot; Mass Spectrometry</td>
<td>Krapivinsky et al., 1998; Partiseti et al., 1998; Döring et al., 1998; Nakamura et al., 1999; Kusaka et al., 2001; Derst et al., 2001; Malinowska et al., 2004; Schindler et al., 2006; Watanabe et al., 2007; Millar et al., 2007; Yang et al., 2008</td>
</tr>
<tr>
<td>GI Tract</td>
<td>Human, Rat, Rabbit</td>
<td>rtPCR; Northern Blot; Immunofluorescence; Western Blot</td>
<td>Krapivinsky et al., 1998; Partiseti et al., 1998; Nakamura et al., 1999; Nakamura et al., 2000; Fujita et al., 2002; Malinowska et al., 2004</td>
</tr>
<tr>
<td>Testis</td>
<td>Human, Rat, Cichlid</td>
<td>rtPCR; Northern Blot</td>
<td>Krapivinsky et al., 1998; Döring et al., 1998; Nakamura et al., 2000; Watanabe et al., 2007</td>
</tr>
<tr>
<td>Ovary</td>
<td>Cichlid</td>
<td>rtPCR</td>
<td>Watanabe et al., 2007</td>
</tr>
<tr>
<td>Liver</td>
<td>Human</td>
<td>rtPCR</td>
<td>Krapivinsky et al., 1998</td>
</tr>
<tr>
<td>Prostate</td>
<td>Human</td>
<td>rtPCR</td>
<td>Krapivinsky et al., 1998</td>
</tr>
<tr>
<td>Lung</td>
<td>Rat, Guiney Pig, Rabbit</td>
<td>rtPCR; Northern Blot</td>
<td>Döring et al., 1998; Derst et al., 2001; Malinowska et al., 2004</td>
</tr>
<tr>
<td>Thyroid</td>
<td>Human, Rat</td>
<td>Northern Blot; Immunofluorescence; Western Blot</td>
<td>Nakamura et al., 1999</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>Human</td>
<td>Northern Blot</td>
<td>Nakamura et al., 1999</td>
</tr>
<tr>
<td>Retinal</td>
<td>Human, Rat, Bovine, Porcine, Monkey</td>
<td>rtPCR; qRT-PCR; Northern Blot; Immunofluorescence; Western Blot</td>
<td>Kusaka et al., 2001; Shimura et al., 2001; Yang et al., 2003; Yasuda et al., 2003; Yang et al., 2008a,b; Hejtmancik et al., 2008</td>
</tr>
<tr>
<td>Pigment</td>
<td>Rat</td>
<td>rtPCR</td>
<td>Kim et al., 2000</td>
</tr>
<tr>
<td>Epithelium</td>
<td></td>
<td>qRT-PCR; SAGE; Microarray</td>
<td>Friedland et al., 2007</td>
</tr>
<tr>
<td>Pancreatic</td>
<td></td>
<td>rtPCR</td>
<td>McCloskey et al., 2014</td>
</tr>
<tr>
<td>Acini</td>
<td></td>
<td>qRT-PCR; Immunohistochemistry; Western Blot</td>
<td></td>
</tr>
</tbody>
</table>
More detailed studies on the cellular localisation of the channel have been performed in the retinal pigment epithelium (RPE), choroid plexus, gastric parietal cells and kidney where it was found to be localised in a polarised way and co-expressed with the Na⁺/K⁺-ATPase (Nakamura et al., 1999; Ookata et al., 2000; Kusaka et al., 2001; Fujita et al., 2002; Yang et al., 2003; Pondugula et al., 2006). There are no previous reports of the expression of Kir7.1 in astrocytes and oligodendrocytes, but protein has been shown to be expressed in Purkinje neurones of the cerebellum and pyramidal neurones of the hippocampus (Krapivinsky et al., 1998).

Their presence in polarised epithelial cells and its co-expression with Na⁺/K⁺-ATPase has led to a hypothesis that Kir7.1 channels play a role in transepithelial K⁺ transport. More specifically, Döring et al (1998) hypothesised that Kir7.1 channels in the choroid plexus are crucial for the clearance of K⁺ from cerebrospinal fluid (CSF), in which [K⁺] is tightly controlled. However, Krapivinsky et al. (1998) demonstrated that Kir7.1 channel protein is expressed in Purkinje and pyramidal neurones and attribute to it a neurone specific function. Kir7.1 are strongly expressed by retinal pigment epithelium (RPE), where it has an important role in the regulation of the subretinal space volume, by facilitating K⁺ efflux from the cells when [K⁺]o is low, i.e. upon illumination and activation of retinal photoreceptor cells, thus enabling water efflux from the RPE. Moreover, due to its weak inward rectification, Kir7.1 maintains a constant K⁺ efflux, thus counteracting and enabling the function of Na⁺/K⁺-ATPase, a function termed "K⁺ recycling" (Wimmers et al., 2007). A loss of function mutation in Kir7.1, results in the autosomal dominant disorder of the eye called Snowflake Vitreoretinal Degeneration (SVD) and manifests itself as a severe impairment of transepithelial ion and water transport via the RPE (Hejtmancik et al., 2008).
1.5. Hypothesis and Aims

The hypothesis underpinning this thesis is that Kir7.1 has unresolved functions in glial cells of the CNS. The overall aim of this PhD project is to characterise the expression and physiological function of the inwardly rectifying potassium channel, Kir7.1, in the mammalian central nervous system.
Chapter 2 - Materials and Methods
2.1. Animals

2.1.1. Declaration

*Animals were humanely killed by cervical dislocation, in accordance with the regulations issued by the Home Office of the United Kingdom under the Animals (Scientific Procedures) Act (ASPA), 1986.*

2.1.2. Animal Strains

Several different strains of mice were used; C57BL/6 and C57BL/10 (Wild type black) and transgenic strains;

- SOX10-lox-GFP-4xPolyA-lox-DTA, which expresses the enhanced green fluorescent protein (EGFP) under transcriptional control of the SOX10 gene (Kessaris et al., 2006; Matsuoka et al., 2005). The SOX10-EGFP transgenic line was a gift from William D Richardson and Nikoleta Kessaris’ lab (UCL).

- TgN (GFAP-EGFP) GFEC-FKi, expressing EGFP under the control of the GFAP gene promoter, resulting in green astrocytes (Nolte et al., 2001).

- TgN (PLP-DsRed1), expressing enhanced red fluorescent protein (DsRed - drFP583, from Discosoma species) under the control of the PLP gene promoter, resulting in red myelin (Hirrlinger et al., 2005). The GFAP-EGFP and PLP-DsRed transgenic lines were a generous gift from Dr. Frank Kirchhoff (Max Planck Institute for Experimental Medicine, Gottingen, Germany).

- NG2-DsRed BAC, in which DsRed is expressed under the control of the NG2 promoter (Ziskin et al., 2007; Zhu et al., 2008). The NG2-DsRed transgenic line was a gift from Dr. Akiko Nishiyama (University of Connecticut, USA).
All transgenic animals used in this study display no unusual phenotype; moreover, their breeding habits and life span are aligned with wild type.

2.1.3. Animal Ages

Mice of both sexes were used at ages P7 (i.e. post natal day 7), P12 or >P35 for the qRT-PCR experiments. For the optic nerve explant cultures, P7-P12 mice were used while adult mice (>P30) were used for brain sections. Optic nerves from P12-P14 mice were used for the oxygen and glucose deprivation and calcium imaging experiments. Finally, new born wild type mice (P0-P2) were used for setting up the cortical neuron and astrocyte cultures.

2.1.4. Tissue Fixation Protocols

Three different fixation protocols have been used in the duration of this project:

a) 1% PFA with 15% Picric acid for 10 mins to fix cultured cells

b) 4% PFA with 15% Picric acid to plunge-fix mouse brains and optic nerves for 24-48 hrs depending on mouse age and size of brain. Brains were cut along the longitudinal cerebral fissure to separate the hemispheres and improve PFA penetration in the tissue.

c) 4% PFA to perfuse adult mice before removing the brain and plunge fix according to fixation protocol b.
2.2. **Cell Culture**

All cell cultures were completed within a routinely sterilized room containing a laminar flow cabinet (Life Technologies) and cell culture incubator (Fisher).

2.2.1. **Cover Slip Preparation**

13mm diameter glass cover-slips were sterilised by immersion in 70% ethanol and placed in 24-well plates (Greiner Bio-One, Manchester, UK) and left to dry in the culture hood. 100 µl of 1% poly L-lysine (Sigma-Aldrich, Dorset, UK) in double distilled sterilised water (ddH2O) was placed on each cover-slip and left overnight at 37°C. Poly L-lysine was removed and replaced with 25 µl of 0.5% laminin (Life Technologies, Paisley, UK) in sterile ddH2O. After two hours, laminin was replaced with 30 µl of sterile ddH2O and the cover-slips were left in the incubator until required. Poly L-lysine coated cover-slips and slides provide higher adhesion, reducing the chance of tissue or cell loss during processing. This pre-preparation of cover-slips is required when culturing cells that do not adhere well.

2.2.2. **Optic Nerve Explant Cultures**

Optic nerve explants were cultured as previously described (Greenwood and Butt, 2003). Briefly, optic nerves were dissected from P7 to P12 mice and placed directly into 50µl dissecting medium consisting of high glucose Dulbecco’s Modified Eagle Medium (DMEM) (Sigma-D5671) containing 10% Fetal Calf Serum (FCS; Life Technologies), L-Glutamine (Sigma) and 0.1% Gentamycin (Life Technologies). Next, they were finely chopped with a scalpel blade and triturated with pipettes of decreasing diameter (P1000 then P200) to further break up the tissue. After adding 50 µl more dissecting medium, the solution was pipetted evenly between cover slips.
(approx. 1 nerve per cover-slip) and left for the initial stick down period. After approximately 24 hrs the dissecting medium was replaced with a low serum (0.5%) modified Bottenstein and Sato (B&S) culture medium (Bottenstein and Sato, 1979) without thyroid hormones in which 10 ng/ml of recombinant human PDGF-a (R&D Systems) was added to encourage OPC proliferation. After 3-4 days in vitro (DIV) the medium was replaced with B&S medium supplemented with 0.5 mM dbc-AMP (Sigma) until DIV10.

2.2.3. Cortical Neuron Cultures

Primary cultured cortical neurons were prepared from C57Bl/6 and C57Bl/10 wild type mice aged between postnatal days P0 to P2. Brains were collected in ice cold HBSS (Life Technologies) containing 1% Pen/Strep (Life Technologies) and cortices were dissected under a dissection microscope (Leica MZ8) and all brain membranes, corpus callosum and hippocampus were carefully removed. The cortical pieces were then placed in fresh ice-cold HBSS in a 15 ml falcon tube. Tissue was left to settle at the base of the tube before HBSS was exchanged with fresh solution. This gentle washing step was repeated three times so that any remaining pieces of brain membranes were washed away. The cortical pieces were then incubated at 37°C for 15-20 mins in NB-A medium (Life Technologies) supplemented with B27, Pen/Strep, L-Glutamine (Sigma) and 10% trypsin (Life Sciences). Following trypsinisation, the media was replaced with FCS (Life Technologies) and incubated at 37°C for 5-10 mins which inactivated any remaining enzyme. The cortical pieces were then washed with NB-A medium and were triturated with a fire polished glass pipette. Finally, the media was filtered through a
70 µm nylon mesh pre-separation filters (Miltenyi Biotec, Bergisch Gladbach, Germany) and brought up to a volume of 5100 µl (5.1 ml). The cells were then counted and seeded on 13 mm coverslips pre-treated with poly-l-lysine and laminin in 24 well plates, as previously described (Section 2.2.1).

2.2.4. Cell Counting

To estimate the percentage of viable cells, cells were counted in a VI-CELL™ Series Cell Viability Analyser (Beckman Coulter). In order to verify cell viability, the VI-CELL™ uses trypan blue, a dye which is only taken up by dead cells which are permeabilised, whereas the live cells which are not permeable to trypan blue are not stained (Marchenko and Flanagan, 2007). To perform a cell count, 100 µl of the re-suspended pellet of cells was placed into a VI-CELL tube with 900 µl of NB-A medium to produce a dilution factor of 10:1. The vial was then placed in the analyser’s carousel for the count.

Finally, cells were further diluted accordingly in order for 10⁶ cells to be seeded per cover-slip for immunostaining, and grown as monolayers in an incubator at 37°C under normoxic culturing conditions (95% air/5% CO₂).
2.3. Immunostaining techniques

2.3.1. Antibodies

All antibodies used during this project are listed in Table 2.1 and 2.2, along with information concerning their epitope localisation, host species and reactivity, working concentration and source.

The secondary antibodies must be raised in the same species as the serum used for blocking, and against the type of animal from which the primary antibody is derived.

Table 2.1: Primary antibodies

<table>
<thead>
<tr>
<th>Antibody (epitope)</th>
<th>Host</th>
<th>ICC/IHC</th>
<th>Western Blot</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP (IC)</td>
<td>Chicken</td>
<td>√</td>
<td></td>
<td>1:500</td>
<td>Chemicon&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>K&lt;sub&gt;ir&lt;/sub&gt;7.1 (EC)</td>
<td>Rabbit</td>
<td>√</td>
<td>√</td>
<td>1:300</td>
<td>Alomone&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>K&lt;sub&gt;ir&lt;/sub&gt;4.1 (IC)</td>
<td>Rabbit</td>
<td>√</td>
<td></td>
<td>1:300</td>
<td>Alomone</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;/K&lt;sup&gt;+&lt;/sup&gt; ATPase a1 (IC)</td>
<td>Mouse</td>
<td>√</td>
<td></td>
<td>1:300</td>
<td>Abcam&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>β3-Tubulin (Tuj1) (IC)</td>
<td>Mouse</td>
<td>√</td>
<td></td>
<td>1:300</td>
<td>Millipore&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse</td>
<td></td>
<td>√</td>
<td>1:3000</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Key: Immunocytochemistry (ICC); Immunohistochemistry (IHC). Intracellular Epitope (IC); Extracellular Epitope (EC)

Table 2.2: Secondary antibodies

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Reactivity</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 488</td>
<td>Gt-a-Rb, Dk-a-Rb</td>
<td>1:400</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Alexa Fluor® 568</td>
<td>Gt-a-Rb, Dk-a-Rb, Gt-a-Ck</td>
<td>1:400</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>DyLight 647</td>
<td>Gt-a-Ms</td>
<td>1:250</td>
<td>Stratech&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>HRP</td>
<td>Gt-a-Ms, Gt-a-Rb</td>
<td>1:5000</td>
<td>Dako&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> Chemicon, Temecula, CA, USA
<sup>2</sup> Alomone, Jerusalem, Israel
<sup>3</sup> Abcam, Cambridge, UK
<sup>4</sup> Millipore, Darmstadt, Germany
<sup>5</sup> Stratech, Suffolk, UK
<sup>6</sup> Dako, Stockport, UK
2.3.2. Immunocytochemistry

Cultured cells were fixed for 10 mins with 1% paraformaldehyde (PFA-TAAB Laboratories Equipment, UK) dissolved with 15% Picric Acid in 0.2 M Phosphate Buffer. After washing 3 x10 mins with PBS (Sigma), cells were blocked for 75 mins at room temperature using blocking solution containing 5% Normal Goat Serum (NGS; Invitrogen) or Normal Donkey Serum (NDS; Invitrogen) in order to prevent non-specific binding of both the primary and secondary antibodies (Abs) to other antigens on the cells. Where primary antibodies targeted an intracellular epitope of a protein (e.g. Kir4.1, GFAP), 0.01% detergent (Triton X, Sigma) was incorporated into the blocking solution, for cell membrane permeabilisation. After blocking, cells were not washed again. Primary antibodies were diluted in PBS. Incubation was performed overnight according to manufacturer’s recommendations at 4°C. Cells were then washed 3 x 10 mins with PBS. The secondary antibody conjugates were diluted in PBS and added to the cells for 1 hr at room temperature on an orbital shaker and protected from light by wrapping the 24-well plate in foil. During this step, the DNA dye Hoechst Blue (Molecular Probes) was added at a concentration of 1:500. After completion of immuno-staining, the cells were once again washed 3 x10 mins with PBS and cover-slips were mounted on clean slides, using mounting medium (Vectashield; Vector Labs or Fluoromount G; Southern Biotech).
2.3.3. Immunohistochemistry

Mouse brains separated into two hemispheres and complete with the cerebellum, pons and medulla oblongata, and optic nerves were dissected and fixed with perfusion and submersion in 4% PFA with 15% picric acid for 24-48 hrs. After fixation, tissues were washed 3 times in PBS and stored at 4°C, in PBS containing 0.05% NaN₃ (Sigma) until used. Brain sections were cut using a vibratome (Leica) at a thickness of 40-70 µm and used immediately or stored at 4°C in PBS/NaN₃ in 24 well plates. The meninges were removed were possible. Tissue was blocked with 10% serum (NGS or NDS) for 1-2 hrs, washed 3 times in PBS, and incubated overnight in the antibody solution which comprised of the primary antibody diluted in blocking solution containing Triton-X. Whole optic nerves were incubated in the primary antibody for 48 hrs. Tissues were then washed 3 times again in PBS and incubated with secondary antibody diluted in blocking solution for 1 hr at room temperature on an orbital shaker, or in the case of whole optic nerves, incubated overnight and protected from the light by wrapping the 24-well plate in foil. Following secondary antibody incubation, tissues were washed 3 times with PBS before being mounted on glass slides and covered with mounting medium and glass coverslips ready for imaging.
2.3.4. Confocal Microscopy

Fluorescence-stained cells and sections were examined under a confocal microscope with the following characteristics (Table 2.3);

Table 2.3: Confocal microscope characteristics

<table>
<thead>
<tr>
<th>Microscope</th>
<th>Zeiss Axiovert LSM 710 VIS405, Zeiss LSM 5 Pascal Axioskop 2</th>
<th>Laser confocal system and LED Light Source (Colibri)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lasers</td>
<td>Argon, helium-neon (HeNe1) and diode lasers</td>
<td>Sequential excitation at wavelengths 488nm, 568nm and 405nm</td>
</tr>
<tr>
<td>Image Acquisition</td>
<td>Zeiss LSM 510NLO confocal scan head</td>
<td>x20(air), or x40, x63 and x100 (oil immersed) objectives</td>
</tr>
<tr>
<td>Image analysis</td>
<td>Zeiss Software</td>
<td>LSM and Zen 9000 Software</td>
</tr>
</tbody>
</table>
2.4. Western Blot

2.4.1. Protein Extraction

Whole cell protein content was extracted from mouse optic nerve, cortex, cerebellum and whole brain. Tissue was dissected from adult mice and immediately frozen in liquid nitrogen and allowed to thaw slowly on ice in homogenisation buffer containing (in mM) 12.5 NaCl (Fisher, Loughborough, UK), 2 Tris·HCl (pH8; Sigma), 0.2 phenylmethylsulphonyl fluoride (PMSF; Sigma) and 1x protease inhibitor cocktail (Roche). Tissues were disrupted and homogenised using the TissueRuptor and disposable probes of approximately 10 mm diameter (Qiagen, Crawley, West Sussex, UK). Disruption was performed in 10 sec bursts to avoid overheating that could cause protein degradation. Samples were then centrifuged at 4°C, at 14000 rpm using a refrigerated table top centrifuge (Fisher) for 4 mins and resuspended in PBS. Homogenates were stored at -80°C.

2.4.2. Bicinchoninic Acid Assay (BCA) for Determination of Protein Content

The bicinchoninic acid assay (BCA assay), also known as the Smith assay, was used to determine the protein concentration of samples. Serial dilutions of a protein standard (1 mg bovine serum albumin/ml in 0.15 M NaCl, 0.05% NaN₃ (Sigma)) were made using sterile ddH₂O. A plate reader (POLAR star OPTIMA, BMG LabTech) was used to measure protein standards in order to generate a standard curve from which the protein concentration of prepared samples was determined. Samples were subsequently diluted with lysis buffer to obtain homogenous protein levels across different samples.
2.4.3. Protein Separation, Transfer and Detection

For SDS polyacrylamide gel electrophoresis (PAGE), 8% running gels (2.31 ml ddH2O; 1.33 ml 30% (v/v) bis-acrylamide; 1.25 ml 1.5M Tris/HCL pH 8.8; 50 µl 10% (w/v) SDS; 50 µl Ammonium Persulfate (0.05 g/500 µl); 5 µl TEMED) with a 5% stacking gel (3.4 ml ddH2O; 830 µl 30% (v/v) bis-acrylamide; 630 µl 0.5M Tris/HCl pH 6.8; 50 µl 10% (w/v) SDS; 50 µl Ammonium Persulfate (0.05g/500µl); 5 µl TEMED) were prepared. Cell lysates were diluted 1:1 in Laemli buffer (Biorad) containing 5% β-mercaptoethanol (Sigma) and were heated for 5 mins at 95°C. 30 µg protein was loaded into each gel lane alongside a pre-stained protein molecular weight marker (Precision Plus Protein Kaleidoscope standard; Biorad, Hercules, California, USA) which was used for protein size indication. The electrophoretic separation was carried out at a constant voltage of 100V (vertical slab gel, 1.5 mm x 14 cm x 14 cm) for 1.5 hrs in electrophoresis buffer containing 25 mM Tris, 190 mM glycine, 0.1% SDS and pH 8. Following this, gels were subjected to semi-dry transfer to a polyvinylidene fluoride (PVDF) membrane (GE healthcare). The gel and membrane were sandwiched between two stacks of filter paper that had been pre-wetted with transfer buffer containing; 25 mM Trizma Base, 192 mM Glycine and 20% Methanol, and ran for 2 hrs at constant Amperage of 75 mA per gel. To confirm protein transfer, the PDVF membrane was stained using Ponceau S (Sigma) following manufacturer instructions. Membranes were washed in distilled water (dH2O) to remove any staining and washed twice for 10 mins in TRIS buffered saline (TBS) at pH 7.4 and incubated for 2 hrs at room temperature (RT) in blocking solution containing 5% milk powder in TBS with 0.1% Tween. Membranes were incubated overnight at 4°C in blocking solution containing primary antibody. After primary incubation, membranes were washed three times with
TBS-Tween for 10 mins followed by a 3 hr incubation in TBS-Tween containing HRP-conjugated secondary antibodies. Finally, membranes were washed twice in TBS-Tween for 10 mins. An enhanced chemiluminescent (ECL) detection mix (GE healthcare) was prepared by mixing enhanced luminal to oxidising agent in a ratio 1:1. The reagent was spread on the membrane and incubated for 5 mins at RT. After removing excess reagent, the membrane was exposed to an ECL detection system (Amersham Biosciences) coupled to a PC and images captured digitally.
2.5. **Oxygen and Glucose Deprivation (OGD)**

Optic nerves from P12–14 transgenic GFAP-eGFP or Sox10-eGFP mice were isolated intact and immediately placed in aCSF for 30 mins at 37°C in a normoxic incubator (95% air/5% CO₂) and allowed to stabilise. Control groups were incubated for a further 1 hr in normal aCSF containing glucose with 5% CO₂/95% O₂ (Oxygen and Glucose Normal; OGN) without the addition of pharmacological modulators. Oxygen-glucose deprivation (OGD) was achieved using the method of Fern and colleagues (Salter and Fern, 2005), by incubating nerves for 1 hr at 37°C in glucose-free aCSF (osmolarity was maintained by replacing glucose with sucrose), and switching the chamber atmosphere to 5% CO₂/95% N₂.

Pharmacological modulators were added to the aCSF solution of test groups during OGN and OGD: the small-molecule Kᵢ,7.1 channel blocker 7,13-bis(4-nitrobenzyl)-1,4,10-trioxa-7,13-diazacyclopentadecane (VU590; 100 µM, Tocris), 6-Nitro-2-[(6-nitro-1H-benzimidazol-2-yl)methoxymethyl]-1H-benzimidazole hydrochloride (VU591; 100 µM, Tocris) or the general Kᵢ, channel blocker Ba²⁺ (Barium Chloride; 100 µM, Sigma).

2.5.1. **Quantification of glia**

At the end of the 60 mins incubation period, optic nerves were fixed and whole-mounted for image capture using a Zeiss LSM 710 metaconfocal microscope (as previously described). Cells expressing the GFAP-eGFP or SOX10-eGFP reporters were visualised at 488 nm using an argon laser and x40 oil immersion lens with high numerical aperture (1.3 nm); images were captured maintaining the acquisition
parameters constant between samples (Figure 5.1A-D). In each nerve, the total number of cells was counted in five FOV along the length of the optic nerve. The FOV comprised a constant volume of 20 x 20 µm in the x-y-plane and 15 x 1 µm optical sections in the z-plane, commencing 15 µm below the pial surface. Cell counts are expressed as mean number of cells per FOV ± SEM (n≥5, where ‘n’ represents the number of nerves), and significance was determined by ANOVA and Newman–Keuls multiple comparison post-hoc analysis, using Graphpad Prism5.0.
2.6. Molecular Biology

All tissues, except the optic nerve tissue, were disrupted and homogenized using the TissueRuptor and disposable probes of approximately 10 mm diameter (Qiagen, Crawley, West Sussex, UK). Due to the abundance of myelin in the optic nerve, Trizol and chloroform protocol was used. A standard table top refrigerated centrifuge (Fisher) was used for all spin protocols.

2.6.1. Total RNA Extraction

Extraction of RNA was performed using the RNeasy Micro Kit (Qiagen) following the manufacturer’s instructions, from approximately 5 mg of whole mouse brain and separately from mouse cortex, cerebellum and optic nerve. When extracting RNA from adult optic nerves, the tissue was placed in 500 µl of ice cold Trizol (Sigma) and mechanically disrupted using a sterile plastic pestle (Fisher) and homogenised by being passed through a 23G syringe 10 times on ice. Homogenate was allowed to rest for 5 mins and then passed through a QIAshredder column (Qiagen) for further homogenisation. 100 µl chloroform (Sigma) was added and the homogenate shaken vigorously for 15 secs before being allowed to rest for 3 mins at room temperature. Then, samples were centrifuged at 12000 g for 5 mins at 4°C. Finally, the top clear, aqueous phase containing the RNA was aspirated and transferred into a new 1.5 ml eppendorf where it was mixed at 1:1 ratio with 70% ethanol. The RNeasy Micro Kit procedure was followed from that point onwards. For whole brain, cortical and cerebellar RNA extraction, approximately 5 mg of tissue was placed in RNA later until it was disrupted and homogenized using the TissueRuptor in a round bottomed collection tube containing a guanidine thiocyanate (GITC) lysis buffer (RLT) and then
centrifuged for 3 mins at 13000 rpm. GITC is a chaotropic salt, i.e. a potent protein
denaturant which inactivates RNases thus allowing for the RNA to be isolated intact.
The supernatant was carefully removed and placed in a new collection tube before
adding 70% molecular grade ethanol at 1:1 ratio. It was then transferred onto an
RNeasy spin column containing a silica-gel membrane upon which the RNA would bind
during subsequent centrifugation at 10000 rpm for 15 secs. A further wash with 350 µl
of a chaotropic salt containing buffer (RW1) was performed at 10000 rpm for 15 secs
in order to wash away any remaining contaminating impurities such as degraded
proteins and a last was with ethanol was utilised to wash away the salts. A dry spin for
5 mins at full speed was performed to remove all ethanol residues from the
membrane. Complete removal of ethanol is essential for high yields of clean eluent. If
there is ethanol remaining on the column then the nucleic acids will not be completely
rehydrated and elution will result in low yields. Finally, high quality total RNA was
eluted in 10µl RNA-free water by centrifuging for 1 min at full speed.

An extra DNase digestion step using DNase I (Qiagen) was performed in order to
eliminate genomic DNA that was released in the lysate, as the homogenisation
protocol disrupted the nucleic as well as the cell membranes. One of the whole brain
samples was left untreated and was used as a positive control in PCR reactions. All RNA
samples were stored at -80°C.

The purity and concentration of isolated total RNA was assessed using a ND-1000
Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) to measure the
UV absorption of samples. The absorption at 260 nm (A_{260}) was used to determine the
concentration of RNA in undiluted samples. The ratio of absorptions at 260 nm and
280 nm (A260/A280 ratio) was used to determine the samples’ purity, where a ratio of >1.8 indicated high RNA purity. As RNA optimally absorbs light at a wavelength of 260 nm due to its high content of nucleotides, while proteins and other impurities that might be contaminating the isolated RNA absorb best at 280 nm, the A260/A280 ratio is the preferred way to assess if an RNA sample is pure. Furthermore, a subset of the samples were separated on a 1% agarose gel by gel electrophoresis in 1x TAE buffer and visualised by ethidium bromide staining subsequent exposure to UV light.

2.6.2. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

First strand cDNA synthesis was carried out using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Burgess Hill, West Sussex, UK). Briefly, a minimum of 1 µg total RNA was incubated in a two-step process with anchored Oligo dT primers, deoxynucleotide mix and the Transcriptor Reverse Transcriptase enzyme to generate full length cDNA following the manufacturers guidelines. High quality cDNA libraries of the whole mouse brain, cortex, cerebellum and optic nerve were stored at -20°C to be used in downstream Polymerase Chain Reactions (PCR) with primers for Kir7.1. The PCR reaction volume was 50 µl (14 µl ddH2O; 1 µl cDNA (1 µg); 25 µl DreamTaq (2x); 5 µl Forward Primer (10 µM); 5 µl Reverse Primer (10 µM)). The primers were designed using the National Center for Biotechnology Information (NCBI) Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesised by Invitrogen (Table 2.4). PCR cycling parameters were as follows; 95°C for 2 mins, followed by 35 cycles of 95°C for 30 secs, 59°C for 30 secs and 72°C for 1 min followed by a final extension of 72°C for 10 mins using a C1000 Touch Thermal Cycler (Biorad, Hercules,
CA). The product amplified by the primers is a 251 bp amplicon spanning exons 2 and 3 of the mouse KCNJ13 gene.

**Table 2.4: PCR primers.**

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACATCACCAGCTTCACAGC</td>
<td>GGTGGGCTATTTGTGAGC</td>
</tr>
</tbody>
</table>

2.6.3. Agarose Gel Electrophoresis

RT-PCR amplicons were separated on a 1% agarose gel containing 1 µg/ml ethidium bromide (Sigma) for visualisation of the bands under UV light. Gel electrophoresis was performed in TAE Buffer (40 mM tris-acetate, 20 mM acetic acid, 1 mM EDTA, pH 8.0) for approximately 45 mins at 5 V/cm. Gel images were captured under UV light using a BioRad Gel Doc EZ System (BioRad).

2.6.4. Quantitative real time PCR (qRT-PCR)

The RT2 First Strand Kit (Qiagen) was used for *in vitro* transcription of extracted RNA into cDNA to be used for qRT-PCR, following the manufacturer’s instructions. The quantity of RNA that was transcribed was the same for all samples (500 ng). 1350 µl of 2x SYBR Green qPCR Mastermix (Qiagen) was mixed with 102 µl cDNA and 1248 µl ultra-pure water (Ambion) and 25 µl was pipetted in each well of the 96 well-plate arrays. All arrays used were purchased from SABiosciences (Qiagen) and thermocycled on a Roche Lightcycler 96 instrument (Roche). The protocol used was the following; 95°C for 15 secs (1 cycle), followed by 60°C for 1 min (45 cycles). Data normalisation was performed using the best housekeeping genes for each experiment. Optimal
housekeeping genes were chosen using the NormFinder logarithm (Andersen et al., 2004) and the standard deviation (SD) method (Mane et al., 2008). The method of Comparative Quantitation was used to study the expression level of ion channels with the following formula;

\[ Ct \text{ Sample} - Ct \text{ Calibrator} = \Delta Ct \]

\[ \text{Relative quantity} = Av(2^{-\Delta Ct}) \]

The fold change in mRNA transcript levels between different ages was calculated using the \( \Delta\Delta Ct \) method and tested for significance using ANOVA and post hoc Bonferroni’s tests. Statistical analysis was performed using the Prism v3.02 software (GraphPad).
2.7. Calcium Imaging

2.7.1. Optic nerve dissection and dye loading

Optic nerves were excised from P10-P14 wild type or PLP1-DsRed transgenic mice and incubated for 1 hr in aCSF containing 4 µM Fluo-4/AM (Molecular Probes) which is a green-fluorescent Ca^{2+} dye in its acetoxymethyl ester form. In this form, an acetoxymethyl ester group renders the dye membrane permeable. Once taken up by a cell, cytosolic esterases remove this ester group and the dye remains intracellular (Garaschuk et al., 2006).

2.7.2. Imaging parameters

Dye loaded nerves were washed in aCSF to remove excess dye, then placed in a perfusion chamber under a Zeiss LSM 5 Pascal Axioskop 2 confocal microscope and continuous perfusion with aCSF in the bath was established. A 20x/0.50 WPh2 Achromplan water immersion lens objective was used to visualise the intracellular Ca^{2+} changes after excitation of the optic nerves at 543 nm for PLP1-DsRed and at 488 nm for Fluo-4. Optical z-sections (typically 7-8 sections at 2–3 µm intervals) were obtained using the Zeiss LSM Image Examiner software (Zeiss, Germany). Regions of interest (ROI) covering single-cell bodies were selected within the z-stacks and the change in fluorescence intensity was measured.
2.7.3. Typical experiment

The nerve was continuously bathed in αCSF via a multitap system that allowed rapid turnover to solutions containing agents of interest, such as ATP or VU590. The health of the optic nerves was assessed by briefly (60 secs) inducing an ATP response at the beginning and end of each experiment and in the cases that [Ca^{2+}]_i increase was not observed in response to ATP, the nerve was excluded from analysis. In any given cell, the response to ATP provided a measure of the maximal rise of glial [Ca^{2+}]_i, irrespective of the level of Fluo4 loading (Hamilton et al., 2008). Thus, quantification of the change in glial [Ca^{2+}]_i could be measured as the change in fluorescence from baseline (ΔF/F), and data expressed relative to the ATP response in the same cell, so that comparison can be made between treatments.

2.7.4. Solutions

- aCSF (in mM): NaCl, 133; KCl, 3; CaCl_2, 1.5; NaH_2PO_4, 1.2; MgCl_2, 1.0; D-glucose, 10; HEPES, 10; pH 7.3 NaOH.
- ZERO-Ca^{2+}aCSF (in mM): NaCl, 133; KCl, 3; NaH_2PO_4, 1.2; MgCl_2, 5; D-glucose, 10; HEPES, 10; EGTA, 1; pH 7.3 NaOH.
- See Table 2.5 for agonists and antagonists.

2.7.5. Statistical analysis

Statistical analysis was carried out using Microsoft Excel and GraphPad Prism 6.0. In all experiments paired t-test or unpaired t-test with Welch’s correction for unequal variances were used (n=5 optic nerves and n>15 ROIs per nerve).
<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration used</th>
<th>Function</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (Adenosine 5 triphosphate)</td>
<td>100µM</td>
<td>P2 purinergic agonist; increases activity of Ca2+ - activated K+ channels; substrate for ATP-dependent enzyme systems</td>
<td>Sigma</td>
</tr>
<tr>
<td>VU590</td>
<td>100µM</td>
<td>Intracellular blocker of Kir7.1 and Kir1.1</td>
<td>Tocris</td>
</tr>
<tr>
<td>VU591</td>
<td>100µM</td>
<td>Intracellular blocker of Kir1.1</td>
<td>Tocris</td>
</tr>
<tr>
<td>BaCl2 (Barium chloride)</td>
<td>100µM</td>
<td>Kir channel blocker (Ba2+)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Verapamil</td>
<td>100µM</td>
<td>L-type calcium channel blocker</td>
<td>Tocris</td>
</tr>
<tr>
<td>BayK</td>
<td>10µM and 100µM</td>
<td>L-type Ca2+-channel activator</td>
<td>Tocris</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>2µM and 10µM</td>
<td>Potent inhibitor of sarco-endoplasmic reticulum Ca2+ - ATPases</td>
<td>Tocris</td>
</tr>
<tr>
<td>2APB (2-Aminooethoxydi phenylborane)</td>
<td>10µM and 50µM</td>
<td>D-myo-inositol 1,4,5-trisphosphate (IP&lt;sub&gt;3&lt;/sub&gt;) receptor antagonist. Stimulates store-operated calcium (SOC) release at low concentrations (&lt; 10 µM) and inhibits it at higher concentrations (up to 50 µM). Blocks TRPC1, TRPC3, TRPC5, TRPC6, TRPV6, TRPM3, TRPM7, TRPM8 and TRPP2 and at higher concentrations stimulates TRPV1, TRPV2 and TRPV3. Also blocks specific gap channel subtypes.</td>
<td>Tocris</td>
</tr>
</tbody>
</table>
Chapter 3 – Potassium Channel Profile of the Mouse Optic Nerve
3.1. Introduction and Aims

It is now well established that astrocytes and oligodendrocytes express many of the same ion channels as neurons (Verkhratsky and Steinhäuser, 2000), although glial cells are electrically inexcitable and the precise roles for many glial ion channels have not been fully elucidated. The exception is the characteristic high expression of plasmalemmal $K^+$ channels, which are the molecular basis of the archetypal strongly negative RMP in glia and their primary physiological function of $K^+$ homeostasis (Butt and Kalsi, 2006). Even here, the precise $K^+$ channels involved are unclear, although a key role for $K_{ir}4.1$ has been demonstrated in astrocytes (Kofuji and Newman, 2004). Furthermore, the maturation of glial functions is related to a developmental shift from predominantly $K_v$ to $K_{ir}$ currents (Olsen and Sontheimer, 2008), although the $K^+$ channels involved have not been clearly defined in vivo. The primary aim of this section was to provide a comprehensive qRT-PCR analysis of developmental changes in potassium ion channel expression in the mouse optic nerve, a typical CNS white matter tract that is used as a model tissue for studying glial cell function physiology (Butt et al., 2004; Bolton and Butt, 2005).
3.2. Results

To determine developmental changes in the transcriptome profile of the main potassium channels in the optic nerve, tissue was analysed in mice aged P9 (pre-myelination), P12 (active myelination) and P35 (post-myelination/mature). mRNA expression levels were determined by qRT-PCR using the Mouse Neuronal Ion Channels RT² Profiler™ qPCR array, which contained the primary potassium channels, and a custom RT² Profiler™ qPCR array for glial channels not included in the neuronal array, such as Kᵢ₄.1 (Sabiosciences, Qiagen). Total RNA was extracted from P9, P12 and P35 tissue and then used to create cDNA libraries for analysis (n=10 optic nerves for each experimental group, run in triplicate). The method of Relative Quantification was used to study the relative expression levels of ion channels and the $2^{ΔΔCt}$ method was used to calculate the fold changes in gene expression between the various ages tested. Ct values were previously normalised to the most stable housekeeping genes (see below). Data are presented as mean ± SEM (n=3) and were tested for significance using ANOVA and post hoc Bonferroni’s tests in Prism v3.02 software (GraphPad). Throughout the results of this section, the gene and protein names are given in the tables. The more commonly used protein names of the ion channels are used in the text and the reader is directed to the tables for the respective gene names.

A limitation of this study was that whole mouse optic nerve extracts were used and hence it was not possible to unequivocally identify the ion channel profiles of specific glial cell types. qRT-PCR on purified glial cells was not feasible in the optic nerve, due to the large number of mice required (the present study utilised 10 mice for
each experimental group to obtain sufficient optic nerve RNA). Nonetheless, it has been previously shown that the vast bulk of the RNA in whole optic nerve extracts is from astrocytes and oligodendrocytes (>90% of cells), together with minor components from microglia and OPCs (together comprise ≤10% of cells), and trace levels from the vasculature and axons (Salter and Fern, 2005); the optic nerve does not contain neuronal somata. Hence, it is reasonable to conclude that the most highly expressed transcripts are the most important functional ion channels in astrocytes and/or oligodendrocytes. An important consideration is the developmental changes in oligodendrocytes, whereby the numbers of OPCs may be greater at P9 and decline thereafter as they differentiate into oligodendrocytes (Butt and Ransom, 1993), although OPCs persist in the adult nerve as a small stable population of ~5% of the cells (Butt et al., 1999).

### 3.2.1. Determination of most stable reference genes

The Mouse Neuronal Ion Channels RT² Profiler™ qPCR Array contained multiple reference genes, and the NormFinder algorithm (Andersen et al., 2004) was used to establish which single gene or combination of genes was the most stable and therefore appropriate to use for normalisation. The top five most stable options are presented in Figure 3.1 and a combination of two genes, Gapdh and Hsp90ab1 with a stability value of 0.004, was determined as the best references for normalisation.
Mane et al. (2008) demonstrated that the ideal reference genes should have a normal distribution across samples and a low standard deviation. In the customised plate for Kᵢ4.1, statistical analysis was performed to identify reference genes with the lowest standard deviation of the Ct values across all optic nerve samples, which confirmed that Gapdh was a suitable stable gene for normalisation in the customised array (Figure 3.2), and allowed comparison with the Mouse Neuronal Ion Channel array, where Gapdh was highly stable (Figure 3.1).

Figure 3.1: Estimation of stability of candidate reference genes using NormFinder. Genes are ranked according to their expression stability across all samples. The lowest value indicates the most stable reference gene or combination of genes (arrow).
3.2.2. Voltage gated K⁺ channels (Kᵥ)

Kᵥ channels are comprised of α and β subunits. The Kᵥ α subunits form the channel conducting pore and there are 12 subtypes, Kᵥα1-12, which are grouped according to their function (e.g. delayed rectifier, A-type, etc.), and subgrouped according to their sequence homology (e.g. Kᵥ1.1/KCNA1, Kᵥ1.2/KCNA2, etc.) (Gutman et al., 2005). The Kᵥ β subunits (e.g. Kᵥβ1/KCNAB1, Kᵥβ2/KCNA2, Kᵥβ3/KCNA3) are auxiliary proteins that do not have the ability to form functional channels in their own right, but associate with α subunits to modulate their function, in an α₄β₄ stoichiometry (Pongs and Schwarz, 2010). The main roles of Kᵥ channels include the determination and maintenance of the RMP, regulation of firing patterns and shaping of action potentials in neurons, as well as the modulation of neurotransmitter release, and they are involved in ion transport in epithelial cells and glia (Hille, 2001). Tables 3.1 and 3.2 display the Kᵥ channels, the mRNA level of which was investigated in the optic nerve.
for the purposes of this project, providing both the gene and protein names, and the results are illustrated in Figure 3.3.

Table 3.1: Voltage Gated K⁺ Channel Alpha Subunits (K,α).

<table>
<thead>
<tr>
<th>Delayed Rectifier (slowly or non-inactivating)</th>
<th>Classification</th>
<th>Family</th>
<th>Gene Name (Protein Name)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K,α1.x</td>
<td>Shaker</td>
<td>Kcna1 (K,1.1), Kcna2 (K,1.2), Kcna5 (K,1.5), Kcna6 (K,1.6)</td>
</tr>
<tr>
<td></td>
<td>K,α2.x</td>
<td>Shab</td>
<td>Kcnb1 (K,2.1), Kcnb2 (K,2.2)</td>
</tr>
<tr>
<td></td>
<td>K,α3.x</td>
<td>Shaw</td>
<td>Kcnc1 (K,3.1), Kcnc2 (K,3.2)</td>
</tr>
<tr>
<td></td>
<td>K,α7.x</td>
<td>-</td>
<td>Kcnq1 (K,7.1), Kcnq2 (K,7.2), Kcnq3 (K,7.3)</td>
</tr>
<tr>
<td></td>
<td>K,α10.x</td>
<td>-</td>
<td>Kcnh1 (K,10.1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A-Type (rapidly inactivating)</th>
<th>Classification</th>
<th>Family</th>
<th>Gene Name (Protein Name)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K,α4.x</td>
<td>Shal</td>
<td>Kcnd2 (K,4.2), Kcnd3 (K,4.3)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Slowly Activating</th>
<th>Classification</th>
<th>Family</th>
<th>Gene Name (Protein Name)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K,α12.x</td>
<td>-</td>
<td>Kcnh3 (K,12.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inwardly Rectifying</th>
<th>Classification</th>
<th>Family</th>
<th>Gene Name (Protein Name)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K,α11.x</td>
<td>ERG</td>
<td>Kcnh2 (K,11.1), Kcnh6 (K,11.2), Kcnh7 (K,11.3)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Modifier/Silencer</th>
<th>Classification</th>
<th>Family</th>
<th>Gene Name (Protein Name)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K,α9.x</td>
<td>-</td>
<td>Kcns1 (K,9.1)</td>
</tr>
</tbody>
</table>
Table 3.2: Voltage Gated K⁺ Channel Beta Subunits (Kβ)

<table>
<thead>
<tr>
<th>Beta Subunits (Modulators)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Classification</strong></td>
</tr>
<tr>
<td>Kβx</td>
</tr>
</tbody>
</table>

Results from the qRT-PCR array signified that the most abundant K⁺ channel subunit mRNA in the mouse optic nerve was the delayed rectifier K\(\text{v}_{1.1}\), 5-times greater than the next most abundant, the delayed rectifier K\(\text{v}_{1.6}\), and a factor greater than all the other transcripts (Figure 3.3). Next in rank order were the transcript levels of the delayed rectifiers K\(\text{v}_{7.3}\) and K\(\text{v}_{1.2}\), together with the A-Type channel K\(\text{v}_{4.3}\), followed by K\(\text{v}_{2.1}\), K\(\text{v}_{7.2}\) and K\(\text{v}_{4.2}\). Also detected at very low levels were the delayed rectifiers K\(\text{v}_{2.2}\), K\(\text{v}_{3.1}\), K\(\text{v}_{3.2}\), and K\(\text{v}_{7.1}\), and K\(\text{v}_{10.1}\), together with the inwardly rectifying K\(\text{v}\) channels K\(\text{v}_{11.1}\), K\(\text{v}_{11.2}\) and K\(\text{v}_{11.3}\). Negative or barely detectable levels of transcript were observed for K\(\text{v}_{1.5}\), which is reported to be expressed by OPCs specifically in the G1 phase of the cell cycle (Chittajallu et al., 2002, Vautier et al., 2004), or the slowly activating K\(\text{v}_{12.2}\) and the modifier K\(\text{v}_\alpha\) subunit K\(\text{v}_{9.1}\). The results are consistent with the literature that the K\(\text{v}_{1.x}\) family are ubiquitously expressed in glia (Olsen and Sontheimer, 2006), and indicate K\(\text{v}\) in optic nerve astrocytes and oligodendrocytes are comprised mainly of K\(\text{v}_{1.1}\), together with K\(\text{v}_{1.6}\) and K\(\text{v}_{1.2}\). Notably, the prominent expression of K\(\text{v}_{7.3}\) may be indicative of OPCs (Wang et al., 2011), whereas K\(\text{v}_{7.x}\) are reportedly lost in differentiated oligodendrocytes and have not been reported in astrocytes; K\(\text{v}_{7.2}/K\text{v}_{7.3}\) are also the principle axonal delayed rectifier K\(\text{v}\) at nodes of Ranvier, although the relative level of axonal expression of
transcripts is most likely a factor lower than that observed for Kv7.3 (0.042 ± 0.002 at P35), as indicated by axonal Na1.6 (0.003 ± 0.0004 at P35; Figure 3.6), which is highly concentrated at nodes of Ranvier and initiates the action potential (Poliak and Peles, 2003). mRNA transcripts encoding for the β subunits Kvβ1 and Kvβ2 were robustly expressed, as reported throughout the brain, where they are expressed with all members of the Kv1α-subunit subfamily (Rhodes et al., 1996), whereas Kvβ3 was barely detectable at any age.

The expression levels of most Kv channel mRNA transcripts were virtually unchanged developmentally, with the exception of Kv1.1, which exhibited a clear developmental upregulation, increasing almost 10-fold between P9 and P35 (Figure 3.3; p<0.01, ANOVA, followed by Bonferroni’s Multiple Comparison test). However, a number of other Kv that were not highly expressed displayed statistically significant developmental regulation (p<0.05, ANOVA’s, followed by Bonferroni’s Multiple Comparison tests); Kv4.2 was markedly downregulated between P12 and P35, whereas Kv7.1 and Kv11.3, which were barely detectable, were respectively downregulated and upregulated.
Figure 3.3: Relative expression of Kv channels in the mouse optic nerve. Channels were grouped according to accepted classification. The relative expression of delayed rectifier Kv1.1 is illustrated in the inset, because expression levels were a factor higher than other transcripts. Tested for significance by ANOVA (**p<0.01) and post hoc Bonferroni's tests (*p<0.05).
3.2.3. Inwardly rectifying K⁺ channels (Kir)

There are seven subfamilies of Kir channels, Kir1 - Kir7, and each subfamily has multiple members (e.g. Kir2.1, Kir2.2, Kir2.3, etc.). In addition, Kir channels can form as homotetramers or heterotetramers, usually between members of the same subfamily (e.g. between Kir2.1 and Kir2.3), although Kir3.1 and Kir5.1 do not form functional homomeric channels and respectively associate with other Kir3.x or Kir4.1 to form functional heteromeric channels (Pessia et al., 1996; Rojas et al., 2007). Glia exhibit prominent and ubiquitous expression of Kir4.1, together with the Kir2.x, Kir3.1 and Kir6.x families, which have a primary function in K⁺ spatial buffering during neuronal activity (Butt and Kalsi, 2006; Kofuji and Newman, 2004). Table 3.3 displays the Kir channels the mRNA level of which was investigated in the optic nerve for the purposes of this project, providing both the gene and protein names, and the results are illustrated in Figure 3.4.

Table 3.3: Inwardly Rectifying K⁺ Channels (Kir).

<table>
<thead>
<tr>
<th>Class</th>
<th>Classification</th>
<th>Gene Name (Protein Name)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical Kir</td>
<td>Kir2.x</td>
<td>Kcnj2 (Kir2.1), Kcnj12 (Kir2.2), Kcnj4 (Kir2.3), Kcnj14 (Kir2.4)</td>
</tr>
<tr>
<td>G-Protein Coupled Kir</td>
<td>Kir3.x</td>
<td>Kcnj3 (Kir3.1), Kcnj6 (Kir3.2), Kcnj9 (Kir3.3), Kcnj5 (Kir3.4)</td>
</tr>
<tr>
<td>K_ATP Channels</td>
<td>Kir6.x/SUR</td>
<td>Kcnj11 (Kir6.2)</td>
</tr>
<tr>
<td>K⁺ Transport Kir</td>
<td>Kir1.x</td>
<td>Kcnj1 (Kir1.1)</td>
</tr>
<tr>
<td></td>
<td>Kir4.x</td>
<td>Kcnj10 (Kir4.1) Kcnj15 (Kir4.2)</td>
</tr>
<tr>
<td></td>
<td>Kir5.x</td>
<td>Kcnj16 (Kir5.1)</td>
</tr>
<tr>
<td></td>
<td>Kir7.x</td>
<td>Kcnj13 (Kir7.1)</td>
</tr>
</tbody>
</table>
The rank order of the most highly expressed \( K_{ir} \) in the Mouse Neuronal Ion Channel array is \( K_{ir}7.1 > K_{ir}2.1 > K_{ir}2.2 > K_{ir}5.1 > K_{ir}3.1 \) (Figure 3.4). The high expression of \( K_{ir}7.1 \) is a novel finding, whereas the remaining \( K_{ir} \) are consistent with previous reports on glia (Verkhratsky and Butt, 2013). \( K_{ir}4.1 \) is not included in the Mouse Neuronal Ion Channel array and was examined using a separate custom qRT-PCR array, and is compared with the Mouse Neuronal Ion Channel array using Gapdh as the housekeeping gene (inset, Figure 3.4). The results indicate \( K_{ir}4.1 \) and \( K_{ir}7.1 \) are expressed at the same level, without developmental regulation, significantly greater than \( K_{ir}2.1 \) and \( K_{ir}5.1 \) \( (p<0.01, \text{ANOVA}, \text{followed by Bonferroni's Multiple Comparison test}) \); notably, \( K_{ir}4.1 \) and \( K_{ir}7.1 \) are expressed at the same levels as \( K_{v}1.1 \) in the early postnatal nerve, but are individually significantly lower than \( K_{v}1.1 \) in the adult nerve. None of the more abundant mRNA transcripts encoding for \( K_{ir} \) channels were developmentally regulated between P9 and P35 \( (p>0.05, \text{ANOVA}) \), although analysis of \( K_{ir}4.1 \) in the cortex indicated a significant developmental upregulation of 4.2-fold between P9 and P35 \( (p<0.01, \text{ANOVA}, \text{followed by Bonferroni's Multiple Comparison test}; \text{not illustrated}) \). \( K_{ir}2.3 \) was expressed at very low levels, but was significantly developmentally downregulated, being almost completely lost between P12 and P35 \( (p<0.01, \text{ANOVA}, \text{followed by Bonferroni's Multiple Comparison test}) \). Similarly, \( K_{ir}3.2 \) was expressed at very low levels, but was apparently increased between P12 and P35, although not statistically significant. The remaining transcript levels for \( K_{ir} \) examined were negligible \( (K_{ir}1.1, K_{ir}2.4, K_{ir}3.3, K_{ir}3.4, K_{ir}6.2) \).
Figure 3.4: Relative expression of K<sub>r</sub> Channels in mouse optic nerve. Inset shows relative expression levels for K<sub>r</sub>.4.1, which was determined on a customised array, compared to the key K<sup>+</sup> channels using Gadph as the housekeeping gene. The remaining K<sub>r</sub> were examined using the Mouse Neuronal Ion Channels RT<sup>2</sup> profiler PCR array, using two genes, Gapdh and Hsp90ab1, for greater accuracy. Data tested for significance by ANOVA and post hoc Bonferroni's tests (*p<0.05, **p<0.01).
3.2.4. Calcium activated K+ channels (K\textsubscript{Ca})

The K\textsubscript{Ca} channel family contains 8 members and they are grouped according to their transmembrane domain homology and biophysical characteristics in three classes; BK (Big Potassium, K\textsubscript{Ca}1.1\textalpha{} and \beta{} subunits \beta{}1-4), characterised by a large potassium conductance, IK (Intermediate Potassium, K\textsubscript{Ca}3.1), and SK (Small Potassium, K\textsubscript{Ca}2.1-K\textsubscript{Ca}2.3), which display very small conductance. The \alpha{} subunits make homo- and hetero-tetrameric complexes and BK channels are composed of at least two different subunits, the pore-forming \alpha{} subunit and modulatory \beta{} subunit. Although K\textsubscript{Ca} channels are named due to the fact that they are activated by increases in intracellular calcium, BK channels are also activated by transmembrane voltage changes independently of Ca\textsuperscript{2+}, unlike SK channels which are solely gated by Ca\textsuperscript{2+}. The efflux of K\textsuperscript{+} ions that occurs when K\textsubscript{Ca} channels are opened leads to hyperpolarisation and, as a result, neuronal excitability is dampened. Glia display BK and SK currents, the latter being mediated K\textsubscript{Ca}3.1 (Tiwari-Woodruff et al., 2001; Schlichter et al., 2010; Longden et al., 2011).

Table 3.4 displays the K\textsubscript{Ca} channels, the mRNA level of which was investigated in the optic nerve for the purposes of this project, providing both the gene and protein names, and the results are illustrated in Figure 3.5.

<table>
<thead>
<tr>
<th>Class</th>
<th>Classification</th>
<th>Gene Name (Protein Name)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK Channel – Alpha Subunit</td>
<td>K\textsubscript{Ca}1.1</td>
<td>Kcnma1 (K\textsubscript{Ca}1.1)</td>
</tr>
<tr>
<td>BK Channel – Beta Subunit</td>
<td></td>
<td>Kcnmb4 (K\textsubscript{Ca} Subunit \beta{}4)</td>
</tr>
<tr>
<td>SK Channel</td>
<td>K\textsubscript{Ca}2.x</td>
<td>Kcnn1 (K\textsubscript{Ca}2.1), Kcnn2 (K\textsubscript{Ca}2.2), Kcnn3 (K\textsubscript{Ca}2.3)</td>
</tr>
</tbody>
</table>
All the $K_{Ca}$ channel transcripts investigated were present in the mouse optic nerve (Figure 3.5), the most prominent being the BK channel $K_{Ca}\beta4$ subunit, which was significantly developmentally downregulated ($p<0.05$, ANOVA, followed by Bonferroni's Multiple Comparison test). At P35 all $K_{Ca}$ channel transcripts tested were expressed at similar levels (Figure 3.5).

**Figure 3.5: Expression of Calcium Activated K⁺ Channels ($K_{Ca}$) in mouse optic nerve.** Tested for significance by ANOVA and post hoc Bonferroni's tests (*$p<0.05$).

### 3.2.5. Voltage gated Na⁺ channels ($Na_v$)

Voltage gated sodium channels ($Na_v$ channels) are generally associated with initiating the neuronal action potential, but are also ubiquitous in astrocytes and oligodendrocytes (Olsen and Sontheimer, 2005; De Biase et al., 2010). $Na_v$ channels comprise an α subunit, which have 9 members ($Na_v1.1$-1.9) and form the channel pore and voltage sensing apparatus, together with associated β subunits ($Na_v\beta1$-$4$), which link the channel to the cytoskeleton and modulate its gating properties. Table 3.5 displays the $Na_v$ channels, the mRNA level of which was investigated in the optic nerve.
for the purposes of this project, providing both the gene and protein names, and the results are illustrated in Figure 3.6.

**Table 3.5: Voltage Gated Na⁺ Channels**

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gene Name (Protein Name)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naᵥ1.x</td>
<td>Scn1a (Naᵥ1.1), Scn2a (Naᵥ1.2), Scn3a (Naᵥ1.3), Scn8a (Naᵥ1.6), Scn9a (Naᵥ1.7), Scn10a (Naᵥ1.8), Scn11a (Naᵥ1.9)</td>
</tr>
<tr>
<td>Beta Subunits</td>
<td>Scn1b (Naᵥ Subunit β1), Scn2b (Naᵥ Subunit β2)</td>
</tr>
</tbody>
</table>

The most abundant Naᵥ channel transcripts in the mouse optic nerve were the β and α subunits Naᵥβ1 and Naᵥ1.2, followed by the Naᵥβ2 subunit and Naᵥ1.3 and Naᵥ1.6 α subunits (Figure 3.6); the remaining Naᵥ channel transcripts tested were barely detectable or not present. We found no significant developmental regulation of Naᵥ channels but there is a downregulation trend; consistent with these findings in the optic nerve, astrocytes and OPCs/oligodendrocytes have been reported to specifically express Naᵥβ1 with Naᵥ1.2, Naᵥ1.3, and Naᵥ1.6, with evidence that Naᵥ1.3 mRNA is downregulated as OPCs differentiate into myelinating oligodendrocytes (De Biase et al., 2010). In addition, axonal mRNA can be detected in optic nerve extracts (Salter and Fern, 2005) and it is possible that some message for Naᵥ reflects their axonal concentration at nodes of Ranvier, where they are responsible for action potential initiation. However, mature nodes are characterised by Naᵥ1.6 (Rasband et al., 1999; Boiko et al., 2001; Kaplan et al., 2001), and the low level of Naᵥ1.6 detected in the P35
samples is consistent with the vast bulk of transcripts in this analysis of optic nerve extracts being glial with an ultralow ‘contamination’ of axonal mRNA.

Figure 3. 6: Expression of Voltage Gated Na⁺ Channels (Naᵥ) in mouse optic nerve. No significant developmental changes were found when tested for significance by ANOVA and post hoc Bonferroni’s tests.

3.2.6. Acid Sensing Ion Channels (ASIC)

Acid sensing ion channels (ASIC) are related to the larger family of Epithelial Sodium/Degenerin Channels (ENaC/Deg). They are voltage independent cation channels with preference for Na⁺ and Ca²⁺ and they are activated by extracellular [H⁺]. There are four known subunits and all but ASIC3 are believed to be expressed in the CNS (Jasti et al., 2007; Carattino, 2011). Table 3.6 displays the ASICs, the mRNA level of which was investigated in the optic nerve for the purposes of this project, providing both the gene and protein names, and the results are illustrated in Figure 3.7.

Table 3. 6: Acid Sensing Ion Channels (ASIC).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gene Name (Protein Name)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asicx</td>
<td>Accn1 (ASIC1), Accn2 (ASIC2), Accn3 (ASIC3)</td>
</tr>
</tbody>
</table>
Asic1 is the most abundant mRNA transcript in the optic nerve, followed by Asic2, whilst mRNA for Asic3 is barely detectable (Figure 3.7). ASIC1 was significantly developmentally downregulated ($p<0.05$, ANOVA, followed by Bonferroni’s Multiple Comparison test) and levels of all ASIC transcripts were very low at P35. Interestingly, Asic1 channel protein and mRNA transcript is expressed by OPCs and is downregulated during their differentiation into myelinating oligodendrocytes (Feldman et al., 2008). Astrocytes have been reported to express all 3 ASICs (Huang et al., 2010).

Figure 3.7: Expression of Acid Sensitive Ion Channels (ASIC) in mouse optic nerve. Tested for significance by ANOVA and post hoc Bonferroni's tests (*$p<0.05$).

3.2.7. Key developmental changes in optic nerve ion channels

Volcano plot analysis (Sabiosciences Rt² Array Data Analysis) was used to identify the ion channels that were significantly (unrelated t-test, $p<0.05$) developmentally regulated greater than 2-fold between P9 and P35 (Table 3.7). The results indicate an overall developmental downregulation ASIC and $K_{Ca}$ and upregulation of both $K_v$ and $K_{ir}$, whereas $Na_v$ were not altered overall (Figure 3.8).
Table 3.7: Developmentally regulated ion channel transcripts in the mouse optic nerve. Data was obtained by qRT-PCR analysis of 3 biological replicates (n=10 optic nerves per group) of acutely isolated optic nerves from mice aged P9 compared to P35. Statistical analysis was performed by volcano plot (Sabiosciences Rt² Array Data Analysis) (unrelated t-test P<0.05; FC>2.0).

<table>
<thead>
<tr>
<th>Ion Channel</th>
<th>Regulation</th>
<th>p-value</th>
<th>Ion Channel</th>
<th>Regulation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K\textsubscript{v}1.1</td>
<td>↑ 9.8</td>
<td>0.0097</td>
<td>K\textsubscript{v}7.1</td>
<td>↓ -3.1</td>
<td>0.0283</td>
</tr>
<tr>
<td>K\textsubscript{v}11.3</td>
<td>↑ 2.3</td>
<td>0.0455</td>
<td>K\textsubscript{v}4.2</td>
<td>↓ -2.9</td>
<td>0.0064</td>
</tr>
<tr>
<td>K\textsubscript{v}7.1</td>
<td>↓ -3.1</td>
<td>0.0455</td>
<td>K\textsubscript{v}10.1</td>
<td>↓ -2.8</td>
<td>0.0145</td>
</tr>
<tr>
<td>K\textsubscript{v}10.1</td>
<td>↑ 2.8</td>
<td>0.0455</td>
<td>K\textsubscript{v}4.2</td>
<td>↓ -2.9</td>
<td>0.0064</td>
</tr>
<tr>
<td>K\textsubscript{v}11.3</td>
<td>↓ -2.8</td>
<td>0.0455</td>
<td>K\textsubscript{v}10.1</td>
<td>↓ -2.9</td>
<td>0.0064</td>
</tr>
<tr>
<td>K\textsubscript{Ca}2.3</td>
<td>↓ -2.7</td>
<td>0.0455</td>
<td>K\textsubscript{Ca}γ4</td>
<td>↓ -2.7</td>
<td>0.0296</td>
</tr>
<tr>
<td>K\textsubscript{Ca}γ4</td>
<td>↓ -2.7</td>
<td>0.0455</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asic1</td>
<td>↓ -2.5</td>
<td>0.0455</td>
<td>K\textsubscript{v}4.2</td>
<td>↓ -2.9</td>
<td>0.0064</td>
</tr>
<tr>
<td>K\textsubscript{v}4.2</td>
<td>↓ -2.9</td>
<td>0.0455</td>
<td>K\textsubscript{v}6.2</td>
<td>↓ -2.1</td>
<td>0.0229</td>
</tr>
<tr>
<td>K\textsubscript{v}6.2</td>
<td>↓ -2.1</td>
<td>0.0455</td>
<td>Na\textsubscript{i}1.3</td>
<td>↓ -2.1</td>
<td>0.0260</td>
</tr>
<tr>
<td>Na\textsubscript{i}1.3</td>
<td>↓ -2.1</td>
<td>0.0455</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.8: Key developmental changes in optic nerve K\textsuperscript{+} and Na\textsuperscript{+} channels. Data are presented as collective relative expression of the different ion channel families, to illustrate the proportional change in gene expression from P9 to adulthood.
3.3. Discussion

Astrocytes and oligodendrocytes have been shown to express a wide range of ion channels in vitro and in vivo using electromolecular biological techniques in a variety of preparations (see Chapter 1 and Verkhratsky and Butt, 2013). An important feature of glial cells is that their ion channel expression is regionally diverse and developmentally regulated. In this section, I have provided the potassium ion channel profile of the postnatal and adult optic nerve, which is summarised in Table 3.8. The optic nerve is widely used as a model of white matter tissue for studying glial cell development and function, because it comprises mainly astrocytes and oligodendrocytes, whose functions are to support and myelinate the axons of retinal ganglion cells (Butt et al., 2004). The results demonstrate that optic nerve glia express a preponderance of delayed rectifier $K_v$ and inward rectifier $K_{ir}$ channels, the mRNA levels of which together comprise $>50\%$ of the transcripts in the optic nerve. $K_{ir}4.1$ was one the most highly expressed transcripts at all ages, supporting a principle role for this channel in glia (Butt and Kalsi, 2006). A key novel finding is an equivalent high level of expression of $K_{ir}7.1$, which has not previously been identified in glia. In addition, there was a marked developmental upregulation of the outwardly rectifying (voltage-dependent) $K_v1.1$ channel. $K_v$ are abundant in astrocytes, but they are generally considered to be down-regulated during astrocyte differentiation, whereas transcripts for $K_v1.1$ are the most highly expressed in the adult optic nerve. This study identifies $K_v1.1$, $K_{ir}4.1$ and $K_{ir}7.1$ as the molecular basis of the characteristic high $K^+$ resting membrane conductance of glia, which determines the strongly negative RMP that underpins glial physiological functions.
3.3.1. Accuracy of the qRT-PCR analysis

There are a number of important considerations when examining the qRT-PCR results, which determine the overall accuracy of the data and their interpretation. Probably the most important methodological variable is differences between samples in terms of the quantity of starting material (RNA) and the efficiency of enzymes (e.g. polymerase). In this project, this variability was addressed by normalisation to housekeeping (reference) genes that were included and ran on the same PCR arrays as the target genes. Other methods of normalisation were not practicable, such as normalisation to the original number of cells, which is only applicable to cultured cells and blood samples (Bustin, 2000), or normalisation to the total RNA quantity, which in itself can lead to inaccuracies due to differences in rRNA/mRNA ratios between samples (Solinas et al., 2001) and spectrophotometric (A260) RNA quantification inaccuracies (Bustin, 2002). Multiple endogenous reference genes were examined (i.e. Gapdh, Hsp90ab1 and Rpl13a), since this addressed the potential issue that any one reference gene may not be expressed constantly and at the same level between samples and experimental conditions (Cook et al., 2009). The Qiagen arrays (Sabiosciences) used in this thesis determined gene expression by the Relative Quantification method, where the quantity of genes of interest are expressed relative to reference genes within the same sample, as opposed to Absolute Quantification, where the exact number of copies of a gene of interest is calculated. The most appropriate reference genes that were constantly present and stably expressed in all samples were selected by the standard deviation method, in combination with the NormFinder algorithm (Casadei et al., 2011). The optimal way to determine the most appropriate reference genes with the lowest difference between samples would be to
use RNA sequencing or to run a variety of candidate reference genes in triplicates for all samples and different experimental conditions. These methods were not feasible in my study because of the large number of mice that would require. Nonetheless, the three reference genes used were expressed in a highly constant and stable manner in all samples and therefore the results were considered accurate. Gapdh is the most widely used reference gene across the literature and has been shown to be the most stable gene in the mouse brain during development and in ageing (Kraemer et al., 2012; Sieber et al., 2010), consistent with the findings of my analysis. In addition, I determined that Hsp90ab1 was optimal in combination with Gapdh in the Mouse Neuronal Ion Channels RT² profiler PCR array; Hsp90ab1 is a member of the heat shock protein 90 family and was previously found to be among the five most stable genes in mouse tissue out of 13 genes tested (Wang et al., 2010).

3.3.2. Relative levels of mRNA as a measurement of functional glial ion channels

The relative quantification and $2^{-\Delta\Delta CT}$ method used here are the simplest and most commonly used methods for accurately determining relative expression levels and changes in expression of transcripts. This technique should only be used if PCR efficiencies for the target and reference genes are close to 100% (Schmittgen and Livak, 2008), and this was confirmed in my samples using three positive PCR controls in each array, run for all replicate samples. However, an important consideration when interpreting the data is how well the relative levels of mRNA reflect expression of functional ion channel proteins. The qRT-PCR method is extremely sensitive, and the presence of a small amount of mRNA is unlikely to represent a functionally important
ion channel. In addition, there are many different post-transcriptional and post-translational regulatory mechanisms that may lead to mismatches between mRNA and protein levels (Greenbaum et al., 2003; Vogel and Marcotte, 2012). For example, functional Kir4.1 expression has been shown to be dynamically regulated by cAMP in optic nerve glia (Bolton et al., 2006; Bolton and Butt, 2006), and multiple regulatory mechanisms regulate protein translation and subsequent targeting of K^+ channels to the plasmalemma, and their biophysical properties will be further regulated by diverse intracellular and extracellular factors including ancillary proteins (Heusser and Schwappach, 2005; Ruppersberg, 2000). Nonetheless, with this caveat in mind, the most highly expressed transcripts are likely to be the most important functional ion channels in optic nerve glia.

A further limitation is that whole mouse optic nerve extracts were analysed and the ion channel profiles of specific glial cell types could not be determined. It was not feasible to perform comprehensive qRT-PCR on purified glial cells using ion channel arrays, due to the large numbers of mice this would require. However, this could now be performed in future studies for the key ion channels identified in my study. Nonetheless, the vast bulk (>90% of cells) of the RNA extracted in whole optic nerves is from astrocytes and oligodendrocytes (Salter and Fern, 2005). Hence, it can be hypothesised that the most highly expressed genes identified in my study, such as Kv1.1 and Kir4.1, are the most important functional ion channel proteins in astrocytes and/or oligodendrocytes. This is supported by an analysis of the relative levels of expression of a group of the known major glial Kv and Kir channels in comparison with
the main axonal channel Na\textsubscript{v}1.6, which is localised to nodes of Ranvier and not reported in glia, together with Ca\textsubscript{v}3.1 and K\textsubscript{Ca}2.3, which are constitutively expressed by OPCs and microglia, respectively, but are not prominent in astrocytes or oligodendrocytes (Figure 3.9). This analysis suggests that a relative expression level of \textasciitilde0.03 represents the lower limit of the main astrocyte and oligodendrocyte channels, whilst channels in OPCs and microglia are detected at relative expression levels of \textgreater0.01, and channels with relative expression values \textless0.005 reflect contamination from non-glial sources. On this basis, the main channels expressed by optic nerve glia are given in Table 3.8. However, it should be noted that some channels reported to be expressed by glia, such as K\textsubscript{v}1.4 and K\textsubscript{Ca}3.1, were not present in the arrays used here, but a recent microarray study performed in our lab did not find either to be significantly expressed in the adult optic nerve (Rivera, 2014).

Figure 3.9: Comparison of relative expression levels of the main glial K\textsubscript{v} and K\textsubscript{Ca} with the main axonal channel Na\textsubscript{v}1.6, together with Ca\textsubscript{v}3.1 and K\textsubscript{Ca}2.3, which are constitutively expressed by OPCs and microglia, respectively, but are not prominent in astrocytes or oligodendrocytes.
Table 3. 8: Main ion channels expressed by optic nerve glia.

<table>
<thead>
<tr>
<th>Channel Family</th>
<th>Astrocytes/Oligodendrocytes</th>
<th>OPC/Microglia</th>
<th>Developmental Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv1.1</td>
<td>Kv1.6 &gt; Kv7.3 &gt; Kv1.2 &gt; Kv2.1 = Kv4.3 &gt; Kv4.2</td>
<td>K1.1 strongly upregulated</td>
<td></td>
</tr>
<tr>
<td>Kir4.1</td>
<td>Kir7.1 &gt; Kir2.1 &gt; Kir2.2 = Kir5.1 &gt; Kir3.1 &gt; Kir3.2</td>
<td>Not regulated</td>
<td></td>
</tr>
<tr>
<td>Kcaβ4</td>
<td>Kcaβ2.3 &gt; Kca2.2</td>
<td>Downregulated</td>
<td></td>
</tr>
<tr>
<td>Nav1β</td>
<td>Nav1.2 &gt; Nav1.3</td>
<td>Downregulated</td>
<td></td>
</tr>
<tr>
<td>ASIC1</td>
<td>ASIC2</td>
<td>ASIC1 downregulated</td>
<td></td>
</tr>
</tbody>
</table>

3.3.3. Voltage Gated Potassium Channels (Kv)

Diverse delayed rectifier and rapidly inactivating A-type Kv were strongly expressed in the optic nerve with a rank order of Kv1.1 >> Kv1.6 > Kv7.3 >> Kv1.2 > Kv2.1 = Kv4.3 > Kv4.2. Only Kv1.1 and Kv4.2 were significantly developmentally regulated, with a marked 6-fold upregulation of Kv1.1, and 3-fold downregulation of Kv4.2 to below the ‘glial threshold’.

With respect to the delayed rectifying Kv, our results are largely consistent with studies in astrocytes showing they mainly express transcripts for Kv1.1 and Kv1.6, as well as Kv1.2, Kv2.1 and Kv7.3 (Smart et al., 1997). These results agree with a microarray study in our lab, in which RNA encoding for Kv1.1 was identified as the most highly expressed among Kv channel transcripts in the adult optic nerve (Rivera, 2014). In addition, Kv1.1 is expressed from early on in development; it has been reported in radial glia of the ventricular zone as early as E9.5, with immunoreactivity peaking in glial precursors and radial glia of the subventricular zone (Hallows and Tempel, 1998). Similarly, transcripts for Kv1.1, Kv1.2, Kv1.5 and Kv1.6 were detected by single cell RT-
PCR in mouse cultured OPCs, and protein expression was confirmed by immunocytochemistry for Kv1.5 and Kv1.6, whereas immunopositivity for Kv1.1 was only seen in a small subset of cells, and Kv1.2 was negative for protein expression (Attali et al., 1997). Moreover, the current profile of OPCs was not found to be dependent on Kv1.1 (Schmidt et al., 1999; Tiwari-Woodruff et al., 2006). At the mRNA level, Kv1.5 and Kv1.6 were downregulated during OPC differentiation, and Kv1.2 protein in mature cultured oligodendrocytes was confirmed by Western Blot (Attali et al., 1997), which correlates with our finding of significant Kv1.2 levels at the mRNA level in the optic nerve. In addition, Kv1.2 and Kv1.6 are expressed at low levels in ‘resting’ microglia, while early migrating microglia express Kv1.1 and Kv1.2 (Kotecha and Schlichter, 1999). It should be noted that Kv1.1 and Kv1.2 are known to be colocalised in high density clusters in the axolemma in the juxtaparanodal region of nodes of Ranvier (Bhat et al., 2001; Robbins and Tempel, 2012), where they have been shown to form heterotetramers (Utsunomiya et al., 2008). Colocalised expression of Kv1.1, Kv1.2 and Kv1.6 in clusters was also found in juxtaparanodes of adult rat optic nerves (Rasband et al., 1999). It is possible that low levels of axonal transcripts for these channels are detected in my analysis, but these are likely to be negligible in relation to glial expression, as indicated by the ultralow levels of nodal Na1.6 detected in my samples (Figure 3.10).

The Kvβ1 and Kvβ2 subunits were also strongly expressed in the optic nerve and are closely associated with Kvα subunits: Kvβ1 associates extensively with Kv1.1 and Kv1.4 in neurons (Rhodes et al., 1997), and Kvβ2 colocalises with Kv1.1 and Kv1.2 at
juxtaparanodal regions, which has been shown in mouse optic nerve (Poliak et al., 2003) and spinal cord (Jukkola et al., 2012). More recently, K\textsubscript{v}β2 was shown to interact with TRPV1 (Bavassano et al., 2013). To our knowledge, glial expression of K\textsubscript{v}β subunits has only been reported in cultured hippocampal astrocytes (Bekar et al., 2005). Since astrocytes and oligodendrocytes express K\textsubscript{v}1.1 and K\textsubscript{v}1.2, it is likely that K\textsubscript{v}β1 and K\textsubscript{v}β2 are associated with these channels and regulate their function/localisation.

In addition, relatively high levels of K\textsubscript{v}2.1 mRNA were detected and were not developmentally regulated. K\textsubscript{v}2.1 protein is absent from axons in the mouse cortex and hippocampus (Du et al., 1998), but forms clusters on neuronal cell bodies adjacent to astrocytic processes, which play a significant role in rapidly removing extracellular K\textsuperscript{+} after an action potential and in neuroprotection during ischaemia (Misonou et al., 2008). It is possible that K\textsubscript{v}2.1 is expressed in a similar functional manner at perinodal astrocyte processes. Oligodendrocytes may also express K\textsubscript{v}2.1, which has been linked with myelination in the PNS (Peretz et al., 2000).

Strong expression of K\textsubscript{v}7.3 mRNA as the third most abundant K\textsubscript{v} in the optic nerve was also found at all ages. Delayed rectifier K\textsubscript{v}7.2 and K\textsubscript{v}7.3 channels have recently been reported in oligodendrocyte lineage cells at all stages of development (Wang et al., 2011), supporting our findings that K\textsubscript{v}7.3 mRNA levels are relatively high, indicating expression in a major glial population. Heteromers of K\textsubscript{v}7.2 and K\textsubscript{v}7.3 are also highly expressed at nodes of Ranvier (Gómez-Posada et al., 2010), and the very low levels of K\textsubscript{v}7.2 detected in my samples may reflect axonal mRNA, whereas the high levels of
Kv7.3 in comparison are likely to be glial. High densities of Kv7.2 and Kv7.3 have been found in the axon initial segment (AIS) of CA1 hippocampal neurons, where they are bound to Ankyrin G thus colocalising with Na⁺ channels (Chung et al., 2006; Pan et al., 2006; Rasmussen et al., 2007). These subunits form the low-threshold voltage-gated K⁺ channel originally termed “M-channel” due to the fact that it is inhibited by muscarinic acetylcholine receptor (mAChR) agonists. Due to their activation at sub-threshold potentials (≈ -60mV) and the fact that they do not inactivate, they are considered to be involved in stabilizing both the neuron’s resting membrane potential and to regulate the action potential threshold thus controlling over-excitability. Expression of Kv7.3 in glia could also be an indication of its involvement in regulating the resting membrane potential, especially in immature oligodendrocytes which have not yet developed the Kir channel profile that characterizes the fully differentiated myelinating oligodendrocytes (Brown and Passmore, 2009). Kv7.1 has been studied in the choroid plexus epithelium (Roepke et al., 2011), in the heart, where it has been linked with cardiac arrhythmias (Wang et al., 1996) and in the stria vascularis of the cochlear duct (Yanget al., 2013) but it is not among the axonal Kcnqx subtypes (Padilla et al, 2009). The low levels of Kv7.1 that we found in the optic nerve could actually be from its expression in the vascular endothelium.

The rapidly inactivating A-type channel Kv4.3 was also amongst the top Kv channels expressed at the mRNA level in the optic nerve. This correlates well with reports of astrocyte Kv4.3 expression (Bekar et al., 2005). Bekar et al. (2005) showed that 70% of total inactivating currents in cultured rat hippocampal astrocytes are associated with
the Kv4.x family and intense staining for Kv4.3 was demonstrated throughout the astrocytic cell somata and processes (Bekar et al., 2005). The Kv4.2 protein has not been reported on glia, but is found on somato-dendritic regions of neurons and not axons (Kerti et al., 2012). Neurons are not present in the optic nerve, and so the relatively high level of mRNA encoding for Kv4.2 detected in the optic nerve seems likely to reflect glial expression. Moreover, the significant downregulation of Kv4.2 transcript post-myelination suggests that it may be expressed by developing OPCs.

Notably, the slowly-activating Kv12.2 and inwardly rectifying Kv11.x (ERG) channels were absent or barely detectable in the optic nerve. ERG-type currents have been detected in hippocampal astrocytes, where they were implicated in $[K^+]_o$ clearance, and there is immunohistochemical and RT-PCR evidence that cortical astrocytes specifically express Kv11.1 (Emmi et al., 2000). However, these channels do not appear to be highly expressed in the optic nerve, with only Kv11.3 being slightly upregulated post-myelination. It is likely that inwardly rectifying Kv are regionally expressed, as demonstrated for many other astrocyte channels, including Kv4.1 in astrocytes, and reflects the different $K^+$ clearance requirements between different regions (Olsen et al, 2007).

The functional role of Kv channels in astrocytes and oligodendrocytes is not absolutely clear, but they are generally considered to be negatively linked with differentiation (Sontheimer et al., 1989; Berger et al., 1991; Gallo et al., 1996; Knutson et al., 1997). A key finding is that we did not observe an overall developmental
downregulation of \( K_v \), due to the prominence of \( K_v1.1 \), which was markedly upregulated with development. Nonetheless, we did observe a 3-fold downregulation of \( K_v4.2 \) between P12 and P35, which is worthy of further study, since it has been reported in astrocytes and is implicated in epilepsy (Aronica et al., 2009). Interestingly, \( K_v1.5 \) is reported to be selectively upregulated with \( K_v1.3 \) in OPCs and plays a central role in promoting their proliferation (Chittajallu et al., 2002; Vautier et al., 2004; Soliven et al., 2003), but \( K_v1.5 \) was not detected in the optic nerve at any age; \( K_v1.3 \) was not included in the arrays used in my study, but a microarray study performed in our lab supported the finding of this section and called \( K_v1.3 \) and \( K_v1.5 \) as absent or barely detectable (Rivera, 2014). These results suggest many of the \( K_v \) may be downregulated prior to P9, when the majority of astrocytes and oligodendrocytes are generated (Butt and Ransom, 1993; Barres and Raff, 1993). These possibilities should be examined in younger nerves. Nonetheless, the developmental upregulation of \( K_v1.1 \) is a completely novel finding and it seems highly likely that \( K_v1.1 \) are important for maintaining the RMP of optic nerve glia and in their primary function of \([K^+]_o\) regulation, which future studies should examine using immunohistochemical and electrophysiological techniques.

### 3.3.4. Inward Rectifying Potassium Channels (\( K_{ir} \))

Most of the \( K_{ir} \) examined were detected in the optic nerve, with a rank order of \( K_{ir}4.1 \approx K_{ir}7.1 > K_{ir}2.1 > K_{ir}2.2 > K_{ir}5.1 > K_{ir}3.1 > K_{ir}3.2 \). The strong and widespread expression of the \( K_{ir}4.1 \) channel subunit throughout the CNS first indicated a key role for this channel in glial function (Butt et al., 2005; Hibino et al., 2010). Further studies proved beyond doubt that astrocytes and oligodendrocytes of white matter tracts such
as the optic nerve express the $K_{ir}4.1$ channel from P5 (Li et al., 2001; Ishii et al., 2003; Kalsi et al., 2004), with western blot and qPCR results showing concomitant protein and transcript up-regulation during development (Nwaobi et al., 2014). Positive immunolabelling and mRNA expression has been established in astrocytes and oligodendrocytes (Olsen and Sontheimer, 2008; Osawa et al., 2009; Bichet et al., 2003; Higashi et al., 2001; Greenwood et al., 2005; Poopalasundaram et al., 2000), as well as in retinal glia (Kofuji et al., 2002). The results of this section corroborate these earlier findings that $K_{ir}4.1$ is an archetypal glial $K^+$ channel.

The most novel finding of this study was the high expression of $K_{ir}7.1$, which has not previously been reported in astrocytes or oligodendrocytes. $K_{ir}7.1$ expression has been demonstrated in cerebellar Purkinje neurons and pyramidal neurons of the hippocampus, and a role in $K^+$ transport in the choroid plexus has been established (Krapivinsky et al., 1998). It is also known that $K_{ir}7.1$ plays a significant role in $K^+$ transport in the retinal pigment epithelium (Yang et al., 2008). In my study, mRNA transcripts for $K_{ir}7.1$ displayed the highest relative expression with $K_{ir}4.1$, and second only to transcripts encoding for $K_{v}1.1$ in the adult nerve. No significant developmental regulation of $K_{ir}7.1$ was evident, suggesting it plays a prominent role in glial cell physiology throughout life.

High mRNA levels for the strongly rectifying $K_{ir}2.1$ and $K_{ir}2.2$ channels was also detected in the optic nerve, which have been previously reported in retinal Müller glia and hippocampal astrocytes at the mRNA level (Schröder et al., 2002). Protein
expression has been shown for Kir2.1 in Müller glia (Kofuji et al., 2002) and Kir2.2 in Bergmann glia and cerebellar astrocytes (Leonoudakis et al., 2001). Heterogeneous expression of Kir2.1, Kir2.2 and Kir2.3 was also reported in astrocytes of the forebrain and hindbrain (Stonehouse et al., 1999), while expression of Kir2.3 has been reported in hippocampal astrocytes in situ (Schröder et al., 2002) and in vitro (Perillán et al., 2000). In my study, mRNA levels for Kir2.3 were very low and they are unlikely to be important in optic nerve glia. Kir2.1 and Kir2.3 have previously been reported to be extensively expressed in somata of RGCs but not in dendrites or axons (Chen et al., 2004), which supports high levels of Kir2.1 in the optic nerve being exclusively glial. Interestingly, Kir2.1 is also reported to form heteromers with Kir4.1 and this has been detected in our lab by immunolabelling and co-immunoprecipitation (Brasko, 2013).

The detection of Kir5.1 also agrees with findings from past studies in retinal and brain astrocytes (Raap et al., 2002). The Kir5.1 subunits do not form functional homomeric channels, but selectively heteromerise with Kir4.1 to form functional channels with distinctive rectification and kinetic properties (Hibino et al., 2004; Pessia et al., 1996; Casamassima et al., 2003). Double immunolabelling for Kir4.1 and Kir5.1 performed in our laboratory indicated that these subunits are colocalised to a high degree in optic nerve astrocytes and oligodendrocytes in situ and in vitro (Brasko, 2013).

The G-protein coupled channels Kir3.1 and Kir3.2 were expressed at very low levels, indicative of OPCs and microglia, or very low expression in astrocytes and
oligodendrocytes. Immunopositivity as well as electrophysiological evidence for Kir3.1 has been shown in astrocytes *in vitro* (Britz et al., 2005), but expression was negative for Kir3.1, Kir3.2 and Kir3.3 at the protein level in Müller glia *in situ* (Skatchkov et al., 2002). Kir3.2 expression has been previously reported on RGC axons (Chen et al., 2004), so it is possible that the low mRNA levels we observed for this channel reflect axonal transcripts. Inwardly rectifying K⁺ currents were reported in microglia more than two decades ago (Brockhaus et al., 1993; Walz et al., 1993; Nörenberg et al., 1994a; Fischer et al., 1995), but Kir are not expressed in resting microglia and they have come to be considered as an early marker of microglial activation (Lyons et al., 2000).

The primary function of Kir channels in glia is in the regulation of K⁺ (Kofuji and Newman, 2004). Specifically, studies using global knock-out and targeted deletion in astrocytes have demonstrated Kir4.1 are critical for K⁺ regulation (Olsen and Sontheimer, 2008; Bay and Butt, 2012; Chever et al., 2010). Moreover, Kir4.1 is essential for generating the glial RMP and its ablation results in hypomyelination (Neusch et al., 2000). In addition, my analysis identified Kir7.1 as potentially being important in these glial functions. Moreover, Kir were not developmentally upregulated between P9 and P35, consistent with a previous Kir4.1 expression study in the optic nerve (Kalsi et al., 2004). A developmental increase in Kir currents is important in the maturation of oligodendrocytes and astrocytes in other brain regions (Knutson et al., 1997; Sontheimer et al., 1989; Seifert et al., 2009), and we observed upregulation of Kir4.1 in the cortex (not illustrated), where glia mature later than in the optic nerve. Developmental changes in Kir may occur prior to P9 in the optic nerve,
during the major period of glial differentiation (Butt and Ransom, 1993; Barres and Raff, 1993), which requires further examination.

### 3.3.5. Calcium Activated Potassium Channels (K\(_{ca}\))

The K\(_{ca}\) channel β4 subunit was strongly expressed in the optic nerve, together with the SK α subunits K\(_{ca}\)2.2 and K\(_{ca}\)2.3, whereas the BK K\(_{ca}\)1.1 subunit was expressed at a low level, equivalent to axonal Na\(_v\)1.6. This suggests optic nerve glial K\(_{ca}\) comprise mainly SK2 and SK3 channels, although the high level of β4 subunits implies multimeric K\(_{ca}\)α1.1/β4 BK channels may be functional in the optic nerve; the auxiliary β4 subunit is the primary neuronal subunit and by associating with K\(_{ca}\)α1.1 endows it with greater Ca\(_{2+}\) sensitivity, and shifts the voltage range of current activation to more negative potentials than the auxiliary β1 subunit (Behrens et al., 2000), which would make these channels in glia more active under physiological conditions. Pharmacological studies in the optic nerve did not indicate K\(_{ca}\) in regulating action potential conduction (Gordon et al., 1988; Gordon et al., 1990), and so the low levels of K\(_{ca}\) transcripts most likely reflect glial expression. Astrocytes exhibit both BK and SK currents in vitro and in situ in postnatal brain slices, and K\(_{ca}\)2.3 (SK3) expression has been shown in astrocytes of the supraoptic nucleus (Armstrong et al., 2005). The BK channel β4 subunit has been reported in cultured astrocytes (Gebremedhin et al., 2003) and it is known to interact with the BK α subunit K\(_{ca}\)1.1, which was expressed at very low levels in the optic nerve and seems unlikely to be of major importance in astrocytes. The relatively low levels of K\(_{ca}\) transcripts in the whole optic nerve tissue might obscure high expression in a small population of cells, in which case the developmental downregulation observed could
be very important in this specific cell population. This is consistent with evidence that OPCs express $K_{\text{Ca}}$ currents that diminish as they differentiate into oligodendrocytes (Bevan et al., 1987; Sontheimer et al., 1989; Verkhratsky and Steinhäuser, 2000). In addition, microglia constitutively express $K_{\text{Ca}2.3}$ (SK3) \textit{in situ} in the healthy adult striatum, and their blockade inhibits their activation (Schlichter et al., 2010).

3.3.6. Voltage Gated Sodium Channels ($Na_v$)

Glial cells express $Na_v$ channels with similar properties as those found in neurons. The function of glial $Na_v$ channels has not been resolved, but their downregulation in mature astrocytes and oligodendrocytes, as well as their high levels of expression in tumours of glial origin are thought to suggest an involvement in the control of cell proliferation, differentiation and even migration (Karadottir et al, 2008; Verkhratsky and Butt, 2013). As observed for $K_{\text{Ca}}$, mRNA transcripts encoding for the $\beta$ subunits of $Na_v$ were more highly expressed than the $\alpha$ subunits, which had a rank order of $Na_v1.2 >> Na_v1.3 > Na_v1.6$, although $Na_v1.3$ transcript levels were greater in postnatal nerves. $Na_v1.6$ is the main $Na^+$ channel isoform at adult nodes of Ranvier, whereas $Na_v1.2$ and the $\beta2$ subunit, but not $Na_v1.6$ or the $\beta1$ subunit, are clustered in developing nodes (Kaplan et al., 2001). The clustering of $Na_v1.6$ channels is induced by myelination and occurs after P9 (Kaplan et al., 2001; Rasband et al., 1999). Equivalent developmental changes were not detected in optic nerve transcript levels of $Na_v1.2$ and $Na_v1.6$ or their $\beta1$ and $\beta2$ subunits, indicating the transcripts are of glial origin. It cannot be excluded that the low level of $Na_v1.6$ transcript in the optic nerve may reflect axonal mRNA, since $Na_v1.6$ has not been reported in glia, but $Na_v\alpha1.2/\beta1$ seems likely to
represent glia (Verkhratsky and Butt, 2013). Voltage-gated Na\(^+\) currents have been recorded \textit{in situ} and \textit{in vitro} in astrocytes isolated from hippocampus, spinal cord and the optic nerve (Sontheimer et al., 1996), and Na\(_v\),1.2 mRNA transcripts have been demonstrated in rat astrocytes \textit{in vitro} and \textit{in vivo} (Oh et al., 1994; Steinhäuser and Seifert, 2002). \textit{In situ}, immunopositivity was found for Na\(_v\),1.2 in astrocytes (Black et al., 1994), consistent with my findings in the optic nerve. Furthermore, immunopositivity for Na\(_v\) has been detected in astrocyte perinodal processes (Sontheimer et al., 1996), suggesting they are involved in ion regulation at nodes of Ranvier. In addition, the relatively low level of expression and the developmental downregulation of Na\(_v\),1.3 is consistent with the loss of voltage-gated Na\(^+\) currents in OPCs when they differentiate into oligodendrocytes (Káradóttir et al., 2008; Chen et al., 2008; De Biase et al., 2010).

Glia are electrically inexcitable and the function of Na\(_v\) is unknown. Intracellular Na\(^+\) fluctuations in which Na\(_v\) are involved may be important for the ability of astrocytes to clear K\(^+\) from the extracellular space during action potential propagation (Kofuji and Newman, 2009). An early hypothesis was that Na\(^+\) influx through Na\(_v\) channels might be important for maintaining the activity of the Na\(^+\)/K\(^+\) pump (Sontheimer et al., 1994), although reports are contradictory on whether Na\(_v\) channels allow significant Na\(^+\) influx into astrocytes under resting conditions (Verkhratsky and Steinhäuser, 2000; Rose and Karus, 2013). In addition, the Na\(_v\),β1 subunit was highly expressed and as well as modulating Na\(_v\) function, serves as a cell adhesion molecule (CAM) (Chen et al., 2007). Na\(_v\),1β has been shown to mediate neurite outgrowth in the CNS postnatally (Brackenbury et al., 2008) and knock out mice were shown to have less severe EAE symptoms compared to wild type due to axonal sparing (O'Malley et al., 2009). Additionally, Na\(_v\),β1 expression was demonstrated in cultured rat astrocytes.
after treatment with cAMP (Oh et al., 1997), raising the possibility that it may act as a CAM in these cells.

### 3.3.7. Acid Sensing Ion Channels

Asic1 and Asic2 were expressed in the optic nerve, and the former was significantly and markedly developmentally downregulated, so that both were barely expressed in the adult nerve. The results are consistent with studies showing expression of ASICs in OPCs and their downregulation in mature oligodendrocytes (Feldman et al., 2008). Lin et al. (2010) provided evidence for proton sensitive currents and immunopositivity for Asic1 in hippocampal NG2+ OPCs (Lin et al., 2010). ASIC are activated by extracellular [H+] and allow the passage of mainly Na+, although they are also permeable to Ca²⁺, and they may be important in intracellular pH regulation in OPCs (Ro and Carson, 2004). It has also been hypothesised that Ca²⁺ influx through ASICs might be involved in oligodendrocyte cell death during ischaemia (Feldman et al., 2008). Importantly, a recent study by Vergo et al. (2011) implicated Asic1 up-regulation in oligodendrocytes with increased axonal and myelin damage in a rodent MS model, and demonstrated neuro- and myelo-protection when blocking Asic1 with amiloride (Vergo et al., 2011). A similar study in the Asic1 knockout mouse supported the hypothesis that these channels contribute to axonal degeneration (Friese et al., 2007). Nuclear expression of Asic1-Asic3 has also been detected in situ and in cultured rat astrocytes, using immunocytochemistry and western blot (Huang et al., 2010), whereas there is no evidence of ASIC expression in microglia.
3.4. Summary and Conclusions

The overall aim of this section was to provide an insight into the $K^+$ channel profile of optic nerve glia, using qRT-PCR, and the key findings are summarised in Figure 3.10. It is evident from the results that $K^+$ channels are most abundantly expressed in glia and the data indicate that astrocytes and oligodendrocytes are dominated by $K_v1.1$, $K_{ir}4.1$ and $K_{ir}7.1$. Key novel findings are the hitherto unrecognised high expression of mRNA encoding for $K_{ir}7.1$ in glia, and the marked developmental upregulation of $K_v1.1$, indicating an important role for these two $K^+$ channels in addition to the recognised role of $K_{ir}4.1$ in glial physiological functions of $K^+$ homeostasis and myelination. A developmental shift in the RMP of optic nerve glia has been shown to occur between P9 and P15 and has been considered to be largely dependent on the upregulation of $K_{ir}$ channels (Bolton et al., 2006; Bolton and Butt, 2006; Bay and Butt, 2012). A corresponding developmental shift in $K_{ir}$ transcripts was observed (Figure 3.8), which is considered as one of the functional hallmarks of differentiation in both oligodendrocytes (Kettenmann et al., 1991, Williamson et al, Knutson et al., 1997, 1997, Attali et al., 1997) and astrocytes (MacFarlane and Sontheimer, 2000). An important question that should be addressed in future studies is whether developmental changes in $K_v1.1$ are important in the maturation of glial biophysical properties.
Figure 3.10: Profile of the major K\(^+\) and Na\(^+\) channels expressed by optic nerve glia.
Chapter 4 - Expression of $K_{ir7.1}$ in the mouse CNS
4.1. Introduction and Aims

Glial cells in the CNS express a wide variety of K⁺ channels and many Kir subtypes. Most notably, Kir4.1 is almost exclusively “glial” in the CNS and has been shown to be the main channel mediating inward currents in retinal Müller glia, grey and white matter astrocytes, cerebellar Bergmann glia and oligodendrocytes (Kalsi and Butt, 2006). However, the qRT-PCR results of Chapter 3 revealed for the first time high expression of Kir7.1 in the mouse optic nerve, a typical white matter tract that contains mainly astrocytes and oligodendrocytes that support retinal ganglion cell axons. The expression of Kir7.1 has not previously been identified in glia, and in the CNS has only been reported in cerebellar Purkinje neurones and pyramidal cells of the hippocampus (Krapivinsky et al., 1998), in the choroid plexus (Döring et al., 1998; Nakamura et al., 1999), and in spinal cord DRG neurones (Zhu and Oxford, 2011). In addition, Kir7.1 is prominent in K⁺ transporting epithelial cells, most notably the retinal pigment epithelium (RPE) (Kusaka et al., 2001; Shimura et al., 2001; Yang et al., 2003), small intestine (Partiseti et al., 1998), gastric parietal cells (Fujita et al., 2002; Malinowska et al., 2003), kidney (Ookata et al., 2000; Derst et al., 2001; Suzuki et al., 2003), and thyroid follicular cells (Nakamura et al., 1999). The primary aim of this chapter was to examine expression of Kir7.1 in astrocytes and oligodendrocytes, using immunolabelling in mouse brain sections and in vitro in cultures of neurones and glia.
4.2. Results

Immunohistochemical and western blot analysis of Kir7.1 expression was performed using an anti-Kir7.1 antibody (aKir7.1) raised in rabbit against the peptide (C)EMNGDLEIDHVPPE, corresponding to amino acid residues 80-94 of the extracellular loop domain of the rat Kir7.1 channel, which is 100% identical to the same domain in mouse Kir7.1 (Alomone Labs, Jerusalem, Israel). Negative controls comprised pre-incubation with the blocking peptide against which the antibody was raised and omission of the primary antibody, and the absence of staining confirmed the specificity of immunolabelling. Immunohistochemistry was performed on 70 µm thick sections of brains, immersion fixed in 4% PFA, unless otherwise stated. Expression of Kir7.1 in neurones and glia was identified by double immunofluorescence labelling with well-characterised cell-specific antibodies, such as GFAP for astrocytes and β3 Tubulin (TuJ1) for neurones, and using transgenic reporter mice to identify glial cell types: GFAP-eGFP for astrocytes, Sox10-eGFP for all oligodendrocyte lineage cells (OPCs and oligodendrocytes), PLP1-DsRed for differentiated oligodendrocytes, and NG2-DsRed for OPCs and pericytes; these mouse strains have been characterised extensively in our lab (Azim and Butt, 2011; Hawkins and Butt, 2013; Leoni et al., 2009). Confocal images were captured using a Zeiss LSM 710 confocal microscope, with x20 (air) or x40, x63, x100 (oil immersion) objectives (numerical aperture 0.5, 1.3, 1.4 respectively). Fluorescence was detected using excitation wavelengths of 488nm (green), 568nm (red), 633nm (far red) and 405nm (blue), with an argon, HeNe1 and diode lasers, respectively. Images were captured using optimal settings for pinhole diameter (0.13-0.3 µm), detector gain and offset acquisition, to detect the positive signal with minimal background. Z-stacks contained 4-15 series of 1024x1024 pixel images were used to
generate three dimensional images (voxel size 43-76 nm xy/ 76-283 nm z). Multi track image capture was used with 2, 3 and 4 channels so that different wavelengths could be imaged separately with minimal overlap in excitation spectra. Identical settings were then used to image negative controls. Images were projected using Zen 2009 Light software (Zeiss) and images were imported to Volocity 6.1.1 (Perkin Elmer) for iterative restoration (deconvolution), to remove out of focus fluorescent scatter from labelled structures above and below the focal plane. In this study, co-expression of the various cell markers and the aK\textsubscript{ir}7.1 immunostaining is identified when emission of fluorescence from the two channels is simultaneous, for example when a red pixel and a green pixel are less than 10 pixels apart, co-expression appears yellow, but without this meaning that the two proteins are colocalised (Zinchuk and Zinchuk, 2009); colocalisation was not determined, because aK\textsubscript{ir}7.1 labels the extracellular loop of the plasmalemmal ion channel, whereas all the cell-specific markers used are intracellular, e.g. Tuj1 and GFAP.
4.2.1. Antibody Validation

For the validation of the specificity of the primary and secondary antibodies used in this project, a range of techniques were used, as summarised in Table 4.1.

Table 4.1: List of techniques and antibody validation methods.

<table>
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<tr>
<th>Technique</th>
<th>Validation Method</th>
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<tr>
<td>rt-PCR</td>
<td>Expression in brain and optic nerve</td>
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<tr>
<td>Protein blast search</td>
<td>Protein Alignment Check</td>
</tr>
<tr>
<td>Western Blot</td>
<td>Expression in mouse cortex and cerebellum</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>Application of antibody on known non-expressing tissue (sk.muscle)</td>
</tr>
<tr>
<td></td>
<td>Application of antibody on known expressing tissue (Cerebellum and Choroid plexus)</td>
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<tr>
<td></td>
<td>Primary antibody pre-bound with peptide</td>
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<td></td>
<td>Omission of primary antibody</td>
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First, prior to using the antibody to analyse cellular localisation of $K_r7.1$, the expression of $K_r7.1$ mRNA in the mouse brain and optic nerve was confirmed using rt-PCR (Figure 4.1). Total RNA was extracted from adult mouse brain (using the RNeasy kit, Qiagen) and optic nerves (using Trizol, Invitrogen), and 1 µg of total RNA was used to synthesise cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR was performed on 1 µg of the cDNA with primers for $K_r7.1$ on a c1000 Touch Thermal Cycler (Biorad, Hercules, CA). The $K_r7.1$ primers used were designed by the National Center for Biotechnology Information (NCBI) Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Invitrogen: forward, CACATCACCAGCTTCACAGC, reverse GGTGGCCATCTTTGTGAGC. The results demonstrate $K_r7.1$ product detected at the expected 251 bp band, and no band was detected in the absence of cDNA in the reaction mix (Figure 4.1).
The specificity of the PCR primers was demonstrated by the amplification of the expected \( K_{ir}7.1 \) product and the detection of a 251 bp band. No band was detected in the negative control in the absence of cDNA in the reaction mix.

Next, a BLAST (Basic Local Alignment Search Tool) search and western blot was performed on the Alomone aKir7.1 antibody (Figure 4.2). The antibody is raised against the peptide sequence EMNGDLEIDHDVPE on the extracellular loop of the C-terminus of the \( K_{ir}7.1 \) protein, corresponding to amino acid residues 80-94 of rat \( K_{ir}7.1 \). The BLAST search of the peptide sequence of the antibody was performed using the National Centre for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and confirmed that the \( K_{ir}7.1 \) protein sequence of the rat is identical to the mouse protein and, moreover, that there are no significant peptide alignments with any mouse proteins other than \( K_{ir}7.1 \). The closest target sequence match was found to be the olfactory receptor 1491 [Mus musculus], with query coverage of 60%, but which includes a 3 amino acid gap and therefore should not bind the aKir7.1 antibody (Figure 4.2A). To further confirm the specificity of the \( K_{ir}7.1 \) antibody, we performed western blot analysis of total protein lysates in the brain of adult mice, which identified three bands at approximately 40.5, 46 and 49 kDa, and positive bands were eliminated by the competitive peptide (Figure 4.2B). The densest band detected corresponds to the predicted molecular weight of 40.5 kDa,
and the higher molecular weight bands are likely to be related to the extent of glycosylation and phosphorylation of the channels (Kusaka et al., 2001).

Figure 4.2: Validation of the Alomone aKir7.1 primary antibody. (A) BLAST search of the peptide against which the Kir7.1 antibody was raised identified no significant peptide alignments with any other mouse protein, and the closest target sequence match was the olfactory receptor 1491, with query coverage of 60% and a 3 amino acid gap. (B) Western Blot analysis revealed that the aKir7.1 antibody recognised three major antigens with apparent molecular weights of 40.5, 46 and 49 KDa. (C) The full Western Blot for Kir7.1 in the mouse cortex and cerebellum.

Finally, a number of positive and negative immunohistochemistry controls were performed using the Alomone aKir7.1 antibody (Figures 4.3 and 4.4). Kir7.1
immunolabelling using other antibodies has been demonstrated previously in Purkinje neurones of the cerebellum (Krapivinsky et al., 1998) and choroid plexus epithelial cells (Nakamura et al., 1999). In contrast, skeletal muscle has been found to be negative for Kir7.1 expression at the transcript level, using Northern Blot and rtPCR (Partiseti et al., 1998; Nakamura et al., 1999; Döring et al., 1998; Shimura et al., 2001; Derst et al., 2001). Therefore, immunohistochemistry using the Alomone aKir7.1 antibody was performed on sections from the adult mouse brain and skeletal muscle (Figure 4.3). We observed strong aKir7.1 immunolabelling of Purkinje neurones (Figure 4.3A) and the choroid plexus epithelium (Figure 4.3B), whereas no Kir7.1 immunolabelling was detected in skeletal muscle (Figure 4.3C). In negative controls, immunolabelling in the cerebellum was abolished by pre-incubating sections with the blocking peptide against which the aKir7.1 antibody was raised (Figure 4.3A inset) or by omitting the primary antibody (not illustrated), and equivalent results were observed in other brain regions and cell cultures (not illustrated). In the absence of Kir7.1 knock-out tissue, the results provide the strongest evidence of the specificity of the aKir7.1 Alomone antibody used in this thesis.
Figure 4.3: Specificity of K<sub>v</sub>7.1 immunolabelling in mouse tissue. (A) K<sub>v</sub>7.1 immunolabelling (green) identifies Purkinje cells of the cerebellum in brain sections from adult PLP1-DsRed mice, in which the reporter identifies myelinated white matter and oligodendrocytes (red). In negative controls, no immunolabelling was observed following pre-incubation of the primary antibody with the complimentary peptide (inset A) or omission of the primary antibody (not illustrated); Hoechst Blue was used to stain the cell nuclei. (B) K<sub>v</sub>7.1 immunolabelling (red) of the choroid plexus epithelium of adult mice; Hoechst Blue (HB) was used to stain the cell nuclei. (C) K<sub>v</sub>7.1 immunolabelling (green) was not detected in skeletal muscle section from adult mouse; Hoechst Blue was used to stain cell nuclei and the inset illustrates collagen immunolabelling in the same section (red). Scale Bars=100µm in A and C, and 20µm in B.
4.2.2. \(K_{ir}7.1\) expression in the forebrain

Immunofluorescence labelling of the adult mouse brain revealed wide expression of \(K_{ir}7.1\) in the cortex and hippocampus (Figure 4.4). The most intensely labelled area of the cortex appeared to be Layer IV of the somatosensory cortex, the posterior parietal association area and extending all the way to area 4 of the visual cortex. In the hippocampus, areas CA2 and CA3 showed the most expression along with the molecular (mo) and polymorph (po) layer of the dentate gyrus (DG). Moreover, noticeable expression was revealed for the caudoputamen area of the striatum and the dorsal thalamus. High expression of \(K_{ir}7.1\) was also seen in the ependymal cell layer of the Lateral Ventricle (LV) and the glia limitans which delineates the body of the Corpus Callosum.

To investigate astrocyte expression of \(K_{ir}7.1\), immunohistochemistry was performed on brain sections from adult GFAP-eGFP mice, focusing on the hippocampus and cortex. First, the overall distribution of \(K_{ir}7.1\) in the different layers of the hippocampus was examined in relation to that of astrocytes (Figure 4.5). In the dentate gyrus (DG), \(K_{ir}7.1\) expression was seen in the molecular (mo) and polymorph (po) layers but not in the granule cell layer. The lacunosum and molecular layer (slm) also displayed widespread expression of \(K_{ir}7.1\); this layer, along with the molecular and polymorph layers of the dentate gyrus, contained large numbers of astrocytes. Moreover, the stratum oriens (so) and stratum radiatum (sr) displayed less intense, punctate expression which is interesting as the stratum oriens contains the basal dendrites of pyramidal neurones which receive input from pyramidal...
neurones, septal fibres and commissural fibres from the contralateral hippocampus; the stratum radiatum also contains septal and commissural fibres. It is possible that the punctate labelling reflects expression of $K_{ir}7.1$ at the nodes of Ranvier of these axons, which requires further examination. Finally, the pyramidal neuron layer was also positive for $K_{ir}7.1$ expression, but with higher intensity in the CA2 and CA3 areas and less so in the CA1 (Figure 4.5C). Higher magnification resolved intense $K_{ir}7.1$ immunopositivity on the cell somata and processes of astrocytes in the dentate gyrus of the hippocampus (Figure 4.6Ai-ii), and double immunofluorescence labelling with the neuronal marker TuJ1 demonstrated $K_{ir}7.1$ expression on the neuronal somata, primary dendrites and axons of pyramidal neurones of the CA1 region (Figure 4.6Bi-iii).

In the frontal cortex, $K_{ir}7.1$ appeared to be localised mainly on astrocyte cell bodies, while the strongest expression was found on the somata of cortical neurones and their axons (Figure 4.7Ai-iii). Immunohistochemistry for $K_{ir}7.1$ in GFAP-eGFP transgenic mouse brain sections revealed intense $K_{ir}7.1$ immunolabelling on astrocytic perivascular end-feet (Figure 4.7Bi-iii). In contrast, NG2-positive pericytes that surround blood vessels in the mouse cortex did not display $K_{ir}7.1$ immunopositivity (Figure 4.7Ci-iii), and neither did cortical oligodendrocytes (not illustrated). Neuronal expression of $K_{ir}7.1$ in the adult mouse cortex was demonstrated using the neuronal marker TuJ1, with the pyramidal cell somata, primary dendrites and axons being immunopositive for $K_{ir}7.1$ (Figure 4.7Di-iii).
Figure 4.4: Expression of Kᵦ7.1 in the adult mouse forebrain. Confocal low magnification photomicrograph of sagittal brain sections, immunolabelling for Kir7.1 (green) and counterstained with the nuclear dye Hoechst blue. Kᵦ7.1 expression in the adult mouse cortex (Layers I - VI) and hippocampus (Regions CA1-CA3 and DG) are illustrated in the overlay (A) and individual green (B) and blue (C) channels. Scale Bars: 300µm. Kᵦ7.1 immunostaining was absent in negative controls (Not illustrated).
**Figure 4.5: Expression of Kir7.1 in the mouse hippocampus.** Confocal low magnification photomicrograph of sagittal brain sections from adult GFAP-eGFP mouse, immunolabelled for Kir7.1 (red); expression of the eGFP reporter identifies astrocytes (green). The overlay (A) and individual green (B) and red (C) channels are illustrated. Important anatomical structures are specified; SUB – Subiculum, CA – Cornu Ammonis (Ammon’s Horn); so – stratum oriens, sp – stratum pyramidale (pyramidal neuron layer), sr – stratum radiatum, slm – stratum lacunsum/moleculare, DG – Dentate Gyrus; mo – molecular layer, sg – stratum granulosum (granule cell layer), po – polymorph layer. Scale Bars: 300µm. Kir7.1 immunostaining was absent in negative controls. (Not illustrated).
Figure 4.6: Kir7.1 expression in hippocampal astrocytes and neurones. Confocal photomicrograph of sagittal brain sections from adult mouse. (A) Double immunofluorescence labelling for Kir7.1 (red); expression of the eGFP reporter identifies astrocytes (green). Kir7.1 expression was found on astrocyte cell bodies (arrows) and processes (arrowheads); astrocytes and Kir7.1 immunolabelling are most dense in molecular (mo) and polymorph (po) layers, while the granule cell layer (sg) is largely devoid of astrocytes and appears immunonegative for Kir7.1 (asterisks). (B) Double immunofluorescence labelling for Kir7.1 (green) and Tuj1 (red) identifies Kir7.1 expression on the cell bodies (asterisks) and axons (arrows) of hippocampal pyramidal neurones; Kir7.1 Immunopositivity can also be seen in Tuj1-negative cells, most likely astrocytes (arrowheads). Individual channels and the overlay are illustrated. Kir7.1 immunostaining was absent in negative controls (Not illustrated). Scale Bars: 50µm
**Figure 4.7: *Kv7.1* expression in the adult mouse cortex.** Confocal photomicrograph of sagittal brain sections from adult mouse. (A) Astrocytes were identified by expression of the GFAP-eGFP reporter (green) and immunolabelling for *Kv7.1* (red) indicated expression on their cell bodies (arrows); neuronal somata (asterisks) and axons (arrowheads) were also immunopositive for *Kv7.1*. (B) Astrocyte perivascular endfeet (green, arrows) are immunopositive for *Kv7.1* (red), and co-expression appears yellow in the overlay (Bl). *Kv7.1* immunolabelling is also evident in the somata of astrocytes (arrowhead) and neurones (asterisks). (C) Pericytes identified by expression of the NG2-DsRed reporter are seen to line the blood vessels (red) but are immunonegative for *Kv7.1* (green). (D) Double immunofluorescence labelling for *Kv7.1* (green) and Tuj1 (red) demonstrates cortical neurones express *Kv7.1* on their cell bodies (asterisks) and axons (arrows); co-expression appears yellow in the overlay (Di) and *Kv7.1* immunopositivity can be seen in Tuj1-negative cells, which are likely to be astrocytes (arrowheads). Individual channels and the overlay are illustrated. *Kv7.1* immunostaining was absent in negative controls (not illustrated). Scale Bars=20µm in all panels.
4.2.3. *K*<sub>i</sub>*<sup>7.1</sup> expression in forebrain white matter

To investigate the expression of *K*<sub>i</sub>*<sup>7.1</sup> in astrocytes and oligodendrocyte lineage cells in white matter of the adult forebrain, immunohistochemistry was performed on 70 µm sagittal and coronal brain sections from SOX10-eGFP and GFAP-eGFP transgenic mice. Intense immunolabelling for *K*<sub>i</sub>*<sup>7.1</sup> could be observed in the choroid plexus and ependymal cells of the lateral ventricle, as well as in the cerebral cortex above the corpus callosum and the caudate putamen, but the myelinated fibre tracts that make up the corpus callosum (CC) and striatum were not as strongly immunopositive (Figure 4.8). Higher magnification confocal images were acquired to examine the expression of *K*<sub>i</sub>*<sup>7.1</sup> in white matter astrocytes of GFAP-eGFP mice (Figure 4.9A) and oligodendrocytes of Sox10-eGFP mice (Figure 4.9B, C). Strong somatal expression was observed in most astrocytes of both the corpus callosum and striatum (Figure 4.9A). In contrast, only a small proportion of Sox10-eGFP+ oligodendrocyte lineage cells were found to be positive for *K*<sub>i</sub>*<sup>7.1</sup> expression in the corpus callosum (Figure 4.9B). The same variable expression in oligodendrocytes was observed in the striatum, where some oligodendrocytes within the myelinated axon bundles were immunopositive for *K*<sub>i</sub>*<sup>7.1</sup> (some indicated by arrows in Figure 4.9C), whereas OPCs within the neuronal parenchyma appeared immunonegative for *K*<sub>i</sub>*<sup>7.1</sup> (some indicated by arrowheads in Figure 4.9C); *K*<sub>i</sub>*<sup>7.1</sup> immunopositive striatal neurones were apparent between the myelinated axon bundles in the striatum (Figure 4.9B, C).
Figure 4.8: K<sub>7.1</sub> expression in adult mouse myelinated tracts. Low magnification confocal images of coronal brain section from adult Sox10-eGFP mouse to identify oligodendrocyte lineage cells (green) immunolabelled for K<sub>7.1</sub> (red). Strong immunopositivity for K<sub>7.1</sub> is detected in the choroid plexus and ependyma of the lateral ventricle (LV). The myelinated fibres of the corpus callosum (CC) and striatum are clearly identified by the dense expression of the Sox10-eGFP reporter (green) but are not strongly immunopositive for K<sub>7.1</sub> (red). Individual channels and the overlay are illustrated. K<sub>7.1</sub> immunostaining was absent in negative controls (not illustrated). Scale Bars: 200µm.
Figure 4.9: Kᵦ,7.1 expression in white matter of the adult mouse forebrain. Confocal images of brain sections from adult GFAP-eGFP mouse (A) and Sox10-eGFP mouse (B, C) immunolabelled for Kᵦ,7.1. (A) Sagittal section illustrating the corpus callosum (CC) from a GFAP-eGFP reporter mouse, in which most astrocytes (green) are seen to be immunopositive for Kᵦ,7.1 (red), some indicated by arrows; Kᵦ,7.1 immunostaining can also be seen in GFAP-eGFP-negative cells, that are probably oligodendrocytes (arrowheads) and striatal neurones (asterisks). (B) Sagittal section illustrating the corpus callosum (CC) from a Sox10-eGFP reporter mouse, in which a small proportion of oligodendrocytes (green) are seen to be immunopositive for Kᵦ,7.1 (red), some indicated by arrowheads, together with Sox10-eGFP-negative cells that are most likely astrocytes (arrows); the strongest Kᵦ,7.1 immunolabelling was observed in the striatal neurones (asterisks), interpolated amongst the myelinated axon bundles (green). (C) Coronal section of the striatum from a Sox10-eGFP reporter mouse illustrating the greatest Kᵦ,7.1 immunolabelling (red) in the neurones (asterisks) surrounding the bundles of myelinated axons that contained the majority of oligodendrocytes (green). Only a small number of oligodendrocytes present clear somatal expression of Kᵦ,7.1 (arrows), whereas OPCs present within the neuronal somata appeared immunonegative for Kᵦ,7.1 (arrowheads). Individual channels and the overlay are illustrated. Kᵦ,7.1 immunostaining was absent in negative controls (not illustrated). Scale Bars: 100µm.
4.2.4. *K*<sub>r</sub>7.1 expression in the cerebellum and pons (Metencephalon)

The expression of *K*<sub>r</sub>7.1 in the cerebellum is illustrated by immunolabelling of 70µm sections from adult PLP1-DsRed and GFAP-eGFP reporter mice (Figures 4.10-4.12). The strongest expression of *K*<sub>r</sub>7.1 was found in the choroid plexus and the Purkinje neurones (Figure 4.10). *K*<sub>r</sub>7.1 immunolabelling was also evident in the deep cerebellar nuclei, as well as the molecular cell layer and the granule cell layer (Figure 4.10A and B). Less intense immunolabelling for *K*<sub>r</sub>7.1 was observed along the myelinated fibre tracts (Arbor Vitae) (Figure 4.10A, B), but high magnification confocal images of PLP1-DsRed transgenic mouse cerebellar sections revealed *K*<sub>r</sub>7.1 immunolabelling on myelin, but not apparently on oligodendrocyte cell somata (Figure 4.11A). In contrast to oligodendrocytes, Bergmann glia displayed somatal expression of *K*<sub>r</sub>7.1, whereas the immunopositivity seen in the molecular cell layer (MCL) seemed to be due to high levels of *K*<sub>r</sub>7.1 expression in the elaborate dendritic trees of Purkinje neurones and not apparently on Bergmann glial processes (Figure 4.11B). Indeed, closer examination of Purkinje neurones confirmed intense immunolabelling for *K*<sub>r</sub>7.1 on their soma (Figure 4.12A), their axons (Figure 4.12B) and dendrites (Figure 4.12C).
Figure 4.10: \( K_{\gamma}7.1 \) expression in the adult mouse cerebellum. Low magnification confocal images of sagittal cerebellar sections from adult PLP1-DsRed mouse (A) and GFAP-eGFP mouse (B) immunolabelled for \( K_{\gamma}7.1 \). The strongest expression of \( K_{\gamma}7.1 \) is observed in the choroid plexus (CP) and the Purkinje cell layer (arrows), with prominent immunolabelling in the molecular layer (MCL), where the radial processes of Bergmann glia are prominent. Positive expression can also be seen in the deep cerebellar nuclei (CBN) and to a decreasing extent in the granule cell layer (GCL) and the myelinated fibre tracts in the cerebellar white matter (WM). Individual channels and the overlay are illustrated. \( K_{\gamma}7.1 \) immunostaining was absent in negative controls (Not illustrated). Scale Bars: 300µm.
Figure 4.11: Kir7.1 expression in oligodendrocytes and astrocytes of the mouse cerebellum. High magnification confocal images of brain sections from adult PLP1-DsRed mouse (A) and GFAP-eGFP mouse (B) immunolabelled for Kir7.1. (A) In the cerebellar white matter, Kir7.1 immunolabelling (green) appears to be expressed on myelin, or axons, but not oligodendrocyte somata (red). (B) In the Purkinje cell layer and molecular layer, Kir7.1 immunolabelling (red) is detected on the cell somata of Bergmann glia (green, co-expression appears yellow), together with the somata and dendritic tree of Purkinje neurones (asterisks). Individual channels and the overlay are illustrated. Kir7.1 immunostaining was absent in negative controls (Not illustrated). Scale Bars= 50µm.
Figure 4.12: Kir7.1 expression in Purkinje neurones of the adult mouse cerebellum. Confocal images of sagittal cerebellar sections from adult mouse immunolabelled for Kir7.1 (green). (A) Low magnification illustrating the dense immunolabelling for Kir7.1 in Purkinje cell bodies compared to the other layers of the cerebellum. (B) High magnification illustrating Kir7.1 immunopositive Purkinje cell bodies, axons (arrows) and primary dendrites (arrowhead). (C) High magnification of the dendritic tree of Purkinje neurones, strongly immunopositive for Kir7.1. Kir7.1 immunostaining was absent in negative controls (not illustrated). Scale Bar= 50µm in A and 20µm in B and C.
4.2.5. $K_{ir}7.1$ expression in the pons

Widespread immunolabelling for $K_{ir}7.1$ immunolabelling was observed in the pons, with the exception of the pyramidal tracts, which include the corticospinal and corticobulbar tracts (Figure 4.13). These largely $K_{ir}7.1$ immunonegative tracts contain the myelinated axons originating from the cerebral cortex, passing through the pons and terminating in the spinal cord and the brainstem, respectively. Higher magnification confocal images of sections from the GFAP-eGFP reporter mouse revealed that astrocytes in the pons express $K_{ir}7.1$ on their cell somata and processes, while a few larger cell somata, most likely neurones, were also $K_{ir}7.1$ immunopositive (Figure 4.14).
Figure 4.13: Kir7.1 expression in the mouse pons. Low magnification confocal images of sagittal sections from adult GFAP-eGFP reporter mouse (astrocytes are green) immunolabelled for Kir7.1 (red). Kir7.1 is widely expressed in the pons, with intense expression in the choroid plexus of the third ventricle (CP), whilst the corticospinal tract appears immunonegative for Kir7.1 (arrows). Individual channels and the overlay are illustrated. Kir7.1 immunostaining was absent in negative controls (not illustrated). Scale Bars: 300µm.
Figure 4.14: Kir7.1 expression in astrocytes in the pons. High magnification confocal images of sagittal sections from adult GFAP-eGFP reporter mouse (astrocytes are green) immunolabelled for Kir7.1 (red). Astrocytes express Kir7.1 on their cell somata and processes (arrows), with apparent enrichment of Kir7.1 on perivascular endfeet (star), while the larger Kir7.1 immunopositive cell somata are most likely neurones (asterisks). Individual channels and the overlay are illustrated. Kir7.1 immunostaining was absent in negative controls (not illustrated). Scale Bars: 50µm.
4.2.6. *Kir7.1* expression in optic nerve astrocytes and oligodendrocytes

*Kir7.1* immunolabelling was performed on 14 µm optic nerve cryosections from adult GFAP-eGFP and PLP1-DsRed reporter mice. *Kir7.1* immunolabelling was widespread in the optic nerve and was localised to astrocyte somata (Figure 4.15A) and discrete interfascicular rows of oligodendrocyte somata (Figure 4.15B).

![Figure 4.15](image)

**Figure 4.15: *Kir7.1* expression in the adult mouse optic nerve.** Immunolabelling for *Kir7.1* in optic nerve sections from adult mice. (A) *Kir7.1* expression (red) in astrocytes (green) expressing the GFAP-eGFP reporter (arrows); rows of GFAP-eGFP-negative/*Kir7.1*-positive cells are most likely oligodendrocytes. (B) *Kir7.1* expression (green) in oligodendrocytes (red) expressing the PLP1-DsRed reporter (asterisks). Individual channels and the overlay are illustrated. *Kir7.1* immunostaining was absent in negative controls (not illustrated). Scale Bars: 50µm.
4.2.7. Glia and neurones express \( K_{ir7.1} \) in vitro

Due to the close apposition of cells in brain sections, it is often difficult to clearly resolve cellular localisation of immunolabelling. To confirm that glial cells express \( K_{ir7.1} \), explant cultures were prepared from optic nerves from P7-P12 wild type and SOX10-eGFP reporter mice and examined after 10 days in vitro (DIV) (Figure 4.16 and 4.17). In addition, mixed neuron-astrocyte cultures were prepared from cerebral cortex isolated from P0-P2 wild type mice and examined after DIV14 (Figure 4.18). Cultured optic nerve oligodendrocytes identified by their expression of the Sox10-eGFP reporter are shown to express \( K_{ir7.1} \) protein on their cell somata and processes (Figure 4.16A), and immunolabelling for GFAP demonstrates \( K_{ir7.1} \) expression in astrocytes (Figure 4.16B). For the \( K_{ir7.1} \) to be functional they should be localised to the cell membrane, and double immunofluorescence labelling for \( K_{ir7.1} \) with the plasmalemmal marker Na\(^+\)/K\(^+\)-ATPase (Figure 4.17A) and main glial channel \( K_{ir4.1} \) (Figure 4.17 B) indicated co-expression with \( K_{ir7.1} \) on the astrocytic cell membrane. In addition, cultured cortical astrocytes identified by GFAP immunolabelling are also shown to display intense \( K_{ir7.1} \) expression (Figure 4.18A), and strong immunopositivity was observed for cultured cortical neurones, identified by Tuj1 immunolabelling (Figure 4.18B), in agreement with our findings in vivo (Figure 4.7).
Figure 4.16: Kir7.1 expression in optic nerve glial cultures. Optic nerve explant cultures from P7-P12 mice were analysed for Kir7.1 immunocytochemistry after DIV10. (A) Immunolabelling for Kir7.1 (red) is evident on the cell somata and processes of oligodendrocytes identified by expression of the SOX10-eGFP reporter (green). (B) Double immunofluorescence labelling for Kir7.1 (green) and GFAP (red) demonstrates Kir7.1 expression in astrocytes in vitro on their cell somata and processes. Individual channels and the overlay are illustrated, and co-expression appears yellow in the overlays (Ai, Bi). Kir7.1 immunostaining was absent in negative controls (not illustrated). Scale Bars: 20µm.
Figure 4.17: Kir7.1 is localised to glial cell membranes. Optic nerve explant cultures from P7-P12 mice were analysed for Kir7.1 immunocytochemistry after DIV10. Double immunofluorescence labelling for Kir7.1 (green) and the plasmalemmal marker Na⁺/K⁺-ATPase (red, A) and glial channel Kir4.1 (red, B) indicates Kir7.1 is expressed on glial cell membranes. Individual channels and the overlay are illustrated, and co-expression appears yellow in the overlays (Ai, Bi). Kir7.1 immunostaining was absent in negative controls (not illustrated). Scale Bars: 20µm.
Figure 4.18: \(K_{\text{ir}}7.1\) expression in cortical neuron-glial cultures. Cells were isolated from P0-P2 mouse cortex and analysed for \(K_{\text{ir}}7.1\) immunocytochemistry after DIV14. Double immunofluorescence labelling for \(K_{\text{ir}}7.1\) (green) and the astrocyte marker GFAP (red, A) or neuronal marker Tuj1 (red, B) identifies expression of \(K_{\text{ir}}7.1\) in cortical astrocytes and neurones; in (B) asterisks indicate Tuj1-negative/ \(K_{\text{ir}}7.1\)-positive cells that are most likely astrocytes, as indicated in (A). Individual channels and the overlay are illustrated, and co-expression appears yellow in the overlays (Ai, Bi). \(K_{\text{ir}}7.1\) immunostaining was absent in negative controls (not illustrated). Scale Bars: 50µm.
4.3. Discussion

The functional expression of the Kir7.1 channel subtype is poorly understood in the CNS, with a single study demonstrating immunolabelling in Purkinje neurones and pyramidal cells of the hippocampus (Krapivinsky et al., 1998). The immunohistochemical results of this chapter show for the first time that glial cells also express Kir7.1 throughout the brain, although the level of immunolabelling for Kir7.1 in glia was heterogeneous. Astrocytes are shown to express Kir7.1 in the cortex, which was confirmed in vitro, as well as in the hippocampus, pons, and white matter tracts, such as the corpus callosum and optic nerve. Astroglial Kir7.1 expression was especially prominent on perivascular endfeet and in Bergmann glial cell bodies. Oligodendrocytes exhibited relatively high expression in the optic nerve, which was confirmed in culture, while cortical and cerebellar oligodendrocytes appeared largely immunonegative, and in the corpus callosum and striatum immunopositivity was apparent only in a small proportion of oligodendrocytes. These novel results provide a new perspective on the widespread but heterogeneous expression of Kir7.1 in neurones and glia, which implies an uncharacterised important function for the channel in specific populations of cells.

4.3.1. Antibody Validation

Antibodies raised against proteins are among the most important tools for studying ion channels and receptors in neuroscience research. The necessity for antibody validation is therefore essential when using an antibody for the first time so that new findings are unambiguous. Although there are no universally accepted validation guidelines for best practice, there have been a number of publications on
the subject, and an assortment of recommendations is available in the literature and online (Deutsch et al., 2008 – MISFISHIE). Ideally, it is preferable to compare multiple antibodies to the same protein and to use tissue from knock-out mice to test the specificity of the antibody. In the absence of alternative sources of the K<sub>ir</sub>7.1 antibody and K<sub>ir</sub>7.1 knock-out mice, we used a range of widely accepted techniques to confirm the specificity of the Alomone aK<sub>ir</sub>7.1 antibody used in this Chapter.

RT-PCR confirmed previous studies that K<sub>ir</sub>7.1 is expressed in the brain at the mRNA level and confirmed the qRT-PCR results of Chapter 3 demonstrating expression of K<sub>ir</sub>7.1 mRNA in the optic nerve. Furthermore, western blot using the Alomone aK<sub>ir</sub>7.1 antibody confirmed the protein was also expressed in total extracts of adult mouse cortex and cerebellum. The predicted size for the K<sub>ir</sub>7.1 protein is 40.5 kDa, based on its amino acid sequence, and the results show the Alomone aK<sub>ir</sub>7.1 antibody recognised a dense band at ~40.5 kDa, together with two further bands at ~46 and ~49 kDa. Reported sizes for the K<sub>ir</sub>7.1 protein vary in the literature depending on the organ system that is being studied; protein size in the rat kidney and RPE has been reported to be ~52 kDa (Ookata et al., 2000; Kusaka et al., 2001; Yang et al., 2003; Suzuki et al., 2003), whereas other reports found the protein in rat thyroid and small intestine to be ~54 kDa (Nakamura et al., 1999), the same as the human K<sub>ir</sub>7.1 protein in RPE (Pattnaik et al., 2013). However, K<sub>ir</sub>7.1 protein extracted from rat brain was found to be ~46 kDa (Döring and Karschin, 2000). The various sizes for the K<sub>ir</sub>7.1 protein reported in literature as well as the bands identified in my study are most likely due to post-translational modifications, such as glycosylation and phosphorylation, since K<sub>ir</sub>7.1 possesses a potential N-glycosylation site, Asn-His-Thr at residues 95-97, and protein
kinase C phosphorylation sites, Ser-Gln-Arg at residues 14-16, Ser-Ile-Arg at residues 169-171, and Ser-Val-Arg at residues 201-203 (Kusaka et al., 2001). The implications of such modifications for the functionality of the protein are uncertain, however it is important to note that these are unlikely to affect the antibody binding site and therefore affect the interpretation of the immunohistochemical analyses detailed throughout the rest of the chapter.

It has been previously shown by immunohistochemistry using different antibodies that Kir7.1 is most strongly expressed in cerebellar Purkinje neurones (Krapivinsky et al., 1998) and the choroid plexus epithelium (Döring et al., 1998; Nakamura et al., 1999). In addition, multiple-tissue Northern blot studies in human tissues (Partiseti et al., 1998; Nakamura et al., 1999) and rat tissues (Döring et al., 1998), and cDNA PCR screening in multiple human tissues (Shimura et al., 2001) and guinea pig tissues (Derst et al., 2001) have all demonstrated that Kir7.1 is expressed in the brain but is absent from skeletal muscle. Here, this knowledge was used in order to provide confirmation of the specificity of the Alomone antibody, and the results demonstrated the most prominent immunolabelling for Kir7.1 in Purkinje neurones and the choroid plexus epithelium, and complete absence of immunolabelling in skeletal muscle. Moreover, pre-incubation in the blocking peptide completely abolished immunolabelling in tissue sections and in western blot, demonstrating the aKir7.1 antibody is specific to the peptide sequence against which it was raised, and a BLAST search confirmed the peptide sequence was specific to Kir7.1.
Thus, the results provide the strongest evidence that Kir7.1 is widely expressed in glial cells as well as neurones throughout the CNS. Although epitope localisation may be altered by the fixation method (Schnell et al., 2012), and caution needs to be exercised when comparing cellular localisation patterns of the Kir7.1 protein between differently fixed tissues, no noticeable differences in the expression pattern was evident in the results of this section or compared to previous studies.

4.3.2. Glial expression of Kir7.1

The utilisation of reporter mice which express fluorescent proteins under the control of astrocytic and oligodendrocytic specific genes have been a great aid in identifying astrocytes and oligodendrocytes throughout the CNS. Glial expression of Kir channels is diverse in the CNS and mRNA and protein expression has been demonstrated in different degrees for all subfamilies (Hibino, 2010). The most prominently expressed and therefore most studied in glia are the Kir4.1 and Kir2.x subfamilies, and the functional importance of the strictly glial Kir4.1 is widely accepted (Kalsi and Butt, 2006). Extensive studies over the last decades have demonstrated the significance of Kir channels, and especially Kir4.1, for the maintenance of the strongly negative resting membrane potential of glia and in turn the ability of these cells to facilitate K⁺ transport between neurones and blood vessels alongside its key role in K⁺ buffering involving the redistribution of K⁺ ions from areas with increased neuronal activity (and therefore high [K⁺]₀) to areas with low [K⁺]₀ (Orkand et al., 1966; Newman et al., 1984). Moreover, Kir4.1 knock-out mice display hypomyelination due to the block of oligodendrocyte differentiation (Neusch et al., 2000), demonstrating another important role for this channel in the exit of glia from the cell cycle and their
maturation (Kalsi and Butt, 2006; Verkhratsky and Butt, 2013). In some brain regions, it was shown that other mechanisms contribute to potassium homeostasis; for example, removal of \([K^+]_o\) in the rat hippocampus was found to be dependent on glial and axonal \(\text{Na}^+/{\text{K}}^-\text{ATPase}\) (D'Ambrosio et al., 2002; Meeks and Mennerick, 2007) and similarly in the optic nerve \(K^+\) clearance under physiological conditions was not dependent on \(\text{Ba}^{2+}\)-sensitive \(K_{ir}\) channels (Ransom et al., 2000). Moreover, synaptic transmission and normal rhythmic activity in the hippocampus is not affected in \(K_{ir}4.1\) knock-out mice (Neusch et al., 2006; Djukic et al., 2007). It is possible that other \(K_{ir}\) channels such as \(K_{ir}7.1\), which is not sensitive to \(\text{Ba}^{2+}\), facilitate \(K^+\) buffering in these studies.

Expression studies of \(K_{ir}7.1\) revealed specific patterns of localisation in polarised cells. *In situ* hybridisation studies revealed that \(K_{ir}7.1\) mRNA is expressed in the secretory epithelial cells of the choroid plexus and its expression is restricted to the apical membrane of the epithelial cells (Döring et al., 1998). Furthermore, in the retina, the channel is also expressed at the apical membrane of the pigment epithelial cells (Hughes and Takahira, 1996; Kusaka et al., 2001). On the other hand, \(K_{ir}7.1\) immunoreactivity was detected at the basolateral membrane of thyroid follicular cells and of renal epithelia in the distal convoluted tubule, proximal tubule and collecting duct (Ookata et al., 2000; Derst et al., 2001). The epithelial cells of the choroid plexus and the retinal pigment epithelia have a unique polarity that is opposite to usual types of epithelial cells. These two types of epithelial cells are known to express the \(\text{Na}^+/{\text{K}}^-\text{ATPase}\) at their apical membrane (DiBona and Mills, 1979; Masuzawa et al., 1981; Caldwell and McLaughlin, 1984; Masuzawa et al., 1985; Ernst et al., 1986). In contrast,
thyroid follicular cells and renal epithelial cells harbour the pump at their basolateral membrane (Hebert et al., 2005). The co-expression of Kir7.1 channels with the Na+/K+-ATPase in the RPE, kidney, choroid plexus and thyroid epithelia is indicative of a possible role of Kir7.1 in regulating potassium transport and in turn sodium expulsion from the cell. The function of Kir7.1 has been closely studied in the retinal pigment epithelium (RPE), where it has an important role in the regulation of the subretinal space volume by facilitating K+ efflux from the cells when [K+]o is low, i.e. upon illumination and activation of retinal photoreceptor cells, thus enabling water efflux from the RPE. Moreover, due to its weak inward rectification, it maintains a constant K+ efflux, thus counteracting and enabling the function of Na+/K+-ATPase, a function termed "K+ recycling" (Wimmers et al., 2007). A loss of function mutation in Kir7.1, results in the autosomal dominant disorder of the eye called Snowflake Vitreoretinal Degeneration (SVD) and manifests itself as a severe impairment of transepithelial ion and water transport via the RPE (Hejtmancik et al., 2008).

Water and potassium transport is an important physiological function of astrocytes and is implicated in osmolarity changes associated with K+ buffering. Astrocytes contacting blood vessels, ventricles and the pia show high expression of aquaporins (AQP, water channels) that mediate water transport (Nielsen et al., 1997). Aquaporins have been shown to be co-expressed with Kir4.1 and it is hypothesised that there is a functional relationship between the maintenance of the strongly negative glial RMP, which drives outward bicarbonate transport via the Na+-HCO3− transporter, and ultimately maintains the driving force for passive water fluxes through AQP4.
(Newman and Astion, 1991; Nagelhus et al., 2004). The water homeostasis functions performed by astrocytes are similar to the water transport facilitated by the RPE and renal epithelial cells in which Kir7.1 has been shown to play a role; therefore it can be hypothesised that the astrocytic Kir7.1 has analogous importance in CNS glia. Further support of this hypothesis is the finding that Kir7.1 is co-expressed on the glial cell membrane with the known glial inward rectifier Kir4.1 and Na⁺-K⁺-ATPase.

The question remains as to the possible function of Kir7.1 in oligodendrocytes, although with the exception of the optic nerve, expression was more heterogeneous than in astrocytes, suggesting it may have a more important function in the optic nerve than elsewhere in the CNS. The function of oligodendrocytes is myelination, and so it is reasonable to assume that Kir7.1 are involved in this function, as has been shown for Kir4.1 (Neusch et al., 2000). Oligodendrocyte maturation and myelination are dependent on the development of a strongly negative RMP, which is related to a loss of Kv and upregulation of Kir (Knutson et al., 1997; Neusch et al., 2003). The results of this chapter indicate Kir7.1 may be an important aspect of these changes. Moreover, oligodendrocytes are exposed to large shifts in ions and water during axonal action potential propagation (Kettenman et al., 1990; Berger et al., 1991; Menichella et al., 2006), so it seems likely Kir7.1 play a similar role in ion and water homeostasis in oligodendrocytes as hypothesised for astrocytes.
4.3.3. Neuronal expression of Kir7.1 in the murine CNS in situ and in vitro

The staining pattern for Kir7.1 in hippocampal and cerebellar neurones observed in this study is consistent with the findings of Krapivinsky et al. (1998), although my results disprove their conclusion that Kir7.1 is a purely neuronal channel. More specifically, in their paper, Krapivinsky et al. show labelling of the pyramidal cell layer in the rat hippocampus as well as less intense labelling in the dentate gyrus, stratum oriens, and stratum radiatum. Due to the lack of double staining for glial and neuronal markers, it is not possible to decipher from their data whether the less intense staining was due to neuronal or glial expression. Nevertheless, my results are in complete agreement about the areas of the hippocampus that display Kir7.1 protein expression. Moreover, my observation that there is localisation of Kir7.1 equally on neuronal cell bodies and their primary dendrites is also in agreement with the data published by Krapivinsky et al. Finally, my study confirmed previous findings that Kir7.1 protein is expressed in cerebellar Purkinje neurons, with the most intense staining on their large cell bodies and still prominent but less intense expression on their elaborate dendritic arbor (Krapivinsky et al., 1998).

The intrinsic electrical properties of neurones are established by and dependent on the unique expression of potassium channels on their cell membranes. A key feature in neurones is the prominent expression of Kir channels and their function in maintaining neuronal excitability, with important roles in stabilising the neuronal membrane potential and repolarisation of axons during the action potential which determines their excitability (Armstrong and Hille, 1998). The localisation of Kir7.1 on the neuronal cell somata and dendrites of pyramidal and Purkinje neurones as well as
the initial segment of their axons, suggests a role for Kir7.1 in the summation of electrical signals received by these neurones and axon potential propagation. Pyramidal neurons receive and transmit both inhibitory (GABA) and excitatory (Glutamate) synaptic inputs. Purkinje neurons are GABAergic neurons and their dendrites receive excitatory glutamatergic signals from parallel and climbing fibres as well as inhibitory GABAergic signals from stellate cells, while basket cells provide further inhibitory (GABAergic) inputs on the purkinje neuron axon initial segment (Ito, 2012). In their paper, Krapivinsky et al. (1998) mention the expression of Kir7.1 in both GABAergic and non-GABAergic neurons and the results shown here suggest that Kir7.1 may have a role in the regulation of postsynaptic excitability of specific populations of neurones in the brain. The GIRK/Kir3.x subfamily has been shown to be most widely and prominently expressed in the postsynaptic membrane of CNS neurones (Inanobe et al., 1999; Koyrakh et al., 2005), and to be intimately involved in the modulation of neuronal excitability by coupling to GABA\textsubscript{\textit{\text{b}}} receptors in CA1 hippocampal and substantia nigra neurones (Koyrakh et al., 2005). Expression of the Kir2.x subfamily has also been studied and found to be expressed mainly on the cell somata and dendrites of neurones which receive excitatory signals in the olfactory bulb (Inanobe et al., 2002). Moreover, Kir channels are hypothesized to be powerful modulators of excitatory postsynaptic potentials (EPSPs) due to their maintenance of the dendritic RMP (John and Manchanda, 2011). It would be interesting to investigate potential coupling of Kir7.1 with GABA and NMDA receptors using pharmacology and electrophysiology in order to determine whether the same receptor interactions apply as with GIRK and classical Kir channels. Patch clamping of pyramidal and Purkinje neurons using hippocampal and cerebellar slices respectively could provide answers
about the functional implications of the postsynaptic expression of Kir7.1 channels in these cells.

The partnering of Kir7.1 channels with the Na<sup>+</sup>/K<sup>+</sup>-ATPase in the RPE, kidney, choroid plexus and thyroid epithelia is indicative of a possible role of Kir7.1 in regulating potassium and sodium movements in neurones. The basal intracellular Na<sup>+</sup> concentration ([Na<sup>+</sup>]) in rat hippocampal neurones is around 8 mM and the function of Na<sup>+</sup>/K<sup>+</sup>-ATPase is to quickly remove Na<sup>+</sup> ions from the cytoplasm following an action potential, which induces only small increases in the [Na<sup>+</sup>]. However, upon inhibiting the Na<sup>+</sup>/K<sup>+</sup>-ATPase with ouabain, the activity of ion channels and transporters of the neuron may increase [Na<sup>+</sup>] levels up to 50 mM within minutes (Yu and Salter, 1998). Thus, it becomes clear that failure of the Na<sup>+</sup>/K<sup>+</sup>-ATPase to maintain the Na<sup>+</sup> and K<sup>+</sup> gradients can lead to loss of neuronal excitability as well as a reduction of the driving force for a variety of Na<sup>+</sup> coupled transport mechanisms, including the Na<sup>+</sup>/H<sup>+</sup> exchange and Na<sup>+</sup>/Ca<sup>2+</sup> exchange, resulting in intracellular acidification and Ca<sup>2+</sup> overload. This sequence of events has been implicated in the pathogenesis of neurodegenerative diseases (Brines et al., 1995; Ellis et al., 2003; Wang et al., 2003; Dobretsov and Stimers, 2005). Specific localisation patterns of Kir channels and their coupling to selective apparatus serve in regulating particular cell functions and in neurones they play a role in unidirectional transport of K<sup>+</sup> and in the organization of signal transduction.
4.4. Summary and Conclusions

The key finding of this chapter is that astrocytes and oligodendrocytes express Kir7.1 channels. In addition, the results provide new information on the pattern of Kir7.1 expression in neurones, with a novel finding being the expression in cortical pyramidal neurones. The highest expression of Kir7.1 was observed in the choroid plexus epithelium, ependymal lining of the ventricles and in Purkinje neurones of the cerebellum. On the whole, astrocytes exhibited immunopositivity for Kir7.1 similar to neurones and greater than oligodendrocytes in most of the brain. Oligodendroglial expression of Kir7.1 was heterogeneous throughout the brain, although they appeared to be the main cells expressing the channel in the optic nerve. Astrocytes expressed Kir7.1 ion their cell somata and processes, with high expression in astrocytic perivascular end-feet, consistent with a role in K⁺ and water transport at blood vessels. The physiological function of Kir7.1 in neurones and glia is unknown, but its pattern of cellular localisation supports a potential role in neuronal excitability and K⁺ and water homeostasis in both neurones and glia. Future electrophysiological studies utilising the recently discovered Kir7.1 blocker VU590 will be invaluable in determining the function of these channels in neurones and glia.
Chapter 5 - Role of Kir7.1 in Ischaemia Mediated Disruption of Optic Nerve Glia
5.1. Introduction and Aims

Glial cells express a variety of Kir subtypes with multiple functions (Kalsi and Butt, 2006). In sections 3 and 4, I demonstrated for the first time that Kir7.1 is prominently expressed by astrocytes and oligodendrocytes in the optic nerve, as well as elsewhere throughout the CNS. Although their precise functions in glia are unknown, Kir7.1 channels have been previously shown to be particularly sensitive to acidification, suggesting Kir7.1 channels could be important during glial ischaemia and hypoxia (Yuan et al., 2003; Hughes and Swaminathan, 2008). Oligodendrocytes are highly susceptible to hypoxic/ischaemic damage (Fern and Möller, 2000), and a recent study in our lab provided evidence that potassium channels contribute to oligodendrocyte damage in hypoxia (Hawkins and Butt, 2013). The high expression of Kir7.1 in oligodendrocytes may therefore be pivotal to their susceptibility to hypoxic injury (Back et al., 2007; Dewar et al., 2003). Therefore, the aim of this chapter was to investigate the effects of Kir7.1 pharmacological blockade on oligodendrocytes in hypoxic/ischaemic conditions, using the small-molecule Kir7.1 channel blocker 7,13-bis(4-nitrobenzyl)-1,4,10-trioxa-7,13-diazacyclopentadecane (VU590) (Lewis et al., 2009). Although VU590 also inhibits Kir1.1 (Bhave et al., 2011), these channels are not present in the optic nerve (Section 3.2.3), whereas Kir4.1 and Kir2.1 are prominent in the optic nerve (Section 3.2.3), but are not affected by VU590 at micromolar concentrations (Lewis et al., 2009).
5.2. Results

The potential function of K\textsubscript{ir7.1} during hypoxia/ischaemia was examined using the oxygen and glucose deprivation (OGD) model (Salter and Fern, 2005; Hawkins and Butt, 2013). In brief, P12-P14 SOX10-eGFP and GFAP-eGFP reporter mice were killed humanely and the optic nerve rapidly isolated intact and immediately placed in normal aCSF to stabilise for 30 min at 37°C in a normoxic incubator (95% O\textsubscript{2}/5% CO\textsubscript{2}). Optic nerves were then incubated for a further 1 h at 37°C in normal oxygen and glucose (OGN; 95%O\textsubscript{2}/5%CO\textsubscript{2}) or in zero O\textsubscript{2} and glucose (OGD; 95%N/5%CO\textsubscript{2}). Ion channel blockers VU590 or BaCl\textsubscript{2} were added directly to the aCSF to a final concentration of 100 µM. At the end of the incubation period, optic nerves were immersion fixed in 4% PFA for 1 hr at RT and following washes were whole-mounted on microscope slides in Fluoromount G. Cells expressing the GFAP-eGFP or SOX10-eGFP reporters were visualised at 488 nm using an argon laser and images captures on a Zeiss LSM 710 metaconfocal microscope, using a x40 oil immersion lens with high numerical aperture (1.3 nm). Images were captured maintaining the acquisition parameters constant between samples. In each nerve, the total number of cells was counted in five FOV along the length of the optic nerve. The FOV comprised a constant volume of 20 x 20 µm in the x-y-plane and 15x1 µm optical sections in the z-plane, commencing 15 µm below the pial surface. Cell counts are expressed as mean number of cells per FOV ± SEM (n≥5, where ‘n’ represents the number of nerves), and significance was determined by ANOVA and Newman–Keuls multiple comparison post-hoc analysis, using GraphPad Prism5.0.
The function of $K_{ir}7.1$ in maintaining glial integrity was examined using the $K_{ir}7.1$ blocker VU590, compared to the general $K_{ir}$ blocker BaCl$_2$, and the $K_{ir}1.1$ blocker VU591. Full concentration curves were not practical, since this would have required a very large number of reporter mice, which were not available within the time-frame. Hence, I selected a concentration of 100 µM for the inhibitors based on the literature and the following reasons. Studies in our lab have shown that bath application of 100µM BaCl$_2$ results in near complete inhibition of $K_{ir}4.1$, the primary glial $K_{ir}$ channel in the optic nerve (Bay and Butt, 2012), whereas other $K_{ir}$ subtypes, including $K_{ir}7.1$ and $K_{ir}2.1$, have been shown to require ≥2mM BaCl$_2$ for effective blockade in HEK cell cultures (Lewis et al., 2009). Similarly, complete inhibition of $K_{ir}7.1$ by VU590 in HEK cell cultures has been shown to require 10 µM in one study (Lewis et al., 2009) and 100 µM in another (Rouhier et al., 2009), whilst $K_{ir}4.1$ and $K_{ir}2.1$ were unaffected at a concentration 10 µM in HEK cell cultures (Lewis et al., 2009). Similarly, VU591 has been shown to specifically inhibit $K_{ir}1.1$ and have no effect on $K_{ir}7.1$ at concentrations up to 100 µM in C1 cell cultures (Bhave et al., 2011). Furthermore, the pial surface of the nerve acts as a permeability barrier and in order to overcome this restriction and affect cells within the nerve parenchyma, numerous studies in our lab have shown that agents need to be applied at 10-fold the concentration used in single cell cultures (Bolton and Butt, 2005; Hamilton et al., 2008; Azim and Butt, 2011; Bay and Butt, 2012). On this basis, a concentration of 100 µM of BaCl$_2$, VU590 and VU591 was selected to specifically inhibit $K_{ir}4.1$, $K_{ir}7.1$ and $K_{ir}1.1$, respectively.
5.2.1. Blockade of Kir7.1 induces loss of oligodendrocytes in OGN and OGD

The function of Kir in maintaining oligodendrocyte integrity was examined using 100 µM VU590 to block Kir7.1 (Lewis et al., 2009; Rouhier et al., 2009). Compared to control nerves incubated in O₂ and glucose (Figure 5.1A), OGD resulted in a marked loss of Sox10-eGFP positive oligodendrocytes (Figure 5.1B), which was statistically significant (Figure 5.1E; p<0.001, ANOVA and Newman-Keuls post-hoc tests). Notably, blockade of Kir7.1 resulted in a significant loss of oligodendrocytes in nerves incubated in normal O₂ and glucose, OGN (Figure 5.1C, E; p<0.05, ANOVA followed by Newman-Keuls multiple comparisons test), and a marked loss of oligodendrocytes in OGD (Figure 5.1D, E; p<0.001, ANOVA followed by Newman-Keuls multiple comparisons test). The number of oligodendrocytes was reduced by half in OGD+VU590 compared to OGN, and by one-third compared to OGD in the absence of VU590 (Figure 5.1E).
Figure 5.1: Kir7.1 inhibition induces loss of oligodendrocytes. Optic nerves from P12-P14 Sox10-eGFP reporter mice were exposed to 1 h OGD in the absence and presence of the Kir7.1 blocker VU590 (100 µM) and compared to controls incubated in normal αCSF with O2 and glucose (OGN). (A–D) Representative images of optic nerves from SOX10-eGFP reporter mice, which identifies oligodendrocytes, incubated in OGN (A), OGD (B), OGN plus VU590 (C), or OGD plus VU590 (E). Quantification of the number of SOX10-eGFP positive cells (mean ± SEM; n ≥ 5 nerves per experimental group in all cases) shows a significant loss of oligodendrocytes during OGD, compared to OGN, and cell loss was significantly greater following treatment with VU590 in both OGN and OGD (ANOVA with Newman–Keuls multiple comparison post-hoc analysis; *p<0.05, **p<0.01, ***p<0.001).
5.2.2. Blockade of Kir4.1 induces loss of oligodendrocytes in OGN and OGD

To test whether the effects on oligodendrocyte integrity are specific to Kir7.1 blockade, optic nerves were treated with 100 µM BaCl₂ to block Kir4.1 in OGN and OGD conditions, to serve as a positive control, since Kir4.1 are the primary potassium channels in optic nerve glia (Butt and Kalsi, 2006; Bay and Butt, 2012; Section 3.2.3). In addition, the effects of the Kir1.1 inhibitor VU591 were examined in OGD to serve as a negative control, since Kir1.1 were not detected in the optic nerve (Section 3.2.3) and VU591 does not inhibit Kir7.1 or Kir4.1 (Bhave et al., 2011). As observed above for Kir7.1, blockade of Kir4.1 resulted in a significant loss of oligodendrocytes in OGN (Figure 5.2A, C, E; p<0.001, ANOVA followed by Newman–Keuls multiple comparisons test), and to a lesser extent aggravated the loss of oligodendrocytes in OGD (Figure 5.2B, D, E; p<0.05, ANOVA followed by Newman–Keuls multiple comparisons test). In contrast, the Kir1.1 blocker VU591 had no significant effect on the loss of oligodendrocytes in OGD (inset Figure 5D and Figure 5E; p>0.05, ANOVA followed by Newman–Keuls multiple comparisons test). Although VUS90 inhibits both Kir7.1 and Kir1.1, the lack of effect of VU591 confirms the effect of VUS90 on oligodendrocyte integrity is mediated by Kir7.1, and the similar action as BaCl₂ in OGN and OGD suggests Kir7.1 are as important as Kir4.1 in maintaining oligodendrocyte integrity.
Figure 5.2: Kir4.1 inhibition induces loss of oligodendrocytes. Optic nerves from P12-P14 Sox10-eGFP reporter mice were exposed to 1 h OGD in the absence and presence of the Kir4.1 blocker BaCl2 (100 µM) or Kir1.1 blocker VU591 (100 µM), and compared to controls incubated in normal αCSF with O2 and glucose (OGN). (A–D) Representative images of optic nerves from SOX10-eGFP reporter mice, which identifies oligodendrocytes, incubated in OGN (A), OGD (B), OGN plus BaCl2 (C), OGD plus BaCl2 (D) or OGD plus VU591 (inset F). Quantification of the number of SOX10-eGFP positive cells (mean ± SEM; n ≥ 4 nerves per experimental group in all cases) shows a significant loss of oligodendrocytes during OGD, compared to OGN, and cell loss was significantly greater following treatment with BaCl2 in both OGN and OGD, whereas VU591 had no effect on oligodendrocyte number in OGD (ANOVA with Newman–Keuls multiple comparison post-hoc analysis; *p<0.05, **p<0.01, ***p<0.001).
5.2.3. Blockade of Kir7.1 does not induce the loss of astrocytes

Compared to oligodendrocytes and neurons, astrocytes are generally considered to be less sensitive to ischaemic injury, which results in reactive astrogliosis (Ridet et al., 1997; Davies et al., 1998), although irreversible ischaemic damage eventually leads to astrocyte cell death (Bondarenko and Chesler, 2001; Thomas et al., 2004; Giffard and Swanson, 2005). Astrocytes may be more sensitive in developing white matter during the period of myelination (Salter and Fern, 2008). Astrocytes in the developing mouse optic nerve during myelination (P10) have been shown to be sensitive to OGD (Shannon et al., 2007; Salter and Fern, 2008). We therefore examined the effects of Kir7.1 blockade and OGD in P12-14 nerves from GFAP-eGFP mice. The results indicated that compared to OGN (Figure 5.3A), the number of astrocytes was not decreased in OGD (Figure 5.3B), and blockade of Kir7.1 with VU590 had no effect on astrocyte numbers in OGN (Figure 5.3C) or OGD (Figure 5.3D), confirmed by statistical analysis of cell counts (Figure 5.3E; p>0.05, ANOVA). However, qualitative examination indicated a marked decrease in the overall density of astrocyte processes following incubation in VU590, in both OGN (Figure 5.3C) and OGD (Figure 5.3D). Previous studies have shown a loss of processes from astrocytes under ischaemic conditions (Davies et al., 1998; Hulse et al., 2001; Kraig and Chesler, 1990; Thomas et al., 2004; Salter and Fern, 2008), and the present results indicate that blockade of Kir7.1 has an equivalent effect in normoxic and ischaemic conditions.
Figure 5.3: $\kappa_\text{n}7.1$ inhibition does not induce loss of astrocytes. Optic nerves from P12-14 GFAP-eGFP reporter mice were exposed to 1 h OGD in the absence and presence of the $\kappa_\text{n}7.1$ blocker VU590 (100 µM) and compared to controls incubated in normal αCSF with $O_2$ and glucose (OGN). (A–D) Representative images of optic nerves from GFAP-eGFP reporter mice, which identifies astrocytes, incubated in OGN (A), OGD (B), OGN plus VU590 (C), or OGD plus VU590 (D). (E) Quantification of the number of GFAP-eGFP positive cells shows no significant difference in astrocyte numbers between the treatment groups (data are mean ± SEM; n ≥ 5 nerves per experimental group; tested for significance by ANOVA with Newman–Keuls multiple comparison post-hoc analysis; *$p<0.05$, **$p<0.01$, ***$p<0.001$).
5.2.4. Blockade of Kir4.1 does not induce the loss of astrocytes

Kir4.1 is the predominant ion channel in optic nerve astrocytes and is essential for maintaining their RMP (Bay and Butt, 2012). It was therefore pertinent to examine the effects of 100 µM BaCl₂ on astrocytes, which has been shown to almost completely inhibit inward currents in optic nerve astrocytes (Bay and Butt, 2012). Blockade of Kir4.1 had no significant effect on astrocyte cell numbers or processes in OGN or OGD (Figure 5.4; \( p>0.05 \), ANOVA). The results indicate Kir7.1 blockade specifically results in the loss of astrocyte processes, and that the effects of OGD and Kir4.1 or Kir7.1 blockade on cell number is specific to oligodendrocytes, consistent with their being more sensitive to ischaemic injury than astrocytes.
Figure 5.4: K_\text{ir}4.1 inhibition does not induce loss of astrocytes. Optic nerves from P12-14 GFAP-eGFP reporter mice were exposed to 1 h OGD in the absence and presence of the K_\text{ir}4.1 blocker BaCl_2 (100 µM) and compared to controls incubated in normal αCSF with O_2 and glucose (OGN). (A–D) Representative images of optic nerves from GFAP-eGFP reporter mice, which identifies astrocytes, incubated in OGN (A), OGD (B), OGN plus BaCl_2 (C), or OGD plus BaCl_2 (D). (E) Quantification of the number of GFAP-eGFP positive cells shows no significant difference in astrocyte numbers between the treatment groups (data are mean ± SEM; n ≥ 5 nerves per experimental group; tested for significance by ANOVA, p>0.05).
5.2.5. *K* _ir7.1_ blockade activates cell death pathways

The results above show that *K* _ir7.1_ blockade results in the loss of oligodendrocytes in normoxic conditions, indicative of cell death. To examine this further, I investigated by qRT-PCR the mRNA expression levels of key genes that are important for the central mechanisms of cellular death (apoptosis, autophagy, and necrosis), using the Cell Death Pathway Finder RT² profiler PCR array (Sabiosciences, Qiagen). Ct values of the genes of interest were normalised to two reference genes, a combination of Actb and Gapdh (variation value 0.005) (Figure 5.5), which is more accurate than using a single reference gene (Gimeno et al., 2014). Relative quantification was performed (\(2^{-\Delta\Delta Ct}\) Method) followed by calculation of the fold change in gene expression (\(\Delta\Delta Ct\) Method).

![CDPF Reference Genes](image)

*Figure 5.5: Estimation of stability of candidate reference genes using NormFinder.* Genes are ranked according to their expression stability across all samples. The arrow indicates the most stable combination of reference genes.

qRT-PCR identified a number of genes in the Cell Death Pathway array that are significantly altered by the *K* _ir7.1_ blocker VU590 in nerves under normoxic conditions.
Table 5.1). Ccdc103, Bcl2 and Ifng were the three most regulated genes in VU590, Ccdc103 and Bcl2 being upregulated and Ifng being downregulated. Mutations in Ccdc103 are a diagnostic marker for Primary Ciliary Dyskinesia (PCD) (Zariwala et al., 2007 and 2013) and the protein serves as an anchoring factor for dynein, a motor protein that uses ATP energy to move cell cilia (Panizzi et al., 2012). Ccdc103 has no reported function in the brain or in glia, but astrocytes express primary cilia and these regulate their survival under stressed conditions (Yoshimura et al., 2011). Hence, the 10.8 FC upregulation of Ccdc103 in VU590 suggests they may be important in the survival of astrocytes and their changes in morphology following blockade of K\textsubscript{i}7.1. Similarly, Bcl2 is a major anti-apoptotic factor (Rossé et al., 1998) and was upregulated 7.031 fold in VU590. Also upregulated were the apoptosome complex gene Apaf1 (FC1.402), which binds with cytochrome C and is responsible for caspase activation and the initiation of apoptotic cell death in the nervous system (Yuan and Yankner, 2000), and caspase 3 (FC 1.231), a pro-apoptotic regulator in oligodendrocytes (Prineas and Parratt, 2012). Interestingly, caspase 3 is also responsible for cleavage of Huntingtin (Goldberg et al., 1996), which was significantly upregulated in VU590 (FC 1.341) and indirectly activates caspase 8, another pro-apoptosis factor (Gervais et al., 2002). In addition, Abl1 was upregulated in VU590 (FC 1.799) and encodes the ubiquitously expressed nonreceptor tyrosine kinase ABL, which is activated in oxidative stress and can stimulate cell survival or death (Li, 2005; Wang, 2014), although it has no reported function in glia or the brain.

The top genes in the Cell Death Pathway array that were significantly (p<0.05, unrelated t-tests) downregulated in VU590 treated nerves under normoxic conditions include Ifng (IFN-\textgamma), which is pro-inflammatory in for glia (Neumann, 2001), and the
poliovirus receptor (PVR), which is a transmembrane glycoprotein belonging to the IgG superfamily, and mediates cell attachment to the ECM molecule vitronectin, which regulates glial growth and activation (Bouslama-Oueghlani et al., 2012; Milner et al., 2007). The remaining downregulated genes were related to cell survival and autophagy and have no reported functions in glia. Autophagy functions as a cell death mechanism or as a stress response, mediating prominent cytoprotective effects (Salminen et al., 2013; Cecconi and Levine, 2008), and so the downregulation of key autophagy related genes ATG5 and Beclin1 (Beclin 1) in VU590 indicates these are important in the effects of Kir7.1 blockade on glial responses in the optic nerve. Moreover, the Bcl2 family can interact with Beclin1 to inhibit autophagy (Salminen et al., 2013), and so there would be potentially opposing effects of upregulation of Bcl2 (see above) and downregulation of Bcl2a1 (bcl-2 related a1) in VU590. Bcl2a1 is also known as Bfl1 and is a highly regulated NF-κB target gene that exerts important pro-survival functions (Vogler, 2012). The remaining gene regulated by VU590 was the cell cycle regulator and tumour suppressor gene TP53 (Table 5.1).
Table 5.1: Effects of Kir7.1 blockade on cell death related genes expressed during OGN, determined by qRT-PCR using the Cell Death Pathway Finder RT² profiler PCR array (Sabiosciences, Qiagen). Data are fold-change (FC) determined in nerves incubated for 1h in normal O₂ and glucose conditions (OGN), comparing nerves in the absence or presence of the Kir7.1 blocker VU590 (n≥6 optic nerves per treatment group, sun in triplicate). Statistical analysis was performed by volcano plot (unrelated t-test $p<0.05$; $FC>1.0$).

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Fold Change</th>
<th>Location</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccdc103</td>
<td>↑ 10.805</td>
<td>Cytoplasm</td>
<td>Other</td>
</tr>
<tr>
<td>Bcl2</td>
<td>↑ 7.031</td>
<td>Cytoplasm</td>
<td>Transporter</td>
</tr>
<tr>
<td>Abl1</td>
<td>↑ 1.799</td>
<td>Nucleus</td>
<td>Kinase</td>
</tr>
<tr>
<td>Apaf1</td>
<td>↑ 1.402</td>
<td>Cytoplasm</td>
<td>Other</td>
</tr>
<tr>
<td>HTT</td>
<td>↑ 1.341</td>
<td>Cytoplasm</td>
<td>Transcription Regulator</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>↑ 1.231</td>
<td>Cytoplasm</td>
<td>Peptidase</td>
</tr>
<tr>
<td>Ifng</td>
<td>↓ -3.775</td>
<td>Extracellular Space</td>
<td>Cytokine</td>
</tr>
<tr>
<td>Bfl1 (Bcl2a1)</td>
<td>↓ -1.670</td>
<td>Cytoplasm</td>
<td>Other</td>
</tr>
<tr>
<td>Pvr</td>
<td>↓ -1.092</td>
<td>Plasma Membrane</td>
<td>Other</td>
</tr>
<tr>
<td>Atg5</td>
<td>↓ -1.062</td>
<td>Cytoplasm</td>
<td>Other</td>
</tr>
<tr>
<td>TP53</td>
<td>↓ -1.033</td>
<td>Nucleus</td>
<td>Transcription Regulator</td>
</tr>
<tr>
<td>Becn1</td>
<td>↓ -1.021</td>
<td>Cytoplasm</td>
<td>Other</td>
</tr>
</tbody>
</table>

Thus, it is evident that multiple genes related to cell death are significantly altered by the Kir7.1 blocker VU590 in nerves under normoxic conditions. Given this, data was further analysed using IPA pathway analysis (Ingenuity Systems), which predicted the activation of caspase-dependent apoptotic signalling (Figure 5.6). The results presented here indicate that the blockade of Kir7.1 with the blocker VU590 induces significant oligodendrocytic cell death via caspase-dependent apoptotic pathways.
Figure 5.6: Apoptosis signalling genes in the optic nerve altered by VU590 under normoxic conditions.

A complement system pathway generated by IPA. Proteins with increased expression are marked in red; proteins with decreased expression are marked in green.
5.3. Discussion

The aim of this chapter was to examine the possible functional role of the novel Kir7.1 subtype in optic nerve glial physiology. In the previous chapters, evidence was provided for the high expression of Kir7.1 in optic nerve glia on both the transcriptome and protein levels. The effects of Kir7.1 blockade on glial cell survival in the myelinating mouse optic nerve during normal (OGN) as well as oxygen and glucose deprivation (OGD) conditions were investigated using the OGD model in combination with pharmacology. Results showed that Kir7.1 inhibition caused significant oligodendrocytic cell death in normal conditions and exacerbated cell death in OGD conditions. In contrast to oligodendrocytes, astrocytes were not as sensitive to hypoxic cell death and Kir7.1 inhibition did not adversely affect their survival in either OGN or OGD. In order to determine the type of cell death that is induced in oligodendrocytes when treated with the specific Kir7.1 blocker VU590, RNA was extracted from optic nerves incubated for 30mins in a normal incubator or for a further 1 hr in OGN conditions with and without VU590 and qRT-PCR was performed using the Cell Death Pathway Finder RT² Array. Pathway analysis of the cell death related genes that were significantly regulated in the VU590 treated groups highlighted a caspase-dependent apoptotic pathway.

5.3.1. Kir7.1 blockade induces oligodendrocytic cell death

Oligodendrocytes are responsible for the myelination of neuronal axons; loss of myelination and, as a consequence axonal functional degeneration, is involved in the pathogenesis of a variety of conditions such as cerebral palsy, stroke, multiple sclerosis
and Alzheimer’s disease. Oligodendrocyte lineage cells, like neurons but not astrocytes, are very vulnerable to ischaemic/hypoxic injury and the investigation of the cellular mechanisms that are responsible for this high sensitivity has been central in scientific studies for many years (Salter and Fern, 2005). Here, the Oxygen and Glucose Deprivation (OGD) model of the optic nerve was selected in order to study the potential involvement of Kir7.1 in glial cell survival during hypoxic conditions. Incubation time of 60 mins was deemed the most appropriate, since this time period has been shown to be sufficient for the observation of significant oligodendrocytic process damage and oligodendrocyte cell death in previously published studies (Salter and Fern, 2005; Hawkins and Butt, 2013). Cell counts of optic nerve oligodendrocytes in normal oxygen and glucose (OGN) versus OGD conditions confirmed that within 60 mins, a significant reduction of oligodendrocyte lineage cells occurred in the model and I also showed that blockade of Kir7.1 with the specific blocker VU590 resulted in significantly increased oligodendrocyte cell death after 60 mins in OGD conditions. Moreover, it was observed that incubation of optic nerves with VU590 caused oligodendrocyte cell death even in OGN conditions, which indicates an important function of Kir7.1 in oligodendrocyte physiology. The pharmacological blocker VU590 has been shown to effectively block Kir7.1 at micromolar concentrations, whereas it has no effect on Kir4.1 and Kir2.1 (Lewis et al., 2009).

Ischaemic/hypoxic injury is mediated by a combination of mechanisms; the disruption of ionic homeostasis due to energy depletion and the accumulation of excessive glutamate are both important factors leading to white matter cell death (Stys, 2004; Tekkök et al., 2007). The significant sensitivity of differentiating oligodendrocytes to ischaemia/hypoxia and oxidative stress has been linked to their
limited anti-oxidative defences (Back et al., 2002; Fern and Möller, 2000) along with their expression of Ca\(^{2+}\) permeable ionotropic glutamate receptors and more specifically N-Methyl-D-Aspartate (NMDA) receptors (Káradóttir et al., 2005; Back et al., 2007). Moreover, the depolarisation of the resting membrane potential as a result of inhibition of oxidative phosphorylation, reduction of ATP levels and subsequent failure of the Na\(^+\)/K\(^+\) pump is one of the perpetrators of ischaemic neuronal cell death (Clarke and Sokoloff, 1994; Lipton, 1999). Since oligodendrocytes have a highly negative resting membrane potential, which is maintained principally via the expression of K\(_i\) channels (Kalsi and Butt, 2006), it is possible that blockade of K\(_i\) would result in exacerbation of ischaemic injury cascades. In addition, blockade of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) during anoxia of the rat optic nerve was found to have a protective effect as it was shown that a reversal of its function was responsible for the large Ca\(^{2+}\) influx that led to white matter cell death (Stys et al., 1991; Fern et al., 1993). Nitric Oxide (NO) production by microglia and \([\text{Na}^+]_o\) increase during hypoxia has been shown to be linked with the reversal of NCX from efflux to influx of Ca\(^{2+}\) but the exact mechanisms have not been elucidated (Boscia et al., 2013; Stys et al., 1992).

Finally, the acid-sensing ion channels (ASICs), which are highly permeable to Ca\(^{2+}\), could also be acting as excitotoxicity mediators in ischaemic/hypoxic conditions since under these conditions extracellular pH becomes acidic enough for these channels to be activated (Barber et al., 2003; Krishtal, 2003). Expression of ASIC at the transcriptome level in optic nerve glia and especially Asic1 was demonstrated in chapter 3 and their expression at protein level has been shown in previous studies in OPCs (Feldman et al., 2008; Lin et al., 2010). The mechanism of Ca\(^{2+}\) -induced excitotoxicity is glutamate-independent and can trigger both necrotic and apoptotic
cell death (Stys et al., 1992) and removal of extracellular calcium resulted in greater recovery of young adult white matter following OGD but not aging white matter (Tekkök et al., 2007; Baltan et al., 2008).

Barium (Ba\(^{2+}\)) is a known nonspecific blocker of K\(_{ir}\) channels; therefore, treatment of optic nerves with barium chloride (BaCl\(_2\)) in OGN and OGD was used as a positive control for oligodendrocyte K\(_{ir}\) blockade. The K\(_{ir}4.1\) subtype, which sets the resting membrane potential in oligodendrocytes, is very sensitive to Ba\(^{2+}\) blockade, but at the concentration used here, it might also partially block other K\(_{ir}\) channels in the mouse optic nerve such as K\(_{ir}2.x\) or K\(_{ir}3.x\) (Bolton et al., 2006; Bay and Butt, 2012). However, K\(_{ir}7.1\), are not blocked by Ba\(^{2+}\) at this concentration (Krapivinsky et al., 1998). In fact, the K\(_{ir}7.1\) subtype displays several unique features compared to the rest of the K\(_{ir}\) channels such as its very low single channel conductance (approximately 50 fS), its independence on internal block by Mg\(^{2+}\) as well as its low sensitivity to block by external Ba\(^{2+}\) and Cs\(^+\). The sensitivity of K\(_{ir}7.1\) to Ba\(^{2+}\) and Cs\(^+\), common blockers of K\(_{ir}\) channels, is very low with IC\(_{50}\) values of 1 mM and ~10 mM, respectively, which are ~10 times higher than the IC\(_{50}\) values for other K\(_{ir}\) channels (Partiseti et al., 1998) therefore the barium concentration used in this study (100 \(\mu\)M) should only block non-K\(_{ir}7.1\) inward rectifier channels, and mainly K\(_{ir}4.1\). Another important biophysical characteristic of K\(_{ir}7.1\) is that its inward rectification is independent of [K\(^+\)]\(_o\). It is possible therefore, that the extracellular K\(^+\) accumulation that occurs during hypoxia, renders K\(_{ir}7.1\) as the main inward rectifier that maintains the resting membrane potential in oligodendrocytes.
This hypothesis is further supported by the finding that while both Ba\textsuperscript{2+} and VU590 cause oligodendrocyte cell death in normal oxygen and glucose conditions, the effect of Ba\textsuperscript{2+} and therefore inhibition of \(K_{ir}4.1\), was more detrimental than the inhibition of \(K_{ir}7.1\). However, the opposite was noted for oxygen and glucose deprivation conditions, during which \(K_{ir}7.1\) blockade had a more significantly damaging effect to oligodendrocytes than \(K_{ir}4.1\) inhibition. Due to its biophysical properties it was hypothesised that \(K_{ir}7.1\) could help set the membrane potential in cells in which it is expressed (Krapivinsky et al., 1998). As it has been previously established that the most important contributor to setting the very negative glial resting membrane potential (RMP) is \(K_{ir}4.1\) (Butt and Kalsi, 2006), it is unlikely that \(K_{ir}7.1\) plays a major role in normal conditions. A conditional CNS knockout of \(K_{ir}7.1\) would answer this question and in combination with OGD it could help elucidate on the role of \(K_{ir}7.1\) in oligodendrocytic survival during hypoxic conditions. It is important to note that incubation of mouse optic nerves with 100 µM BaCl\textsubscript{2} in OGN conditions for 1hr caused significant oligodendrocyte cell death but since we did not perform a time course we are unable to say with certainty how long after the introduction of BaCl\textsubscript{2} the cells started to die.

In contrast to the dramatic effect that VU590 had on oligodendrocytes, astrocytic cell death was not exacerbated in OGN or OGD. Moreover, no significant ischaemic/hypoxic astrocytic death was observed after incubation of optic nerves in OGD conditions. Astrocytes are generally more resilient than oligodendrocytes and neurons and they can survive longer in oxygen and glucose deprivation. Even in the absence of oxygen, astrocytes can still produce ATP (anaerobic metabolism) which allows them to maintain intracellular ionic homeostasis as well as continue the
clearance of $[K^+]_o$ that is increased in ischaemic/hypoxic conditions thus protecting the brain. Astrocytes are protective against ischaemic damage in other ways as well, by clearing excess glutamate and Reactive Oxygen Scavengers (ROS) from the extracellular space, sustaining neurons by producing lactate, by releasing neuroprotective factors such as vascular endothelial growth factor (VEGF) and by forming the glial scar thus isolating damaged areas (Verkhratsky and Butt, 2013). However, there is a likelihood that $K_r$ blockade could compromise astrocytic functions such as $[K^+]_o$ clearance and consequently resulting in an exacerbation of the effects of OGD on oligodendrocytes.

Finally, application of VU591 did not have any effect in glial cell death in the mouse optic nerve during OGD. VU591 was identified by Bhave et al as a $K_r1.1$ inhibitor that is more specific than VU590 as it does not block $K_r7.1$ at concentrations up to 10 µM (Bhave et al., 2011). The potential effects of VU591 on $K_r7.1$ at the concentration used here (100 µM) were not examined electrophysiologically, therefore it is possible that some blockade occurred, however it was not high enough that it caused an effect on cell survival during OGD.

5.3.2. Cell Death Pathways

Cell death can happen in any of the three different forms; apoptosis, autophagy or necrosis (Lipton, 1999). Here, the Cell Death Pathway Finder qRT-PCR Array (Qiagen) was used in order to investigate the involvement of key molecules from all three cell death modes that may participate in the observed oligodendrocytic cell death after incubation with VU590, which blocks $K_r7.1$. Volcano plot analysis identified the
upregulation Bcl2, Abl1, Apaf1 and Caspase 3, which are key players in the initiation of caspase dependent apoptotic cell death. The family of caspases contains at least 14 members in mammalian cells, where they are present as inactive pro-caspases and are proteolytically processed into active caspases. They are activated either through engagement of cell surface receptors such as the tumour necrosis factor alpha receptor (TNFa) and Fas or by the release of cytochrome c from mitochondria (Kroemer et al., 1998). Release of cytochrome c from the mitochondria depends on the integrity of the mitochondrial outer membrane and more specifically on the permeability of the mitochondrial voltage dependent anion channel (VDAC) which is regulated by the Bcl-2 family of proteins (Shimizu et al., 1999). Mitochondria can also be induced to release cytochrome c through various weak stress signals emanating from the interior of the cell such as DNA damage, premature mitotic arrest or unfolded protein response (Green and Reed, 1998; Zamzami and Kroemer, 2001). The redistribution of cytochrome c into the cytosol results in the formation of the apoptosome with Apaf1, which in turn activates caspase-9 in the presence of dATP (Li et al, 1997; Du et al, 2000). Caspase-9 then activates the effector caspase-3 that is responsible for many of the morphological and biochemical features of apoptosis (Los et al., 1999; Salvesen and Dixit, 1997). It is important to note that the family of Bcl-2 contains both pro- and anti-apoptotic molecules that can either directly affect the integrity of the outer mitochondrial membrane (protein-membrane interaction) or heteromerise with each other (protein to protein interaction). Moreover, it has been hypothesised that the fate of a cell will ultimately depend on the relative balance of pro- and anti-apoptotic signals, but the exact mechanisms and interactions of Bcl-2 family proteins are still being studied. Multiple models have been hypothesised over
the last 3 decades to describe the regulation of mitochondrial apoptosis by Bcl-2 proteins and it appears that the specific functions of each member depend on the specific combination of BH regions (Bcl-2 Homology regions), as cleavage of specific regions and alternative splicing can determine whether a Bcl-2 related protein will protect or condemn a cell, and all these mechanisms comprise of multiple stages (Chi et al., 2014).

Besides the well-established role of mitochondria in the induction of cell death, the disruption of the function of endoplasmic reticulum has also been implicated in the pathophysiology of various diseases involving cell death (Kim et al., 2006; Hetz et al., 2013). Moreover, membrane contact sites (MCSs) have been discovered between the ER and mitochondria, with important implications about the involvement of ER in mitochondria-induced apoptosis (Kornmann, 2013). The ER extends all through the cytoplasm and it is primarily involved in protein production and transportation, Ca^{2+} homeostasis as well as lipid and membrane biosynthesis. Importantly, it also serves as the initiating locus for autophagy and is able to initiate signals that regulate cell survival (Szegezdi et al., 2006; Martinez et al., 2010). The autophagy related gene Beclin1 (Becn1) is located on the ER membrane as well as other Bcl-2 related proteins (Sinha et al., 2008) and the cell death signals initiated from the ER and transmitted to the mitochondria are aimed at both the transcriptional and post-transcriptional level (Puthalakath et al., 2007) as well as the regulation of Ca^{2+} via trans-ER-membrane channels including IP_{3} receptors (Wang et al., 2011).

An interesting finding was that the Ccd3c103 transcript was significantly upregulated in the VU590 treated optic nerves. Generally very little is known about the
possible expression and distribution of Ccdc103; mRNA was found to be highly expressed in all cilia bearing cells of the zebrafish and protein expression was shown in the cytoplasm and the axonemes (cytoskeleton) of cilia as well as in human respiratory epithelium (Panizzi et al., 2012) but it is not known whether it is expressed physiologically in the CNS. Ccdc103 transcription is regulated by the Foxj1a transcription factor which in turn is a target of Hedgehog signalling in the floor plate (FP) of both mouse and chick embryos (Yu et al., 2008; Cruz et al., 2010). Expression of Foxj1 has been previously found in mouse respiratory and reproductive epithelium, T-cells, as well as pancreatic b-cells but it has never been investigated in the CNS (Hackett et al., 1995; Park et al., 2007; Lin et al., 2004; Kang et al., 2009). Interestingly, T-cell populations from Foxj1<sup>−/−</sup> chimeras were found to produce up to 7fold more interferon-γ (IFN-γ) than their Foxj1<sup>+/−</sup> counterparts (Lin et al., 2004) whereas expression of Ccdc103 was negative in foxj1a-deficient embryos (Panizzi et al., 2012). IFN-γ is a cytokine that belongs to the family of interferons and it is known to activate the JAK-STAT system which is a major signalling alternative to the second messenger system that involves IP<sub>3</sub> and Ca<sup>2+</sup> among others (Holla et al., 2014). Here, Ccdc103 transcript expression was greatly upregulated (11fold) and IFN-γ almost 4fold downregulated in VU590 treated optic nerves when compared to the untreated. This is very interesting since astrocytes and neural stem cells express primary cilia and these regulate their survival under stress conditions (Yoshimura et al., 2011). Further investigations into the physiological function of Ccdc103 in CNS astrocytes would possibly provide information about its potential significance in astrocytic/neural stem cell motility and astrocytic activation for the formation of glial scar.
The expression and function of Fas/Fasl in glial cells has been investigated in several studies in vitro and the association of Fas activation with apoptosis is controversial for astrocytes. IFN-γ significantly upregulates microglial Fas/Fasl expression whereas cytokine treatment has been shown to have no effect on astrocytic Fas/Fasl in mice (Lee et al., 2000). However, treatment with IFN-γ upregulated Fas/Fasl in human embryonic astrocytes (Choi et al., 1999) and Fas upregulation promoted apoptosis in astrocytic cell lines (Saas et al., 1999). Fas induced apoptosis and Bcl2 downregulation that is enhanced by INF-γ treatment has been also shown in cultured microglia (Spanaus et al., 1998). In oligodendrocytes, Fas dependent cell death was demonstrated in MS that was described as atypical apoptosis (Thilenius et al., 1999) and a more recent study showed that IFN-γ and Fas induced oligodendrocytic cell death was mediated by Caspase 11 (Hisahara et al., 2001). Additionally, studies have shown that Fas/Fasl activation is associated with decreased levels of mitochondrial anti-apoptotic proteins such as Bcl2 and Bcl2a1a (Li et al., 1998; Werner et al., 2002). IFN-γ is also involved in the process of reactive astrogliosis (Balasingam et al., 1994). Microglial cytotoxicity against oligodendrocytes can be enhanced by treatment with IFN-γ (Peck et al., 1989). Fasl is a member of the Tumour Necrosis Factor (TNF) family and can be either a transmembrane protein or be produced by cells and exist in the extracellular space and is an inducer of apoptosis by interacting with the Fas receptor (Kearns et al., 2014). Interestingly, studies have shown that Fas/Fasl activation is associated with decreased levels of mitochondrial anti-apoptotic proteins such as Bcl2a1a (Li et al., 1998; Werner et al., 2002).

From the above, it can be hypothesized that in my study of the optic nerve, IFN-γ production by microglia may play a role in the oligodendrocyte cell death observed...
after application of VU590. These results are also consistent with oligodendrocyte lineage cells being vulnerable to oxidative stress which can trigger mitochondrial apoptosis (Sánchez-Gómez et al, 2003), and the analysis of VU590 treated optic nerves may have identified early changes in these cells. As described in section 5.2.1, significant oligodendrocytic cell death has already occurred after 1hr of incubation with VU590. It can be therefore assumed that the mRNA transcript levels that indicate there is ongoing caspase-dependent apoptosis are a reflection of the intracellular signalling in the remaining oligodendrocytes that have not yet perished. A time course of VU590 incubation of 30 mins, 90 mins and 120 mins incubation with the blocker could provide additional information about the cell death relevant genes altered that might aid in the formulation of a more complete cell death model for oligodendrocytic lineage cells.
5.4. Summary and Conclusions

The results provide novel evidence that $K_{ir}7.1$ are important for the survival of oligodendrocytes and this is greater when they are under hypoxic stress. The equivalent effect of VU590 and $Ba^{2+}$ indicates $K_{ir}7.1$ are as important as $K_{ir}4.1$, an essential channel for the survival of oligodendrocytes and their primary function of myelination (Neusch et al., 2000). In addition, the results suggest a unifying mechanism by which blockade of $K_{ir}4.1$ and $K_{ir}7.1$ and consequent oligodendrocyte depolarization compromises their integrity, via caspase-dependent pathways.
Chapter 6 - $K_{ir}^{7.1}$ effects on glial calcium: a role for store operated calcium entry
6.1. Introduction and Aims

In Chapter 5, I provided evidence that Kir7.1 are cytoprotective for oligodendrocytes. Furthermore, their blockade resulted in oligodendrocyte death in normoxic conditions and abrogated their loss in ischaemia. In contrast, blockade of Kir7.1 did not induce a loss of astrocytes, but there was evidence of a loss of their processes, which has been reported in ischaemia and may be an early indication of astroglial injury response (Salter and Fern, 2008). Calcium is a key mechanism mediating oligodendrocyte cell death in ischaemia (Fern et al., 2014) and in regulation of astrocyte plasticity and reactive astrogliosis (DeBock et al., 2014). Furthermore, cell death pathway analysis in Chapter 5 revealed that cell integrity maybe compromised via caspase-dependent pathways, which have been shown to mediate oligodendrocyte cell death as a result of Ca^{2+} dyshomeostasis (Alberdi et al., 2005). Kir have been shown to be critical for establishing the RMP of optic nerve glia (Bolton and Butt, 2006; Bolton et al., 2006; Bay and Butt, 2012), providing a potential mechanism by which Kir7.1 blockade could cause sustained depolarization and therefore unregulated Ca^{2+} influx through plasmalemmal voltage-operated Ca^{2+} channels (Ca_{v} channels). In addition, recent studies have identified that store operated calcium entry (SOCE) may play a key role in Ca^{2+} movements in astrocytes via TRPC1 channels (Verkhratsky and Parpura, 2014), although it is not known whether this is a widely expressed mechanism in the CNS. Oligodendrocytes have not been studied in this context, but SOCE has been demonstrated in OPCs via TRPC1 (Paez et al., 2011). The molecular identity of glial SOCE has not been identified unequivocally, but the balance of evidence indicates Orai is predominantly expressed in microglia, whereas astrocytes and oligodendrocytes rely more on TRP channels (Verkhratsky and Parpura, 2014). Studies in our lab have shown
that optic nerve glia display ‘calcium excitability’ involving a range of G-protein coupled receptors (GPCR) (Hamilton et al., 2008, 2010), and indicated that SOCE is an important mechanism for replenishment of intracellular stores following IP$_3$-mediated rises in [Ca$^{2+}$]$_i$ (James and Butt, 2001). The aims of this section are to determine whether blockade of Kv7.1 triggers a rise in glial [Ca$^{2+}$]$_i$ and examine the potential mechanisms mediating plasmalemmal Ca$^{2+}$ fluxes in optic nerve glia, using calcium imaging and qRT-PCR.
6.2. Results

6.2.1. Calcium imaging of glia in isolated intact optic nerves

Chapter 5 shows that blockade of Kᵢ7.1 channels has pathological implications for glia. Calcium dyshomeostasis is a primary physiological mechanism in glial pathology (Fern et al., 2014; DeBock et al., 2014), suggesting that the effects of Kᵢ7.1 blockade may be mediated by a rise in glial \([\text{Ca}^{2+}]_\text{i}\) which is normally tightly regulated at nanomolar levels. We therefore examined the potential role of Kᵢ7.1 in \(\text{Ca}^{2+}\) fluxes in optic nerve glia using Fluo4 calcium imaging, as described previously (Hamilton et al., 2008, 2010). In brief, P10-P14 nerves were isolated intact and loaded with Fluo-4/AM, a green-fluorescent \(\text{Ca}^{2+}\) dye that in its AM (acetoxymethylester) form is membrane permeable and, once taken up by the cell, cytosolic esterases remove the ester group and the dye is rendered membrane impermeable and thus remains intracellular (Garaschuk et al., 2006). Fluo4-loaded nerves were placed in a perfusion chamber under a Zeiss LSM5 Pascal Axioskop 2 confocal microscope and nerves imaged using a 20x/0.50 WPh2 Achromplan water immersion lens. Fluo4 was visualised by excitation at 488 nm and optical z-sections were obtained, typically 7-8 sections at 2–3 µm intervals. Changes in Fluo4 fluorescence were measured using the Zeiss LSM Image Examiner software (Zeiss, Germany) in glial cell bodies, selected as regions of interest (ROI). Figure 6.1 illustrates an experiment on a P14 optic nerve, where PLP-DsRed-positive oligodendrocytes are identified in z-stacks by excitation at 543 nm (Figure 6.1A), Fluo4 loading is detected at 488 nm (Figure 6.1B), and the overlay identifies Fluo4-loaded PLP-DsRed-positive oligodendrocytes and PLP-DsRed-negative cells (Figure 6.1C). Oligodendrocytes comprised 52% of Fluo4-loaded cells (n=250 cells from
and the bulk of the remaining 48% were astrocytes, based on their large cell bodies (Figure 6.1D), although a minor population of small diameter cells are likely to be OPCs and possibly microglia (Hamilton et al., 2008, 2009), but we did not attempt to analyse these separately. Subsequent continuous recordings of Fluo4 were performed using excitation at 488 nm alone, to increase the rate of image acquisition, and analysed using a grayscale (Figure 6.1B). It was not possible to perform equivalent experiments in GFAP-eGFP reporter mice to unequivocally identify astrocytes, because eGFP is detected at the same wavelength as Fluo4, and experiments using alternative red calcium dyes have not been successful in our hands. Furthermore, it was not possible to perform all experiments on PLP1-DsRed nerves, because of insufficient numbers, and so most experiments were performed on wild-type mice, unless otherwise stated.
Figure 6.1: Identification of oligodendrocytes for calcium imaging. Live cell confocal imaging of isolated intact optic nerves from P10-P14 PLP1-DsRed mice loaded with the calcium dye Fluo4. Images are stacks of 8x2µm z-sections and illustrate PLP1-DsRed positive oligodendrocytes detected at 543 nm (A, C) and Fluo-4 positive cells detected at 488 nm (B, C), in greyscale with representative examples of the ROIs (B) and in green in an overlay of 488 nm and 543 nm channels (C). Examples of Fluo4-loaded oligodendrocytes are indicated by arrowheads, while asterisks indicate PLP-DsRed-negative cells, which from their large somata are identified as astrocytes. (D) Mean percentage of oligodendrocyte ROIs (52.26%) versus astrocyte ROIs (47.74%); n=250 cells from 12 optic nerves.
6.2.2. \( K_{\text{ir}} \) blockade induces \([\text{Ca}^{2+}]_i\) fluctuations in optic nerve glia

A typical experiment is illustrated in Figure 6.2. The nerve was continuously bathed in \( \alpha \text{CSF} \) via a multitap system that allowed rapid turnover to solutions containing the agents of interest, such as ATP or VU590, as illustrated (Figure 6.2A, B). Fluo4 is extremely sensitive to changes in \([\text{Ca}^{2+}]_i\), but as a single wavelength dye it is not possible to measure the absolute \([\text{Ca}^{2+}]_i\), and it has a number of limitations compared to dual wavelength dyes, such as: cells take up the dye to a variable extent, and so generally it is not possible to compare absolute values of fluorescence between cells and between nerves; moreover, fluorescence may decline with time due to bleaching. In order to compensate for these limitations, in each nerve a ‘positive control’ was performed, whereby a 60s pulse of 100 \( \mu \text{M} \) ATP was administered at the beginning and the end of the experiment. In any given cell, the response to 100 \( \mu \text{M} \) ATP provides a measure of the maximal rise of glial \([\text{Ca}^{2+}]_i\), irrespective of the level of Fluo4 loading (Hamilton et al., 2008). Thus, quantification of the change in glial \([\text{Ca}^{2+}]_i\), can be measured as the change in fluorescence from baseline (\( \Delta F/F \)), and data expressed relative to the ATP response in the same cell, so that comparison can be made between treatments, independent of dye-concentration and absolute level of fluorescence. Furthermore, an ATP response is tested at the end of the experiment, to ensure the responsiveness and viability of the cell.

Hence, in a typical experiment for the analysis of VU590 illustrated in Figure 6.2:

1. fluorescence was measured continuously for \( \geq 60 \text{s} \) to provide a baseline and then 100 \( \mu \text{M} \) ATP was applied for 60s, followed by a recovery period of 10 min in \( \alpha \text{CSF} \),
during which fluorescence was measured for the first 120s and, to minimise the exposure of the nerve to the lasers, excitation and recording was paused (indicated by // in the figures);

(2) after 10 min recovery in aCSF, fluorescence recording was recommenced to provide a baseline in aCSF and then the agent of interest, in this case the K\textsubscript{ir}7.1 blocker VU590 (100 µM), was applied for ≥120s to completely replace the solution in the chamber; depending on the duration of exposure to the agent, perfusion and recording may be stopped, as in the example illustrated, where the optic nerve was incubated in VU590 for 60 min, during which time fluorescence was recorded intermittently, to minimise the exposure of the nerve to the lasers;

(3) at the end of the test period; there was a 10 min recovery in aCSF (indicated by // in the figures), and a final 60s pulse of ATP was applied to confirm the viability of the cells.

Notably, VU590 evoked fluctuations in glial [Ca\textsuperscript{2+}]\textsubscript{i} that are generally termed ‘calcium oscillations’ and appeared as spontaneous and transient rises in [Ca\textsuperscript{2+}]\textsubscript{i} (Volterra et al., 2014) (Figure 6.2A). In some cases, cell death was observed, which was often associated with an initial rise in [Ca\textsuperscript{2+}]\textsubscript{i} followed by a decay in fluorescence before the cell fluorescence disappeared (Figure 6.2B). However, not all cells responded to VU590 and to analyse this, 30 cells that displayed a robust response to ATP were randomly selected in a constant field of view (FOV) in each nerve (unless otherwise stated, this method of analysis was used throughout). During exposure to VU590 for 60mins, an analysis of \( n=140 \) cells from 5 nerves revealed that 23.5±3.1% of cells displayed oscillations while 76.5±3.1% did not display a clear-cut response (Figure 6.2C).
Figure 6.2: Calcium responses in optic nerve glia in response to Kir7.1 blockade. (A, B) Confocal images of Fluo4 fluorescence intensity (illustrated in rainbow false colour) and representative traces of individual glial cells illustrate the changes in fluorescence in response to 100 µM ATP (60s) and 100 µM VU590 (60 min). During exposure to VU590, glial cells exhibited oscillations in $[\text{Ca}^{2+}]_i$ (A), and in some cases cell death (B). (C) Histogram of the mean (±SEM) percentage of cells displaying calcium oscillations in response to VU590 (n=140 cells from 5 nerves).
6.2.3. Temporal effects of Kir7.1 blockade on glial [Ca\textsuperscript{2+}] fluctuations

In order to assess whether the effect of VU590 on optic nerve glia required long term administration, VU590 was applied for 10 min and the results compare to 60 min exposure (Figure 6.3). VU590 for 10 min evoked [Ca\textsuperscript{2+}] oscillations in 36.7±3.4% of cells (n=30 cells from 2 nerves; Figure 6.3A), greater but not significantly different to 23.5±3.1% of cells during 60 min exposure (n=140 cells from 5 nerves; Figure 6.3B; unpaired t-test). Administration of VU590 for 10 min did not result in cell death, indicating the detrimental effects of Kir7.1 block occurs over longer periods. From this point on, VU590 was routinely administered for 10 min to examine the mechanisms of the calcium oscillations.

Figure 6.3: Temporal effects of Kir7.1 blockade on glial [Ca\textsuperscript{2+}]. (A) Representative trace illustrating calcium oscillations during 10 min exposure to VU590. (B) Histogram of the mean (±SEM) percentage of cells displaying calcium oscillations during exposure to VU590 for 10 min (n=30 cells from 2 nerves) or 60 min (n=140 cells from 5 nerves). p>0.05, unpaired t-test.
6.2.4. Comparison of effects on glial $[Ca^{2+}]_i$ of VU590 and other $K_{ir}$ blockers

The primary glial $K_{ir}$ subtype $K_{ir}4.1$ is rapidly blocked by $Ba^{2+}$ ions (100 µM $BaCl_2$) in an irreversible or very slowly reversible manner, resulting in depolarization of astrocytes and oligodendrocytes in the optic nerve (Bolton and Butt, 2006; Bolton et al., 2006; Bay and Butt, 2012). In contrast, $K_{ir}7.1$ channels are insensitive to 100 µM $BaCl_2$ and are blocked by VU590, which does not act on $K_{ir}4.1$ (Lewis et al., 2009). VU590 also inhibits $K_{ir}1.1$, and this was tested for using the specific $K_{ir}1.1$ blocker VU591 (Bhave et al., 2011). Application of 100 µM $BaCl_2$ for 10 min had no effect on glial $[Ca^{2+}]_i$ (Figure 6.4A, B; n=73 cells from 5 nerves). Similarly, in the vast majority of cells (~90%) VU591 had no effect on glial $[Ca^{2+}]_i$ (Figure 6.4B), but $[Ca^{2+}]_i$ oscillations were observed in a small number of cells during administration of VU591 (Figure 6.4C). The proportion of cells responding to VU591 was 6.6±3.6%, significantly less than 36.7±3.4% of cells that exhibited $[Ca^{2+}]_i$ oscillations in VU590 (Figure 6.4D; unpaired t-test). Transcripts for $K_{ir}1.1$ were not detected in the optic nerve (Chapter 3) and VU591 did not affect glial integrity (Chapter 5), hence the significance of the calcium response in VU591 is unclear, but its infrequency suggests it could reflect low levels of $K_{ir}1.1$ expression in a subset of glia or a non-specific effect of VU591 on $K_{ir}$ channels (Bhave et al., 2011). Overall, the lack of effect of barium and minor effect of VU591 indicate the $[Ca^{2+}]_i$ fluctuations evoked by VU590 are a specific effect of $K_{ir}7.1$ blockade.
Figure 6.4: Comparison of effects of \( K_r \) blockers on glial \([Ca^{2+}]_i\). Fluo4 fluorescence was measured during administration of \( BaCl_2 \) to block \( K_r 4.1 \) and VU591 to block \( K_r 1.1 \). (A) \( BaCl_2 \) did not evoke a change in glial \([Ca^{2+}]_i \) (n=73 cells from 5 nerves). (B, C) In most cases, VU591 did not evoke a change in glial \([Ca^{2+}]_i \) (B), although a subset displayed \([Ca^{2+}]_i \) oscillations in response to VU591 (C). (D) Histogram of the mean (±SEM) percentage of cells displaying calcium oscillations in VU590 and VU591; **p<0.01, unpaired t-test.
6.2.5. Effects of K_{r}7.1 blockade on glial [Ca^{2+}]_{i} are independent of extracellular Ca^{2+}

Removal of extracellular Ca^{2+} (ZERO-[Ca^{2+}]_{o}) for 10 min prior to application of VU590 is illustrated in Figure 6.5. Perfusion of the optic nerve with ZERO-[Ca^{2+}]_{o} resulted in a slow decline in [Ca^{2+}]_{i} in all cells analysed, to a new stable baseline, and did not completely block the VU590-evoked oscillations in glial [Ca^{2+}]_{i} (Figure 6.5A), but the number of cells displaying VU590-evoked oscillations was significantly decreased (p<0.05, unpaired t-test) from 36.7±3.4% of cells in αCSF to 13.1±3.5% of cells in ZERO-[Ca^{2+}]_{o} (n=75 cells from 5 nerves, Figure 6.5B). The results indicate that the response of glial cells to VU590 is largely dependent on extracellular Ca^{2+}.

Figure 6.5: Effects of extracellular Ca^{2+} removal on VU590-evoked changes in glial [Ca^{2+}]_{i}. (A) Representative trace showing ZERO-[Ca^{2+}]_{o} resulted in a gradual decline in glial [Ca^{2+}]_{i} but this did not block VU590-evoked oscillations in all cells. (B) Histogram of the mean (±SEM) percentage of cells displaying calcium oscillations in VU590 in αCSF and VU590 in ZERO-[Ca^{2+}]_{o}; *p<0.05, unpaired t-test.
6.2.6. Role of Ca\textsubscript{v} channels in mediating glial [Ca\textsuperscript{2+}]\textsubscript{i} fluctuations

There is evidence that L-type Ca\textsubscript{v} channels may participate in astroglial [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (Parri and Crunelli, 2003). To examine this in the optic nerve, the effects of the potent L-type Ca\textsubscript{v} channel activator BayK 8644 and blocker Verapamil were examined (Figure 6.6). BayK did not elicit a [Ca\textsuperscript{2+}]\textsubscript{i} response in cells that displayed responses to ATP, when applied at 10 µM or 100 µM (n=50 cells from 2 nerves), indicating activation of Ca\textsubscript{v} channels alone does not induce Ca\textsuperscript{2+} oscillations (Figure 6.6A). In the presence of the L-type Ca\textsubscript{v} channel blocker verapamil (100 µM), most cells did not display VU590-evoked [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (Figure 6.6B), although they persisted in a small proportion of cells (Figure 6.6C). Overall, verapamil resulted in a decrease in the percentage of cells displaying VU590-evoked [Ca\textsuperscript{2+}]\textsubscript{i} oscillations from 36.7±3.4% in αCSF to 8.5±7.2% in verapamil (n=70 cells from 4 nerves), but this was not statistically significant (Figure 6.6D; p>0.05, unpaired t-test). The results suggest Ca\textsubscript{v} channels participate in [Ca\textsuperscript{2+}]\textsubscript{i} oscillations evoked in response to K\textsubscript{i}r7.1 blockade, but they were not abolished in verapamil, indicating other mechanisms are involved, such as release from intracellular stores and SOCE.
Figure 6.6: Role for Ca\textsubscript{v} channels in mediating glial [Ca\textsuperscript{2+}]i fluctuations. (A) The potent L-type Ca\textsubscript{v} channel activator BayK did not elicit a rise in glial [Ca\textsuperscript{2+}]i when applied at 10 µM or 100 µM. (B, C) Administration of the L-type Ca\textsubscript{v} channel blocker verapamil decreased the number of cells displaying oscillations in response to VU590 (B), although oscillations were still prominent in some cells (C). (D) Histogram of the mean (+SEM) percentage of cells displaying calcium oscillations in VU590 in αCSF and VU590 plus verapamil; p<0.05, unpaired t-test).
6.2.7. Evidence of SOCE in optic nerve glia

SOCE was activated using the SERCA pump blocker thapsigargin to induce depletion of intracellular Ca\(^{2+}\) stores (ER-Ca\(^{2+}\)-depletion), as previously reported in astroglia (Singaravelu et al., 2006; Moreno et al., 2012). Figure 6.7A shows a trace of the mean (+SEM) change in Fluo4 fluorescence (\(\Delta F/F\)) in response to thapsigargin, normalized to the ATP-response in the same cells (n=37 cells from 3 nerves). Administration of thapsigargin caused an initial rise in [Ca\(^{2+}\)]\(_i\), due to SOCE in response to SERCA blockade (Figure 6.7A, arrow). In ZERO-[Ca\(^{2+}\)]\(_o\), there is depletion of [Ca\(^{2+}\)]\(_i\), largely reflecting uptake of Ca\(^{2+}\) into intracellular stores in the absence of replenishment from the extracellular milieu (Figure 6.7A,B), and this [Ca\(^{2+}\)]\(_i\)-depletion was significantly reduced following blockade of uptake into ER by thapsigargin (Figure 6.7A,B; \(p<0.01\), unpaired t-test with Welch’s correction). When the solution bathing the nerve is returned to 2.24mM-[Ca\(^{2+}\)]\(_o\), there is a rapid replenishment of [Ca\(^{2+}\)]\(_i\), due to SOCE, which overshoots the baseline and is significantly greater in thapsigargin (Figure 6.7A,C, indicated by red arrow; \(p<0.05\), unpaired t-test). The SOCE inhibitor 2-aminoethoxydiphenylborane (2APB, 50 µM) (Singaravelu et al., 2006; Abdullaev et al., 2008) is shown to completely abolish the initial thapsigargin-induced rise in [Ca\(^{2+}\)]\(_i\) in optic nerve glia (Figure 6.8A, arrow). In addition, 2APB significantly blocked the thapsigargin-induced reduction in [Ca\(^{2+}\)]\(_i\)-depletion in ZERO-[Ca\(^{2+}\)]\(_o\) (Figure 6.8B; \(p<0.001\), unpaired t-test with Welch’s correction) and [Ca\(^{2+}\)]\(_i\)-replenishment was significantly less on return to 2mM-[Ca\(^{2+}\)]\(_o\) (Figure 6.8C; \(p<0.001\), unpaired t-test with Welch’s correction). Even though 50 µM 2APB effectively inhibits SOCE, it has been shown to stimulate SOCE at lower concentrations (Salmon and Ahluwalia, 2010), and administration of 10 µM 2APB in normal 2mM-[Ca\(^{2+}\)]\(_o\) αCSF evoked a transient rise in
Moreover, the transient \([\text{Ca}^{2+}]_i\) increase induced by 10 µM 2APB was not significantly different to that caused by 10 µM Thapsigargin (Figure 6.9B; \(p>0.05\), unpaired t-test with Welch's correction).

Together, these results provide evidence of SOCE in optic nerve glia (Singaravelu et al., 2006; Moreno et al., 2012).
Figure 6.7: SOCE after Ca\(^{2+}\) store depletion by thapsigargin. (A) Trace of the response to thapsigargin (2µM), each point and error bars representing the mean Fluo4 fluorescence ± SEM (n=37 cells from 3 nerves for thapsigargin and n=81 cells from 5 nerves for aCSF). Blockade of uptake into ER by thapsigargin causes an initial small accumulation of [Ca\(^{2+}\)]\(_i\) (black arrow), followed by [Ca\(^{2+}\)]\(_i\)–depletion in ZERO-[Ca\(^{2+}\)]\(_o\) in the absence of replenishment from the extracellular milieu. On return to 2mM-[Ca\(^{2+}\)]\(_o\) there is a rapid replenishment of [Ca\(^{2+}\)]\(_i\) due to SOCE (red arrow). (B, C) Histograms of mean (±SEM) [Ca\(^{2+}\)]\(_i\)-depletion and [Ca\(^{2+}\)]\(_i\)–replenishment in the presence and absence of thapsigargin (*p<0.05; **p<0.01, unpaired t-test with Welch's correction).
Figure 6.8: SOCE is blocked by 50 µM 2APB. SOCE induced by the SERCA blocker Thapsigargin (black) and in the presence of the SOCE blocker 2APB (red). (A) 2APB (50 µM) abolished the Thapsigargin-induced transient rise in [Ca^{2+}]_{i} (black arrow). (B) [Ca^{2+}]_{i}-depletion in ZERO-[Ca^{2+}]_{o} was significantly greater in 2APB (**p<0.001, unpaired t-test with Welch’s correction). (C) [Ca^{2+}]_{i}-replenishment on return to 2mM-[Ca^{2+}]_{o} was significantly decreased in 2APB and [Ca^{2+}]_{i} did not recover to the original baseline. (**p<0.001, unpaired t-test with Welch’s correction).
Figure 6.9: SOCE is stimulated by 10 µM 2APB. (A) Application of 10 µM 2APB induced a transient [Ca\textsuperscript{2+}] increase (arrow) (n=65 ROIs from 5 nerves). (B) The transient [Ca\textsuperscript{2+}] increase induced by 10 µM 2APB was not significantly different from the one caused by 10 µM Thapsigargin (p>0.05, unpaired t-test with Welch's correction).
6.2.8. Evidence of SOCE in the VU590-evoked glial [Ca^{2+}]_i oscillations

The effects of thapsigargin (10 µM) and 2APB (50 µM) on the VU590 (100 µM) response is illustrated in Figure 6.10. The VU590-evoked oscillations were inhibited in a significant number of cells (65.3±9.2%) in the presence of thapsigargin (p<0.05, unpaired t-test; n=116 cells from 5 nerves) (Figure 6.10Ai, Aii;), and thapsigargin also significantly decreased the response to ATP (Figure 6.10Aiii; p<0.001, paired t-test).

Inhibition of SOCE with 50 µM 2APB (Figure 6Bi, red line and symbols) also inhibited the VU590-evoked oscillations in a significant number of cells (77.0±6.3%; p<0.001, unpaired t-test) (Figure 6.10Bi inset and Figure 6.10Bii). Moreover, 50 µM 2APB significantly decreased the [Ca^{2+}]_i increase in response to ATP (Figure 6.10Biii, p<0.001, paired t-test; n=83 cells from 5 nerves). Notably, the glial response to ATP is primarily mediated by P2Y_1 GPCR (James and Butt, 2000) and it was significantly decreased by 2APB (Figure 6.9), supporting a role for SOCE in the IP_3-mediated rise in [Ca^{2+}]_i and indicating it is irreversibly or only slowly reversibly inhibited by 2APB. Taken together, the results provide further evidence to indicate a significant role for SOCE in glial response to ATP and [Ca^{2+}]_i oscillations evoked in response to Kir7.1 blockade.
Figure 6.10: Activation of SOCE by Kir7.1 blockade. (A) Thapsigargin (10 µM) significantly decreased the VU590 induced oscillations (inset Ai and mean data in Aii) and the ATP response (Aiii); n=116 cells from 5 optic nerves, ***p<0.001, unpaired t-test in Aii and paired t-test in Aiii). (B) Blockade of SOCE with 50µM 2APB (red trace) significantly decreased the VU590 induced oscillations (inset Bi, mean data in Bii); n = 83 cells from 5 optic nerves, ***p<0.001, unpaired t-test with Welch's Correction). (Biii) 50 µM 2APB significantly decreased the response to ATP (***p<0.001, paired t-test).
6.2.9. qRT-PCR identification of Ca\textsubscript{v} channels in the mouse optic nerve

Taken together, the effects of verapamil above provide functional evidence of Ca\textsubscript{v} channels in optic nerve glia. To inform on their possible molecular identity, I performed qRT-PCR, using the Mouse Neuronal Ion Channels RT\textsuperscript{2} Profiler™ qPCR array and a custom RT\textsuperscript{2} Profiler™ qPCR arrays (Sabiosciences, Qiagen). For technical reasons, and for efficient Fluo4 loading, the calcium imaging experiments were performed on P9-12 optic nerves. However, there is evidence that glial cells may lose calcium channels during maturation (Verkhratsky and Butt, 2013), which raises the question of whether studies in the postnatal tissue can be extrapolated to the adult. Therefore, developmental changes in the calcium channel profile was analysed in mice aged P9 (pre-myelination), P12 (active myelination) and P35 (post-myelination/mature), as described in Chapter 3. Results are expressed as relative gene expression using the $2^{-\Delta\Delta CT}$ method, normalised to Gapdh. Ca\textsubscript{v} channels are grouped functionally into the High Voltage Activated (HVA), including the N-type, P/Q-type and L-type channels, and Low Voltage Activated (LVA) T-type channels, all of which have been reported in glia (Verkhratsky and Butt, 2013). Ca\textsubscript{v} channels are structurally and functionally diverse due to their multimeric composition; the $\alpha$1 subunit that forms the ion conducting pore, and associated subunits ($\alpha$2$\delta$, $\beta$1-4, and $\gamma$), which modulate channel activity, gating and cellular localisation (Simms and Zamponi, 2014). Table 6.1 displays various Ca\textsubscript{v} channel subunits, the mRNA level of which was investigated in the optic nerve for the purposes of this project, providing both the gene and protein names, and the results are illustrated in Figure 6.11.
### α1 Subunits

<table>
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<th>Voltage</th>
<th>Type</th>
<th>Gene Name (Protein Name)</th>
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<td>P/Q Type</td>
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<tr>
<td></td>
<td>N Type</td>
<td>Cacna1b (Ca\textsubscript{v}2.2)</td>
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<tr>
<td></td>
<td>L Type</td>
<td>Cacna1c (Ca\textsubscript{v}1.2), Cacna1d (Ca\textsubscript{v}1.3)</td>
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<tr>
<td>Low Voltage Activated (LVA)</td>
<td>T Type</td>
<td>Cacna1g (Ca\textsubscript{v}3.1), Cacna1h (Ca\textsubscript{v}3.2), Cacna1i (Ca\textsubscript{v}3.3)</td>
</tr>
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</table>

### β Subunits – Gene Name (Protein Name)

- Cacnb1 (Ca\textsubscript{β}1)
- Cacnb2 (Ca\textsubscript{β}2)
- Cacnb3 (Ca\textsubscript{β}3)

### γ Subunits – Gene Name (Protein Name)

- Cacng2 ((Ca\textsubscript{γ}2 - Stargazin), Cacng4 (Ca\textsubscript{γ}4))

The Ca\textsubscript{v} channel transcripts with the most abundant expression levels in the mouse optic nerve are illustrated in Figure 6.11. Prominent expression of Ca\textsubscript{v}β\textsubscript{1} and Ca\textsubscript{v}γ\textsubscript{4} was observed, together with a wide range of α subunits, most prominently the P/Q-Type Ca\textsubscript{v}2.1, L-Type Ca\textsubscript{v}1.2 and T-Type Ca\textsubscript{v}3.1 (Figure 6.11); other subunits were expressed to a lesser extent and the T-Type LVA Ca\textsubscript{v}3.2 and Ca\textsubscript{v}3.3 were not detected. Generally, mature glial cells do not exhibit measurable Ca\textsubscript{v} currents, although Ca\textsubscript{v}1.2, Ca\textsubscript{v}2.1 and Ca\textsubscript{v}3.1 are detected in OPCs and cultured astrocytes (Verkhratsky and Steinhäuser, 2000; Paez et al., 2009). This is consistent with findings in the optic nerve, where there was a general developmental downregulation of Ca\textsubscript{v}, most markedly for Ca\textsubscript{v}β\textsubscript{3} and Ca\textsubscript{v}γ\textsubscript{4} (Figure 6.11; \( p < 0.01 \) and \( p < 0.001 \) respectively, ANOVA, followed by Bonferroni's Multiple Comparison test).
Figure 6.11: Expression of voltage-gated Ca$^{2+}$ channels (Ca$_v$) in mouse optic nerve. Tested for significance by ANOVA and post hoc Bonferroni's tests ($^* p<0.05$, $^{**} p<0.01$, $^{***} p<0.001$).
6.2.10. qRT-PCR identification of Orai/Stim

The calcium imaging results indicated glial ‘Ca\textsuperscript{2+} signalling’ involves Ca\textsuperscript{2+} release from the ER stores by activation of IP\textsubscript{3}R, and replenishment via store-operated calcium channels (SOCC). The best characterised SOCC are the Ca\textsuperscript{2+} release activated Ca\textsuperscript{2+} channels (CRAC) in which the Stromal Interaction Molecule (STIM) acts as the Ca\textsuperscript{2+} sensor in the ER, which upon Ca\textsuperscript{2+} depletion activates Ca\textsuperscript{2+} influx through the Orai proteins, which form the plasmalemmal CRAC channel pore (Frischauf et al., 2008).

Table 6.2 displays the SOCC channels, the mRNA level of which was investigated in the optic nerve by qRT-PCR, using a custom RT\textsuperscript{2} Profiler\textsuperscript{™} qPCR array (Sabiosciences, Qiagen), and expressed as relative gene expression normalised to Gapdh; gene and protein names are given and the results are illustrated in Figure 6.12.

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<tr>
<th>Type</th>
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<tbody>
<tr>
<td>IP\textsubscript{3} Receptors</td>
<td>Itpr1 (IP3R1), Itpr2 (IP3R2)</td>
</tr>
<tr>
<td>Calcium Release-Activated</td>
<td>Orai1*, Orai2*, Orai3*</td>
</tr>
<tr>
<td>Channels (CRAC)/Store Operated Ca\textsuperscript{2+} Channels (SOCC)</td>
<td>Stim1*, Stim2*</td>
</tr>
</tbody>
</table>

IP\textsubscript{3}-mediated Ca\textsuperscript{2+} signalling is very prominent in the mouse optic nerve (Butt, 2006, 2012), and this is supported by high expression of IP3R2 mRNA and to a lesser extent IP3R1 (Figure 6.12). Notably, Orai1 and Stim1/2 were robustly expressed and similar results were obtained in the cortex and cerebellum, although IP3R1 was more strongly expressed than IP3R2, and Orai1, Orai2 and Orai3 were expressed at similar levels (not illustrated). Interestingly, in the optic nerve Orai2 and Orai3 appeared to
increase and decrease with age, respectively, although these changes were not statistically significant (p>0.05, ANOVA). Orai1 however, was significantly downregulated between P9 and P35 (Figure 6.12; p<0.05, ANOVA, followed by Bonferroni's Multiple Comparison test). Orai1 and Stim1 have been detected in cortical astrocytes (Moreno et al., 2012), whereas microglia mostly express Orai3 (Ohana et al., 2009), but otherwise little is known about CRAC in glia. The results indicate Orai and Stim may be important for CRAC in the optic nerve.

Figure 6.12: Expression of channel protein transcripts involved in Ca^{2+} release from intracellular ER stores in mouse optic nerve. Tested for significance by ANOVA and post hoc Bonferroni's tests (*p<0.05).
6.2.11. qRT-PCR identification of TRP channels in the mouse optic nerve

The calcium imaging results indicated an important role for SOCE in optic nerve glia. TRP channels are the primary mechanisms of SOCE in astrocytes, and these were analysed by qRT-PCR, using the Mouse Neuronal Ion Channels RT²Profiler™ qPCR array and a custom RT² Profiler™ qPCR array (Sabiosciences, Qiagen), and results are expressed as relative gene expression normalised to Gapdh. The superfamily of TRP channels are non-selective cation channels that are expressed in many cell types and can be activated by various intra- and extracellular signals (Venkatachalam and Montell, 2007). TRP allow both Na\(^+\) and Ca\(^{2+}\) ions to enter the cell in both excitable and non-excitable tissues (Ramsey et al., 2006). TRP channels constitute at least seven subfamilies, TRPA (TRP-ankyrin), TRPC (TRP-canonical), TRPM (TRP-melastatin), TRPV (TRP-vanilloid), TRPP (TRP-polycystin), TRPML (TRP-mucolipin) and TRPN (TRP-NOMPC) which is not found in mammals. Table 6.3 displays the TRP channels, the mRNA level of which was investigated in the optic nerve for the purposes of this project and the results are illustrated in Figure 6.13.

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<th>Sub-Family</th>
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<td>TRPM (Melastatin)</td>
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<tr>
<td>TRPV (Vanilloid)</td>
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The most abundant mRNA transcripts in the mouse optic nerve were TRPM3, TRPM7 and the ‘canonical’ TRPC1 (Figure 6.14), which is the prominent TRP channel in astrocytes and OPCs/oligodendrocytes (Malarkey et al., 2008; Paez et al., 2011). In rank order, TRPM3 > TRPM7 > TRPC1 > TRPV2 > TRPC3 ≥ TRPM6, whereas the known astrocytic ‘Ankyrin’ TRPA1 was present, but less highly expressed, as were TRPV3, TRPV4, TRPC6 and TRPM2, whereas TRPV1 and TRPM8 were not detected. Developmentally, TRPV3 was barely detected in the P9 optic nerve and was significantly increased at P35 (p<0.05, ANOVA, followed by Bonferroni’s Multiple Comparison tests); there were significant changes in TRPA1, but this channel was barely detectable at any age.
Figure 6.13: Expression of TRP channel transcripts in mouse optic nerve. Tested for significance by ANOVA and post hoc Bonferroni's tests (*p<0.05).
6.2.12. **Key developmental changes in optic nerve calcium channels**

Volcano plot analysis (Sabiosciences Rt² Array Data Analysis) was used to identify significant changes in SOCE mechanisms (unrelated t-test, \( p<0.05 \)) developmentally regulated greater than 2-fold between P12 and P35 (Table 6.5). The results indicate the overall trend was for a developmental upregulation of SOCE.

**Table 6.4: Developmentally regulated Ca\(^{2+}\) channel transcripts in the mouse optic nerve.** Data was obtained by qRT-PCR analysis of 3 biological replicates (n=10 optic nerves per group) of acutely isolated optic nerves from mice aged P12 compared to P35. Statistical analysis was performed by volcano plot (Sabiosciences Rt² Array Data Analysis) (unrelated t-test, \( p<0.05; \text{FC}>2.0 \)).

<table>
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</table>
6.3. Discussion

Calcium is a key molecule in glial cell physiology and pathology, including ischaemia. In Chapter 5, I showed that blockade of $\text{Kir}_7.1$ with VU590 induced pathological changes in optic nerve glia, most notably oligodendrocyte death. Here, I show that blockade of $\text{Kir}_7.1$ evokes $[\text{Ca}^{2+}]_i$ fluctuations and cell death in optic nerve glia, using calcium imaging of Fluo4 -loaded isolated intact optic nerves. In addition, I provide pharmacological evidence that $\text{Kir}_7.1$ blockade triggered $\text{Ca}^{2+}$ influx involving plasmalemmal SOCE and possibly $\text{Ca}_v$ channels. qRT-PCR identified Orai/Stim transcripts at high levels and hence likely underlying the primary molecular basis of SOCE in optic nerve glia, together with TRP channels, with prominent expression of TRPM3, which is expressed by oligodendrocytes (Hoffmann et al., 2010), TRPC1, which have a demonstrated role in astrocyte SOCE (Parpura et al., 2011), and TRPM7, which has only been reported in microglia and was highly expressed in the optic nerve (Jiang et al., 2003). Expression of functional $\text{Ca}_v$ channels in mature glial cells is contradictory, but the qRT-PCR analysis of optic nerves reinforced calcium imaging evidence that optic nerve glia express L-type $\text{Ca}_v1.2$ (James and Butt, 2000; Hamilton et al., 2008), in addition to P/Q-Type $\text{Ca}_v2.1$ and T-Type $\text{Ca}_v3.1$. Thus, the results of this chapter identify a novel function for $\text{Kir}_7.1$ in $\text{Ca}^{2+}$ homeostasis in glial cells and provide SOCE as the potential molecular basis of $\text{Ca}^{2+}$ fluxes that mediate the pathological effects of $\text{Kir}_7.1$ blockade (Table 1). Notably, CRAC and TRP channels are developmentally upregulated, indicating a persistent role for SOCE in the adult.
Table 6.5: Main calcium ion channels expressed by optic nerve glia.

<table>
<thead>
<tr>
<th>Calcium Ion Channels</th>
<th>Astrocytes/Oligodendrocytes</th>
<th>OPC/Microglia</th>
<th>Developmental Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(_v)</td>
<td>Ca(_v)β3 &gt; Ca(_v)γ4 &gt;&gt; Ca(_v)3.1 &gt; Ca(_v)β1 &gt; Ca(_v)2.1 &gt; Ca(_v)1.2</td>
<td>Downregulated</td>
<td></td>
</tr>
<tr>
<td>TRP</td>
<td>TRP3 &gt; TRP7 &gt; TRPC1 &gt; TRPV2 &gt; TRPC3 &gt; TRPM6</td>
<td>TRPC1 and TRPM6 upregulated; TRPC3 downregulated</td>
<td></td>
</tr>
<tr>
<td>CRAC</td>
<td>ORAI1 = STIM1 ≥ STIM2 &gt; ORAI2</td>
<td>Not regulated</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6.14: Key developmental changes in optic nerve calcium ion channels. Data are presented as collective relative expression of the different ion channel families, to illustrate the proportional change in gene expression from P9 to adulthood.

6.3.1. Identification of cell types in calcium imaging experiments

A major limitation of the calcium imaging experiments in the isolated intact optic nerve is that it was not possible to identify the specific glial cell types from which recordings were made. Unfortunately, both the GFAP-eGFP and the SOX10-eGFP strains emit at 488 nm and could not be used with Fluo4, and loading of calcium dyes that emit in the red wavelength was not successful. Furthermore, although Fluo4 recordings were possible with PLP-DsRed reporter mouse, there were insufficient mice available during the course of the project. Nonetheless, analysis of Fluo4 loading in PLP1-DsRed reporter mice indicated that the dye was taken up into astrocytes and oligodendrocytes in equal proportions; therefore calcium imaging measurements will include both cell types. However, analysis of the calcium response to VU590 was
heterogeneous, with one-third of cells responding by Ca\(^{2+}\) oscillations, approximately one-third dying, and the remainder not displaying a clear response; the viability of the non-responders was confirmed by demonstrating a robust response to ATP. The cells that die in response to VU590 are oligodendrocytes (Chapter 5), and the identity of the oscillating-cells versus non-responders may reflect a difference between \(K_{ir7.1}\) and SOCC expression in oligodendrocytes and astrocytes. Immunolabelling for \(K_{ir7.1}\) was strongest in oligodendrocytes (Chapter 4) and TRPM3, which is the most highly expressed TRP channel transcript in the optic nerve, has not been reported in astrocytes but mediates Ca\(^{2+}\) influx in oligodendrocytes (Hoffmann et al., 2010). Indeed, blockade of \(K_{ir7.1}\) induced greatest changes in oligodendrocytes (Chapter 5). Further studies are required to resolve the cellular identity and the precise mechanisms of action of VU590, but overall the results are consistent with deregulation of Ca\(^{2+}\) involving TRPM3 being a primary mechanism in the loss of oligodendrocyte integrity following \(K_{ir7.1}\) blockade.

### 6.3.2. VU590 induced deregulation of glial \([\text{Ca}^{2+}]_i\): specificity of action on \(K_{ir7.1}\)

The results provide clear evidence that application of VU590 induces Ca\(^{2+}\) oscillations and cell death in a large proportion of cells. Notably, \(K_{ir4.1}\) blockade did not affect glial \([\text{Ca}^{2+}]_i\), whilst blockade of both \(K_{ir4.1}\) and \(K_{ir7.1}\) caused a loss of oligodendrocytes, indicating they have different physiological functions and induce oligodendrocyte cell death by different mechanisms. Barium has been shown to depolarise optic nerve astrocytes and oligodendrocytes (Bolton et al., 2006; Bolton and Butt, 2006; Bay and Butt, 2012), and blockade of \(K_{ir7.1}\) may have the same effect, but
only VU590 induced glial \([\text{Ca}^{2+}]_i\) -oscillations, indicating that depolarisation is not the primary cause. It is notable that the \(K_r1.1\) blocker VU591 also induced \(\text{Ca}^{2+}\)-oscillations in a very small population of glial cells. Although transcripts for \(K_r1.1\) were not detected in the optic nerve, splice variants of \(K_r1.1\) (also known as the renal outer medullary \(K^+\), ROMK, channel) have been described in the kidney and ROMK2 and is localized to the apical tips of a glial cells in mouse taste buds (Dvoryanchikov et al., 2009). Hence, it cannot be excluded at present that a small population of optic nerve glia may express \(K_r1.1\), but they have never been described in CNS glia, and it seems more likely that at 100 µM VU591 partially inhibits \(K_r7.1\). In addition, VU591 may partially inhibit \(K_v\) at high micromolar concentrations, by <10% (Bhave et al., 2011). The possible off-target effects of VU590 and VU591 on \(K_v1.1\) channels, which were the most highly expressed transcript in the optic nerve, warrants further investigation, using blockers such as tetraethylammonium ions (TEA) and agitoxin-2. Nevertheless, it seems unlikely that partial blockade of \(K_v1.1\) compared to complete blockade of \(K_r7.1\) is the mechanism of VU590-evoked glial \([\text{Ca}^{2+}]_i\) -oscillations. Overall, the effects of ZERO-\([\text{Ca}^{2+}]_o\), thapsigargin and 2APB indicate VU590 acts on \(K_r7.1\) to deregulate glial \([\text{Ca}^{2+}]_i\) homeostasis involving \(\text{Ca}^{2+}\) release from intracellular stores and SOCE.

### 6.3.3. A major role for SOCE in glial \([\text{Ca}^{2+}]_i\) homeostasis

A striking finding of this chapter was the dependence of glial \(\text{Ca}^{2+}\)homeostasis on an apparent continuous \(\text{Ca}^{2+}\) influx from the extracellular milieu, since removal of \([\text{Ca}^{2+}]_o\) resulted in a rapid and marked depletion of \([\text{Ca}^{2+}]_i\) within minutes. The effects of thapsigargin and 2APB indicate this is largely dependent on SOCE and uptake into ER stores via SERCA pumps, as summarised in Figure 6.16. Moreover, blockade of SOCE
with 2APB resulted in an irreversible deregulation of \([\text{Ca}^{2+}]_i\), and future experiments should determine whether this effects glial integrity.

The mechanisms of SOCE have been most extensively studied in astrocytes than other types of glia (Kirischuk et al., 1996; Golovina, 2005; Malarkey et al., 2008; Pivneva et al., 2008; Lo et al., 2002; Singaravelu et al., 2006). Stim1 and Orai1 expression and function has been demonstrated in rat cortical astrocytes \textit{in vitro} (Moreno et al., 2012), and a role for TRP channels and especially TRPC1 and TRPC3 in SOCE has also been substantiated (Pizzo et al., 2001; Grimaldi et al., 2003). In contrast, the only experimental evidence of oligodendroglial SOCE is for TRPM3 (Hoffmann et al., 2010) and association of the Golli protein with Stim1 and TRPC1 in OPCs (Paez et al., 2007; Walsh et al., 2010). The evidence provided here in the optic nerve indicates functional expression of SOCE has a widespread importance in astrocytes and oligodendrocytes.
Figure 6.15: Diagram summarising the data showing a key role for SOCE in glial [Ca^{2+}]_i homeostasis.
My finding that thapsigargin induced an increase of \([\text{Ca}^{2+}]_i\) in optic nerve glia is in agreement with literature reports that the blockade of SERCA results in ER \(\text{Ca}^{2+}\) store depletion which causes an influx of \(\text{Ca}^{2+}\) into the cytosol. Furthermore, I showed that the \([\text{Ca}^{2+}]_i\) increase caused by thapsigargin was abolished by 50 µM 2APB, which is a potent inhibitor of ORAI/STIM CRAC and TRPC channels (Prakriya and Lewis, 2001; Trebak et al., 2002; Peinelt et al., 2008), all of which I show are expressed in the optic nerve. In addition, 2APB has been found to cause inhibition of IP₃R and inhibit \(\text{Ca}^{2+}\) efflux from the ER lumen into the cytoplasm (DeHaven et al., 2005). My finding that 2APB caused a more significant depletion of \([\text{Ca}^{2+}]_i\) in combination with thapsigargin is in agreement with previous experiments performed in rat parotid acinar cells (Soltoff and Lannon, 2013). Soltoff and Lannon also demonstrated that after the initial \([\text{Ca}^{2+}]_i\) increase that followed re-introduction of \(\text{Ca}^{2+}\) to the extracellular solution, inhibitory concentrations of 2APB induced an immediate decrease of \([\text{Ca}^{2+}]_i\), whereas in the cells that were only treated with 2 µM of thapsigargin, \([\text{Ca}^{2+}]_i\) levels returned to baseline but decreased no further. My results demonstrated the same action for 2APB in optic nerve glia. In contrast, <10 µM 2APB has been shown to rapidly activate CRAC channels (DeHaven et al., 2005). Moreover, Salmon and Ahluwalia (2010) demonstrated that 10µM 2APB consistently stimulated \(\text{Ca}^{2+}\) influx which was even more significant in combination with thapsigargin. In agreement with their findings, my data demonstrated a transient increase of \([\text{Ca}^{2+}]_i\) following the application of 10 µM 2APB, thus indicating that at this concentration \(\text{Ca}^{2+}\) influx into the cytosol is induced via activation of SOCE.
In addition to SOCE and uptake into ER via SERCA pumps, calcium homeostasis involves multiple mechanisms, including Ca\(^{2+}\) extrusion by Na\(^+\)/Ca\(^{2+}\)-exchangers (NCX) and ATP-dependent plasma membrane Ca\(^{2+}\) pumps (PMCA), uptake by mitochondria, mainly via the mitochondrial Ca\(^{2+}\) uniporter (MCU) and mitochondrial NCX (Verkhratsky and Butt, 2013). PMCA may be main contributors to glial [Ca\(^{2+}\)]\(_i\) homeostasis (Nett and Deitmer, 1998) and have been described in astrocytes (Fresu et al., 1999) and oligodendrocytes (Kirischuk et al., 1995). Plasmalemmal NCX are regarded as main contributors to clearing large increases in [Ca\(^{2+}\)]\(_i\), such as occur following receptor-mediated transients (Deitmer et al., 1998), and their expression and function has been clearly defined in glial cells (Goldman et al., 1994; Alberdi et al., 2002 and 2005). In astrocytes, NCX are also up-regulated in hypoxic conditions (Takuma et al., 2013), while oligodendrocytic NCX are believed to be involved in cell differentiation and myelin production (Boscia et al., 2013).

Block of K\(_i\)7.1 may also cause glial depolarisation, but as discussed above this is unlikely to be a mechanism of action of VU590, since barium is known to massively depolarise optic nerve glia and did not cause Ca\(^{2+}\) influx. Another example of the K\(_i\)/[Ca\(^{2+}\)]\(_i\) relationship was demonstrated when over-expression of K\(_i\) channels resulted in reduced calcium spikes in neurons of the developing nervous system (Rosenberg and Spitzer 2011). In addition, K\(_i\)4.1 channels can be permeable to Ca\(^{2+}\) (Härtel et al., 2007) and if the same is true for K\(_i\)7.1, then the decrease in Ca\(^{2+}\) influx following their blockade would trigger Ca\(^{2+}\) release from intracellular stores and activate SOCE to maintain [Ca\(^{2+}\)]\(_i\). Interestingly, K\(_i\)7.1 blockade did cause a decrease in
[Ca$^{2+}$], in some cells (not illustrated), which paradoxically caused Ca$^{2+}$ overload and death, termed the “Ca$^{2+}$ paradox” of ischaemia-reperfusion (Wei et al., 2007). In addition, verapamil depressed VU590-evoked [Ca$^{2+}$]$_i$ oscillations in optic nerve glia, consistent with a role for L-type Ca$_v$ channels (Hoehn et al., 1993). However, verapamil also blocks T-type Ca$_v$ channels at micromolar concentrations (Begson et al., 2011), implicating both L-type and T-type Ca$_v$ channels in the VU590-evoked [Ca$^{2+}$]$_i$ oscillations, likely to be Ca$_v$2.1 and Ca$_v$3.1 based on the qRT-PCR. Interestingly, Ca$_v$3.1 are open at more negative membrane potentials and it would be interesting to further investigate their involvement in glial oscillations.

6.3.4. qRT-PCR analysis of Ca$_v$ in optic nerve glia

The correlation between qRT-PCR and calcium imaging data provides evidence for functional expression of Ca$_v$ channels, CRAC and TRP channels in astrocytes and oligodendrocytes. Moreover, our arrays included all of the main Ca$_v$ channels, TRP channels and CRAC shown to be expressed by glia (Verkhratsky and Butt, 2013). On this basis, the relative expression levels identify the key channels expressed by optic nerve astrocytes and oligodendrocytes, and the low expression of some transcripts suggests either low expression in macroglia (e.g. Ca$_v$2.1), or possibly significant expression in minor populations of OPC and microglia, such as Ca$_v$1.2 which is constitutively expressed by OPCs (Table 6.6). In addition, the results indicate a general developmental downregulation of Ca$_v$ channels, whereas CRAC and TRP channels are developmentally upregulated, supporting a key role for these channels in mediating Ca$^{2+}$ fluxes in mature glia (Figure 6.15).
The Caβ3 and Caβ4 subunits were strongly expressed in the optic nerve, although the α subunits were expressed at lower levels, with a rank order of Ca3.1 > Ca2.1 > Ca1.2. Astrocytes are not considered to express CaV channels in situ (Parpura et al., 2011), and although oligodendrocytes express CaV channels during myelination (Paez et al., 2009), there is little evidence for functional CaV channels in differentiated oligodendrocytes. Taken together with the calcium imaging data, the low transcript levels of CaV and their marked developmental downregulation are suggestive of OPCs. CaV channels currents have been demonstrated in OPCs in vitro and in situ (Paez et al., 2009a), and they regulate important developmental processes in OPCs, such as cell migration and proliferation (Paez et al., 2010; Fulton et al., 2010). Nonetheless, CaV channels currents and protein expression for CaV2.1 (P/Q Type), CaV2.2 (N-Type) and CaV1.2 (L-Type) have been reported in astrocytes and oligodendrocytes (Parri and Crunelli, 2003; Fern, 1998; Akopian et al., 1996; Paez et al., 2009), and their expression in optic nerve astrocytes and oligodendrocytes is supported by the effect of verapamil in our calcium imaging experiments. CaV channels may be highly localized to process terminals, and therefore difficult to detect unless they are activated by mechanisms such as Kir7.1 inhibition.

In addition, Caγ subunits, including Caγ4, which was detected in the optic nerve, are type I transmembrane AMPA receptor (AMPA-R) regulatory proteins (TARPs) and regulate trafficking and gating of AMPA-R (Körber et al., 2007). Interestingly, astrocytes, oligodendrocytes and OPCs express all four types of AMPA-R subunits (GluA1-4) and their activation causes raised intracellular Ca2+ (Hamilton et al., 2008,
2009), and makes OPCs and oligodendrocytes vulnerable to hypoxic-ischaemic damage (Salter and Fern, 2005; Zonouzi et al., 2011). The relatively high expression of Ca\textsubscript{v}4 in the postnatal nerve and its subsequent downregulation may be related to changes in glial AMPA-R expression. Furthermore, optic nerve axons have been shown to express Ca\textsubscript{v} channels and these are able to support axonal conduction in premyelinated axons, involving L-type Ca\textsubscript{v} prior to P5 and P/Q-type Ca\textsubscript{v} up to P12 (Alix et al., 2008). In my study, L-Type channels (Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3) were downregulated only after P12, and P/Q-type channels (Ca\textsubscript{v}2.1) was not significantly downregulated, supporting the conclusion that they predominantly reflect glial expression rather than axonal.

6.3.5. qRT-PCR evidence of CRAC in optic nerve glia

In physiological conditions, SOCE play important roles in the sustained phase of the [Ca\textsuperscript{2+}]\textsubscript{i} signal observed following stimulation of GPCR in glial cells (Verkhratsky and Parpura, 2014). The results of this chapter demonstrate a role for SOCE in ATP-mediated calcium signalling in optic nerve glia, which has previously been shown to be primarily from IP\textsubscript{3}-mediated release of Ca\textsuperscript{2+} from intracellular ER stores (James and Butt, 2001; Hamilton et al., 2008). Replenishment of ER stores depends on SOCE involving CRAC comprising ORAI, which form the plasmalemmal Ca\textsuperscript{2+} channels, and STIM proteins, which act as the sensors of Ca\textsuperscript{2+} depletion (Soboloff et al., 2012). More recently, the canonical sub-family of TRP channels (TRPC) is reported to be implicated in the construction and function of SOC channels (Huang et al., 2006; Cahalan, 2009). In this chapter, the qRT-PCR identified predominance of IP3R2, Orai1/2, Stim1/2 and a wide range of TRP channels in the mouse optic nerve, and the calcium imaging supports a role for these channels in regulating Ca\textsuperscript{2+} fluxes in astrocytes and
oligodendrocytes. The high relative expression of IP3R2 is consistent with it being the predominant astrocytic IP3R involved in calcium signalling in response to GPCR activation (Fiacco and McCarthy, 2006; Kanno and Nishizaki, 2012; Zhao et al., 2000). IP3-mediated Ca2+ signalling is also present in OPC, oligodendrocytes and microglia, although the IP3R involved are unresolved (Butt, 2006; Kettenmann et al., 2011). ORAI are most associated with microglia (Verkhratsky and Parpura, 2014), and the main microglial ORAI is Orai3 (Ohana et al., 2009), transcript levels for which were the lowest ranked in the optic nerve. Expression has also been demonstrated in astrocytes (Akita and Okada, 2011; Motiani et al., 2013), where Orai1/Stim1 may predominate (Moreno et al., 2012). The levels of Orai1/2 and Stim1/2 mRNAs detected in the mouse optic nerve are comparable to those for IP3R, and together with the calcium imaging data demonstrate a previously unrecognised important role of CRAC in optic nerve astrocytes and oligodendrocytes. It is possible that OPCs also express CRAC, since Golli, a member of the MBP family of proteins, has been shown to regulate SOCE in OPCs (Paez et al., 2007) and to bind Orai1/Stim1 complexes to negatively regulate SOCE (Walsh et al., 2010).

6.3.6. qRT-PCR evidence of TRP channels in optic nerve glia

TRP channels are non-selective cation channels and important routes of Ca2+ and Na+ influx (Bon and Beech, 2013). In astrocytes, TRPC1 channels appear to primarily mediate SOCE (Golovina, 2005), which is consistent with the calcium imaging data of this chapter and the qRT-PCR showing TRPC1 transcript being highly expressed in the adult nerve. The transcript level of TRPV2 was also high, supporting a recent study showing TRPV2 functional expression in cerebellar astrocytes (Shibasaki et al., 2013).
In addition, TRPM3 was the highest transcript detected and is of interest because in the brain they are specifically localised to oligodendrocytes (Hoffmann et al., 2010), indicating that TRPM3 function as a Ca\(^{2+}\)-permeable channel in optic nerve oligodendrocytes. TRPM7 was the second highest transcript level and, to our knowledge, has not been described in astrocytes or oligodendrocytes and has only been reported in microglia (Jiang et al., 2003). However, the level of expression of TRPM7 seems unlikely to reflect the minor population of microglia, hence future experiments should determine whether it is also expressed in astrocytes and oligodendrocytes. In hippocampal neurons, TRPM7 channels detect decreases in \([\text{Ca}^{2+}]_o\) that paradoxically causes \(\text{Ca}^{2+}\) overload and death, termed the “Ca\(^{2+}\) paradox” of ischaemia-reperfusion (Wei et al., 2007). A similar function for TRPM7 in oligodendrocytes may be important in their loss in OGD and following blockade of Kir7.1.

The other main TRP channels detected were TRPM6 and TRPC3, which were respectively up- and downregulated with development. Previous studies have shown extremely low expression of TRPM6 in various CNS regions, but did not include the optic nerve (Lee et al., 2012). Notably, TRPM6 is central to Mg\(^{2+}\) homeostasis (Runnels, 2011), and Mg\(^{2+}\) deficiency results in hypomyelination in the optic nerve (Gong et al., 2003). Our finding that TRPM6 mRNA is prominent in the optic nerve and developmentally upregulated is therefore of some interest, since it suggests oligodendroglial TRPM6 may have a previously unrecognised Mg\(^{2+}\) regulatory function during myelination, which requires further investigation. In the case of TRPC3, positive
mRNA and protein expression for TRPC3 has been shown in cultured mouse astrocytes (Akita and Okada, 2011), but immunolabelling for TRPC3 in the brain was specific to oligodendrocytes and labelling intensity decreased with age (Fusco et al., 2004). This is consistent with my findings, and suggests a developmental role for TRPC3 in optic nerve oligodendrocytes.

Although TRP channels are prominent in microglia and play an important role in SOCE (Verkhratsky and Parpura, 2014), of the main TRP channels reported in microglia, only TRPM7 was detected at significant levels in the optic nerve, and TRPV1, which is reported to be found solely in microglia and no other glia, was barely detectable in the optic nerve. TRPV2 has also been shown to increase phagocytic activity in microglia, but may be less important in resting microglia (Hassan et al., 2014).
6.4. Summary and Conclusions

The overall aim of this section was to examine the possibility that Kir7.1 have a role in glial calcium homeostasis. The results of calcium imaging and qRT-PCR indicate blockade of Kir7.1 induces SOCE involving ORAI1/STIM1 CRAC and TRP channels, principally TRPM3 in oligodendrocytes and TRPC1 in astrocytes (Figure 6.16). In addition, high expression of TRPM7 suggests a major role in microglia or unresolved in optic nerve macroglia. The results also indicate a lesser functional role for Ca\textsubscript{v}, comprising predominantly Ca\textsubscript{v.3.1}/β3/γ4 subunits that may be developmentally downregulated.

Figure 6.16: Profile of the major calcium ion channels expressed by optic nerve glia and their role in deregulation of [Ca\textsuperscript{2+}], and oligodendrocyte cell death induced by Kir7.1 blockade.
Chapter 7 - General Discussion
7.1. Summary of Key Findings

The overall aim of this thesis was to provide an insight into the ion channel expression and function in optic nerve glia, with a focus on the inward rectifier subtype Kir7.1. The optic nerve is widely used as a model of white matter tissue for studying glial cell development and function, because it comprises mainly astrocytes and oligodendrocytes, whose functions are to support and myelinate the axons of retinal ganglion cells (Butt et al., 2004). The key findings of the optic nerve ion channel profile are summarised in Figure 7.1. It is evident from the results in Chapter 3 that potassium channels are abundantly expressed in glia and the data indicated that optic nerve astrocytes and oligodendrocytes are dominated by Kv1.1, Kv4.1 and Kir7.1. A key novel finding was the high expression of Kir7.1 and Chapter 4 demonstrated this is major feature of oligodendrocytes in the optic nerve, and astrocytes throughout the brain. The physiological function of Kir7.1 in glia is unresolved, but Chapter 5 identified Kir7.1 as critical for maintaining the integrity of oligodendrocytes in the optic nerve and their blockade results in pathological loss of oligodendrocytes under normal conditions and in ischaemia models/hypoxia. Chapter 6 identified SOCE as a potential mechanism for the pathological effect of Kir7.1 blockade by causing deregulation of calcium homeostasis in optic nerve glia. In addition, Chapter 6 identified important functions for CRAC and TRP channels in mediating SOCE and what has been termed ‘calcium excitability’ in optic nerve glia. This study has provided a new insight into the ion channel expression profile and function in optic nerve glia, which is relevant to fundamental functioning of the CNS and to its pathology, including ischaemia, stroke and demyelination.
Figure 7.1: Profile of the major ion channels expressed by optic nerve glia.

7.2. Antibodies and VU590 as tools for studying Kir7.1

Kir7.1 expression and function were examined using commercially available antibodies and the small molecule inhibitor of Kir7.1, VU590. A Kir7.1 knock out animal model would be invaluable for validating the antiKir7.1 antibody and VU590, and elucidating the significance of Kir7.1 in neuronal and glial function and integrity. Notwithstanding this, these tools have been validated in homologous cell systems, using transfection gain- and loss-of-function techniques (Lewis et al., 2009; Bhave et al., 2011; McCloskey et al., 2014). Hence, it is reasonable to conclude that the results of this thesis represented an accurate measurement of Kir7.1 expression and function. Nonetheless, the specificity of VU590 for Kir7.1 remains an important consideration. At concentrations of 10 µM in vitro, VU590 inhibits Kir7.1 and Kir1.1, but not other Kir subtypes, and is largely ineffective on Kv (Lewis et al., 2009). Here however, VU590 was used at 100 µM, on the basis that the pial surface of the nerve acts as a permeability...
barrier and to be effective in the intact optic nerve, pharmacological agonists and antagonists are applied at 10-times the concentration used \textit{in vitro} (Bolton and Butt, 2005), and this concentration has been shown to fully block $K_{ir}7.1$ (Rouhier et al., 2014). Potential off-target effects of VU590 would most likely be due to $K_{ir}4.1$ and $K_{v}1.1$, the most highly expressed ion channels in glia alongside $K_{ir}7.1$. It seems likely that the marked effects of VU590 on optic nerve glia reflect its complete block of $K_{ir}7.1$, rather than partial inhibition of $K_{ir}4.1$ or $K_{v}1.1$. This conclusion is supported by the differences in action of 100 $\mu$M $Ba^{2+}$ ($BaCl_2$), which completely blocks $K_{ir}4.1$, but at this concentration does not inhibit $K_{ir}7.1$ channels. Together with electrophysiological studies performed by various groups (Lewis et al., 2009; McCloskey et al., 2014), the results of this thesis provide novel evidence of functional expression of $K_{ir}7.1$ in astrocytes and oligodendrocytes.

7.3. \textbf{Glia ion channel toolbox in the mouse optic nerve}

An important feature of glial cells is that their ion channel expression is regionally diverse and developmentally regulated (Verkhratsky and Butt, 2013). The results here demonstrated that optic nerve glia express diverse ion channels, with a developmental trend of increased $K^{+}$ channel expression and a decrease in $Na^{+}$ and $Ca^{2+}$ channels (Figure 7.2). Developmental analysis of the ion channels, grouped according to class, provides further insight into the primary functional importance of delayed rectifier $K_{v}$ and inward rectifier $K_{ir}$ channels, the mRNA levels of which together comprise $>50\%$ of the transcripts in the adult optic nerve (Figure 7.3). This study identified highest expression levels of mRNA encoding for $K_{v}1.1$, followed by $K_{ir}4.1$ and $K_{ir}7.1$, which indicates these channels are the molecular basis of the strongly negative RMP that
underpins glial physiological functions. The expression of $K_{ir}7.1$ protein was confirmed in astrocytes and oligodendrocytes, and immunolabelling with $\text{Na}^+$/K$^+$-ATPase supported plasmalemmal expression of $K_{ir}7.1$ with $K_{ir}4.1$. Together with evidence that $K_{ir}7.1$ is potentially as important for oligodendrocyte integrity as $K_{ir}4.1$, this thesis demonstrates a previously unrecognised important function of $K_{ir}7.1$ in glia, in particular oligodendrocytes. Moreover, the combined qRT-PCR and calcium imaging approach identified CRAC and TRP channels that mediate SOCE in glial $\text{Ca}^{2+}$ signalling, and demonstrated that they persist in the adult nerve. These results have important implications for glial physiology and pathology.

Figure 7.2: Key developmental changes in optic nerve ion channels. Data are presented as collective relative expression of the different ion channel families, to illustrate the proportional change in gene expression from P9 to adulthood.
Figure 7.3: Developmental expression (percentile) of ion channels in the optic nerve grouped by class. Data are presented as collective relative expression of the different ion channel classes, to illustrate the proportional expression within the ages studied.
7.4. **Kir7.1 expression and function in glia**

The main novel finding of this thesis was the high expression levels of Kir7.1 detected in glia. These channels have been mainly studied in epithelial cells of the RPE and kidney (Pattnaik et al., 2013; Suzuki et al., 2003), and in the brain were considered to be restricted to the choroid plexus, Purkinje neurones of the cerebellum and pyramidal neurones of the hippocampus (Krapivinsky et al., 1998; Döring et al., 1998). The highest glial expression of Kir7.1 was found in optic nerve oligodendrocytes. Oligodendroglial expression was variable in the rest of the mouse brain, while astrocytes showed a fairly uniform and ubiquitous expression, notably in their perivascular endfeet. The specific localisation of Kir7.1 on perivascular astrocytic endfeet is consistent with a function in ion and water homeostasis. The co-expression of Kir7.1 with Kir4.1 on the cell membrane is interesting, since it implies that they have different roles in K⁺ transport, as they do in epithelial cells where they are also co-expressed, such as in the kidney (Zhang et al., 2008). Kir4.1 is known to have a major function in setting the glial RMP and K⁺ spatial buffering, which is the movement of K⁺ away from areas of high neuronal activity to areas of low activity, such as blood vessels (Kalsi and Butt, 2004). At physiological [K⁺]o, Kir7.1 have an almost linear I-V relationship between -100 and 0 mV, indicating they are particularly well-suited to mediate K⁺ efflux. Notably, a recent study in our lab showed that in optic nerve, glial K⁺ spatial buffering via Ba²⁺-sensitive Kir4.1 was only significant during large shifts in [K⁺]o in response to intense neuronal activity and pathology (Bay and Butt, 2012). In contrast, K⁺ uptake during physiological neuronal activity and subsequent K⁺ efflux for redistribution to axons was largely insensitive to Ba²⁺, suggestive of Kir7.1. Moreover, Kir7.1 are essential for K⁺ recycling to maintain the activity of the Na⁺/K⁺-pumps, which
are a primary mechanism for K⁺ uptake during neuronal activity (Bay and Butt, 2012; Ransom et al., 2000). In addition, the Na⁺/K⁺-pumps establish and maintain the Na⁺ concentration gradient across the cell membrane that drives multiple plasmalemml transport mechanisms in glia, most notably glutamate uptake, which is a primary function of astrocytes and is essential for glutamatergic neuronal activity (Verkhratsky and Butt, 2013). Notably, astroglial glutamate uptake protects oligodendrocytes from glutamate-mediated cytotoxicity, and the results of Chapter 5 implied a protective role for Kir7.1 in oligodendrocytes (see below). These studies indicated Kir4.1 and Kir7.1 play distinct functional roles in glia that are essential for maintaining white matter function and integrity.

Figure 7.4: Scheme of mechanisms contributing to K⁺ transport in glia. Adapted from studies in the proximal kidney tubule (Derst et al., 2001).
7.5. **Kir7.1 expression and function in neurones**

The identification of Kir7.1 in cortical neurones is novel. The localisation of Kir7.1 on the primary dendrites of cortical neurones and the dendritic tree of Purkinje neurones, as well as the somatal expression in these cell types, implicates the channel in the regulation of postsynaptic excitability. The "classical" Kir2.x channels are also mainly found in neuronal cell somata and dendrites and their blockade resulted in membrane depolarisation and action potential initiation in cortical neurones, as well as enhancement of dendritic excitability in striatopallidal neurones (Day et al., 2005; Shen et al., 2007). It is interesting though that no obvious neuronal abnormalities are reported in Kir2.1 and Kir2.2 knockout mice (Zaritsky et al., 2000), which was postulated to be due to compensation by other Kir channels in these mutant mice (Prüss et al., 2005), which my results show includes Kir7.1.

7.6. **Kir7.1 and Kir4.1 are essential for maintaining oligodendrocyte integrity**

Based on the hypothesis that Kir7.1 is involved in the maintenance of ion homeostasis in glial cells, which is essential for glial integrity under normal physiological conditions and in particular during stress (Stys, 2004; Tekkök et al., 2007), the next step was to examine the effects of Kir7.1 inhibition on optic nerve glial survival by treating the nerves with the specific Kir7.1 blocker VU590. Kir7.1 blockade induced oligodendrocytic cell death in control conditions via activation of caspase-dependent apoptotic pathways, and it severely exacerbated cell death in OGD. Moreover, inhibition of Kir4.1 with Ba^{2+} was also detrimental for oligodendroglial survival during
hypoxic stress, although the effect was less pronounced than with VU590. These results demonstrated that both Kir4.1 and Kir7.1 sustain oligodendrocyte integrity. Kir4.1 is essential for maintaining the RMP of oligodendrocytes (Bolton and Butt, 2006; Neusch et al., 2000), and Kir7.1 may have a similar function. Hence, severe depolarisation may be the mechanism of oligodendrocyte demise following the block of these channels, which could be tested using high [K+]o to directly depolarise glia. Cell death was observed during live cell calcium imaging, and the qRT-PCR results provided evidence of functional expression of Cav channels; the above are consistent with membrane depolarisation being involved in the loss of oligodendrocytes during OGN, which could be tested using verapamil and other Cav channel blockers. In addition, a classical picture of calcium-mediated caspase activation and excitotoxic cell death is illustrated in Figure 7.5, which involves astroglial depolarisation causing a reversal of glutamate uptake (see above), with a concomitant glutamate build up in the extracellular space activating oligodendroglial AMPA-R and NMDA-R, resulting in a cytotoxic rise in [Ca²⁺]i (Benerroch, Neurology 2009, 1779-1785). This could be examined using AMPA-R and NMDA-R inhibitors to protect oligodendrocytes from VU590-induced death. Other mechanisms of oligodendroglial demise include reversal of NCX, which normally extrudes Ca²⁺ from the cell, but a rise in [Na⁺]i induced by depolarisation and run-down of Na⁺/K⁺-pumps causes reversal of NCX and a cytotoxic influx of Ca²⁺, which could be tested in future experiments using ouabain to block Na⁺/K⁺-pumps and NCX inhibitors, such as KB-R7943 and SEA0400 (Akabas, 2004). Further experiments are required to investigate these multiple potential effects of Kir4.1 and Kir7.1 blockade, which due to time limitations were outside the scope of my
project, which focused on the mechanisms of Ca\(^{2+}\)-oscillations evoked by K\(_{ir7.1}\) blockade.

Figure 7.5: Classical model of glutamate-mediated cytotoxicity and Ca\(^{2+}\)-dependent activation of caspase-mediated mechanisms of oligodendrocyte cell demise. (Adapted from Benerroch, 2009).

7.7. \(K_{ir7.1}\) blockade results in glial \([Ca^{2+}]_i\) deregulation

This study has provided clear evidence that application of VU590 induced Ca\(^{2+}\) oscillations and cell death in a large proportion of cells. Notably, Ba\(^{2+}\) did not affect glial \([Ca^{2+}]_i\), indicating differences in the functions of K\(_{ir4.1}\) and K\(_{ir7.1}\) in glia. Calcium imaging studies revealed that K\(_{ir7.1}\) blockade induced glial \([Ca^{2+}]_i\) oscillations that are dependent on SOCE, mediated most likely via both Orai1/Stim1 and Trpm3/Trpc1 channels. There was evidence that Ca\(_v\) channels may also be involved in glial \([Ca^{2+}]_i\),
oscillations, but since blockade of Kir4.1 with Ba^{2+} is known to cause glial depolarisation but did not induce glial [Ca^{2+}]_i oscillations, they are unlikely to be dependent on Ca_v channels. Overall, the results suggest a specific role for Kir7.1 in SOCE, which provides a potential mechanism by which a pathological rise in [Ca^{2+}]_i results in caspase-dependent cell death in oligodendrocytes, although as noted above this is unlikely to be the sole mechanism and further experiments are required to demonstrate a causal link between these events (Figure 7.5).

Figure 7.6: Potential mechanism of effect of Kir7.1 blockade on deregulation of [Ca^{2+}]_i and oligodendrocyte cell death.
7.8. Clinical implications of findings

The results of this thesis indicate a protective role for Kir7.1 in ischaemic/hypoxic conditions, since their blockade aggravated oligodendrocyte cell loss. The high expression of Kir7.1 in neurones and glia supports a potential role in membrane depolarisation, which is one of the causes of neuronal and glial stress during a hypoxic/ischaemic event that eventually leads to cell demise. Moreover, transient ischaemia resulted in a marked decrease in glial Kir4.1 (Iandiev et al., 2006), and it would be of interest to investigate whether changes in Kir7.1 expression are involved in the sensitivity of neurones and oligodendrocytes to ischaemia/hypoxia. In addition, acidification increased Kir7.1 conductance, which could be important in helping to maintain the neuron/glial RMP in oxidative stress (Hughes and Swaminathan, 2008). Furthermore, Kir4.1 mutations are associated with increased seizure activity, such as SESAME/EAST Syndrome (Williams et al., 2010), and a similar function for Kir7.1 is likely, since KCNJ13, the gene encoding for Kir7.1, is one of the genes associated with epileptogenicity in a functional genomics study on chronic epilepsy (Winden et al., 2011).
7.9. Summary and Conclusions

The results presented in this thesis have shed some light on the expression and function of K\(_{ir}7.1\) in glia. However, these experiments created more questions on the functions of K\(_{ir}7.1\) in glia and neurones, as might be expected in the study of a novel ion channel. My findings lead to many further potential avenues of research that may be of interest and importance. Moreover, high expression of mRNA for K\(_{v}1.1\) and the K\(_{v}\beta1\) and K\(_{v}\beta2\) subunits is of particular interest, because K\(_{v}\beta\) subunits provide a direct mechanistic link between cell membrane functionality, and thus ionic flux, and cellular metabolic status in their role as aldo-ketoreductases linked to membrane voltage-gated potassium channel subunits, and are known to be closely associated in neurones and axons (Rhodes et al., 1997; Poliak et al., 2003; Jukkola et al., 2012), but have not been demonstrated in glia. The high expression of these transcripts in the optic nerve are not consistent with axonal expression and implies a major role in mature glia, which is completely novel; immunolabelling and electrophysiology experiments would be invaluable in order to determine its function, e.g. with pharmacological modulators such as \(\kappa\)-Dendrotoxin, a selective blocker of homomeric and heteromeric K\(_{v}1.1\) channels (Hatton et al., 2010; Hao et al., 2013). In conclusion, this study has demonstrated that glia express a remarkable variety of ion channels, equivalent to neurones, and elucidating their precise functions in physiological and pathological processes is an immense task, but will provide a solid foundation for future development of strategies to combat the deleterious consequences of glial cell pathology.
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### UPR 16
Research Ethics Review Checklist

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<td><strong>First Supervisor:</strong></td>
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If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University’s Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study.

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

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