ATP elicits electrophysiological and migratory responses in cells of the spinal cord stem cell niche

Claudia Catherine MacLean

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

There are multiple similarities between the central canal region of the spinal cord and the adult neurogenic niches in the mammalian brain. The central canal region displays increased proliferation after injury with migration of the newly-proliferated cells to the site of injury and differentiation into astrocytes and oligodendrocytes. This study will look at the role of purinergic signalling in the ependymal cells and CSF-contacting cells (CSFcCs) as the CSFcCs present in this area express P2X receptors containing the $P2X_2$ subunit.

Local application of ATP elicited fast depolarisations via activation of suramin-sensitive channels in a subgroup of CSFcCs comprising both spiking and non-spiking subtypes of CSFcC (types 1, 2 and 3). The $P2X_{2/3,3}$-specific antagonist A-317491 had no effect in the majority of CSFcCs but produced a reduction in depolarisation in a subgroup of CSFcCs predominantly located ventral to the central canal, with the ventral expression of the $P2X_3$ subunit supported by immunohistochemistry.

Ependymal cells and the remainder of CSFcCs produced a suramin-insensitive hyperpolarisation to the application of ATP or UTP, while producing a small depolarisation to the dinucleotide polyphosphate Ap4A.

Modulation of purinergic signalling had no effect on proliferation rate in spinal cord slices over a 4 hour time period, nor did it affect the survival rate of the newly-proliferated cells over 5 days in organotypic slice cultures. In this model, inhibition of purinergic signalling with suramin reduced the migration of newly-proliferated ependymal cells away from the central canal, while inhibition of the breakdown of ATP by ARL 67156 facilitated this migration.

The presence of fast acute responses to ATP including spatial variation in receptor subtypes indicates the importance of purinergic signalling in this area and the release of ATP known to be triggered by spinal cord injury could now have a role in the necessary migration of newly-proliferated ependymal cells.

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Abbreviations

aCSF – artificial cerebrospinal fluid
AADC – L-amino acid decarboxylase
ACh – acetylcholine
ADP – adenosine diphosphate
Ap4A – diadenosine tetraphosphate
ATF3 – activating transcription factor 3
ATP – adenosine triphosphate
BDNF – brain-derived neurotrophic factor
BLBP – brain lipid binding protein
BMSC – bone marrow-derived mesenchymal stromal cell
BrdU – bromodeoxyuridine
CNS – central nervous system
COX-2 – cyclooxygenase-2
CSFcC – cerebrospinal fluid-contacting cells
CSP – cysteine string protein
CXCL12 – C-X-C Motif Chemokine Ligand 12
CXCR4 – C-X-C Motif Chemokine Receptor 4
DA – dopamine
DAB – diaminobenzidine
DAPI - 4',6-diamidino-2-phenylindole
Dcx – doublecortin
DIC – differential interference contrast
DOPA – dihydroxyphenylalanine
EAP – extravidin peroxidase
EdU – 5-Ethynyl-2´-deoxyuridine
EGF – epidermal growth factor
EPSP – excitatory post-synaptic potential
epSPCi – ependymal stem/progenitor cell from injured spinal cord
epSPC – ependymal stem/progenitor cell
FGF2 – fibroblast growth factor 2
GABA – γ-aminobutyric acid
GAD – glutamate decarboxylase

GPR17 – G protein-coupled receptor 17
hMSC – human mesenchymal stem cell
IGF-2 – insulin-like growth factor 2
IPSP – inhibitory post-synaptic potential
IR – input resistance
KCC2 – K-Cl co-transporter 2
LTS – low threshold spike
MRI – magnetic resonance imaging
nAChR – nicotinic acetylcholine receptor
NeuN – neuronal nuclei
NKCC1 – Na-K-Cl co-transporter 1
NMDA – N-methyl-D-aspartate
NMDG – N-methyl-D-glutamine
NTPDase – nucleoside triphosphate diphosphohydrolase
OP – oligodendrocyte precursors
PDGFRα – platelet-derived growth factor receptor α
PNMT – phenylethanolamine N-methyltransferase
PNS – peripheral nervous system
PPADS – pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
PSA-NCAM – polysialated-neural cell adhesion molecule
RMP – resting membrane potential
RT-PCR – real time polymerase chain reaction
SCI – spinal cord injury
SDF1 – stromal cell-derived factor 1
SK – small-conductance Ca^{2+}-activated K^{+} channels
SOC – store operated calcium channel
SVZ – subventricular zone
TEA – tetraethylammonium
TH – tyrosine hydroxylase
TRP – transient receptor potential
TTX – tetrodotoxin
UDP – uridine diphosphate
UTP – uridine triphosphate
VIP – vasoactive intestinal peptide
ZEB1 – zinc finger E-box binding protein 1

Chapter 1 - General Introduction

1.1 Introduction to the central canal region of the spinal cord

The spinal cord is a bundle of neural fibres and cells which runs down the vertebral column, extending from the medulla oblongata in the brainstem to the lumbar region. The primary function of the spinal cord is to relay messages between the peripheral nervous system (PNS) and the central nervous system (CNS), however there are also several other functions independent of the brain. The spinal cord has many neural circuits, controlling both reflexes and central pattern generators for locomotion.

The core of the spinal cord is made up of the cellular grey matter, surrounded by the neuronal tracts of the white matter (De Leener et al., 2016). Through the centre of the grey matter is the central canal through which the cerebrospinal fluid flows. There are 31 segments in the human spinal cord, 8 cervical segments, 12 thoracic segments, 5 lumbar and 5 sacral segments, with each segment having two dorsal and two lateral roots which combine to form a single pair of spinal nerves. The dorsal roots consist of a bundle of the axons of sensory neurons with their cell bodies in the dorsal root ganglia. The ventral roots have efferent fibres containing axons from autonomic pre-ganglionic neurons and motor neurons with their cell bodies sitting in the ventral horn. There are also many interneurons and non-neuronal cells within the spinal cord.

The spinal cord is contained within 3 protective layers of membrane, known as the meninges. The innermost of these is the pia mater which closely wraps around spinal cord and roots. The next layer is the arachnoid mater, with the space between the pia and the arachnoid mater called the subarchnoid space. Outside the arachnoid mater is a final tough protective layer, called the dura mater (Russi and Brown, 2015).

The grey matter of the spinal cord is organised into 10 layers called the Rexed laminae (Rexed, 1952; Molander et al., 1984) (Figure 1.1). Laminae I-VI are located in the dorsal horn, lamina VII forms the intermediate zone, laminae VIII and IX are in the ventral horn and lamina X sits around the central canal. This work focuses on the area immediately surrounding the central canal and the cell types and characteristics of this area will be expanded on below.

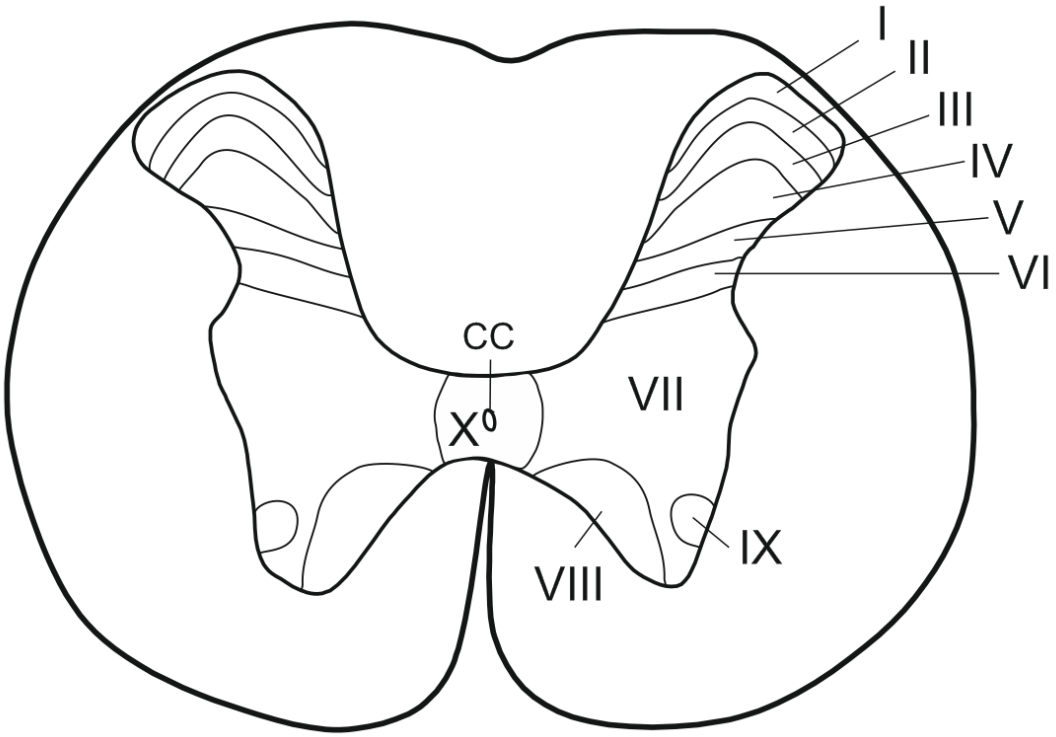


Figure 1.1 Rexed laminae of the spinal cord showing the central canal in the centre of lamina X

Diagram of a transverse spinal cord section showing the white matter surrounding the grey matter. The grey matter is divided into 10 layers (I-X) as identified by Bror Rexed, with each layer corresponding to a column of cells defined by their cell structure. The central canal (CC), through with the CSF flows, is shown in the centre of lamina X.

1.2 The components of the central canal region

The key components of the central canal region are the cells that form the ependymal cell layer that line the central canal, the cerebrospinal fluid (CSF) which flows through the central canal and the tanycytes and CSF-contacting cells which sit within or just outside the ependymal cell layer (Figure 1.2).

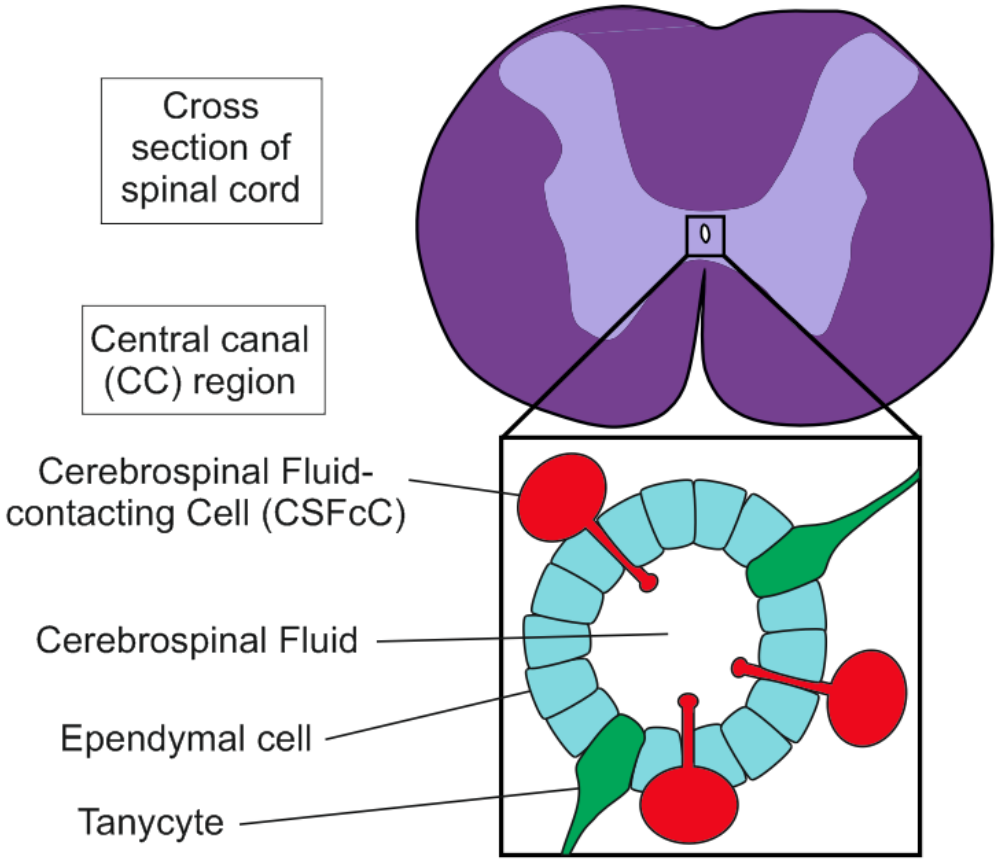


Figure 1.2 The components of the spinal cord central canal region

Schematic of the location and cellular components in the central canal as shown on a transverse view of the spinal cord. The close view of the central canal shows ependymal cells surrounding the central canal through which the CSF flows. Interspersed in the ependymal layer and just outside it are cerebrospinal fluid contacting cells (CSFcCs) and tanycytes.

1.2.1 The cerebrospinal fluid

The cerebrospinal fluid (CSF) is part of the extracellular fluid in the central nervous system (CNS). CSF is secreted by the choroid plexus of the cerebral ventricles (Spector et al., 2015). The choroid plexus is a highly vascularised region of the ventricles with modified ependymal cells which are highly tight-junction coupled. Plasma is filtered from the blood through the choroid plexus cells, which then also actively transport many small molecules into the CSF.

The net flow of CSF is caudal, from the choroid plexus predominantly in the lateral ventricles, to the 4th ventricle and down the central canal of the spinal cord and

through the caudal opening; however a large proportion is absorbed by the lateral apertures of Luschka into the sub-arachnoid space without going down the central canal. The CSF that flows down the spinal cord is subject to two biphasic tides, the caudal flow and the cephalic ebb (Whedon and Glassey, 2009).

In adult humans, approximately 500 ml is produced each day with a maximum of 150 ml present at any point. Of this, only about 30 ml is present within the ventricles and central canal. The remaining 110 ml sits within the subarachnoid space, between the dura and pia maters. In mice, the total CSF volume is only approximately 40 µl (Oshio et al., 2005).

Although there are turbulences and ebbs, the CSF has been measured to flow at approximately 1 cm/minute (Cifuentes et al., 1994). At the filum terminale, one or several openings allow the CSF to pass into the subarachnoid space (Nakayama, 1976). Whilst some CSF is absorbed into the spinal cord tissue, most is released into lymphatic vessels or veins (Maillot, 1990).

Despite the tight junctions in the choroid plexus, the majority of the ependymal cell layer lining the central canal of the spinal cord does not possess tight junctions, and thus allows for considerable flow between the CSF and the extracellular fluid of the spinal cord tissue. The level of permeability is lower in foetal stages and increases into adulthood to the extent that it is permeable to 70 kDa plasma proteins (Whish et al., 2015). Approximately 75 % of the CSF is produced by the choroid plexuses in the lateral ventricle, while the other 25 % passes into the CSF system through the ependymal surfaces in the brain and spinal cord after being produced by the CNS parenchyma (Veening and Barendregt, 2010).

The CSF contains ions (Na^+, Cl^-, HCO_3^-, and lower concentrations of K^+, Mg^{2+} and Ca^{2+}), peptides, proteins, and micronutrients such as vitamin C and folate (Spector et al., 2015) with the overall concentration of salts high and the protein concentration relatively low. The CSF also carries signalling molecules such as hormones (e.g. leptin) and growth factors (e.g. brain-derived neurotrophic factor). Some neuropeptides are produced by the choroid plexus such as the thyroid hormone carrier, transthyretin (Veening and Barendregt, 2010). Cells of the choroid plexus bordering the CSF also release neuropeptides into the CSF, which could then have actions on other parts of the CNS (Nilsson et al., 1992). Many fibres are observed in

the sub-ependymal layer of the lateral ventricles, such as serotonergic fibres, and are also thought to be releasing substances into the CSF.

The main functions of the CSF, in addition to signalling, are buoyancy, reducing the effective mass of the brain from 1200 g to 45 g (Spector et al., 2015) and thus preventing the damage that would occur with that weight of brain tissue, protection from impacts and chemical and fluid stability. Both the volume and the composition of the CSF can adapt to the needs of the central nervous system (Johanson et al., 2008), providing homeostasis for the extracellular fluid in the brain and spinal cord. A last important function of the CSF is as the clearance mechanism for the CNS. As the CNS lacks a lymphatic clearance system, metabolites and toxins are cleared by the CSF and either via active absorption by cells or by draining of the CSF into venous and lymphatic systems (Johanson et al., 2008). The CSF is also involved in multiple signalling procedures. The flow of the CSF is involved in directing the migration of newly proliferated cells from the adult subventricular zone (SVZ) (Sawamoto et al., 2006).The constituents of the CSF also have signalling roles for example insulin-like growth factor (IGF-2) levels stimulate stem cell production in development and glioblastomas (Lehtinen et al., 2011). These indicate that the CSF fulfils a range of roles and is involved in signalling with the CNS cells by a multiple methods.

1.2.2 CSFcCs

1.2.2.1 Distribution and structure of CSFcCs

CSFcCs represent a population of cells which have been identified in a range of species from marine chordates such as lancelets to mammals including humans (Bruni and Reddy, 1987; Lamotte, 1987; Bjugn et al., 1988; Vigh et al., 2004) and are present along the length of the ventricular system and the central canal.

These cells sit in the ependymal cell layer or subependymally and send a dendritic process into the internal CSF. These processes bear a number of stereocilia, with the number of the stereocilia varying between species. Many of these cells also possess axons extending away from the central canal and making contact with the external cerebrospinal fluid in the subarachnoid space (Lamotte, 1987; Stoeckel et al., 2003). The number of CSFcCs is not consistent between species, with far lower numbers in mammals compared to fish or amphibians (Vigh and Vigh-Teichmann, 1998; Vigh et al., 2004). Non-mammalian vertebrate CSFcCs bear a cilium in addition to the

stereocilia, however mammalian CSFcCs do not possess this (Vigh et al., 1977). Cilia are supported by a cytoskeletal structure called the axoneme which is composed of microtubules. The formation of the axoneme differs between different types of cilia. The cilia typically have a circle of 9 doublets (9x2) or triplets (9x3) microtubules with motile cilia also having a pair of central microtubules (9x2+2). The type of cilium present changes between ventricular CSFcCs and medullo-spinal CSFcCs. While ventricular CSFcCs have a 9x2+0 cilium which is characteristic of chemosensory cells, medullo-spinal CSFcCs express a 9x2+2 kinocilium which is typical of mechanosensory cells such as those in the inner ear and the lateral line organ of fish. This kinocilium has been seen to be associated with Reissner's fibre in the carp, which is the fibre from the subcommissural organ that runs through the ventricular system and suggests a role in detection of movement of the spinal cord (Vigh et al., 2004). Whilst the lack of kinocillia in the mammalian system makes this role for CSFcCs less likely, there are a number of other factors suggesting a range of roles involved in detection of the internal CSF and intercellular solution.

1.2.2.2 CSFcC expression profile

CSFcCs express immature neuronal markers and markers of plasticity

CSFcCs do not have the characteristics of fully mature neurons, instead maintaining expression of immature neural markers, doublecortin (Dcx), a neuronal migration protein, and neuronal RNA-binding proteins HuC/D into adulthood (Marichal et al., 2009). Dcx expression is typical of migrating neuroblasts (Kuhn and Peterson, 2008), with expression of Dcx ceasing as they become electrophysiologically mature neurons and become positive for the mature neuronal marker NeuN (Neuronal Nuclei)(Mullen et al., 1992). Hu proteins are expressed in the very early stages in newborn neurons (Marusich et al., 1994). CSFcCs do not express NeuN (Marichal et al., 2009), and whilst NeuN is not a definitive marker for mature neuronal cells with cell types including cerebellar Purkinje and Golgi cells of the cerebellum (Mullen et al., 1992; Tulke et al., 2016) devoid of NeuN in maturity, this supports the conclusion of an immature neuronal profile suggested by the expression Dcx and HuC/D by CSFcCs. In addition to expression during immaturity, many of these markers are also expressed during times of neuronal plasticity and remodelling. Hu proteins (Okano and Darnell, 1997; Deschênes-Furry et al., 2006) and Dcx (Nacher et al., 2001; Brown et al., 2003) are expressed in mature neurons during neuronal remodelling

and sprouting as shown in neurons of the piriform cortex (Nacher et al., 2004). Other markers associated with neuronal plasticity and neural development which CSFcCs express include Polysialylated Neural Cell Adhesion Molecule (PSA-NCAM) and Growth Associated Protein 43 (GAP-43; a protein expressed in high levels in neuronal growth cones during development and during axonal regeneration and plasticity) (Theodosis et al., 1994; Benowitz and Routtenberg, 1997; Cremer et al., 2000; Stoeckel et al., 2003; Studeny et al., 2005; Marichal et al., 2009; Reali et al., 2011). The polysialylated form of NCAM is more commonly found in immature neurons (Seki and Arai, 1993), with expression maintained into adulthood within proliferative areas such as the subventricular zone (SVZ) (Bonfanti and Theodosis, 1994) and where cells are undergoing plasticity. However CSFcCs are not proliferative past embryological periods. They do not express proliferating cell nuclear antigen, an endogenous cell cycle protein expressed in all stages of the cell cycle of proliferating cells (Marichal et al., 2009). Additionally, when a marker for proliferating cells, EdU or BrdU (thymidine analogues that are incorporated into any DNA undergoing DNA replication) was applied between P0 and P5 no CSFcCs were labelled, with all CSFcCs being labelled when the thymidine analogue was applied before E17 (Marichal et al., 2009; Petracca et al., 2016). This indicates that all CSFcCs are born embryologically and the expression of these molecules above suggests that they are either still undergoing development into adulthood, or are in a state of dormant plasticity.

Subcellular components and synaptic equipment

The process of CSFcCs which passes through the ependymal cell layer ends in a globular expansion containing numerous vesicles of varying size and shape and many mitochondria (Alfaro-Cervello et al., 2012). They were distinguishable from the surrounding ependymal cells by their less electron-dense cytoplasm, lack of intermediate filaments and a wealth of rough endoplasmic reticulum and ribosomes. CSFcCs were shown to receive axosomatic synaptic contacts (Alfaro-Cervello et al., 2012) and have axons that lead away from the central canal (Stoeckel et al., 2003). These axons are present in CSFcCs caudal to mid thoracic levels and are very small and unmyelinated (Stoeckel et al., 2003). These axons run around and through the ependymal cell layer to the ventral end of the central canal and form axon bundles which run ventrally from the central canal and through the corticospinal tract and then along the sides of the ventral median fissure. The presence of visible bundles only in

the more caudal regions of the spinal cord suggests that they may also run caudally down the spinal cord but this has not been confirmed. These axons are immunoreactive for GAP-43, which is involved in neurite outgrowth by regulating cytoskeletal organisation (Benowitz and Routtenberg, 1997).

Whilst the CSFcC axons express a number of synapse-associated proteins in mammals, these are not arranged in discrete synapses in the mammalian spinal cord (Stoeckel et al., 2003). The axonal tracts were strongly labelled with synaptotagmin and more lightly labelled with both synaptophysin and cysteine string protein (CSP). Diffuse labelling of synaptic proteins has been observed in neurons undergoing differentiation or preceding synapse establishment (Rene et al., 1996). However in non-mammalian species functional synapses are observed. In *Xenopus* spinal cord, CSFcC axons form endings with dense-cored vesicles and small, clear vesicles, usually into the external CSF (Vigh et al., 1977). The axons in the non-mammalian CSFcCs also run ventrally, terminating on the ventrolateral surface between glial endfeet. These terminals contain synaptic and granular vesicles and attach by hemidesmosone-like structures to the basal lamina surrounding the spinal cord. Hemidesmosomes are an integrin-based structure that functions to attach cells to basal membranes. Vesicle-containing endfeet were observed in a range of non-mammalian species including fish, reptiles and birds (Vigh et al., 1977). These findings again suggest an immature or plastic nature to mammalian CSFcCs and greater indications for settled roles for CSFcCs in the non-mammalian spinal cord. However findings by Lamotte (1987) suggest that there is considerable variation amongst mammalian species, as well as between mammalian and non-mammalian CSFcCs. Lamotte (1987) looked at CSFcCs positive for the peptide hormone vasoactive intestinal polypeptide (VIP; discussed in detail below) and observed that from the basal end of CSFcCs within the thoracic region of the cat spinal cord there were many long dendrites and thin varicose axons running for long distances parallel to the central canal but also running ventrally and ventralaterally to reach the pial surface of the spinal cord. The varicosities on the CSFcC axons in the cat contained small round clear vesicles and dense core vesicles. In rat spinal cord, no varicosities were observed on the CSFcC axons which were positive for $P2X_2$, GAD and GAP-43 (Stoeckel et al., 2003) suggesting different functions of the CSFcC axons in these species or investigation of different types of CSFcC with other non-investigated CSFcCs showing parallel structures.

Expression of neurotransmitter-related molecules

Whilst the contents of these synaptic and granular vesicles have not been identified in the mammalian spinal cord, there are a number of neurotransmitter-related proteins that have been observed in spinal cord CSFcCs. One neurotransmitter that has been shown to produce responses in CSFcCs indicating the presence of functionally active receptors is γ-aminobutyric acid (GABA). GABAergic terminals have been identified in close apposition to the cell bodies of CSFcCs, identified by their apical process projecting into the central canal (McLaughlin et al., 1975) and the CSFcCs are themselves immunoreactive for glutamic acid decarboxylase (GAD; the enzyme which synthesises GABA) (Barber et al., 1982; Stoeckel et al., 2003). The expression of GAD in CSFcCs is a conserved characteristic of these cells, with expression demonstrated in the lancelet (Cephalochordata) spinal cord where they constitute a large portion of the GABAergic neurons (Anadón et al., 1998). In rat spinal cord, the highest numbers of GABAergic CSFcCs were observed in caudal to mid thoracic regions, similar to the distribution of CSFcC axons (Stoeckel et al., 2003).

Application of GABA has been shown to produce a response in CSFcCs as demonstrated in perforated patch and whole cell patch clamp electrophysiological recordings (Marichal et al., 2009; Corns, 2012), mediated by $GABA_A$ receptors as indicated by the sensitivity of the response to the selective $GABA_A$ receptor antagonist gabazine. However CSFcCs in the rat spinal cord have also been shown to be immunoreactive for the $GABA_B$ receptor (Margeta-Mitrovic et al., 1999). The actions of the metabotropic $GABA_B$ receptor on K^+ channels result in a much more hyperpolarised reversal potential than that of ionotropic $GABA_A$ receptors, which are caused by an increase Cl^- conductance (Kuriyama et al., 1993). $GABA_B$ is thought to act as an autoreceptor as well as having post-synaptic actions, to inhibit further release of GABA from a cell (Waldmeier et al., 1993). The presence of functional GABAergic signalling in the mammalian CSFcCs suggests that they are integrated into to ependymal circuitry and possibly the wider spinal cord circuitry.

However not all receptors identified in non-mammalian CSFcCs have been shown to be present in mammalian CSFcCs. A role for catecholaminergic signalling has been shown in CSFcCs in *Xenopus* spinal cords as the CSFcCs were shown to be immunoreactive for tyrosine hydroxylase (TH), a protein involved in the production of

catecholamines by the conversion of tyrosine to L-DOPA (Heathcote and Chen, 1994). In the fish spinal cord (eel and trout), a population of CSFcCs was immunoreactive for both TH and dopamine (DA) and was distinct from the population of CSFcCs shown to express GABA by immunohistochemical methods (expressed as GABA-positive henceforth) (Roberts et al., 1995). Neither population of CSFcCs was immunoreactive for choline acetyltransferase (ChAT; the enzyme responsible for producing acetylcholine). These two populations of CSFcCs in the fish occupied different locations around the central canal (Roberts et al., 1995). The dopaminergic CSFcCs were located predominantly on the ventral aspect of the central canal, usually close to the midline, while the GABAergic CSFcCs were more likely to be located laterally. The GABAergic CSFcCs were also more numerous than the dopaminergic CSFcCs. In mammalian spinal cord, no catecholaminergic immunoreactivity has been detected, unlike in the fish and amphibian spinal cord, CSFcCs do not express TH (Stoeckel et al., 2003). Mammalian CSFcCs (rat and mice) do not express several other monoamines or related proteins including DA, serotonin and phenylethanolamine N-methyltransferase (PNMT; an enzyme which converts norepinephrine to epinephrine) (Jaeger et al., 1983; Nagatsu et al., 1988). However, despite the lack of expression of any catecholamines, rat and mice spinal cord CSFcCs do express the enzyme aromatic L-amino acid decarboxylase (AADC) (Jaeger et al., 1983; Nagatsu et al., 1988). This enzyme acts in a number of decarboxylation reactions, including the production of dopamine from L-DOPA, of serotonin (5-hydroxytryptamine) from 5-hydroxytryptophan, the production of histamine from L-histadine, and in the production of a number of trace amine neuromodulators such as tryptamine and phenylethylamine. It could be possible that this enzyme functions to produce one of these trace amine neuromodulators, although this hasn't been reported, or it could be that AADC expression is a remnant of a functioning catecholamine production system previously expressed in these cells further back in the evolutionary timeline.

There are several species differences in the proteins expressed by CSFcCs. One protein that has been observed expressed in CSFcCs of the cat and monkey is vasoactive intestinal polypeptide (VIP) (Lamotte, 1987). VIP is a peptide hormone that also acts as a signalling molecule between cells in certain brain regions such as within the suprachiasmatic nuclei where VIP release plays a critical role in circadian cycle regulation (Fan et al., 2015). In the subrachiasmatic nucleus the VIP-positive

neurons have been shown to co-release GABA and VIP (Castel and Morris, 2000). Methionine-Enkephalin-Arg^{6}-Gly^{7}-Leu^{8} has also been shown to be expressed by CSFcCs of the rat (Shimosegawa et al., 1986) with the number of Met-Enk-Arg-Gly-Leu-positive CSFcCs varying along the rostrocaudal extent of spinal cord. The highest numbers of Met-Enk-Arg-Gly-Leu-positive CSFcCs was in thoracic regions, with slightly lower numbers in the cervical and lumbar regions and no Met-Enk-Arg-Gly-Leu-positive CSFcCs in the brainstem. This indicates that there are expression differences between medullary and spinal CSFcCs. The expression of these proteins gives indications as to the roles played by CSFcCs with spatial and species differences suggesting possible differences in these functions.

Expression of receptors affected by changes in pH

Mammalian CSFcCs do express 2 functional receptors that share in common a response to changes in pH. The first of these is polycystic kidney disease 2-like 1 protein (PKD2L1) which is expressed by CSFcCs in mouse spinal cord (Huang et al., 2006; Petracca et al., 2016) and the macaque and zebrafish spinal cords (Djenoune et al., 2014). PKD2L1 is a member of the transient receptor potential (TRP) family (Delmas, 2005). It is a non-selective cation channel that can be activated by small decreases in extracellular pH (Huang et al., 2006), a property that has been identified in CSFcCs (Marichal et al., 2009; Petracca et al., 2016). While PKD2L1 is also involved in the detection of pH (sour tastes) in the taste receptor cells, where typical pH levels being detected are <pH 5, CSFcCs are able to respond to a much smaller change in pH levels. A reduction from the control pH of 7.4 to pH 6.9 produced a significant increase in action potential frequency, and more so at pH 6.5 (Huang et al., 2006). However, from their work on CSFcCs in the mouse brainstem Orts-Del'Immagine et al. (2016) argued that PKD2L1 could be more effectively activated by alkalinisation (pH 8.8) than by extreme acidification (pH 2.8) and that P2K2L1 was more involved in the OFF response upon release of acidification with alkalinisation able to generate depolarisations large enough to trigger action potentials. There were considerable differences in the age of the animals, the CNS region from which the CSFcCs were being assessed and the pH level of the acidification. However in all these experiments, PKD2L1-expressing CSFcCs were sensitive to changes in extracellular pH and therefore may have a sensory role in regulation of the pH of the intercellular solution or CSF.

In mouse CSFcCs PKD2L1 is expressed with PKD1L2, a complementary protein thought to act in a regulatory manner for PKD2L1 (Petracca et al., 2016). In zebrafish, all PKD2L1-positive CSFcCs were GABAergic (Djenoune et al., 2014), however in mice only 80% of PKD2L1-positive CSFcCs were immunoreactive for GAD (Petracca et al., 2016). The PKD2L1 staining did not colocalize with either the glial marker glial fibrillary acidic protein (GFAP) or the ependymal cell marker vimentin (an intermediate filament) (Petracca et al., 2016) showing that the expression of PKD2L1 was confined to CSFcCs but was robustly expressed by the entire CSFcC population and well conserved throughout species.

Another protein expressed by mammalian CSFcCs is $P2X_2$, which although not directly stimulated by changes in pH, does have its response to its agonist modulated by changes in pH (Stoeckel et al., 2003). $P2X_2$ is a purinergic non-selective cation channel that opens in response to extracellular Adenosine Triphosphate (ATP). Both the cell bodies of CSFcCs and the axons have been shown to be immunoreactive for $P2X_2$ in the rat spinal cord. $P2X_2$ labelling in both the cell body and axons is of a punctate nature and around the cell body is often observed to be membranous staining (Stoeckel et al., 2003). $P2X_2$ staining was also observed in the apical process with the same punctate distribution. Electron microscopy revealed that the CSFcCs displayed finger-like projections at the basal end of the cell body, intermingling with the synapses between the CSFcCs and the surrounding neuropil. These finger-like projections were particularly dense in $P2X_2$ labelling (Stoeckel et al., 2003). Unlike other P2X receptors, the response of $P2X_2$ receptors to ATP can be potentiated by lower pH levels where the affinity of the channel for ATP was increased up to 5 fold with the maximum response at pH 6.5 (King et al., 1997; Stoop et al., 1997; Ding and Sachs, 1999). $P2X_2$, in addition to other purinergic receptors, will be examined in detail further on in this introduction.

It may well be that as Orts-Del'Immagine et al. (2016) hypothesised for CSFcCs in the brainstem, an alternative mechanism, perhaps through $P2X_2$ receptors, is capable of detecting small changes in levels of acidification and PKD2L1 detects release of acidification. While $P2X_2$ is predominantly responds to levels of extracellular ATP, the fact that the amplitude of the response to ATP is modulated by pH suggests that both $P2X_2$ and PKD2L1 may respond to pH changes in CSFcCs, with $P2X_2$ possibly playing a significant role in times of high extracellular ATP concentration.

1.2.2.2 Variation in CSFcCs

From a range of measures and multiple different factors, spinal cord CSFcCs are a non-homogenous cell population, even within the same species. In lamprey spinal cord, 2 types of CSFcC with different shapes, expression patterns and electrophysiological characteristics have been identified (Jalalvand et al., 2014), which they labelled as type 1 and type 2. Type 1 CSFcCs were positioned close to the central canal and had a very short apical process ending with a large spherical bulb. These cells had active neuronal properties, displaying spontaneous action potential firing, excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs). They depolarised to application of GABA and glutamate and were immunoreactive for GABA and somatostatin. The type 2 CSFcCs identified in the lamprey spinal cord had a longer, thinner apical process and were located further away from the central canal. They did not display any active neuronal properties and were not immunoreactive for GABA or somatostatin but did show immunoreactivity for taurine. Jalalvand et al. (2014) suggest that the type 2 CSFcCs in lamprey spinal cord may be a type of glial cell, indicating some of the difficulties in defining the CSFcC population. Despite some of the uncertainty as to the CSFcC nature of these cells, other findings show significant differences between clearly defined CSFcC populations.

In mammalian spinal cord Barber et al. (1982) also noted differences in the shape of CSFcCs. They observed that the GAD-positive CSFcCs around the central canal could be categorised into two groups based on the shape of the cell body. One group had spherical cell bodies, measuring 10-12 μm in diameter and were found on the outer edge of the ependymal cell layer, while the other group had a cell body that was a fusiform shape and were longer than the other group, measuring 20 μm by 5-8 μm. CSFcCs of the second type were observed in many different positions through the ependymal cell layer. When Golgi's silver staining method was used to stain CSFcCs, it stained CSFcCs that had a similar shape and location to the second type of CSFcCs observed with GAD staining, which were found throughout the ependymal cell layer. CSFcCs bearing the spherical shape were not observed using this method (Barber et al., 1982). This may suggest that there may be another CSF-contacting cell population which are not neuronal and as such do not take up the neuronal Golgi silver stain. It is for this reason that CSF-contacting cells are here referred as such rather than CSF-contacting neurons (CSFcNs) as is done in the majority of work.

As was mentioned above, Roberts et al. (1995) detected two populations of CSFcCs within the fish spinal cord, one of which was GABAergic and the other dopaminergic. The GABAergic CSFcCs made up the larger population of CSFcCs and were predominantly located laterally, although some were located ventrally. The dopaminergic population of CSFcCs was lower in number and restricted to the ventral end of the central canal.

A strikingly similar pattern was observed in mammalian spinal cord where it was found that in spinal cord that there were two distinct populations of PKD2L1-positive CSFcCs coming from different embryonic origins (Petracca et al., 2016). This was investigated by detection of the transcription factors Gata2 and Gata3 in the population of PKD2L1-expressing CSFcCs in the mouse spinal cord. All of these CSFcCs were shown to be born between E14.5 and E16.5, an embryological period associated with the production of astrocytes and oligodendrocytes. Embryological neurogenesis occurs earlier, with some of the latest interneurons differentiating between E10 and E12 (Zhou et al., 2000; Francius et al., 2013). Of the CSFcCs, the vast majority (90 %) were found to derive from the late-p2 domain and the dorsal half of the oligodendrogenic (pOL) domain. These CSFcCs were termed CSFcN' and were found predominantly laterally. The second population was termed CSFcN'' and made up only 10 % of PKD2L1+ CSFcCs. This CSFcN'' population was derived from precursors in the proximity of the embryological floor plate and occupied an exclusively ventral position around the central canal at E15.5. There were also differences observed between these two populations in the electrophysiological profiles. Amongst the CSFcN' population, the majority of the cells were able to fire multiple spikes (80 %) but none of the CSFcN'' population could produce multiple spikes (approximately 50 mV in amplitude), instead only producing a single spike of similar amplitude in response to a 10 pA depolarising current step.

Whilst the lack of catecholaminergic immunoreactivity in mammalian CSFcCs suggests that there may only be the GABAergic CSFcCs conserved into mammalian spinal cords, this connection between the two populations and numerical distribution and locations suggests that they may be the same two populations in mammalian spinal cord as in non-mammalian spinal cord but in the mammalian spinal cord the two populations bear more similarity than they do in the non-mammalian spinal cord.

In addition to the multiple spiking and single spiking CSFcCs observed by Petracca et al. (2016) in mouse spinal cord, a non-spiking population of CSFcCs has also been previously observed in the rat spinal cord (Marichal et al., 2009; Corns, 2012). Both of these studies described spiking behaviour in CSFcCs as a fast event with a depolarising and repolarising event. In comparison to action potentials, these spikes have smaller amplitude (amplitude of spikes was 38-56 mV (Marichal et al., 2009)) and longer half-amplitude duration (3.8-6 ms (Marichal et al., 2009)). This work will use the same definition of spikes to facilitate discussion of events that do not satisfy the criteria for action potentials.

Marichal et al. (2009) described three populations of CSFcC. The first of these produced a slow regenerative potential (a reduction in the extent of depolarisation caused by a positive current pulse over the duration of its application) upon application of depolarising current pulses, which was abolished when in the presences of 3 mM Mn^{2+} indicating that this response to current injection was a Ca^{2+} conductance. The majority of the activity in these cells was insensitive to the Na^{+} channel blocker tetrodotoxin (TTX) indicating that Na^{+} conductances did not cause the depolarisation. However there was an initial element to the response that was sensitive to TTX showing a small level of involvement of voltage-gated Na^{+} channels. These cells also showed a small rebound spike upon release of hyperpolarising current that was sensitive to the T-type Ca^{2+} channel blocker 300 µM Ni^{2+}.

The second type of CSFcC identified by (Marichal et al., 2009) had smaller input resistances than the first and produced a single spike with a mean amplitude of 38 mV and a half-amplitude duration of 6 ms in response to a depolarising current pulse. For an event to be considered an action potential it must fulfil a number of commonly-accepted criteria. These include having a rapid depolarising then a rapid repolarising phase usually followed by an after-hyperpolarisation, a duration as measured by the half-amplitude duration of < 3 ms and a peak amplitude of over 60 mV at resting membrane potential. The term spike is used when the event has rapid depolarising then hyperpolarising phases but does not fulfil one or multiple of the other criteria.

Some of these cells had a slow depolarising potential similar to those observed without the spike, whilst others had a rapid repolarising phase but none of these cells displayed a slow after-hyperpolarisation. These spikes were all sensitive to TTX indicating that they were produced by opening of voltage-gated Na^{+} channels.

Amongst this group of CSFcCs there was considerable variation in the shape of the cell body and the complexity and reach of basal processes.

The third group of CSFcCs in this study was able to produce a train of spikes and in some cases spikes that would be considered action potentials (Marichal et al., 2009). The spikes produced by these CSFcCs had larger amplitude (56 mV) and shorter half-amplitude duration (4 ms), than those of the single spiking cells. Some of the CSFcCs that could produce multiple spikes displayed increasing half-amplitude duration in the later spikes. Others were able to fire more robustly, with smaller differences from spike to spike. In these robustly firing cells there was a defined slow after-hyperpolarisation. As with the single-spiking cells, the spikes in the multiple-spiking cells were sensitive to TTX. Only in a small proportion of the cells was there a TTX-resistance depolarisation indicating that the more robust the spiking, the less the contribution of calcium conductances present in that cell. These multiple-spiking CSFcCs were observed to have greater complexity of basal processes than the single-spiking CSFcCs displayed but still did not express NeuN (Marichal et al., 2009).

Previous work from our group (Corns, 2012) has seen the same variation as has been previously reported. Additionally, they observed a population of CSFcCs within the rat spinal cord that showed passive, glial-like electrophysiological responses to current injection reminiscent of the glial-like type 2 CSFcCs observed in lamprey spinal cord (Jalalvand et al., 2014). Few of the non-spiking CSFcCs with slow Ca^{2+}-mediated depolarisations were observed (Corns, 2012), although cells producing a slow depolarisation after a single spike were more frequently observed. This work defined subtype 1 cells (CSFcC 1s) as the CSFcCs with glial-like non-spiking characteristics, subtype 2 cells (CSFcC 2s) as those that could produce a single spike and subtype 3 cells (CSFcC 3s) as those that could produce > 1 spike. The work presented in this thesis will use this classification of CSFcCs throughout.

1.2.2.3 Functions of CSFcC

There is little consensus on the primary function of CSFcCs with inter-species differences and within species differences suggesting that CSFcCs may fulfil a range of functions. There are multiple aspects of CSFcC structure that indicate that in many cases, they are likely to fulfil sensory roles; however these sensory roles are not always sensing the same physiological aspect.

The first physiological aspect that may be being detected by CSFcCs is movement of the spinal cord or flow of the CSF. In many non-mammalian species, the CSF-contacting bouton of CSFcCs displays a 9x2+2 cilium that is characteristic of kinocilia (Vigh and Vigh-Teichmann, 1998; Vigh et al., 2004). This differs from ventricular CSFcCs which show are more likely to have a 9x0+2 cilia (Vigh-Teichmann and Vigh, 1983). These kinocilia have been observed in contact with Reissner's fibre in the lancelet (a marine chordate) (Vigh-Teichmann and Vigh, 1983). This has been suggested to be able to detect movement of the spinal cord. Whilst mechanically sensitive neurons, called edge cells, have been identified in the lamprey spinal cord (Grillner et al., 1984), these cells were not comparable to CSFcCs.

CSFcC have also been shown to be mechanosensitive via a mechanism that is dependent on the expression of the PKD2L1 channel (Bohm et al., 2016). In 5 day old zebrafish, genetic ablation of CSFcCs (named Kolmer-Agduhr cells in this study) reduced spontaneous forward swimming (Wyart et al., 2009). Further investigation showed that the CSFcCs modulate components of the central pattern generator (CPG) by synapsing on glutamatergic interneurons (Fidelin et al., 2015). The response of CSFcCs to active and passive movement of the spinal cord was absent in the PKD2L1-knockout mutant zebrafish (Bohm et al., 2016). Although there are many similarities between the CSFcCs in the zebrafish and in mammalian systems, expressing PKD2L1, GABA and the olig2 transcription factor, differences in cilia structure and axonal characteristics mean that this functioning is not necessarily conserved into mammals.

The CSFcCs have variable numbers of stereocilia from the bouton on the apical CSF-contacting process (Vigh et al., 2004). These stereocilia are similar to those found in the inner ear, where they sense the flow of the endolymph fluid (Zetes and Steele, 1997). Both mammalian and non-mammalian spinal CSFcCs have stereocilia on the ends of their apical process, although there are few on mammalian CSFcCs (Vigh-Teichmann and Vigh, 1983). This leaves open the possibility that one role for CSFcCs conserved between species is detection of CSF flow. Whilst it has not been demonstrated that CSFcCs possess this role, maintaining the flow of CSF is very important, particularly in the spinal cord, where it is more prone to stasis (Whedon and Glassey, 2009). CSF stasis is associated with hydrocephalus and intracranial hypertension and thus detection is likely to be important.

As mentioned above, another aspect of CSF and the local environment around the CSFcCs that they are well equipped to detect is changes to pH. Expression of both PKD2L1 and $P2X_2$ receptors (via modulation of the response to ATP) enables CSFcCs to detect changes to pH levels and whilst $P2X_2$ has not been as widely identified, PKD2L1 appears to be one of the more conserved aspects of the CSFcC expression profile (Stoeckel et al., 2003; Studeny et al., 2005; Huang et al., 2006; Marichal et al., 2009; Djenoune et al., 2014). This could be detection of the pH of either the intercellular fluid or the CSF as the receptors identified which can sense pH are present throughout the cell and CSF-contacting process.

The CSFcCs could also be sensing signals released from nearby cells. CSFcCs receive synaptic inputs in the form of PSPs recorded from single cells abolished by TTX (Russo et al., 2004; Marichal et al., 2009) and respond to a number of neurotransmitters. CSFcCs from rat and turtle spinal cord have been shown to respond to GABA via $GABA_A$ receptors. This produced either a depolarisation or a hyperpolarisation, which has been suggested to be due to differences in expression of chloride transporters amongst CSFcCs (Reali et al., 2011; Chamma et al., 2012). Mammalian CSFcCs also respond to cholinergic signalling occurring through nicotinic acetylcholine receptors (Corns et al., 2015). The ionotropic purinergic receptor $P2X_2$ is also expressed in CSFcCs from P2 to adulthood in mammalian spinal cord as mentioned above (Stoeckel et al., 2003; Studeny et al., 2005). $P2X_2$ receptors primarily respond to levels of extracellular ATP. Purinergic signalling is involved in a range of functions including interactions between neuronal and glial cells (Fields and Burnstock, 2006; Lohr et al., 2014), and roles in stem cell signalling and development (Ulrich et al., 2012; Jiang et al., 2016). It may therefore be that the presence of $P2X_2$ receptors on CSFcCs enables purinergic signalling as part of the wider spinal cord circuitry.

The next point to consider is outcome of the detection of any of the above factors. CSFcCs are connected to the neuropil surrounding the central canal via the multitude of basal processes. There are many axons and neuronal cell bodies that run near the central canal, particularly at the ventral end (Bruni and Reddy, 1987). Synapses have been observed between CSFcCs and these neuronal cells (Stoeckel et al., 2003). This indicates that any information detected by the CSFcCs could therefore be transferred to these neighbouring cells by these connections.

Alternatively, in CSFcCs where the CSFcC axons are present, is transmission of the information to cells near the pial surface. However in most species such as in the rat spinal cord the synaptic proteins are diffusely dispersed throughout the axons and not concentrated at synapses (Stoeckel et al., 2003). This suggests that information release is not the primary function of these axons as this diffuse labelling of synaptic proteins is more normally associated with neurons during development before the attainment of functional synapses (Rene et al., 1996). In other species such as the *Xenopus* spinal cord there do appear to be functional vesicles but these do not appear to be functioning as synapses, releasing instead into the external CSF, rather than onto cells so they could therefore be acting as a hormonal signalling mechanism for the substances to act on a wide range of cells.

This release into the external CSF could function as a communication system, but it could also function as a trafficking system. The fact that the CSFcCs in *Xenopus* spinal cords are in contact with both the internal and the external CSF make them uniquely placed to modulate the compositions of both of these solutions.

There is some evidence for each of these suggested functions however the differences between species and the frequent variation within species make it unlikely that any one function is undertaken by all CSFcCs. It is likely that CSFcC are involved in a range of functions and may also have features of other roles that they no longer perform.

1.2.3 Ependymal cells

1.2.3.1 Distribution and expression of ependymal cells

Ependymal cells around the central canal are predominantly pseudostratified cuboidal ependymal cells forming the lining of the channel through which the cerebrospinal fluid flows (Bruni and Reddy, 1987)(Figure 1.2). This layer of ependymal cells is only a single cell thick in the majority of the rat spinal cord, however in some places it is thickened such that it contains multiple layers of ependymal cells. The cells in the single layer of ependymal cells are consistent in shape and orientation, with slight differences at the dorsal and ventral poles of the ependymal cells where the cells are more elongated in shape (Bruni and Reddy, 1987). The ependymal cells of the mammalian spinal cord are surrounded by collagens I and III and fibronectin which act as a basement membrane (Azzi et al.,

1990). Under electron microscopy ependymal cells have large nuclei and electron dense cytoplasm rich in intermediate filaments (Bruni and Reddy, 1987; Alfaro-Cervello et al., 2012). They also have numerous mitochondria, lipid droplets towards the apical membrane and Golgi apparatus in a horse-shoe shape (Alfaro-Cervello et al., 2012).

In studies of the mouse spinal cord, ependymal cells usually have 2 motile (9+2) cilia which were approximately 8 µm long and were invaginated at their ends (Alfaro-Cervello et al., 2012). The cilia of the ependymal cells were associated with electron-dense basal bodies that differed from those associated with cilia in any of the ciliated ventricular ependymal cells. The basal bodies were also not associated with a daughter centriole as has been observed with one type of ventricular cell (Doetsch et al., 2002; Mirzadeh et al., 2008; Alfaro-Cervello et al., 2012). A small percentage of cells (<7 %) had only one cilium but the type and basal body were similar to those with 2 cilia (Alfaro-Cervello et al., 2012). Similarly small populations of ependymal cells had 3 or 4 cilia and those with 4 cilia were larger and possessed 2 nuclei (Alfaro-Cervello et al., 2012). There were no large differences in cilia presentation of ependymal cells along the rostro-caudal axis of the spinal cord. The motility of the cilia has been demonstrated by the deposition of fluorescent microbeads which became attached to the cilia and revealed their back and forth motion (Alfaro-Cervello et al., 2012). Some ependymal cells had basal processes leading away from the central canal. These cells have predominantly been identified at the dorsal and ventral poles of the central canal with the processes leading along the dorsal-ventral axis (Bruni and Reddy, 1987; Alfaro-Cervello et al., 2012).

Ependymal cells are connected to neighbouring ependymal cells via a number of connections. Long zonulae adherens junctions run along the lateral sides of the ependymal cells (Alfaro-Cervello et al., 2012). These junctions are formed of cadherins (Ferreri and Vincent, 2008) and are linked to the actin cytoskeleton (Guo et al., 2007).

The second type of junctional connection through which the ependymal cells are connected is gap junctions. Expression of connexin 43 (Belliveau and Naus, 1995; Ochalski et al., 1996; Marichal et al., 2009), connexin 45 (Chapman et al., 2013) and connexin 50 (Rodriguez-Jimenez et al., 2016) has been observed in ependymal cells in the mammalian spinal cord. Additionally connexion 30 has been identified in

ventricular ependymal cells (Kunzelmann et al., 1999) but this is thought not to be expressed in spinal cord as Cx30-Cre recombinase reporter mice did not label any ependymal cells, only astrocytes within the grey and white matter (Barnabé-Heider et al., 2010). The expression of these connexins has been shown to produce functionally active gap junctions between the cells as demonstrated during electrophysiological experiments by the increase in input resistance upon application of gap junction blockers (Corns, 2012; Corns et al., 2013). Application of gap junction blockers also increased the voltage change seen in response to agonist application (Corns et al., 2013) indicating that this could be a way ependymal cells modulate their responses to signalling.

The expression profile of ependymal cells

Ependymal cells in the mammalian spinal cord express a number of markers associated with stem cells. Sox2, a transcription factor essential for the maintenance of pluripotency in undifferentiated stem cells and a related protein, Sox9, are widely expressed in ependymal cells around the central canal (Hamilton et al., 2009; Barnabé-Heider et al., 2010). Sox2 expression has been observed in a range of species including rat (Hamilton et al., 2009), mouse (Meletis et al., 2008) and human spinal cord (Dromard et al., 2008). Nestin is expressed in a subset of ependymal cells, predominantly found dorsal to the central canal and usually those with long basal processes leading dorsally away from the central canal, occasionally they are also seen ventrally or laterally (Hamilton et al., 2009; Alfaro-Cervello et al., 2012). Nestin is an intermediate filament found in developing cells and cells after injury, further suggesting that the ependymal cells around the central canal may act as stem cells within the spinal cord.

The majority of ependymal cells contain vimentin, an intermediate filament associated with mesenchymal cells, and the calcium binding protein S100β which is predominantly found in astrocytes (Hamilton et al., 2009). Further, ependymal cells express CD24, a glycoprotein expressed by mature ependymal cells (Mirzadeh et al., 2008; Alfaro-Cervello et al., 2012) and CD133 (Alfaro-Cervello et al., 2012) which is observed in ependymal cells and astrocytes. Ependymal cells can also be identified by their expression of the transcription factor FoxJ1 which acts as a marker for ciliated epithelia and cells of ependymal lineage (Huang et al., 2003; Meletis et al.,

2008; Alfaro-Cervello et al., 2012). There are further proteins expressed in a subset of ependymal cells which will be discussed below.

1.2.3.2 Proliferative abilities of ependymal cells

Spinal cord ependymal cells show proliferative properties that can be modulated by a number of factors. Under physiological control situations ependymal cells replicate at a low rate and self-duplicate to maintain ependymal cell numbers with minimal differences between different regions along the rostro-caudal axis of the spinal cord (Horner et al., 2000; Alfaro-Cervello et al., 2012). Whilst this proliferation occurs in all aspects of the central canal, a largest proportion of the newly-proliferated cells have been observed dorsally and dorsolaterally with the lowest proportion laterally and intermediate levels in ventral regions (Alfaro-Cervello et al., 2012). The cells produced were almost all ependymal cells as they still expressed CD24 (96%) and were ciliated (Alfaro-Cervello et al., 2012). In young mice there was a higher rate of proliferation of ependymal cells which correlated with growth of the spinal cord (Alfaro-Cervello et al., 2012) resulting in a low rate of self-duplication that was maintained into adulthood in mice (Barnabé-Heider et al., 2010) and rats (Horner et al., 2000). These newly proliferated cells were observed to stay in the location of the central canal.

There are physiological and pathological factors that can modulate central canal proliferation. Enhanced physical activity by use of a running wheel, increased the level of proliferation around the central canal in rat spinal cord by 2.1-2.6 fold after 4 days (Cizkova et al., 2009) but this difference decreased to closer to the control levels by 14 days suggesting that it is a short-lived response and not on-going. Cizkova et al. (2009) also observed re-expression of nestin in previously nestin-negative ependymal cells with increased physical activity.

A range of spinal cord insults including contusion and incision insults (Cizkova et al., 2009; Barnabé-Heider et al., 2010; Lacroix et al., 2014) also produced a considerable increase in proliferation rate of ependymal cells. This response was not seen when the functioning of the spinal cord including locomotion was damaged by demyelinating lesions. Neither chemically induced demyelination nor autoimmune-related demyelination produced any change in the rate of ependymal cell proliferation (Lacroix et al., 2014). This is very different from injured spinal cord, where the increased rate of proliferation peaked at 3 days after injury in the adult mouse spinal

cord, followed by a wave of proliferation moving rostrally up the spinal cord such that the segment rostral to the injury had peak proliferation 14 days after injury and cervical segments showed peak proliferation after 21 days (Lacroix et al., 2014). In the rat spinal cord, proliferation was observed to increase 2.6 fold, compared to control levels (Cizkova et al., 2009).

Increases in proliferation rate could also be triggered by growth factors, which when infused into the 4th ventricle, increased the number of newly proliferated cells within the 4th ventricle and around the central canal in the spinal cord (Martens et al., 2002). The increase in proliferation around the central canal was seen with the triple infusion of epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2) and heparin. This caused approximately a 33-fold increase in the numbers of newly-proliferated cells around the central canal. The newly-proliferated cells produced by this treatment were predominantly around the central canal. Martens et al. (2002) suggested that the proliferating cells may not be ependymal cells as the thymidine analogue marker for newly-proliferated cells (BrdU) did not co-localise with S100β, which is known to be expressed by ependymal cells (Hamilton et al., 2009). This lack of co-localisation between the marker for newly-proliferated cells and S100β was also observed under physiological conditions (Horner et al., 2000) however neither study used any other ependymal cell markers to confirm this finding and ensure that proliferating ependymal cells express S100β. S100β is a calcium binding protein expressed in mature ependymal cells and there may be changes to expression during proliferation. Alternatively there could be multiple proliferating cell types around the central canal, however S100β is also expressed by astrocytes so reducing the pool of potential other dividing cell types. In a similar study with rat spinal cord (Kojima and Tator, 2000), EGF and FGF2 increase the rate of proliferation in cells that were described as likely to be ependymal cells due to their location clearly within the ependymal cell layer and their lack of expression of GFAP, an astrocytic marker not widely expressed in ependymal cells (Bruni et al., 1985).

Using electrophysiological experiments, ependymal cells were shown to respond to acetylcholine (ACh) application and modulation of cholinergic signalling potentiated the electrophysiological response of ependymal cells to ACh and increased the numbers of newly-proliferated cells around the central canal in an organotypic slice culture model using mouse spinal cord and *in vivo* (Corns et al., 2015). In this study the newly-proliferated cells marked with an alternative thymidine analogue (EdU) co-

localised with two markers for spinal cord ependymal cells, namely the Na-K-ATPase α1 subunit (NKAα1) and CD24, as well as being positive for the stem-cell marker Sox2. This indicates that in the case of cholinergic enhancement of proliferation around the central canal, ependymal cells are the proliferating cell. In the non-mammalian spinal cord dopamine has a neurogenic role. During spinal cord regeneration in the adult zebrafish endogenous dopamine signalling plays an important role in the production of motor neurons similarly to the pro-motor neurogenic role dopamine signalling plays during development (Reimer et al., 2013). Despite demyelinating disorders not triggering increases in proliferation nicotine signalling increases ependymal cell proliferation (Gao et al., 2015). Experimental autoimmune encephalomyelitis (EAE) decreases the level of proliferation of ependymal cells even lower than is seen in uninjured spinal cord, accompanied by an increase in nestin production. Application of nicotine reduced both of these effects of EAE, reducing the increase in nestin expression and the decrease in proliferation, and also acted to increase the number of oligodendrocytes present and decrease the presence of inflammatory microglia and leukocytes (Gao et al., 2015).

1.2.3.3 Characteristics of newly-proliferated ependymal cells

For ependymal cells to be classified as stem cells rather than progenitor cells, they must fulfil a number of criteria. The first of these is that they are unspecialised. The morphology of ependymal cells is simple cuboidal with relatively few specialisations. The second is that the cells are capable of self-renewal. Self-renewal of stem cells is associated with asymmetric division, which is indicated by a plane of cleavage parallel to the luminal surface of the cell. In ependymal cells after spinal cord injury, in 83% of newly-proliferated ependymal cells with a detectable plane of cleave the plane was parallel to the luminal cell surface (Johansson et al., 1999). The third criterion is that the progeny they produce are specialised cells. This has been demonstrated by (Barnabé-Heider et al., 2010), as discussed below.

Barnabé-Heider et al. (2010) showed that it is not only the ependymal cells that increase their proliferation after spinal cord injury. There were two other cell populations which proliferate in the intact spinal cord and respond to spinal cord injury by increasing their proliferation rate. In the intact spinal cord oligodendrocyte precursors were observed to self-renew and produce mature oligodendrocytes. Ependymal cells were observed just to self-replicate and produce more ependymal

cells in the intact spinal cord, as has been previously observed (Alfaro-Cervello et al., 2012). Similarly, in the intact spinal cord, astrocytes also self-duplicated, producing more astrocytes. Genetic fate mapping was used with FoxJ1-Cre recombinase mice to track ependymal cells, Connexin30-Cre mice to track astrocytes and Olig2-Cre recombinase mice to track cells of oligodendrocyte lineage. In the intact cord the number of newly proliferated ependymal cells was much lower than of astrocytes and oligodendrocytes, but after an incision was made to the dorsal funiculus, the biggest increase in the rate of proliferation was seen in the ependymal cells. After injury, ependymal cell proliferation increases by 4-5 fold, while the proliferation of the other two cell populations increases approximately 2-fold (Barnabé-Heider et al., 2010). These increases in proliferation were not observed in segments adjacent to the injury, where the levels were the same as in intact spinal cords, indicating that the response to the injury was local and not throughout the spinal cord.

The use of genetic fate mapping in this experiment also showed differential localisation of these newly-proliferated cells after incision of the dorsal funiculus (Barnabé-Heider et al., 2010). Unlike in the intact condition, where ependymal cells are found exclusively within the central canal region, after injury the newly-expressed ependymal cells were predominantly found in the centre of the lesion site, in the scar-forming tissue, which in these experiments was in the dorsal funiculus. They accounted for over half the cells within the glial scar. Astrocytic progeny also accumulated near the injury site but unlike ependymal cells which were found at the centre, newly-proliferated astrocytic cells were found on the perimeter of the injury site. Oligodendrocytic progeny were also observed to be more numerous after injury and there were some present within and near the injury site but similar distribution to that observed in the grey matter and other white matter regions. This migration of ependymal cells to the site of injury has also been observed in other studies (Johansson et al., 1999; Meletis et al., 2008) as well as in minimal injury models, where there was insufficient injury to draw the ependymal cells fully to the site of injury, but there was a 8-fold increase in proliferation and migration of ependymal cells in dorsal and ventral directions (Mothe and Tator, 2005).

A further difference observed by Barnabé-Heider et al. (2010) between intact and injured spinal cord was in the phenotype of the newly-proliferated cells. This type of injury produced considerable cell death of neurons and no BrdU labelled cells expressed NeuN (a neuronal marker) indicating that no new neurons were produced.

Of the newly-proliferated cells, those of oligodendrocyte lineage developed as in intact spinal cords to produce oligodendrocyte precursors and mature oligodendrocytes. The cells of astrocytic lineage also predominantly maintained astrocytic features as was seen in intact spinal cords, however there were morphological changes. After injury the newly-proliferated astrocytic cells displayed a more elongated and less ramified shape. In both intact and injured spinal cords, a small number of cells of astrocytic lineage expressed a marker for oligodendrocyte precursors (Olig2), with a lower percentage displaying Olig2 expression after spinal cord injury. Cells of ependymal lineage showed considerable differences in the phenotype after spinal cord injury compared with those in the intact condition. In the intact spinal cord, all newly-proliferated cells of ependymal lineage showed ependymal cell markers (FoxJ1 and Sox9) and were localised to the central canal region. After spinal cord injury <30 % displayed FoxJ1 expression and over 20 % displayed GFAP positivity, where none was observed in the intact cords. Additionally a small percentage of ependymal progeny displayed expression of the oligodendrocyte precursor marker, Olig2. This indicates that after spinal cord injury, ependymal cells are able to differentiate into astrocytes and oligodendrocyte precursors as well as the ependymal cells they produce in the intact spinal cord.

The formation of the glia scar by cells of ependymal lineage has been shown to be beneficial (Sabelström et al., 2013), and not purely detrimental as has been suggested due to its action as a barrier to axonal regrowth (Burda and Sofroniew, 2014). Sabelström et al. (2013) used a mouse model with selective inducible knockout of all Ras genes within the FoxJ1-expressing ependymal cells (as used in Barnabé-Heider et al. (2010)) to assess the outcomes of removing ependymal contribution to the glial scar. Ras genes enable the ependymal cells to proliferate, thus knock out of these genes prior to injury removal of the involvement of ependymal progeny in the response to the injury without disruption of the non-proliferative functions of ependymal cells. In the mice without ependymal contribution there was a higher fibrous component contributed by pericytes, and also a higher contribution to the glial scar by resident astrocytes. After 14 weeks, the mice without scar forming contribution by ependymal cells had far larger and deeper lesions, with thinner remaining spinal cord at the level of the lesion, compared to controls (Sabelström et al., 2013). Blocking the pericytes response didn't have the same effect of deepening the lesions site compared to controls, suggesting that ependymal cell contribution is

particularly important for minimising lesion depth. A secondary factor likely to have contributed to the increased lesion depth in the mice with no ependymal cell contribution is the fact that the ependymal cell progeny acted as a rich source of neurotrophic factors without which there was increased neuronal death (Sabelström et al., 2013). The consequence of this deepened lesion in this experiment using a lesion to the dorsal funiculus was that damage to the corticospinal tract was more frequently observed indicating a protective role for glial scar-forming ependymal cells.

While Barnabé-Heider et al. (2010) showed the progeny of ependymal cells did not include any mature neuronal cells, as no BrdU was observed to co-localise with NeuN, they did observe expression of some markers associated with neural stem cells. Cells of ependymal lineage and astrocytic lineage but only a few of oligodendritic lineage expressed Sox2 while both CD133 expression and nestin second intron enhancer activity were only seen in cells of ependymal lineage. Additionally various *in vitro* and transplantation studies have provided evidence for this capability in spinal cord ependymal cells (Weiss et al., 1996; Shihabuddin et al., 1997; Johansson et al., 1999). Cells from all regions along the rostro-caudal axis of the spinal cord of a 3 month old rat, once cultured in a fibroblast growth factor 2 (FGF-2) produced some passagable neurospheres expressing the neuron-specific microtubule-associated protein 5 (MAP-5) (Shihabuddin et al., 1997). Neurospheres are free-floating spherical expansions of cells that express nestin, which is found in stem cells and neural progenitors (Gage, 2000). Weiss et al. (1996) also observed that both primary culture and secondary expanded clones from all spinal cord regions produced spheres of undifferentiated cells that could be induced to differentiate into either neurons, astrocytes and oligodendrocytes as determined by expression of neurofilament M, GFAP and O4 respectively. However Weiss et al. (1996) found that the greatest yield of multipotent cells was obtained from cells collected from the lumbar/sacral region of the spinal cord. All three lineages, astrocytes, oligodendrocytes and neurons could be produced from cultures from a single founder cell, indicating the multipotency of spinal cord ependymal cells and the process of serial passaging and recloning indicated that *in vitro* they are self-renewing (Shihabuddin et al., 2000).

With respect to the type of neurons produced by these cultures of ependymal cell-derived progenitors, Hamilton et al. (2009) induced neurospheres to differentiate into neurons expressing βIII tubulin and GAD-65. Moreno-Manzano et al. (2009)

demonstrated differentiation of the neurospheres into functional spinal motor neurons as demonstrated by electrophsiological recording of the cells produced and expression of the motor neuron-specific transcription factor HB9 and axons and dendrites labelled with MAP-2. These could represent the same population of GABAergic motor neurons or the differences in culture protocol and culture medium could have induced the stem cells to differentiate into different types of neuron.

It has been shown that these neurospheres and spheres of undifferentiated cells originate from the ependymal cells and not any astrocytes or oligodendrocyte precursors by use of genetic fate mapping in cultured cells (Barnabé-Heider et al., 2010). This showed that astrocyte and oligodendrocyte precursors were not responsible for the production of neurospheres in the culture of spinal cord cells. Instead approximately 80 % of neurospheres were labelled by recombination of FoxJ1-Cre. This was the same proportion of ependymal cells labelled by this method, suggesting that they were all likely to be derived from cells of ependymal lineage.

These *in vitro* experiments demonstrate the multipotency and neurogenic potential of ependymal cells, however not all this potential is seen *in vivo*. Shihabuddin et al. (2000) cultured ependymal cells *in vitro* and expanded a clonal population of these cells that were transplanted into various CNS regions. When these cultured ependymal stem cells were transplanted back into the spinal cord, they did not produce any cells of neuronal lineage shown by no co-localisation of the marker for the cloned cells and NeuN. Instead mainly astrocytes (GFAP expression) and glial precursors (NG2 expression) were produced, with a few oligodendrocytes (Rip or adenomatous polyposis coli expression without GFAP expression). The cells were also transplanted into the hippocampus. When they were located in the neurogenic regions of the hippocampus, the majority of the cells expressed NeuN and almost all of them also expressed calbindin-$_{D28k}$ and some showed morphology of mature hippocampal granule neurons. Many of the remainder of the cells expressed markers of glial progenitors. The cultured cells transplanted into the non-neurogenic region of the hippocampus did not display any co-localisation of the transplanted cell marker and NeuN indicating that none of the transplanted cells differentiated into neurons in this region. Instead the cells displayed markers of glial progenitors, astrocytes and oligodendrocytes with the proportion of these cells reasonably similar to what was observed in the from endogenous ependymal cell populations in the spinal cord but

with fewer GFAP-positive astrocytes (Shihabuddin et al., 2000). This suggests that the spinal cord is a particularly gliogenic environment.

The idea that the spinal cord is a gliogenic environment is supported by a study which showed that transplantation of ependymal-derived cultured stem cells which had been transduced to express the neurogenesis-promoting protein neurogenin-2, still produced very few neurons upon transplantation into the spinal cord (Hofstetter et al., 2005). The neurogenin-2 expression did act to shift the differentiation of the transplanted cells towards an oligodendrocyte phenotype and away from an astrocytic one. This had multiple effects compared to transplantation of non-neurogenin-2 cultured stem cells. Transduction of neuogenin-2 expression increased the positive effects of stem cell transduction after injury, with increased amounts of myelin in the injured area and recovery of hindlimb functioning and sensory responses. Neurogenin-2 expression in the transplanted stem cells also reduced the allodynia observed with transplantation of non-neurogenin-2-treated stem cells (Hofstetter et al., 2005). This indicates that multiple aspects of the potential responses of ependymal cells to spinal cord injury could be beneficial although it may need directed to produce the maximal result.

The capabilities of ependymal cells after spinal cord injury was expanded on by Moreno-Manzano et al. (2009) who cultured ependymal cells in a similar way to the studies mentioned above but with some of the initial cells coming from animals with spinal cord injury. The ependymal stem/progenitor cells from injury (epSPCis) proliferated 10 times faster than those from non-injured spinal cord (epSPCs) and while both epSPCs and epSPCis produced functional, HB9-expressing spinal motor neurons, a better yield was produced by neurospheres formed by epSPCi culture. Acute transplantation of undifferentiated epSPCis or resulting oligodendrocyte precursors into rat model of severe spinal cord contusion injury improved motor functioning 1 week after injury. The transplanted cells migrated a considerable distance (2 mm) from the transplantation site to the injury site. When the outcome of the transplantation of epSPCis or oligodendrocyte precursor cells (OPC) on the motor functioning (assessed by Basso, Beattie, and Bresnahan (BBB) scoring) was compared, it showed that the animals with OPC transplantation performed better than those with epSPCi transplantation. This suggests that whilst the formation of the glial scar is important (Sabelström et al., 2013), additional benefits are gained by more oligodendrocytes and oligodendrocyte precursors.

Infusion of growth factors also improves functional recovery from spinal cord injury (Kojima and Tator, 2002), and as this has been shown to increase ependymal cell proliferation greatly (30-fold) (Kojima and Tator, 2000), this further hints at a beneficial role for ependymal cells and a great importance for understanding their signalling as a potential method to improve outcome from spinal cord injury by utilising the endogenous stem cell capabilities of the spinal cord.

1.2.3.4 Variation of ependymal cells and their role in the neurogenic niche

Ependymal cells have widely been recognised as a heterogeneous group and there are many aspects in which there is variation between ependymal cells. One way in which ciliated ependymal cells expressing FoxJ1 (a protein which identifies cells with motile cilia (Meletis et al., 2008)) differ is the number of the cilia they possess. Most ependymal cells had 2 cilia but ependymal cells with 1, 3 and 4 were also observed (Alfaro-Cervello et al., 2012). The ependymal cells with 4 cilia were noted to be binucleated.

Another structural way that ependymal cells vary is between the cuboidal cells with and without basal processes. Sabourin et al. (2009) used GFAP-GFP mice to show that GFAP was expressed in radial ependymal cells predominantly in the dorsal region of the central canal although a few were observed laterally and ventrally. These cells were observed either within the ependymal cell layer or subependymally with a process into the central canal. These cells were observed to have radial processes leading away from their basal end and towards the pial surface of the spinal cord. As well as expressing GFAP, these cells express the epithelial-mesenchymal-transition zinc finger E-box-binding protein 1 (ZEB1) and a subset also expressed brain lipid-binding protein (BLBP) (Sabourin et al., 2009), a protein proposed to be involved in the establishment of radial fibres (Feng et al., 1994).

The vast majority of neurospheres produced by culturing of central canal cells from the GFAP-GFP mice showed expression of GFP, with those that were GFP negative ascribed to the incomplete expression of GFP in GFAP expressing cells in this transgenic model (Sabourin et al., 2009). This was supported by the fact that all neurospheres expressed BLBP with no BLBP observed in non-GFAP expressing cells (Sabourin et al., 2009). Additionally, use of a transgenic line expressing an inactive form of ZEB1 almost eliminated the production of neurospheres and dramatically increased the amount of cell death, indication that expression of ZEB1

was essential for the survival of neural stem cell functioning *in vitro* (Sabourin et al., 2009). They noted that GFAP expression was only seen with polyclonal antibodies and was not seen with monoclonal antibodies. This may explain some differences in observed level of GFAP expression within the ependymal cell layer and in resultant cell cultures.

Sabourin et al. (2009) observed that a proportion of the GFAP-positive stem cells were located subependymally and put a process into the central canal, accessing the CSF. This morphology is reminiscent of CSFcCs and has also been described in GFAP-positive cells (Alfaro-Cervello et al., 2012), where they were defined as astrocytes. In this experiment the GFAP-positive subependymal astrocytes were not observed to proliferate *in vivo*. This suggests that there may be different populations of stem cells within the spinal cord. It may be that the neurogenic population is not the primary proliferative population that produces the majority of astrocytes and oligodendrocytes. Sabourin et al. (2009) also showed that CD15 was expressed around the central canal and was co-localised with both GFAP and BLBP. CD15 is a stem cell marker (Zhang et al., 2013; Li et al., 2016). Dromard et al. (2008) observed CD15 expression around the central canal in the human spinal cord, with CD15 expressed predominantly dorsal to the central canal and very little in ventral regions. They drew connections between the expression of GFAP, CD15, nestin and Sox2 at the dorsal end of the central canal with the neural stem cells in the subventricular zone (SVZ; an adult neurogenic niche within the brain). The SVZ neural stem cells express Sox2 (Ferri et al., 2004), GFAP and CD15 (Capela and Temple, 2002; Doetsch, 2003).

The studies above have looked at the ability of the GFAP-positive radial cells to form neurospheres in the culture situation however there is evidence that they may also be important in the proliferation seen *in vivo*, in situations such as spinal cord injury. GFAP, nestin and CD15 expression have all been observed predominantly around the dorsal aspect of the central canal, with minimal expression laterally and ventrally. This correlates with where the majority of the proliferation has been observed *in vivo*, where the highest rates are from dorsal regions of the central canal (Hamilton et al., 2009; Sabourin et al., 2009).

It could be that it is just GFAP-positive radial ependymal cells that are stem cells and not cuboidal ependymal cells but it may also not be that clear cut. Antigenic

differences between cuboidal ependymal cells, radial ependymal cells and tanycytes (discussed below), which are also sometimes considered a subclass of ependymal cell, have not been clearly defined and intermediate phenotypes have also been observed, suggesting that perhaps the cells represent a spectrum of ependymal cells (Meletis et al., 2008). There may also be a range of capabilities of the cells, perhaps with only a subpopulation that has neurogenic potential *in vitro*. Mothe and Tator (2005) used DiI labelling to track the ependymal cells as they migrated away from the central canal in response to injury. No co-localisation was seen between DiI and GFAP until 14 days after injury, however after that point the majority of DiI labelled cells also expressed GFAP. This fits with the fact that ependymal cells produce predominantly astrocytes after injury but would suggest that it is a non-GFAP expressing cell population within the ependymal cells that is responsible for the proliferation after injury. Whilst Sabourin et al. (2009) noted difficulties visualising GFAP, other cells are shown to be GFAP positive as are some ependymal cells after 14 days suggesting that this was not an issue. There may be species differences as the cultured GFAP positive ependymal cells were from the mouse spinal cord where the migratory ependymal cells in situ experiments were done in the rat. However it is also possible that there are multiple types of stem cell within the heterogeneous ependymal cell population with different activators and differentiation capabilities.

1.2.4 Tanycytes

The other population of cells in this area are the tanycytes, which are also sometimes referred to as radial ependymal cells (Figure 1.2). These cells provide a link between the internal CSF and the vasculature, as they sit within the ependymal cell layer and contact the CSF via microvillia and a single cilium at their apical end (Rafols and Goshgarian, 1985; Meletis et al., 2008) and from their basal end, they have a process of which terminates on a blood vessel (Rafols and Goshgarian, 1985). Tanycytes have been observed along the length of the spinal cord (Bruni and Reddy, 1987) at an average frequency of 1-3 cells per 0.5 µm section, although the numbers were higher in the lumbar and sacral regions than in cervical and thoracic levels. In addition to the basal process that terminates on blood vessels subjacent, the tanycytic processes also contact neurons (Bruni and Reddy, 1987). Ependymal cells and tanycytes and CSFcCs show immunoreactivity for vasoactive intestinal polypeptide (VIP) (Basbaum and Glazer, 1983; Lamotte, 1987; Chung and Lee,

1988), a hypertensive and vasodilatory peptide found in the CSF (Fahrenkrug et al., 1977; Sharpless et al., 1984).

This section of the introduction has discussed the stem cell niche around the central canal of the spinal cord and the neurogenic potential of the spinal cord ependymal cells. The central canal region is not the only stem cell niche in the adult central nervous system; there are two stem cell niches within the brain which have proven neurogenic potential. These are discussed and contrasted with the central canal region below.

1.3 Adult CNS stem cell niches

Although the vast majority of neurogenesis is completed in embryological development there are two stem cell niches within the adult mammalian brain that maintain neurogenic activity into adulthood. The adult brain neurogenic niche that is best characterised is the subventricular zone (SVZ) of the forebrain (Chojnacki et al., 2009) with the other being the dentate gyrus of the hippocampus (Zhao et al., 2008). Within the SVZ the neural stem cells are of astrocytic type (Hamilton et al., 2009) and sit subependymally. Also subependymally, the SVZ has transit-amplifying progenitors, neuroblasts, tanycytes, microglia and blood vessels all sitting outside the quiescent ependymal cells which line the lateral ventricles and are post-mitotic (Mirzadeh et al., 2008; Hamilton et al., 2009). The astrocytic stem cells (the stem cells are the B1 cell group (Spassky et al., 2005)) generate the transit-amplifying progenitors, that then produce neuroblasts. These neuroblasts migrate to the olfactory bulb and produce new interneurons (Morshead et al., 1994; Doetsch et al., 1999). The morphology of the SVZ astrocytes is such that they have an apical process which contacts the CSF in the lateral ventricle despite their subependymal location, and also a basal process terminating on blood vessels (Mirzadeh et al., 2008; Tavazoie et al., 2008). Under normal physiological conditions, the stem cells of the SVZ replicate at a steady rate producing the progenitors which move along the developmental pathway to become new neurons and also produce oligodendrocyte precursors which mature into oligodendrocytes. This differs from the stem cells in the spinal cord which do not function to produce any other cell types under intact physiological conditions. Spinal cord stem cells then increase their proliferation

dramatically in the injured situation and the SVZ astrocytic stem cells also respond to external situations. When the cells in the SVZ were experimentally depleted, there was a corresponding increase in the proliferation of the stem cells to replace the lost cells (Morshead et al., 1994; Doetsch et al., 1999).

Despite also being an adult CNS stem cell niche, there are multiple differences between the SVZ and the spinal cord ependymal cells. In the spinal cord there is no clear subependymal region, with a few cells positioned subependymally but often of the same cell type as found ependymally (Sabourin et al., 2009). Significantly the cell type acting as the stem cell in these regions differs, despite the expression of GFAP in the spinal cord stem cell (Sabourin et al., 2009) it is widely agreed that ependymal cells are the stem cell in the spinal cord (Meletis et al., 2008), unlike the astrocytic stem cells in the SVZ.

A further difference is the role of PSA-NCAM- and Dcx-containing cells within these stem cell niches. In the spinal cord stem cell niche the Dcx-containing cells are the CSFcCs which have not been observed to proliferate in the adult spinal cord (Marichal et al., 2009), whereas, in the SVZ, Dcx is expressed by the neuroblasts in the SVZ and as they migrate towards the olfactory bulb (Kuhn and Peterson, 2008). These neuroblasts go on to produce new neurons and start expressing the marker for mature neurons, NeuN (van Praag et al., 2002).

Despite the fact that astrocytic SVZ stem cells could repopulate a portion of the SVZ subependymal cells, generally experimental insults to the ventricular ependyma are not reversible and the functioning of the stem cells in this area does not appear to be focussed on response to injury (Garfia et al., 1979). Instead the role of the SVZ stem cells is to produce cells including neurons which integrate into a range of circuits and are involved in pattern separation, olfactory discrimination and memory (Gengatharan et al., 2016; Migaud et al., 2016).

The other brain region that produces new neurons in adulthood is the dentate gyrus of the hippocampus, where the adult-born dentate granule cells integrate into the local hippocampal circuitry (Parent, 2007). The neurogenic ability of the dentate gyrus is important for several learning processes and for dentate gyrus plasticity (Saxe et al., 2006; Winocur et al., 2006; Dupret et al., 2008). Unlike in the subventricular zone where the proliferating cells are multi-potent stem cells, the proliferating cells within the dentate gyrus are progenitor cells (Seaberg and van der Kooy, 2002). Culture of

the proliferative cells from the dentate gyrus *in vitro* showed that there are separate neural and glial progenitors within this region. Despite the fact that the dentate gyrus does not contain stem cells, one similarity between the dentate gyrus and the central canal region is that exercise also increases the rate of cell production in the dentate gyrus (Fabel and Kempermann, 2008). This increase in neurogenesis in the dentate gyrus is also seen with factors such as enrichment of the environment (Van Praag et al., 2000). There are more similarities between the central canal region and the SVZ than with the dentate due to the dentate gyrus not having a location next to the CSF and therefore no ependymal cells.

Despite the fact that the SVZ has ependymal cells, the ependymal cells in the brain do not display the same level of proliferative ability as those in the central canal region. This is highlighted by the proportion of gliomas that ependymomas account for in the two regions. Ependymomas constitute only 4-6 % of intracranial gliomas but 63 % of medullary and spinal gliomas (Rubenstein, 1989).

1.4 Response of the central canal region to damage – Spinal Cord Injury

The responses of ependymal cells to injury to the spinal cord have been discussed above in terms of their proliferation rate, migration and the lineages of cells produced. It is important to put into context the other factors occurring during the injury states where the increase in ependymal proliferation is seen. The predominant way that the increased proliferative abilities of ependymal cells could be activated is by spinal cord injury (SCI). SCI occurs at a worldwide annual incidence rate of between 10 and 40 cases per million people mainly due to vehicle accidents, falls, sports injuries, work related injuries and violence (Lee et al., 2014a). Spinal cord injury affects a range of people but disproportionally affects males and shows a bimodal distribution for age, affecting young adults and the elderly at the highest rates (Furlan et al., 2009).

In mammals, spinal cord injury leads to irreversible loss of function distal to the lesion site. This loss of damage is mediated through several factors including damage to the axons passing through the lesion site, demyelination and death of cells involved in the healthy functioning of the spinal cord such as oligodendrocytes, astrocytes and neurons (Grossman et al., 2001; Hulsebosch, 2002). There are multiple stages of

spinal cord injury which contribute to this damage to axons, death of cells and loss of functioning. The primary mechanical trauma disrupts cells and axons and initiates a cascade of further events, many of which contribute to the damage. Loss of microcirculation around the damage site contributes to local ischemia (Tator and Fehlings, 1991) and together with other factors, expands the injury site. The cavity formed by the rapid necrosis of cells becomes filled by a cyst containing many microglia, fibroblasts and astrocytes and leads to the formation of a glial scar (Iyer et al., 2017). All of these factors play a number of roles which may be both beneficial and detrimental at different times and in different circumstances.

It is therefore likely that a number of therapeutic treatments will be needed to fulfil these varying requirements. One possible therapeutic option under consideration for a role in the response to spinal cord injury is utilisation or modulation of the properties of ependymal cells or epSPCs (Parr et al., 2008; Moreno-Manzano et al., 2009). Both cultured ependymal cells derived from injured and non-injured spinal cords have been shown to have beneficial effects on the functional outcome after spinal cord injury in rats.

Spinal cord-derived neural stem/progenitor cells (NSPCs; likely to be analogous to epSPCs as cultured from the ependymal regions of the rat spinal cord) implanted 9 days after a spinal cord injury was shown to produce an improved functional outcome (Parr et al., 2008). Implantation of bone marrow-derived mesenchymal stromal cells (BMSCs) has previously been shown to be beneficial when implanted after SCI (Chopp et al., 2000; Syková and Jendelová, 2005) however Parr et al. (2008) showed no functional benefit of either BMSCs alone nor when BMSCs and NSPCs were applied, despite showing a reduction in cavity size, with only application of NSPCs alone improving the BBB score of locomotor functioning. These differences in the effectiveness of BMSC transplantation may have been due to timing of transplantations and possibly also the strain and culturing methods of BMSCs. However the implantation of NSPCs alone was able to improve functional recovery as shown by a significantly better performance in both the ladder-walk and open-field tests compared to both control and BMSC treated animal (Parr et al., 2008). Transplanted NSPCs produced a high number of cells expressing APC (adenomatous polyposis coli), a marker for oligodendrocytes (Lang et al., 2013). Additionally, their transplantation was shown to help preserve or promote

regeneration of the descending axons as shown by retrograde labelling to brain regions such as motor nuclei (Parr et al., 2008).

Ependymal-derived stem/progenitors for injury (epSPCis) were shown to proliferate 10 times faster *in vitro* than epSPCs and were shown to aid functional recovery when transplanted into injured rat spinal cords 1 week after the SCI (Moreno-Manzano et al., 2009). The implanted cells showed good survival rates and migrated to the site of injury. Unlike with implantation of epSPCs/NSPCs (Parr et al., 2008), implantation of epSPCis was shown to reduce the size of cysts/cavities around the injury site. Transplantation of epSPCis improved the open-field BBB scores compared to control animals despite minimal differentiation of the transplanted cells being seen (Moreno-Manzano et al., 2009). This lack of differentiation of the transplanted cells suggests that either the transplanted cells are providing an environment that is more conducive to the optimal functioning of endogenous cells or that the transplanted cells can provide positive effects in their undifferentiated state.

1.4.1 Possible methods of detection of damage by ependymal cells

While it is known that spinal cord injury affects the functioning of ependymal cells, it is not known which aspect of the spinal cord injury is being detected by the ependymal cells or if other cells are detecting the damage and signalling to the ependymal cells. One theory is that their response is triggered by the raised levels of extracellular ATP and other nucleotides that are observed after spinal cord injury (Wang et al., 2004), mechanical brain injury (Franke et al., 2006) and even ischemia (Melani et al., 2005). ATP is used as a damage signalling mechanism in other regions of the body such as in the cochlea (Paemeleire and Leybaert, 2000; Lahne and Gale, 2010). In spinal cord injury levels of extracellular ATP have been shown to play important roles in the local response mechanisms, where in addition to the injury site, ATP was shown to be released by the peritraumatic zones for multiple hours after injury (Wang et al., 2004). This raised level of extracellular ATP was a factor in the death of neurons and oligodendrocytes. ATP and several analogues were also shown to be sufficient to cause cell death (10 μM in 2 μL aliquots), with the $P2X_7$ receptor implicated due to similar levels of cell death with the $P2X_7$-specific agonist BzATP (Wang et al., 2004). In addition to the cell death, this raised level of extracellular ATP is likely to affect a range of cells as purinergic receptors are expressed on many cells. ATP is frequently used as an intercellular signalling molecule including in neuron-glia interactions

(Fields and Burnstock, 2006; Lohr et al., 2014). Purinergic signalling is also used by astrocytes which release ATP in order to propagate waves of calcium. These are a response to stimuli such as synaptic activity and traumatic injury (Arcuino et al., 2002; Fields and Stevens-Graham, 2002).

This increase in extracellular ATP levels is implicated in multiple aspects of the response to spinal cord injury such as activation of microglia (Wang et al., 2004; Davalos et al., 2005) and the proliferation of astrocytes (Franke et al., 2001). The modulation of the purinergic signalling may also affect the response of ependymal cells to spinal cord injury. Intrathecal infusion of broad spectrum purinergic antagonists PPADS and suramin after spinal cord injury was shown to decrease the numbers of GFAP positive cells in the injury site (Rodríguez-Zayas et al., 2012). This reduction in GFAP-positive cells after treatment with purinergic antagonists may have been due to reduced proliferation of ependymal cells, reduced differentiation into GFAP-expressing cells, as was seen by Mothe and Tator (2005), or it could have been an impact on the astrocytes which infiltrate the injury site but are not those derived from ependymal cells. The reduction in GFAP+ cells within the injury site was accompanied by a decrease in the amount of spared tissue. However this difference in the tissue spared from the injury did not affect the functional outcome of the injury, with open-field testing showing no difference between the conditions. This work and the expression of purinergic receptors around the central canal of the spinal cord (discussed below) indicate a potential role for purinergic signalling in these cells, particularly after spinal cord injury.

1.4.2 Regenerative potential in non-mammalian species

Unlike in mammals, considerable regeneration is seen in lower vertebrates after injury to the spinal cord (Bruni and Reddy, 1987). Spinal cord regeneration in lower species is seen both through axonogenesis which is able to bridge the gap but not faithfully replicate the structures that were there before injury and through neurogenesis, which together with axonogenesis can produce a more comparable spinal cord structure (Tanaka and Ferretti, 2009). The central canal region plays an early role in the reconnection of the cord after transection in the eel spinal cord (Dervan and Roberts, 2003). This occurs by the central canal cells forming a new canal by producing flat plates that serve as templates for the formation of the lateral and dorsal walls with ependymal cells displaying a modified phenotype which is

thought to have trophic effects on the regenerating axons. In the salamander, the ependymal tube that gives rise to the regenerated spinal cord following tail amputation bears considerable similarity to the early structure of the neural tube in development (Tanaka and Ferretti, 2009). The proliferating cells within these species with regenerative spinal cord capabilities have stem cells that show features of radial glia and are more similar to those seen during development and it is suggested that this presence of functional radial glia that also retain stem cell potential is an essential difference between non-regenerative and regenerative species (Tanaka and Ferretti, 2009). This suggestion that the retention of features associated with development is supported by the high levels of regeneration seen after injury in embryonic mammalian spinal cord (Nicholls and Saunders, 1996), however there may be other factors such as the spinal cord environment adding to the differential regenerative abilities of different species.

1.5 Purinergic signalling

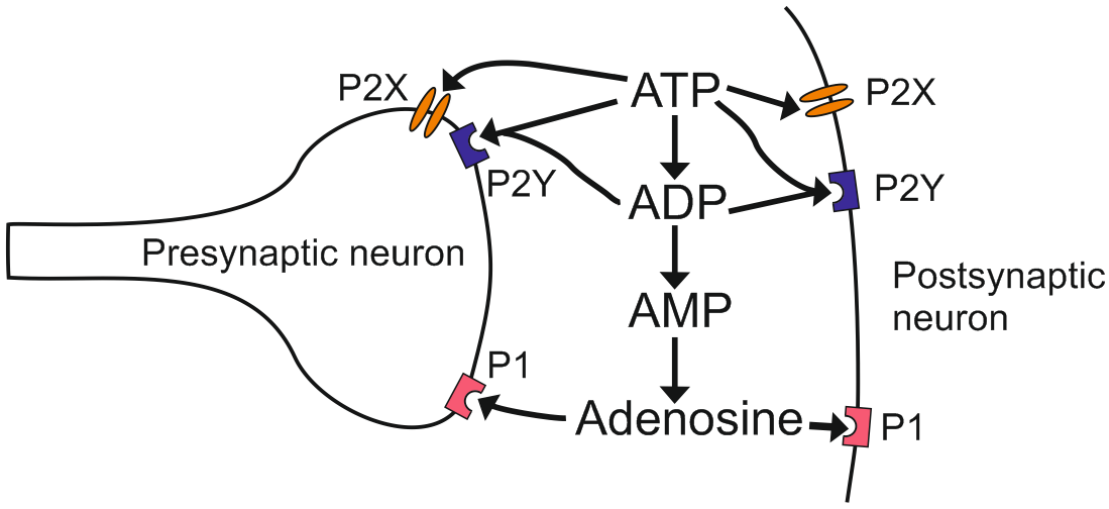


Figure 1.3 ATP and its derivatives act as agonists on pre-synaptic and post-synaptic purinergic receptors.

Schematic showing the extracellular metabolism of ATP to ADP, then AMP and then Adenosine and the actions of these products on purinergic receptors in pre- and post-synaptic neurons. ATP is shown to act on ionotropic P2X receptors, ADP on P2X and metabotropic P2Y receptors and adenosine on metabotropic P1 (or A1) receptors.

As well as acting as an intracellular energy store (Hultman and Greenhaff, 1991), ATP and other purine nucleotides and nucleosides also act as neurotransmitters and are often involved in sensory and autonomic systems via actions on a range of pre-synaptic and post-synaptic receptors (Figure 1.3) (Lohr et al., 2014; Shatarat et al., 2014; Adriaensen et al., 2015). The processing pathway of purine nucleotides and individual receptors are discussed in detail below. Purinergic signalling is involved in a wide range of systems throughout the body and is particularly highly used during development from some of the first stages of development in the fertilised egg (Kupitz and Atlas, 1993). Many embryos respond to ATP signalling, including mammalian embryos. This was shown by electrophysiological recordings of mouse mesodermal stem cell line cells which showed an initial increase in internal Ca^{2+} after ATP application, followed by a hyperpolarisation of the cell mediated by Ca^{2+}-activated K^{+} conductances (Kubo, 1991). Purinergic signalling is utilised by many cell types during development to trigger particular processes and delay others. Modulation can therefore lead to significant changes in outcomes of this development. Application of ATP to dendrites of embryonic hippocampal neurons produced changes in dendritic morphology *in vitro* (Khakh et al., 2001) and acts as a trophic factor on grown axons during development, with an increase in hippocampal connectivity seen after application of purinergic agonists and a reduction with antagonists (Heine et al., 2006). These suggest a role for ATP in the correct development of these processes. During development, purinergic signalling interacts with many other factors such as in the inner ear where brain-derived neurotrophic factor (BDNF)-mediated development of spinal ganglion neurons was inhibited by purinergic signalling in the neonatal rat cochlea (Greenwood et al., 2007). Another way in which purinergic signalling impacts development other than direct action upon the neurons is through actions on the glial cells which play important roles during development. Purinergic signalling is frequently used by radial glial cells to communicate over long distances with other glia or neurons by propagation of calcium waves (Weissman et al., 2004). These ATP-elicited calcium waves act to modulate proliferation in the developing neocortex, with reduced calcium waves decreasing proliferation in the ventricular zone during the peak period of neurogenesis. Myelinating glial cells, Schwann cells in the periphery and oligodendrocytes in the central nervous system, are also affected by purinergic signalling during development (Lecca et al., 2012). ATP release non-synaptically from electrically stimulated axons activates receptors on nearby oligodendrocytes and triggers myelination (Fields and Stevens, 2000). A further role

of purinergic signalling on oligodendrocyte development has been shown via indirect signalling upon astrocytes which release a cytokine, leukaemia inhibitory factor, that then acts on the oligodendrocytes to promote myelination (Ishibashi et al., 2006). Myelination is not the only process that purinergic signalling has been shown to elicit in oligodendrocyte lineage cells. In oligodendrocyte precursor cells (OPs), application of ATP or ADP but not UTP was shown to stimulate OP migration and differentiation (Agresti et al., 2005b). These various roles indicate the importance of purinergic signalling during development on multiple different cell types. There is therefore the question of whether there are similar roles for purinergic signalling in postnatal development and adult niches that retain stem cell features.

Adult post-natal neurogenic niches in the mammalian brain also retain multiple features of purinergic signalling with both B cells of the subventricular zone (SVZ) and of the radial glial cells in the dentate gyrus of the hippocampus expressing nucleoside triphosphate diphosphohydrolase 2 (NTPDase2) (Braun et al., 2003). NTPDase2 is an ecto-ATPase, a protein which hydrolyses extracellular nucleoside triphosphate to the respective diphosphates e.g. ATP to ADP, and therefore acts to control levels of extracellular nucleotide triphosphates, such as ATP, and thus modulate levels of ATP signalling (Langer et al., 2007). Additionally, the radial glia in the dentate gyrus and the B cells of the SVZ express functional purinergic receptors (Shukla et al., 2005; Mishra et al., 2006; Messemer et al., 2013), with SVZ cells expressing another type of purinergic receptor when they mature into immature neurons and migrate to the olfactory bulb. This suggests that the expression of the particular purinergic receptors (metabotropic P1 and P2Y receptors, expanded upon below) is important for the functioning of these regions as stem cell niches. Evidence to support this comes from the responses of SVZ-derived cultured neurospheres to purinergic signalling, where application of agonists increased the rate of proliferation while application of antagonists or knockout of the metabotropic purinergic receptor $P2Y_1$, discussed below, reduced the proliferation rate (Mishra et al., 2006). This increase in the rate of proliferation in SVZ cells with increased purinergic signalling has also been observed *in vivo*. Infusion of ATP into the lateral ventricles increased the numbers of newly-proliferated cells and was decreased by application of a specific antagonist for $P2Y_1$ or $P2Y_1$ knock out (Suyama et al., 2012).

The fact that purinergic signalling has been shown to be involved in a range of processes including development, adult-stem cell niches and responses of multiple

cell types after spinal cord injury makes it a viable candidate for a significant role in central canal functioning. The following section will detail the receptors involved in purinergic signalling and look at their expression around the central canal of the spinal cord.

1.5.1 Extracellular nucleotide metabolism

Adenosine triphosphate (ATP) is composed of adenosine and three phosphate groups. ATP can be dephosphorylated by ecto-nucleotide triphosphate diphosphydrolase (ecto-NTDPase) (Doleski et al., 2016). This produces adenosine diphosphate (ADP), which can be further dephosphorylated by the same enzymes to adenosine monophosphate (AMP) (Figure 1.3). This can then be completely dephosphorylated to adenosine by ecto-5'-nucleotidase enzymes (Street et al., 2013). There are other enzymes which dephosphorylate ATP directly to AMP. Collectively enzymes that are involved in the extracellular metabolism of nucleotides are known as ecto-nucleotidases or ecto-ATPases if they are specific for ATP. Ecto-ATPases are bound to the membranes of many cells and act to metabolise external nucleotides meaning that the levels of extracellular ATP and other nucleotides such as UTP are controlled by multiple factors including release, uptake and metabolism (Zimmermann et al., 2012).

Many of these molecules are able to act as agonists on some purinergic receptors. ATP acts on P2X receptors and a subset of P2Y receptors, ADP acts on a subset on P2Y receptors, as does UTP and UDP-glucose while adenosine acts on P1 (or A1) receptors. The structure and function of these will be expanded upon below.

1.5.2 Structure of purinergic receptors

1.5.2.1 P2X receptors

Ionotropic P2X receptors are ligand-gated ion channels which are bound to the plasma membrane. Individual subunits have 2 transmembrane domains with intracellular N- and C-termini and form trimeric channels. There are 7 subunits ($P2X_{1\text{-}7}$) which can form heteromeric and homomeric assemblies. Heteromeric assemblies of P2X receptors that have been identified include $P2X_{2/3}$, $P2X_{4/6}$, $P2X_{1/5}$, $P2X_{2/6}$, $P2X_{1/4}$ and $P2X_{1/2}$. All subunits form homomeric assemblies except $P2X_6$ (Abbracchio et al., 2009). All 7 P2X subunits are expressed in neural tissue with $P2X_3$

and $P2X_{2/3}$ expressed in sensory and autonomic peripheral neurons (Abbracchio et al., 2009). All of these assemblies are sensitive to ATP, although with different potencies. Specifically homomeric $P2X_7$ has a lower sensitivity to ATP than the other receptors; whilst the other receptors have an EC_{50} of 1-10 μM (Abbracchio et al., 2009), $P2X_7$ has an EC_{50} of 100 μM for ATP (Donnelly-Roberts et al., 2009), but is more sensitive to BzATP for which the EC_{50} is 26 μM (Zhang et al., 2005). They also all form non-specific cation channels through which Na^+, K^+ and Ca^{2+} ions can flow once 3 molecules of ATP are bound (Burnstock, 2006), however the permeability of each channel to calcium varies slightly depending on the subtypes which form the channels (North, 2002).

Despite these similarities, there are multiple differences between ion channels with made up of different P2X subunits. One difference is that some subtypes demonstrate an increase in the size of the pore after prolonged exposure to ATP which allows passage of larger ions such as $NMDG^+$ and the dye YO-PRO-1 (Khakh et al., 1999; Virginio et al., 1999). $P2X_2$, $P2X_{2/3}$, $P2X_4$ and $P2X_7$ all show this biphasic response which is activated by ATP application for longer than 10 seconds. The size of the initial pore opened with application of ATP is about 8-20 Å with the larger pore formed after prolonged application at least 3 Å larger than the initial pore (Eickhorst et al., 2002). This increased pore size changes the permeability of NMDG from 5 % of that of Na^{2+} to 50 % of the permeability of Na^{2+} (North, 2002). This biphasic response allows cells expressing these purinergic receptors to produce 2 different currents, one elicited initially upon detection of ATP, the second after prolonged exposure and it has been suggested that this property may be involved in synaptic plasticity (Khakh et al., 1999).

The desensitisation times of the receptors also vary between receptors made up of different subtypes for example $P2X_1$ and $P2X_3$ homomeric receptors have a fast desensitisation compared to $P2X_2$ homomeric receptors which sustain currents for multiple seconds with sustained application (North, 2016), while heteromeric $P2X_{2/3}$ receptors and homomeric $P2X_4$ receptors showing intermediate desensitisation characteristics (Mackenzie et al., 1999).

The heteromeric channels display a range of characteristics maintaining some characteristics of each of the homomeric channels of the constituent subtypes. For example $P2X_{2/3}$ heteromer channels resemble $P2X_3$ receptors with respect to factors

such as sensitivity to Ap5A (1 μM for $P2X_3$ and $P2X_{2/3}$, and inactive in $P2X_2$ receptors) and lack of effect of high zinc levels (100 μM) on the response size to ATP. Zinc significantly potentiates the response in $P2X_2$ receptors but not in $P2X_3$ or $P2X_{2/3}$ (Liu et al., 2001). Conversely, $P2X_{2/3}$ receptors resemble $P2X_2$ receptors in respect to the desensitisation times (North, 2002). In other characteristics, the heteromeric $P2X_{2/3}$ receptor displays an intermediated profile. $P2X_2$ receptors have their response to ATP potentiated by a decreased pH while acidification has a slight inhibitory effect on $P2X_3$ receptors. In this respect, $P2X_{2/3}$ receptors show a small potentiation of response when the pH is decreased from 7.4 to 6.5 (Liu et al., 2001).

There are some characteristics that are specific to one particular subtype arrangement of P2X receptor. The functioning of the $P2X_3$ receptor is modulated by temperature (Khmyz et al., 2008). Under normal temperatures the $P2X_3$ receptor desensitises rapidly and is slow to return to the non-desensitised state slowly but when the temperature is increased to 40 °C the rate of desensitisation did not change but the rate of recovery increases significantly. Knock out experiments of the $P2X_3$ subunit demonstrated a role for this receptor subtype in non-noxious temperature sensing (Egan et al., 2006).

1.5.2.2 P2Y receptors

Metabotropic P2Y receptors are seven-transmembrane domain G-protein coupled receptors which respond to a range of nucleotides. There are 8 P2Y subtypes, named $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$, with the non-consecutive numbering due to naming of putative new subtypes that were later shown not to be true P2Y receptors e.g. $P2Y_5$ (Pasternack et al., 2008). As with P2X receptors, P2Y receptors are found in a wide range of tissues throughout the body. Whilst the receptors tend to respond to different nucleotide agonists with a range of sensitivities, they can all be classified according to which one or two nucleotides act as their primary agonists. $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ all have the highest sensitivity for adenosine diphosphate (ADP). $P2Y_6$ has the highest sensitivity for UTP, $P2Y_{14}$ for UTP-glucose and $P2Y_{11}$ for ATP. $P2Y_2$ and $P2Y_4$ both respond with high levels of sensitivity to both ATP and UTP (von Kügelgen and Hoffmann, 2016).

There is variation amongst the P2Y receptor population regarding the G proteins coupled to the receptors, with the receptors split into two main groups. The first group contains $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ which couple to the $G_{q/11}$ pathway while

the second group, made up of $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$, couples to the $G_{i/o}$ pathway (Abbracchio et al., 2009). However P2Y receptors have been observed coupling to multiple pathways, sometimes in response to activation by different agonists such as in $P2Y_{11}$ where activation by ATP mobilises inositol triphosphate (IP_3) to produce an increase in intracellular Ca^{2+} concentration, while UTP application raised intracellular Ca^{2+} levels without mobilisation of IP_3 suggesting an agonist-dependent difference in intracellular signalling pathways (White et al., 2003).

Despite P2Y receptors primarily acting as monomers, some P2Y receptors form dimers, sometimes with other P2Y receptors and sometimes with other receptors. $P2Y_1$ has been shown to dimerise with the adenosine receptor A_1 upon activation of both receptors (Yoshioka et al., 2001, 2002) with the resultant dimer demonstrating combined properties of the monomers of the two constituent channels. The $P2Y_1$-A_1 receptor responded to ADPβS, a feature of $P2Y_1$ receptors, but this response was sensitive to the A_1-specific antagonist DPCPX. This indicates the potential complexity in purinergic signalling via P2Y receptors.

1.5.2.3 Adenosine receptors

ATP is dephosphorylated and produces adenosine and this is the primary production route for adenosine. Where the dephosphorylation of ATP is intracellular, the adenosine is more often released by transporters rather than vesicular release (Sheth et al., 2014).

There are also purinergic receptors that respond selectively to adenosine. There are four adenosine receptors (P1 receptors), the A_1, A_{2A}, A_{2B} and A_3 receptors. They are all G-protein coupled receptors. A_1 and A_3 are coupled to $G_{i/o}$ inhibitory intracellular signalling proteins which inhibit adenylyl-cylase to reduce the levels of cyclic AMP and therefore the concentration of intracellular calcium. A_{2A} and A_{2B} receptors are coupled to G_s intracellular signalling proteins which act in an excitatory manner by increasing intracellular calcium levels (Jacobson et al., 2012; Sheth et al., 2014). Adenosine receptors are involved in a range of functions including modulation of neurotransmitter release (Sebastião and Ribeiro, 2000) and neuroprotection (Ferreira and Paes-de-Carvalho, 2001). In addition to the activation of adenosine receptors by release of adenosine, the presence of ecto-ATPases means that where adenosine receptors are present, there could be activation of adenosine receptors when levels of extracellular ATP are increased.

1.5.3 Purinergic signalling in the central canal region

1.5.3.1 Purinergic signalling in CSFcCs

A range of purinergic receptors has been identified in the ependymal cells and CSFcCs of the mammalian spinal cord both under intact physiological conditions and in conditions of spinal cord injury or *in vitro* culturing of epSPCs or epSPCis.

Within CSFcCs, $P2X_2$ expression has been shown at all stages of development (Stoeckel et al., 2003). The expression of $P2X_2$ within CSFcCs appears strong within a large proportion of CSFcCs. When this expression is assessed via electron microscopy, it was shown that $P2X_2$ intensely expressed along the inner membrane of the cell with the strongest concentration in discrete regions. This pattern of $P2X_2$ expression was similar along the apical process and the bouton that sits within the central canal. However $P2X_2$ was particularly intensely expressed in small finger-like projections which intrude into the surrounding neuropil. Within these projections $P2X_2$ expression is in regions which were closely opposed to synaptic boutons outside the cell (Stoeckel et al., 2003). $P2X_2$ expression is also present in the axons leading away from the CSFcCs in ventral then ventrolateral directions. These $P2X_2$ receptors were shown to be functionally active in that they produce a rapid inward current which then shows strong rectification of the current (Marichal et al., 2009). Inward rectification of a channel is when current passes more readily in the inward direction than in the outward direction and is a feature of $P2X_2$ receptors (Zhou and Hume, 1998). CSFcCs also respond to changes in pH (Marichal et al., 2009). This is significant because $P2X_2$ receptors have their responses to ATP potentiated by acidification (King et al., 1997; Stoop et al., 1997; Ding and Sachs, 1999). This may not be the cause of the response in the electrophysiological recordings by Marichal et al. (2009) as another pH sensitive molecule, PKD2L1, has been identified in CSFcCs (Huang et al., 2006; Petracca et al., 2016), however the $P2X_2$ receptors present in CSFcCs could also be playing these roles.

1.5.3.2 Purinergic signalling in radial glia

$P2X_7$ receptors have also been identified around the central canal (Marichal et al., 2016). The first cell type that $P2X_7$ has been identified in is CC-contacting radial glia

which could be activated by either ATP or BzATP, with the response inhibited by Brilliant Blue G (BBG). This expression of $P2X_7$ is along both the apical and basal processes and upon activation of the basal endfoot of the radial glia a wave of Ca^{2+} is propagated along the process towards the cell body and then along the apical process (Marichal et al., 2016).

1.5.3.3 Purinergic signalling in ependymal cells

The other cell type around the central canal that express $P2X_7$ receptors is the ependymal cell (Marichal et al., 2016). Similarly to the responses seen in radial glia, application of either BzATP or ATP produces an inward current in ependymal cells. An increase in intracellular calcium levels was also seen although it did not propagate in a wave as ependymal cells do not display the same processes as radial glial cells. As $P2X_7$ receptors do not show very high sensitivity for ATP, these responses were only seen with a long pulse (>1s) of a relatively high concentration of ATP (1 mM).

Another receptor that has been observed in native ependymal cells is the P2Y-like protein GPR17 (Ceruti et al., 2009). The GPR17 is activated by both uracil nucleotides (UDG-glucose) and also cysteinyl-leukotrienes, such as Leukotriene D4 (LTD_4) and Leukotriene C4 (LTC_4). The GPR17 receptor plays a role in the response of the ependymal cells to injury. GPR17 is upregulated in cells undergoing apoptotic cell death due to an ischemic infarct in the brain and inhibition of GPR17 increases the amount of cell death but at later times GPR17 activation is important in the remodelling and repair responses (Lecca et al., 2008). An effect in spinal cord injury has also been showing using animals with the GPR17 receptor knocked down by use of a specific antisense oligonucleotide. After spinal cord injury, the animals with GPR17 knock down showed both morphological and functional differences. Knock down of GPR17 acted in a neuroprotective manner in spinal cord injury causing a reduced disruption of the myelin structure and higher locomotor function score 7 days after spinal cord injury (Ceruti et al., 2009). A similar role for GPR17 in increasing the sensitivity of cells to nucleotide-mediated cell death has been observed in oligodendrocyte precursor cells (Ceruti et al., 2011). These indicate that GPR17 signalling is involved in multiple aspects of the response to injury including roles in mediating cell death and also in the secondary repair responses to the injury, one or both of which may occur in ependymal cells.

1.5.3.4 Purinergic signalling after spinal cord injury

In addition to roles in the response to spinal cord injury, the expression levels of some purinergic receptors have been shown to be modulated by spinal cord injury. Much of this work has looked at epSPCs and epSPCis. Within epSPCs both ionotropic P2X and metabotropic P2Y receptors were identified. Activation by ATP produces increases in intracellular calcium concentration via $P2X_4$ and $P2X_7$ and $P2Y_1$ and $P2Y_4$ (Gómez-Villafuertes et al., 2015). Analysis of P2X RNA found in epSPCs showed presence of $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ but with $P2X_4$ and $P2X_7$ observed at the highest quantities and expression of these was confirmed by western blot. The contribution of $P2X_7$ was demonstrated by the high levels of activation by the $P2X_7$ specific agonist BzATP. In addition to $P2Y_1$ and $P2Y_4$, a band corresponding to $P2Y_2$ was also identified but was not seen in Western blot analysis. $P2Y_1$ is activated by ADP and $P2Y_2$ and $P2Y_4$ are activated by UTP. Both ADP and UTP highly activate epSPCs. Additionally, UDP and two diadenosine polyphosphates (Ap4A and Ap5A) produce a small response (Gómez-Villafuertes et al., 2015). The expression of the purinergic receptors in epSPCis differs from those in epSPCs with a downregulation of $P2Y_1$ (Gómez-Villafuertes et al., 2015). When the responses of epSPCis were compared to those of epSPCs to various nucleotide agonists, the majority showed very similar pharmacological properties however the response in intracellular calcium levels to ADP application was significantly smaller and the response to Ap5A was slightly larger.

In vivo assessment of $P2X_4$ and $P2X_7$ levels at the site of spinal cord injury has shown that there is an accumulation of $P2X_4$-positive and $P2X_7$-positive cells within the injury site and peaking at 7 days after injury (Schwab et al., 2005). These cells were predominantly microglia and surviving neurons (Schwab et al., 2005; Gómez-Villafuertes et al., 2015). Transplantation of epSPCis to the injury site reduced the injury-induced increases in both $P2X_4$ and $P2X_7$ expression around the central canal which correlated with the increased functional locomotor scores seen with epSPCi transplantation (Gómez-Villafuertes et al., 2015).

Another purinergic receptor which is found at higher concentrations in the grey and white matter after spinal cord injury is $P2Y_2$ (Rodríguez-Zayas et al., 2010). $P2Y_2$ expression is seen in neurons in the ventral horn in uninjured animals and in the white matter $P2Y_2$ is expressed at low levels in glial cells. 14 days after a

compression spinal cord injury, rat spinal cord displayed increased levels of $P2Y_2$ expression as shown by RT-PCR studies. This was largely due to an increase in numbers of $P2Y_2$ expressing cells within the white matter with significantly more $P2Y_2$ positive processes. The cell types expressing $P2Y_2$ after spinal cord injury were shown to be astrocytes, macrophages, oligodendrocytes, neurons and neuronal axons as demonstrated by co-localisation with GFAP, MAB 328, CD68: Biotin, NeuN and NF-H respectively (Rodríguez-Zayas et al., 2010). It is not known whether these $P2Y_2$ expressing cells are derived from ependymal cells or if the $P2Y_2$ expression is due to proliferation of another cell type, infiltration of $P2Y_2$-positive cells or increased $P2Y_2$ expression from cells already present.

Purinergic signalling has also been implicated in other aspects of the response to spinal cord injury via $P2X_7$ and $P2X_4$ receptors which are highly expressed in neurons and immune cells (de Rivero Vaccari et al., 2012). $P2X_7$ has been suggested to have a role in causing cell death. Application of $P2X_7$ antagonists have been shown to have neuroprotective effects including a reduction in neuroinflammation, resulting in an improvement in motor function (Peng et al., 2009) and (Wang et al., 2004) . A similar reduction in neuronal cell death with application of $P2X_4$ modulators has been seen in a model of amytrophic lateral sclerosis (Andries et al., 2007). $P2X_4$ has a critical role in the development of neuropathic pain following spinal cord injury (de Rivero Vaccari et al., 2012) . $P2X_4$ is upregulated after by 24 hours post injury (Tsuda et al., 2013) and application of the histone deacetylase inhibitor valproic acid had an inhibitory effect on the expression of $P2X_4$ in microglia and helped preserve neuronal fibres after spinal cord injury.

These findings detailed above demonstrate the complexity of the spinal cord stem cell niche and the processes it is involved in, particularly in response to injury to the spinal cord tissue. There is also a breadth of evidence to suggest multiple roles of purinergic signalling via a range of purinergic receptors within the cell types around the central canal and more broadly within the mammalian spinal cord.

1.6 Hypotheses and aims

The cells of the central canal region of the spinal cord are involved in the responses of the spinal cord to injury. Purinergic signalling is hypothesised to play a role in the detection of this injury and the responses to it due to the increases of extracellular nucleotide levels after spinal cord injury and the presence of purinergic receptors and ecto-nucleotidases in the cell types in this area. There is currently little understanding of the electrophysiological behaviour of ependymal cells and CSFcCs in response to application of nucleotides and purinergic agonists, particularly in spinal cord slices as opposed to cultured epSPCs. This study will investigate the role of purinergic signalling in the functioning of the cell types in the area over acute and longer time scales.

1.6.2 Hypotheses

This study has 2 specific hypotheses:

1. Activation of purinergic receptors by application of nucleotides and purinergic agonists will produce changes to membrane potential of the ependymal cells and CSFcCs of the central canal region of the mammalian spinal cord.

2. Modulation of purinergic signalling will affect the behaviour of cells within organotypic spinal cord slices in culture.

These hypotheses can be summarised in the overall hypothesis:

Cells within the central canal region of the mammalian spinal cord can respond to application of nucleotides and purinergic agonists via ionotropic and metabotropic purinergic receptors, which affects their functioning as stem cells and migratory cells.

1.6.1 Aims

This study uses whole cell patch-clamp electrophysiological recordings and EdU-labelling of proliferative cells in organotypic slice cultures to:

1. Better understand the electrophysiological responses of ependymal cells to a range of nucleotides and purinergic agonists.

2. Determine any variation in the responses of CSFcCs to application of ATP and if this variation correlates with the CSFcC subtypes previously identified.

3. Determine the effect of modulation of purinergic signalling on the behaviour of cells of the central canal region in organotypic slice cultures, behaviour which is reminiscent of that observed after injury.

Chapter 2 - General Methods

All experiments were carried out as detailed in the Animals Scientific Act (1986) and the ethical standards prescribed by the University of Leeds Ethical Review Committee by Home Office-approved individuals. At all times, efforts were made to reduce the number of animals used and minimise any suffering they experienced.

2.1 Animals

Two species of animal were used for the following experiments. For the electrophysiological experiments, wild-type Wistar rats (p 7-13) were used. For the culture and proliferation experiments wild-type C57bl6 mice (p 12-21) were used. The immunohistochemical analysis was carried out on wild-type Wistar rats both pre-weaner (p7-13) and adult (aged approximately 3 months), and on wild-type C57bl6 mice, pre-weaner (p 12-21) and adult (aged approximately 3 months). All C57bl6 mice were obtained from the in-house breeding facility at the Central Biomedical Services at the University of Leeds. The majority of the Wistar rats were similarly obtained from the in-house breeding facility at the Central Biomedical Services. A small proportion of the Wistar rats were obtained from Charles River Laboratories, UK, through the Central Biomedical Services at the University of Leeds.

2.2 Spinal cord slice preparation

7-13 day old rats as detailed above were anaesthetised by intraperitoneal (I.P.) administration of either pentabarbitone (Euthatal – Merial Animal Health Ltd, UK) or urethane (Sigma-Aldrich, UK) made up in aCSF at doses of 60 mg/kg and 2g/kg of body weight respectively. Minimal acceptable level of anaesthesia was such that the pedal withdrawal reflex in response to a foot pinch was abolished.

After necessary level of anaesthesia was achieved, spring scissors were then used to do a transverse laparotomy. The diaphragm was perforated and vertical incisions made to the clavicle and the ventral rib cage removed. The right atrium was then perforated and 20 ml of ice cold sucrose artificial cerebrospinal fluid (sucrose aCSF; see table 2.1 for constituents) transcardially perfused by injection into the left ventricle. Ice cold sucrose aCSF was used for the preparation of the slices to cool the spinal cord and to reduce the levels of extracellular sodium ions (sucrose takes the place of the sodium ions, see Table 2.1) to minimise cell death by excitotoxicity. The sucrose aCSF was oxygenated by continual bubbling with 95% O_2:5% CO_2 until the time of perfusion. Appropriate level of perfusion was determined by blanching of the liver and limb extremities. Decapitation was then performed using blunt scissors.

A dorsal laminectomy was used to expose the spinal cord. This involved the use of blunt scissors to remove the skin and fatty tissue from over the spinal column. Spring scissors were then used to make incisions on the lateral edges of the vertebrae such that the dorsal portion of the vertebrae could be removed. The nerve rootlets attached to the spinal cord were severed using spring scissors. This allowed the spinal cord to be lifted from the vertebral column and placed into a petri dish. Using a dissecting microscope (SM2 2B, Nikon, Japan) for visualisation, fine forceps were used to remove the dura and pia mater. A segment of the thoracic and upper lumbar regions, approximately 1 cm in length was then selected for sectioning. This segment was set into warm agar (Sigma Aldrich, UK, made at 3% in aCSF; see table 2.1 for aCSF constituents) for stability during sectioning. Once cooled, this agar block was set in front of a larger 4% agar block for further support and superglued in place. This was then placed in a bath of ice cold sucrose aCSF which was oxygenated (95% O_2:5% CO_2) throughout the sectioning process. Transverse slices were cut at 300 µm thickness, using settings of 108 Hz, 0.8 mm amplitude and 0.5 mm/s advance speed (Leica VT1200s, Leica Microsystems, UK with stainless steel blades or Integraslice 7550 PSDS, Campden Instruments, UK with ceramic blades). Slices were placed in a holding chamber containing oxygenated aCSF (for composition see Table 2.1) at room temperature; they were left for 60 minutes to equilibrate.

Table 2.1 Composition of extracellular solutions for electrophysiological experiments

Component	**Sucrose aCSF (mM)**	**aCSF (mM)**
Sucrose	217	
NaCl		124
$NaHCO_3$	26	26
KCl	3	3
$MgSO_4.7H_2O$	2	2
NaH_2PO_4	2.5	2.5
Glucose	10	10
$CaCl_2$	1	2

2.4 Whole cell patch clamp electrophysiology

2.4.1 Experimental set up

Recordings were made after the 60 minute equilibration time. The slice was first transferred to the recording chamber and the border of 3% agar remaining from the sectioning was pinned under a taught thread to immobilise the slice. Oxygenated (95% O_2; 5% CO_2) aCSF flowed at a rate of approximately 4-6 ml/minute through the recording chamber, gravity fed from a flask above. The pH of the aCSF was 7.83 prior to being bubbled with gas and 7.54 after bubbling. Outflow from the recording chamber used a suction system and was not recycled. Recordings were done at room temperature (16-24°C).

The glass electrode was pulled using a Sutter P97 micropipette puller (Sutter Instruments, USA), from borosilicate glass capillary tubes (inside diameter 0.94 mm, and outside diameter 1.2 mm; Warner Instruments, USA). The pulled electrodes had resistance of 5-8 MΩ and a tip diameter of approximately 1-3 µM.

The bath contained a silver chloride pellet connected to a silver wire; this acted as the reference electrode. The recording electrode consisted of a silver wire which sat within a glass electrode containing intracellular solution composed of in (mM): K gluconate (110), EGTA (11), $MgCl_2$ (2), $CaCl_2$ (0.1), HEPES (10), Na_2ATP (2), Na_2GTP (0.3) and additionally 0.5% Neurobiotin (Vector Laboratories, USA) and 0.02% dextran tetramethylrhodamine (rhodamine; Invitrogen, USA). The electrode was filled about a $^1/_3$ of the way with the intracellular solution, such that it just contacted the silver wire and thus electrically connected the electrode tip to the silver wire of the electrode holder (QSW A12P, Warner Instruments, USA).

The electrode holder and the reference electrode, which was placed in the aCSF within the recording chamber, were attached to a headstage (CV-4 1/100U from Axon Instruments, USA). This completed the electrical circuit from inside the cell, measured with the recording electrode, to outside the cell, measured using the bath reference electrode. The electrode holder had a side tube for pressure application. The headstage also acted to preamplify the signal from the recording electrode. The signal was then filtered by a Bessel low pass filter set at 2-5 kHz and passed through the Axopatch 1D amplifier (Axon Instruments, USA). The mains noise was filtered out using a HumBug (Quest Scientific, via Digitimer, UK). The signals were then

converted from analogue signals that leave the amplifier to digital signals by the Digidata 1322A analogue to digital converter (Axon Instruments, USA). These digital signals were then captured by the pClamp 9 software. The acquisition frequencies varied between the recording modes discussed below (2.5), with a frequency of 20 kHz for the sweep mode characterisation recordings and 5 kHz for the continuous mode experimental recordings.

Adjustment of the timing and duration of the current and voltage applied to the cell (in voltage-clamp and current-clamp modes respectively) was provided by the Master-8 stimulator (A.M.P.I., Israel) which connects to the Axopatch 1D amplifier. The amplitude and polarity of the current and voltage steps were set according to the parameters on the Axopatch.

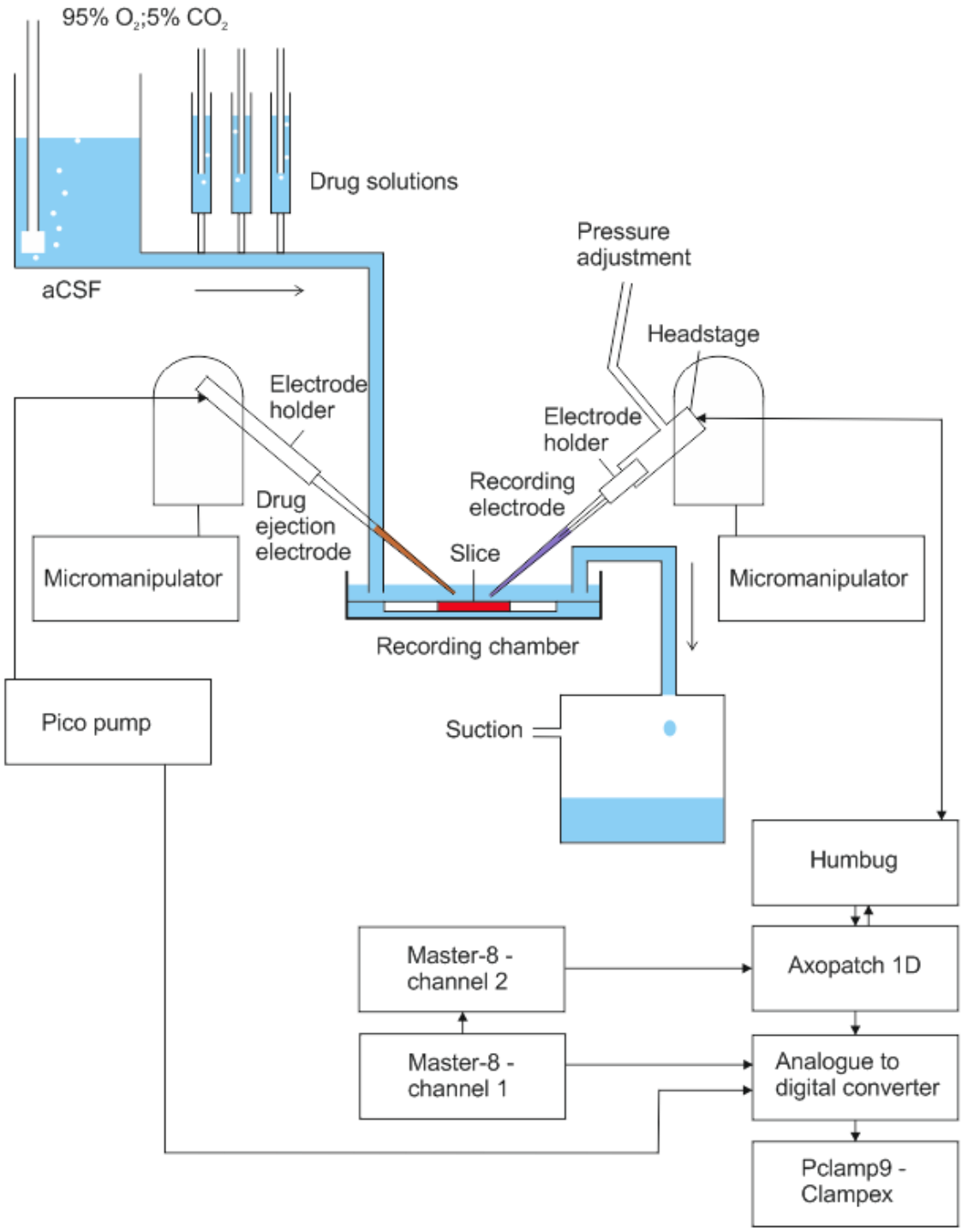


Figure 2.1 Experimental set up for whole cell patch clamp electrophysiology recordings

See 2.4.1 Experimental set up for further details.

2.4.3 Whole cell patch clamp recordings

The recording chamber that the slices were held in was positioned under an upright microscope (Olympus BX50W1). The x10 magnification lens was used to position the central canal in the centre of the field. The central canal was re-identified at x 60 magnification. The visualisation was then switched over to differential interference contrast (DIC) imaging using a camera (QImaging Rolera-XR, QImaging, Canada) attached to the microscope, which transmitted the images to the QCapture Pro software to enable the individual cells to be seen.

2.5.1 Cell identification

DIC imaging was used to identify healthy cells by their even grey tone and visible but not overly distinct or large outline. In many cases cell types could be identified. Prior to recording ependymal cells were often distinguishable by their presence within the ependymal cell layer, their more cuboidal shape and their less distinct cell outline. CSFcCs differed from ependymal cells in that they tended to be slightly larger and were more likely to have a subependymal location. After recording, further morphological characteristics could be viewed due to the inclusion of 0.02 % dextran tetramethylrhodamine (rhodamine; see section 2.4.1) in the intracellular solution. Excitation of the rhodamine by a wavelength of 550 nm allowed visualisation of the recorded cell and was used, in conjunction with the electrophysiological characterisation, to identify the cell type. Presence of a process into the central canal and/or spiking responses to depolarisation or repolarisation indicated that the cell being recorded from was a CSFcC. Ependymal cells were identified by passive, non-spiking profile and an absence of processes into the central canal. Neuronal cells could be identified by the production of action potentials and the presence of many axons and dendrites. Images were captured on the QCapture program (QImaging, Canada).

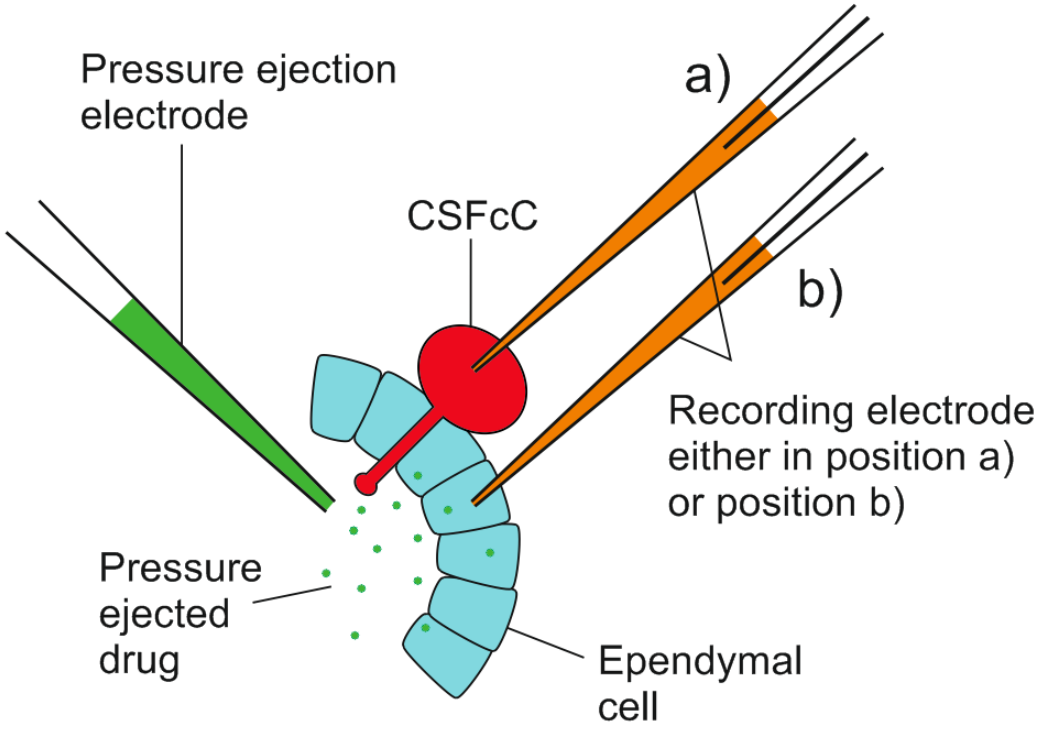


Figure 2.2 Configuration of electrodes for patch-clamp electrophysiology

Illustration of the electrode placement during the electrophysiology recordings. Recording electrode (filled with intracellular solution shown in orange) was attached to and recording from either a CSFcC (a) or an ependymal cell (b). The pressure ejection electrode containing drug to be pressure ejected (shown in green) was positioned near the cell being recorded from but not directly pointing towards it to minimise movement of the cell.

Once a cell of interest was selected, the recording electrode (and drug ejection electrode if used) was brought down to a level slightly above the slice by first using the x10 magnification, then the x60, and positioned over the cell using the micromanipulator. Before being introduced to the aCSF in the recording chamber, a small amount of positive pressure was applied to the recording electrode to reduce the likelihood of blockages. Once in the solution and a small distance above the slice, in current-clamp mode, the offset and resistance of the electrode due to a -250 pA current pulse, were compensated for using the amplifier's offset and the series resistance dials respectively. This brought the baseline voltage reading to 0 mV. The amplifier was then switched to voltage-clamp and track modes with a voltage pulse of -25 mV. The electrode was then brought down towards the cell until a dimple was visible on the cell surface, caused by the positive pressure in the electrode. At this point, the positive pressure was released, and a small amount of negative pressure

was applied if necessary, to form a seal where the resistance was ≥3 GΩ. The amplifier was then switched back to current-clamp and the current pulse reduced to -50 pA. A small amount of additional holding current was also applied to the cells to help stabilise their membrane potentials. The membrane within the tip of the electrode was interrupted by a strong, quick period of negative pressure, to produce a whole cell patch clamp recording. The configuration of electrodes is illustrated in figure 2.2, with the recording electrode represented in position a) if recording from a CSFcC or position b) if recording from an ependymal cell.

Details of the constituents of the pressure ejection and recording electrodes are listed above in table 2.2 and section 2.4.1 respectively.

2.4.4 Electrophysiological cell characterisation

For the characterisation of the electrophysiological behaviour of the recorded cell, 1 s current pulses were applied to the cell with the resulting voltage responses measured and analysed. Positive current pulses (+10 to +50 pA) were used to determine if there was any spiking or action potentials produced in response to the depolarisation created by the current pulse. Negative current pulses (-10 to -50 pA) produced hyperpolarisations. These were then used to calculate the input resistance (IR) of the cell by using Ohm's law (input resistance = voltage response / current injected).

2.4.2 Application of drugs

All drugs apart from ATP and UTP were applied in the bath solution. This was achieved with separate smaller flasks, also oxygenated, containing a dilution of the drug in aCSF, flow of which was controlled via three-way taps.

ATP and UTP were applied in a more focal and rapid way, via local pressure ejection through a glass electrode (resistance 3-5 MΩ, tip diameter 3-4 μm) attached to a PV800 pneumatic pico pump (World Precision Instruments, UK) that was connected to a pressurised gas cylinder. Positioning of the pressure ejection electrode was controlled by another micromanipulator (PatchStar, Scientifica, UK). The PV800 was also connected to the pClamp9 program via the Digidata 1322A, and thus allowed the program to record the timing of the pressure ejection of the drug. The two drug application methods were used in conjunction to apply ATP or UTP via puff ejection before, during and after bath application of antagonist. A list of drugs applied during

the electrophysiological experiments can be found below (table 2.2) with details of the concentrations they were used at, their known functions and the supplier. All drugs for application in the bath solution were stored at -20 °C in stock solutions at $x10^3$ their working concentrations. ATP and UTP were stored in the same concentrations that would be used to fill the ejection electrode. All drugs were dissolved in aCSF solution unless otherwise stated.

Table 2.2 Drugs for electrophysiological experiments

Common name Chemical name	Known function	Final concentration	Justification for concentration	Supplier
Adenosine triphosphate, ATP [[(2R,3S,4R,5R)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy-hydroxyphosphoryl] phosphono hydrogen phosphate	P2X and $P2Y_{2, 4, 6, 11}$ and $_{13}$ agonist	Puff applied at 300 μM	Previously used effectively (Gómez-Villafuertes et al., 2015)	Sigma-Aldrich, UK
Uridine triphosphate, UTP [[(2R,3S,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-3,4-dihydroxyoxolan-2-yl]methoxy-hydroxyphosphoryl] phosphono hydrogen phosphate	$P2Y_{2, 4, 6, 11}$ and $_{14}$ agonist	Puff applied at 300 μM	Slight increase[1] in concentration previously used effectively (Gómez-Villafuertes et al., 2015)	Acros Organics, Belgium
Adenosine diphosphate, ADP [(2R,3S,4R,5R)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methyl phosphono hydrogen phosphate	$P2Y_{1, 12}$ and $_{13}$ agonist	Puff applied at 300 μM	Slight increase[1] in concentration previously used effectively (Gómez-Villafuertes et al., 2015)	Sigma-Aldrich, UK

[1] Slight increase to ensure identification of any response including those with minimal amplitude change.

Suramin 8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid	P2X and P2Y antagonist	50 µM	Previously used effectively (McLaren et al., 1995)	Tocris Bioscience, UK
A-317491 5-([(3-phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydronaphthalen-1-yl]carbamoyl)benzene-1,2,4-tricarboxylic acid	$P2X_3$ and $P2X_{2/3}$ antagonist	1 µM	Previously used effectively (Wang et al., 2014)	Sigma-Aldrich, UK
PPADS pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid	P2X antagonist	10 µM	Previously used effectively (Flores-Soto et al., 2012)	Tocris Bioscience, UK
2-Aminoethoxydiphenyl borate, 2-APB 2-Diphenylboranyloxyethanamine	TRP channel (1, 3, 5, 6, V6, M3, M7, M8 and P2) antagonist, and IP_3 and SOC channel antagonist	100 µM dissolved in 50 % ethanol	Previously used effectively (Akpinar et al., 2014)	Tocris Bioscience, UK
Apamin Amino acid sequence: CNCKAPETALCARRCQQH	SK channel antagonist	100 nM	Previously used effectively (Noble et al., 2010)	Sigma-Aldrich, UK
Dipropylcyclopentylxanthine, DPCPX 8-Cyclopentyl-1,3-dipropylxanthine	Adenosine A1 antagonist	500 nM dissolved in DMSO	Previously used effectively (Hargus et al., 2009)	Sigma-Aldrich, UK

Tetraethylammonium, TEA tetraethylazanium	Potassium channel antagonist (and nAChr antagonist with IC_{50} = 60 µM; (Blanchet and Dulon, 2001)	500 µM	Previously used effectively (Exintaris and Lang, 1999)	Sigma-Aldrich, UK
Niflumic acid 2-([3-(trifluoromethyl)phenyl]amino)pyridine-3-carboxylic acid	Chloride channel antagonist and COX-2 inhibitor and TRPA1 activator	100 µM dissolved in DMSO	Previously used effectively (Camprubí-Robles et al., 2013)	Sigma-Aldrich, UK
18β-glycyrrhetinic acid 3β-Hydroxy-11-oxo-18β,20β-olean-12-en-29-oic acid	Gap junction inhibitor	100 µM dissolved in 50 % ethanol	Previously used effectively (Momose-Sato et al., 2005)	Sigma-Aldrich, UK
Bicuculline (6R)-6-[(5S)-6-methyl-5,6,7,8-tetrahydro[1,3]dioxolo[4,5-g] isoquinolin-5-yl]furo[3,4-e][1,3] benzodioxol-8(6H)-one	$GABA_A$ antagonist (and Ca^{2+}-activated K^+ channel blocker (Khawaled et al., 1999)	50 µM	Previously used effectively in (Park et al., 1999)	Sigma-Aldrich, UK
Ap4A P^1,P^4- Di(adenosine-5') tetraphosphate ammonium salt	$P2X_{1, 2, 3, 4}$ and $P2Y_{2, 4}$ and $_{12}$ agonist	100 µM	(Wildman et al., 1999; Wildman et al., 2003; Chang et al., 2010)	Sigma-Aldrich, UK

2.5 Cell identification

2.5.1 Post fixative identification of cells using Neurobiotin

Neurobiotin was also included in the intracellular solution. It acted to fill the cell in a similar way to rhodamine, however it was visualised after fixation of the slice with 0.1 M phosphate buffer (PB) containing 4 % paraformaldehyde (PFA) and 0.2 % glutaraldehyde for approximately 18 hours at 4°C. Unlike rhodamine (Heyman and Burt, 2008), neurobiotin is able to pass through gap junctions into the coupled cells (Hou et al., 2013; Ponce et al., 2014). The process for visualisation of the neurobiotin filled cells utilised 3, 3'-diaminobenzidine (DAB). Slices were removed from the PFA solution and washed 3 times for 10 minutes in 0.1 M PB. The 3 % agar border was then removed. The membranes were then permeabilised using 50 % ethanol for 30 minutes and the sections were then washed in 0.1 M phosphate buffered saline (PBS; 3 x 10 minutes). Sections were then incubated in extravidin peroxidase (EAP; Sigma-Aldrich, UK; 1:250 in 0.1 M PBS with 0.2 % Triton) for approximately 72 hours at 4 °C to detect the neurobiotin. Following this, they were washed in 0.1 M PBS (2 x 10 minutes) to remove excess EAP. The EAP was then detected using a DAB substrate kit (Vector Laboratories, UK). The slices were then incubated in distilled water (dH_2O) containing Buffer Stock Solution (16.8 µl/ml of dH_2O), DAB Stock Solution (20 µl/ml) and Hydrogen Peroxide Solution (20 µl/ml) for between 2 and 18 minutes. The progress of the reaction was monitored at 2 minute intervals to determine the point at which the EAP marking the recorded cell was identified but before the endogenous peroxidase had also reacted making the slice too dark to identify the recorded cell. The slices were then rinsed in dH_2O to stop the DAB reaction.

2.5.2 Enhanced clarity of visualisation with ClearT

The clarity of the visualisation of the recorded cell and its processes was occasionally enhanced using the ClearT method (Kuwajima et al., 2013) to clear the tissue within the slice. This method uses successive, increasing concentrations of formamide (see table 2.3).

Table 2.3 ClearT method for sections (20-1000 μm)

Solution	**Incubation time (minutes)**
20% formamide	5
40% formamide	5
80% formamide	5
95% formamide	5
95% formamide	15

After being removed from the second incubation in 95% formamide, slices were mounted temporarily onto slides under glass coverslips. They were then imaged on a microscope (Nikon Eclipse E600 microscope, Nikon, Japan) with the images captured on the AcQuis image capture software (Digital Imaging Systems, UK) via a microscope-mounted camera (Micropublisher 5.0 RTV; QImaging, Canada).

2.6 Data analysis

2.6.1 Data collection

Electrophysiological data were collected on the Clampex program of the pClamp software (Molecular Devices, USA). The data were then analysed on the Clampfit program. Averages for a particular group were calculated and expressed as the mean ± standard error of the mean (S.E.). For drugs applied in the bath solution, comparisons were made before application, at the last point of application, and after 10 minutes of drug wash-out. The measurements made were of amplitude of voltage response (MP; measured as a change in membrane potential) and input resistance (IR; as calculated from the current steps using Ohm's law). For drugs applied via focal pressure ejection, current steps could not be maintained during the pressure ejection as the time period of response was too short and would have been lost within a current pulse such that the changes in MP and IR could not be separated. Therefore the current pulses were switched off shortly before each pressure ejection.

The measurements made of the responses to pressure ejected drugs were amplitude of response (MP), onset to start of response (ms) and duration of response (s).

Due to the lack of change in the composition of the intracellular and bath solutions and therefore the consistent influence of the junction potential across experiments, this was not compensated for except in experiments where reversal potentials were calculated. The junction potential for these solutions was calculated using the Clampex junction potential calculator as 13.9 mV. Therefore for reversal potential calculations, the membrane potential was calculated using the following equation: Vm = Vr - 13.9 mV where Vr is the voltage recorded.

2.6.2 Statistical analysis of electrophysiological experiments

Data were tested to determine whether or not they were normally distributed. Those that were not normally distributed but were log-normal distributions were treated as such. The analyses utilised both parametric and non-parametric test according to the distributions of the data being analysed. The tests were selected as laid out in table 2.4 below.

Table 2.4 Statistical tests used in the analysis of the data produced by this study

Testing the difference between:	**Parametric test (test statistic)**	**Non-parametric test (test statistic)**
2 groups or subgroups	Independent samples t test (t)	Mann-Whitney U Test (U)
>2 groups or subgroups	One-way ANOVA (F)	Kruskal-Wallis H test (H)
Before and during/after drug application	Paired samples t test (t)	Wilcoxen ranked sum test (Z)
Between groups/subgroups and drug application	Two-way ANOVA (F)	Non-parametric two-way ANOVA (F)

Parametric tests were performed if the data in each groups being compared all fitted into either normal or log-normal distributions. If any of the groups of data could not be

normalised or if the groups did not fit into the same distribution (i.e. normal or log-normal) then the corresponding non-parametric test was chosen.

2.7 Proliferation experiments

2.7.1 Experimental set up for 1 day proliferation preparation

The slices for the 1 day proliferation preparation were taken from the spinal cords of wild-type C57bl6 mice, pre-weaner (p12-21). The animals were anaesthetised and perfused and the spinal cords removed and sectioned as for the electrophysiological experiments, detailed in 2.2 above. The slices were removed from the agar border as they were being sectioned and were then moved to custom built holding chambers. The slices rested on a mesh membrane, submerged within the beaker of aCSF, such that both surfaces of the slice are in contact with the aCSF. The aCSF was bubbled throughout the procedure with 95 % CO_2: 5 % O_2. The holding chamber was then placed within a water bath, heated to 37 °C; (Figure 2.3).

The slices were kept in the holding chamber within the water bath for the remainder of the experimental period. For the first 60 minutes, the slices were left to acclimatise in a similar way to those used in the electrophysiological experiments. At this point EdU (5-ethynyl-2'-deoxyuridine) made up in medium C (see table 2.5) was added directly to the aCSF in the chamber. The concentration of EdU in the chamber was 10 μM as has been previously shown to maximally label proliferative cells, without inducing toxicity (Chehrehasa et al., 2009).

In the drug conditions, either 50 μM ARL 67156 (ecto-ATPase inhibitor, made up in aCSF; Sigma-Aldrich, UK) or 200 μM suramin (purinergic antagonist, see table 2.2, made up in aCSF; Tocris Bioscience, UK) was added to the aCSF, in addition to EdU. In the control condition, only EdU was added to the aCSF.

EdU is a thymidine analogue which is incorporated into the DNA of dividing cells during S-phase (Taupin, 2007) and therefore acts as a marker of cells that have newly-proliferated during the experimental period (period of EdU application). The method of detection of proliferation traditionally usually used is BrdU (5-bromo-2'-deoxyuridine) (Cavanagh et al., 2011). This is another thymidine analogue and incorporates similar way as EdU to the DNA during S-phase. However, unlike the incorporation method, the detection methods differ, with EdU being detected by click

chemistry (Buck et al., 2008) and BrdU being detected by antibody recognition (Gratzner, 1982). Detection of BrdU must, therefore, involve denaturation of the DNA, either by heat or acid treatment, which degrades the tissue. EdU detection uses the click chemical reaction that covalently bonds an azide-conjugated fluorescent probe to the alkene incorporated into the DNA as part of the EdU. This reaction works on the double-stranded DNA and does not therefore require denaturation. This confers a significant advantage in the use of EdU over BrdU and motivates the decision to use EdU as the proliferation marker in these experiments.

In the drug conditions either 50 μM ARL 67156 (ecto-ATPase inhibitor, made up in aCSF; Sigma-Aldrich, UK) or 200 μM suramin (purinergic antagonist, see table 2.2, made up in aCSF; Tocris Bioscience, UK) was added to the aCSF, in addition to EdU, after the slices had acclimatised for 60 minutes. In the control condition, only EdU was added to the aCSF.

The slices were then bubbled in the aCSF at 37 °C containing EdU and drugs for a further 4 hours; no further EdU or drugs were added during this period. After this point, the slices were removed from the aCSF and fixed in 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (PFA) at 4°C for 1 hour. After 1 hour, they were transferred to 0.1 M phosphate buffered saline (PBS).

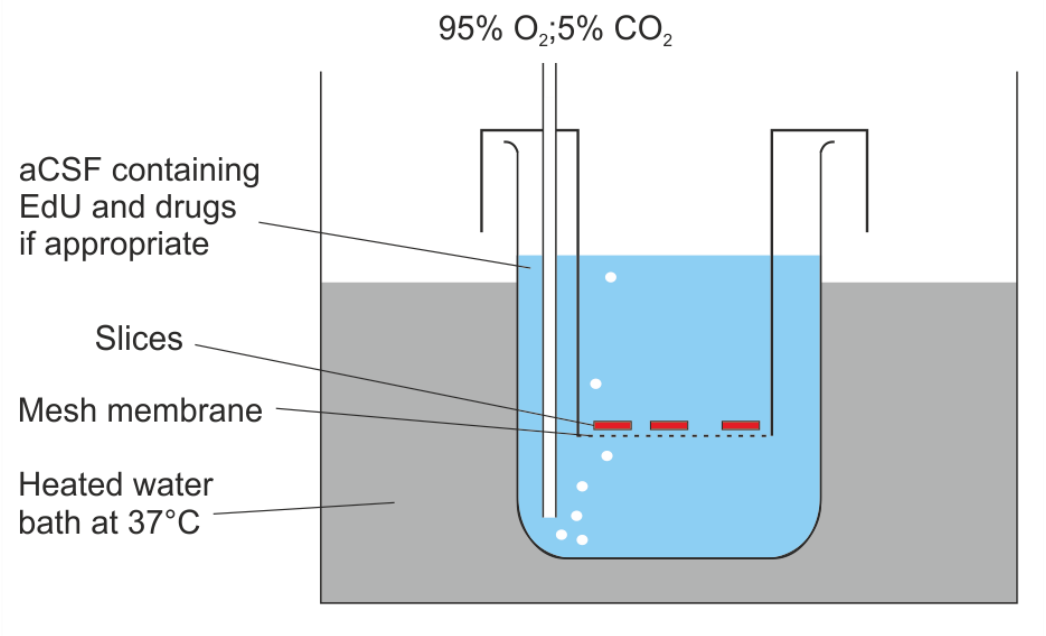


Figure 2.3 Experimental set up for 1 day proliferation preparation

Slices in 1 day proliferation preparation experiments were held inside a plastic tube with a mesh membrane base, suspended within a glass tube containing artificial cerebral spinal fluid (aCSF) and bubbled with a gas mixture of 95% oxygen (O_2) and 5% carbon dioxide (CO_2). Glass tubes were placed in a water bath maintained at 37°C.

2.7.2 EdU detection of proliferation preparations

The fixed slices were washed in 0.1 M phosphate buffered saline (PBS) with 0.2 % Triton for 12 minutes to permeabilise the cell membranes. They were then washed in TBS (2 x 10 minutes; 50 mM Tris, 150 mM NaCl, pH 7.6). The slices were then incubated in a solution of 320 µl distilled water, 25 µl Tris (2 M), 50 µl Cu(II)SO_4 (10 mM), 5 µl biotinylated azide (1 mM) and 100 µl ascorbic acid (0.5 M) for 30 minutes and protected from light from this point onwards. They were then washed again in TBS (2 x 10 minutes) and then a third wash in 0.1 M PBS with 0.2% Triton (1 x 10 minutes). The slices were then incubated with streptavidin^{555} (1:1000 in 0.1 M PBS) for 1 hour before a final set of washes in 0.1 M PBS with 0.2% Triton (3 x 10 minutes). They were then mounted onto slides using VectaMount mounting medium with DAPI (Vector Laboratories, UK) and covered with a cover slip, which was secured in place using nail polish.

The slices were then imaged on a fluorescence microscope (Nikon Eclipse E600 microscope, Nikon, Japan) with the images captured on the AcQuis image capture

software (Digital Imaging Systems, UK) via a microscope-mounted camera (Micropublisher 5.0 RTV; QImaging, Canada).

EdU-positive cells were manually marked onto a schematic representation of the spinal cord and then counted by spinal cord region. The data produced by these counts were statistically analysed according to table 2.4.

2.8 Organotypic slice culture preparation experiments

2.8.1 Experimental set up for organotypic slice culture preparations

Organotypic slice culturing is a method which allows extension of the viability period of neuronal tissue slices from up to 9 hours (Hatton et al., 1980) to maintenance of the electrophysiological and morphological characteristics for over 3 weeks (Davids et al., 2002). The method of organotypic slice culturing used in this experiment was the interface method (Stoppini et al., 1991) whereby slices are held at the interface between medium and air by the fact that they are placed on a semi-porous mesh. This culture method offers the physiological heterogeneity of cell types and cyto-architecture of the spinal cord that is available in *in vivo* experiments, but retains the drug accessibility of cell culture methods. It has therefore become widely used for injury modelling (Labombarda et al., 2013; Llorente et al., 2015) and exploration of cellular development (Heine et al., 2015). This technique also has the advantage that, as the slices could be prepared in a similar way to those used for the electrophysiological and proliferation preparations, there could be greater comparison between the results.

As mentioned above, the slices were prepared as in section 2.2. The animals used for these experiments were wild-type C57bl6 mice (p12-21). As with the proliferation preparations, the slices were removed from their agar border at the time of sectioning. They were then transferred to a sterile Falcon tube containing sterile medium A (see table 2.5 for details of composition of all media used for organotypic slice culture experiments). They were then moved into a laminar flow hood and transferred onto Millicell organotypic filter inserts (Merck Millipore, Germany), with 3-6 slices per filter. They were transferred by means of suction into a widened, sterile tip of a P1000 pipette, and ejection onto the filter insert. The filter inserts sat on medium B such that the medium was in contact with the underside of the filter insert. The medium had been pre-heated to 37 °C. This medium contained foetal bovine serum

as this aids neuronal survival and recovery from trauma (Takeshima et al., 1994). After plating of the slices, the plate was transferred to a sterile incubator at 37 °C with 100 % humidity, 95 % air and 5 % CO_2-enriched environment.

2.8.2 Maintenance and experimental period of 5 day organotypic slice cultures

After 24 hours in the incubator, the culture plate was transferred back to the laminar flow hood, where the medium under the filters was changed for medium C. The removal of serum from the medium, ensured more exact knowledge of the constituents of the medium as animal sera are undefined and have variable composition (van der Valk et al., 2010). Foetal bovine serum (FBS) contains a large and complex mixture of growth factors, vitamins, hormones, proteins and trace elements. Medium C contains a B27 supplement (Brewer et al., 1993) as this has been shown as effective for CNS slice culture and avoids problems with other defined (serum-free) media of fibroblast or astrocyte overgrowth (van der Valk et al., 2010). This protocol whereby a medium containing serum is used for the initial period of culturing, which is then replaced with a defined (serum-free) medium shortly before and during the experimental period (Noraberg et al., 1999) has been found to work well for organotypic CNS cultures.

After 48 hours in culture, the medium in the plate was exchanged for medium C containing 10 μM EdU (made up in medium C). In addition to the EdU, the experimental drugs were also added at this time. The experimental drugs for the organotypic slice culture experiments were the same as for the proliferation preparation experiments above (section 2.6.1), namely 50 μM ARL 67156 (ecto-ATPase inhibitor; Sigma-Aldrich, UK) or 200 μM suramin (purinergic antagonist, see table 2.2; Tocris Bioscience, UK). These were then returned to the incubator for a further 42 hours.

After 42 hours, the filter medium under the filter was removed and replaced with 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (PFA). Further PFA was then added to the top of the filter to ensure complete fixation of both sides of the spinal cord slices. The slices were fixed in PFA for 6 hours at 4 °C. They were then removed from the filter using a fine paint brush and placed in 0.1 M phosphate buffered saline (PBS).

Table 2.5 Composition of media for culture experiments

Medium A – dissecting medium	**Medium B** – serum medium	**Medium C** – serum-free medium
Dulbecco's modified eagles medium (DMEM; Sigma-Aldrich, UK)	Neurobasal A medium (Invitrogen, USA)	Neurobasal A medium (Invitrogen, USA)
1 % penicillin/streptomycin (Sigma-Aldrich, UK)	1 % penicillin/streptomycin (Sigma-Aldrich, UK)	1 % penicillin/streptomycin (Sigma-Aldrich, UK)
	1 % L-Glutamine (Sigma-Aldrich, UK)	1 % L-Glutamine (Sigma-Aldrich, UK)
	10 % foetal bovine serum (Sigma-Aldrich, UK)	2 % B27 supplement (Invitrogen, USA)

2.8.3 EdU detection of 5 day organotypic slice cultures

Detection of EdU on fixed slices from the organotypic slice culture experiments was carried out as per section 2.6.2 for the fixed slices from the proliferation preparation experiments.

2.8.4 Analysis of EdU labelling in 5 day organotypic slice cultures

EdU labelling was too expansive and overlapping for a counting procedure to be reliable. Therefore a fluorescence analysis was carried out to give a reading of the distribution of EdU positive cells within the slice. This involved imaging the fixed and processed slices (as in section 2.6.2) using the AcQuis image capture program (Digital Imaging Systems, UK) on the computer, which captured the images through the camera (Micropublisher 5.0 RTV; QImaging, Canada) mounted onto the microscope (Nikon Eclipse E600 microscope, Nikon, Japan). The images were taken at x 4 and x 20 magnification, with the fluorescence analysis applied to the x 4 images.

Images were then opened in ImageJ image processing software (National Institutes of Health, USA). Within this program, images were adjusted manually to maximise

EdU positive cell fluorescence and minimise background fluorescence. The analysis measured the light intensity of the image over a lateral half of the spinal cord slice, from the midpoint of the central canal across the half width of the spinal cord slice to the lateral edge (see figure 2.4). The midpoint of the central canal was selected using the DAPI nucleic stain to identify the central canal. The half of the slice to be analysed was chosen for completeness of the slice section and lower levels of background fluorescence. Data were expressed as amount of light intensity (in arbitrary units) for each vertical column of pixels in a 300 pixel height strip extending from the midpoint of the central canal to the edge of the slice. These data were then combined to create a simple moving average (15 pixel period) for each of the 3 conditions. The output (over 5 pixels) of this was averaged to create a weighted moving average (over a total period of 19 pixels). This was plotted as light intensity as a function of distance from the central canal, which was then normalised by area under the curve. This allowed comparison of particular pixel distances corresponding to areas of interest between drug and control conditions (statistically analysed as detailed above in table 2.4) as well as comparison of location and spread of light intensity within each condition.

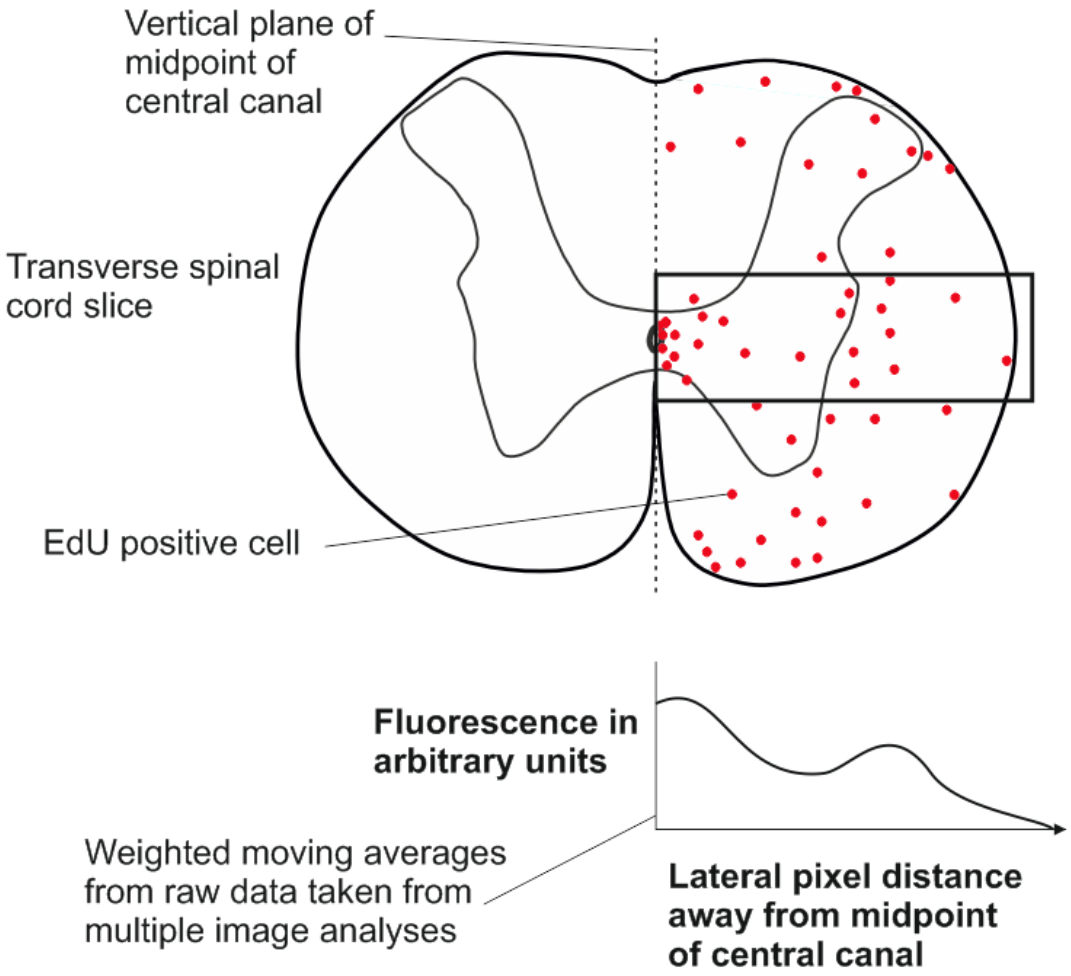


Figure 2.4 Illustrative example of fluorescence analysis for organotypic slice cultures

A transverse section of the spinal cord demonstrating the procedures used for analysis of location of EdU-containing cells. EdU-positive cells are shown in red with the box indicating the area of the spinal cord analysed. The left hand side of the box was positioned on the midpoint of the central canal, the right hand side is outside the limits of the slice. The height of the box was 300 pixels (256 µm) with the central canal at the midpoint of the box height. An example trace is shown after use of weighted moving averages to reduce the spikes corresponding to individual cells.

2.8.5 Maintenance and experimental period of time-point organotypic slice cultures

Similarly to the 5 day cultures, the maintenance of which is detailed above in 2.7.2, the medium was changed after 24 hours, from medium B to medium C. 45 hours after the slices were first prepared the EdU (made up in medium C) was added to the medium C in the wells with an in-well concentration of 10 µM. The slices in the drug

condition also had ARL 67156 added to the well at a final concentration of 50 μM. The slices remained in the presence of EdU for 4 hours. At this point all slices were removed from the EdU containing medium. For this experiment there were two time-point conditions (1 and 3), with a control well and a drug condition well at each time point. After 8 hours in EdU containing medium, the wells in the time-point 1 condition were fixed above and below the filter with 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (PFA) at 4°C for 1 hour. At this time, the wells in time-point 3 had the medium under the filter exchanged for fresh medium C, to which ARL 67156 was again added to the drug condition wells. The wells in the time-point 3 condition were fixed in 4% PFA as above after 48 hours in non-EdU containing medium.

2.8.6 EdU detection of time-point organotypic slice cultures

The EdU was detected using the same click-chemistry protocol that was used above in both the proliferation preparation experiments, and the 5-day organotypic slice culture experiments, see section 2.6.2 above.

2.8.7 Analysis of EdU labelling in time-point organotypic slice cultures

The EdU was only taken up by the cells that underwent S-phase during the 8 hour period in which the slices were in EdU-containing medium. This meant that unlike the slices in the 5 day organotypic slice culture experiments, the number of EdU-positive cells was low enough to be counted using an automated counting program, ImageJ, NIH, US. Therefore the analysis was carried out as was done in the proliferation preparation experiments (section 2.6.2). Slices were viewed on the microscope (Nikon Eclipse E600 microscope, Nikon, Japan) with any images captured using the AcQuis image capture program (Digital Imaging Systems, UK) on the computer, through the microscope-mounted camera (Micropublisher 5.0 RTV; QImaging, Canada).

EdU-positive cells were then manually marked onto the spinal-cord schematic representation and manually counted by spinal cord region. Data from these counts were statistically analysed using the statistical tests laid out above in table 2.4.

2.9 Immunohistochemistry

2.9.1 $P2X_3$ immunohistochemistry on fixed slices

Immunohistochemistry was carried out by Lucy Peers.

Tissue for immunohistochemical analysis of $P2X_3$ expression was prepared by perfusion with 0.1 M phosphate buffer (PB) containing 4 % paraformaldehyde (PFA). After spinal cord dissection, the spinal cord was post-fixed in the same PFA solution. Slices were sectioned at 50 μM on a Leica VT100 S vibratome (UK). After 3 x washes in PBS, slices were incubated in rabbit anti-$P2X_3$ antibody (Alomone Labs, Jerusalem, Israel) in PBS with 0.1 % triton (PBST) overnight, then washed with PBS a further 3 times. The secondary antibody, donkey anti-rabbit IgG Alexa Fluor 488 (Invitrogen, US), was applied at 1 in 1000 in PBS for 2 hours and then washed 3 x with PBS. They were then mounted using VectaMount mounting medium with DAPI (Vector Laboratories, UK) and covered with a cover slip and secured in place using nail polish.

Chapter 3 - CSFcCs respond to purinergic activation with either a hyperpolarisation or a depolarisation

3.1 Introduction

Previous work on the electrophysiological properties of CSFcC suggests that there is considerable variation within the CSFcC population. Cells previously identified include cells which passively responded to current injections and showed no spontaneous activity, through cells which exhibited some spontaneous activity and were able to produce a single spike or action potential in response to depolarising current pulses, and cells which fired a train of action potentials or spikes in response to current injection (Corns et al., 2015). These were labelled CSFcC 1, CSFcC 2 and CSFcC 3 respectively and this nomenclature was continued in this work.

Several differences have been observed in CSFcCs showing different electrophysiological profiles. The ionic contribution to their responses to depolarising current pulses differs, with cells producing slow regenerative potentials or small amplitude, long half-amplitude duration spikes being largely mediated by Ca^{2+} (Marichal et al., 2009). This was demonstrated by their sustained occurrence in the presence of the specific voltage-gated sodium blocker TTX and blockage in the presence of 3 mM manganese (Mn^{2+}), which is a potent calcium channel blocker. There was however a small component of the response blocked by TTX suggesting a small contribution of voltage-gated Na^{2+} channels to otherwise Ca^{2+} mediated currents. These cells also demonstrated a small low threshold spike (LTS) as a rebound response from a hyperpolarising current pulse. Application of 300 µM nickel (Ni^{2+}) blocked this rebound response indicating that it was via T-type Ca^{2+} channels.

Unlike in the non-spiking or minimally spiking CSFcCs, in CSFcCs that could larger spike or action potential, the fast spike was inhibited by TTX indicating that, in these cells, the depolarisation was due to voltage-gated Na^{+} channels (Marichal et al., 2009). The CSFcCs that produced only a single spike did not show the slow after-hyperpolarisation (AHP) typically observed in spinal neurons (Russo and Hounsgaard, 1999; Marichal et al., 2009). However the CSFcCs that were able to produce a train of spikes or action potentials, particularly those with the most robust firing pattern, did display AHPs. The spikes or action potentials in these multi-spiking cells tended to have larger amplitude and shorter half-amplitude duration than single spiking cells. As with the single-spiking cells, no activity was seen in the multi-spiking cells in the presence of TTX suggesting minimal contribution of Ca^{2+} in these responses.

No correlation between the electrophysiological phenotype and the age of the animal was observed (Marichal et al., 2009). There was also no link between the phenotype of the CSFcCs and the embryological period in which they are born. All CSFcCs are born in a late embryological period (E14.5-16.5 in mice)(Petracca et al., 2016), alongside astrocytes and oligodendrocytes. In addition to determining the embryonic age of CSFcCs, Petracca et al. (2016) also showed that CSFcCs are produced from 2 distinct dorso-ventral regions of the spinal cord. Most CSFcCs (90 %) were derived from progenitors in the late-p2 and the oligodendrogenic (pOL) domains, while a subset (10 %) arose from progenitors near the floor plate. In these experiments they used $Gata2^{GFP}$ tissue to identify CSFcCs which expressed the transcription factor Gata2. All of the cells identified in this way produced either a single spike or a train of spikes in response to depolarising current pulses, and they termed these cells CSFcNs. This method allowed targeting of Gata2-positive, PKD2L1-positive cells, which may not represent the entire range of CSFcCs recorded in other studies.

As the cells from the two separate embryonic regions differentially used Pax6 as an embryonic transcription factor, the cells could be traced to show that they adopt separate locations around the central canal (at E18.5) and show different electrophysiological profiles or subtypes (at P1-2) (Petracca et al., 2016). At these times, the subset of cells derived from the floor plate were located ventrally and showed only single spiking, whilst of CSFcCs derived from p2/pOL, 80 % could produce a train of spikes in response to current injection and most were located laterally. These studies show the breadth of electrophysiological profiles of CSFcCs and demonstrate some of the causes of those differences. Whilst the study by Petracca et al. (2016) is in very young and embryonic animals, previous work has demonstrated similar levels of variation amongst the CSFcC population at different ages (Corns, 2012), suggesting this variation is not a temporary feature.

As well as differences in ionic contributions and embryonic origins between CSFcC subtypes, there are also differences in their responses to neurotransmitters. GABA produces a range of responses in CSFcCs via $GABA_A$ receptors (Marichal et al., 2009; Corns, 2012). Both hyperpolarisations and depolarisations are produced, with a range of reversal potentials from -80 mV to -43 mV in gramicidin-perforated patch clamp experiment, which could be due to differences in internal Cl^- levels due to K-Cl co-transporter (KCC2)/ N-K-Cl co-transporter (NKCC1) expression (Marichal et al., 2009; Kaila et al., 2014), however both hyperpolarisations and depolarisations have

been observed in whole cell patch clamp experiments (Corns, 2012). In whole cell patch clamp experiments, the internal Cl^- concentration should be set by the composition of the intracellular solution in the electrode. There are also differences in the responses of CSFcCs to acetylcholine (ACh) (Corns et al., 2015). Whilst all CSFcCs depolarised to ACh, a different composition of receptors was responsible for the effects. Both non-α7* nicotinic ACh receptors and α7* nAChrs were shown (by use of selective antagonists) to contribute to the response but to varying extents in different subtypes. Non-spiking CSFcC 1s mainly responded to ACh via α7* nAChrs but with contribution from non-α7* nAChrs, whilst CSFcC 2s predominantly, and CSFcC 3s almost exclusively, responded via non-α7* nAChrs (Corns et al., 2015). This indicates differential receptor make-up between CSFcC subtypes.

It has been shown that CSFcCs express $P2X_2$ receptors and that they can respond to extracellular ATP and produce a depolarisation (Marichal et al., 2009). It is not yet know if the differences in receptor composition, ion balance and electrophysiological profiles of CSFcCs will produce significant differences in their responses to ATP.

3.1.1 Hypothesis

The hypotheses of this chapter are that CSFcCs around the central canal of the spinal cord would:

1. Fall into subtypes which would be distinguishable by their level of activity in the voltage response to current pulses or other changes in membrane potential
2. Acutely respond to ATP application with a depolarisation
3. That ATP would produce the response by acting via the ionotropic P2X receptor, $P2X_2$.

3.1.2 Aims

This chapter uses patch-clamp electrophysiology in addition to fluorescence and post-fixation DAB imaging following intracellular dye-loading to:

1. Identify and categorise the CSFcCs in the area surrounding the central canal of the spinal cord
2. Investigate whether there is an acute response to ATP in the CSFcCs and look for differences between subtypes
3. To investigate the receptors involved in producing this response.

3.2 Results

The data in this chapter come from recordings made using spinal cord slices from Wistar rats, aged 7-13 days.

3.2.1 CSFcCs have varied phenotypes which can be sub-classified

Electrophysiological experiments revealed CSFcCs to display considerable variation in phenotype. This extended from a passive phenotype which was indistinguishable from that of ependymal cells to a profile which produced multiple spikes and bore closer similarity to the behaviour of some neurones. As used by Marichal et al. (2009), the term spike is used to refer to a fast event with depolarising and repolarising phases, but one which does not fulfil the criteria to be classified as an action potential. Spikes have lower amplitude than action potentials, not going above 0 mV. Spikes also have larger half-amplitude duration than would be expected for action potentials. There was a continuum of responses of CSFcCs to depolarising current pulses between those with a passive phenotype which are unable to produce any spontaneous or evoked electrical activity and those that could fire repetitively.

Due to this range of responses to the depolarising current pulses, these could not be used to confidently identify CSFcCs from the recordings from ependymal cells. Therefore visualisation of the cell, either by post-recording fluorescent imaging or post-fixation DAB imaging, was used for this purpose. Specifically, the phenotypic feature that was used to identify CSFcCs was the presence of a process (closed arrowheads, Figure 3.1 Aiii, Biii and Ciii) which extended towards the lumen of the central canal, the direction and boundaries of which were identified by infrared DiC imaging (Figure 3.1 Aii, Bii and Cii).

Despite the continuum of responses, CSFcCs were categorised into 3 subtypes dependent on their voltage response to the depolarising current pulses applied during the characterisation of the cell. These subtypes enabled analysis of the different responses to the drugs and allowed correlation between response size or type and extent of spontaneous or evoked electrical activity. The subtypes used for this analysis have been applied previously (Corns et al., 2015).

The CSFcC 1 subtype was comprised of cells which showed a passive electrophysiological profile and were unable to produce any spikes or action potentials when depolarised with a positive current pulse (Figure 3.1 Ai). These cells

also displayed no spontaneous activity. CSFcC 1s had a relatively low input resistance compared with the other subtypes of CSFcC (194.19 ± 34.35 MΩ; n = 37; N = 32) but the resistances appeared to have a bimodal distribution (Figure 3.3 A). Type 1 CSFcCs had a resting membrane potential (RMP) of -64.23 ± 4.01 mV (n = 39; N = 39). CSFcC 1 was the most common subtype in the recorded CSFcCs (48.2 % of CSFcCs (39 of 81); Figure 3.2 C).

CSFcCs were classified as CSFcC 2s if they were able to produce a single spike in response to a depolarising current pulse (Figure 3.1 Bi). CSFcC 2 had a larger IR than CSFcC 1 (891.88 ± 101.98 MΩ; n = 25; N = 21) and a less hyperpolarised RMP (-36.3 ± 2.80 mV; n = 31; N = 28). CSFcC 2s were less frequently observed than CSFcC 1s (38.3 % of recorded CSFcCs (31 of 81)).

CSFcC 3s were capable of producing a train of spikes or action potentials in response to a depolarising current pulse (Figure 3.1 Ci). CSFcC 3s are less common than CSFcC 1 and 2s, comprising only 13.6 % of CSFcCs (11 of 81) in the recorded population. In terms of RMP and IR, CSFcC 3s had an RMP of -58.22 ± 9.77 mV (n = 11; N = 10) and an IR of 995.20 ± 344.13 MΩ (n = 7; N = 6).

For both IR and RMP, the data for all CSFcC subtypes were not normally distributed as assessed by the Shapiro-Wilk test. Therefore comparisons used the Kruskal-Wallis test and indicated that there were significant differences between the CSFcC subtypes for both characteristics (RMP: $P < 0.001$, Figure 3.2 A; IR: $P < 0.001$, Figure 3.2 B).

Bonferroni post-hoc analysis showed that CSFcC 1s have a significantly more negative RMP than CSFcC 2s ($P < 0.001$), however neither group were significantly different to CSFcC 3s ($P = 0.889$ for CSFcC 1s; $P = 0.674$ for CSFcC 2s). In similar analysis of IR, CSFcC 1s had a significantly lower IR than both CSFcC 2s ($P < 0.001$) and CSFcC 3s ($P = 0.002$) while the IRs of these subtypes were not significantly different from one another ($P = 1.000$).

There was an uneven distribution of cells of each CSFcC subtype around the central canal (Figure 3.2 D), with a higher proportion of CSFcC 3s around the ventral end of the spinal cord compared to the other regions of the ependymal zone. CSFcC 1s and CSFcC 2s were more evenly distributed.

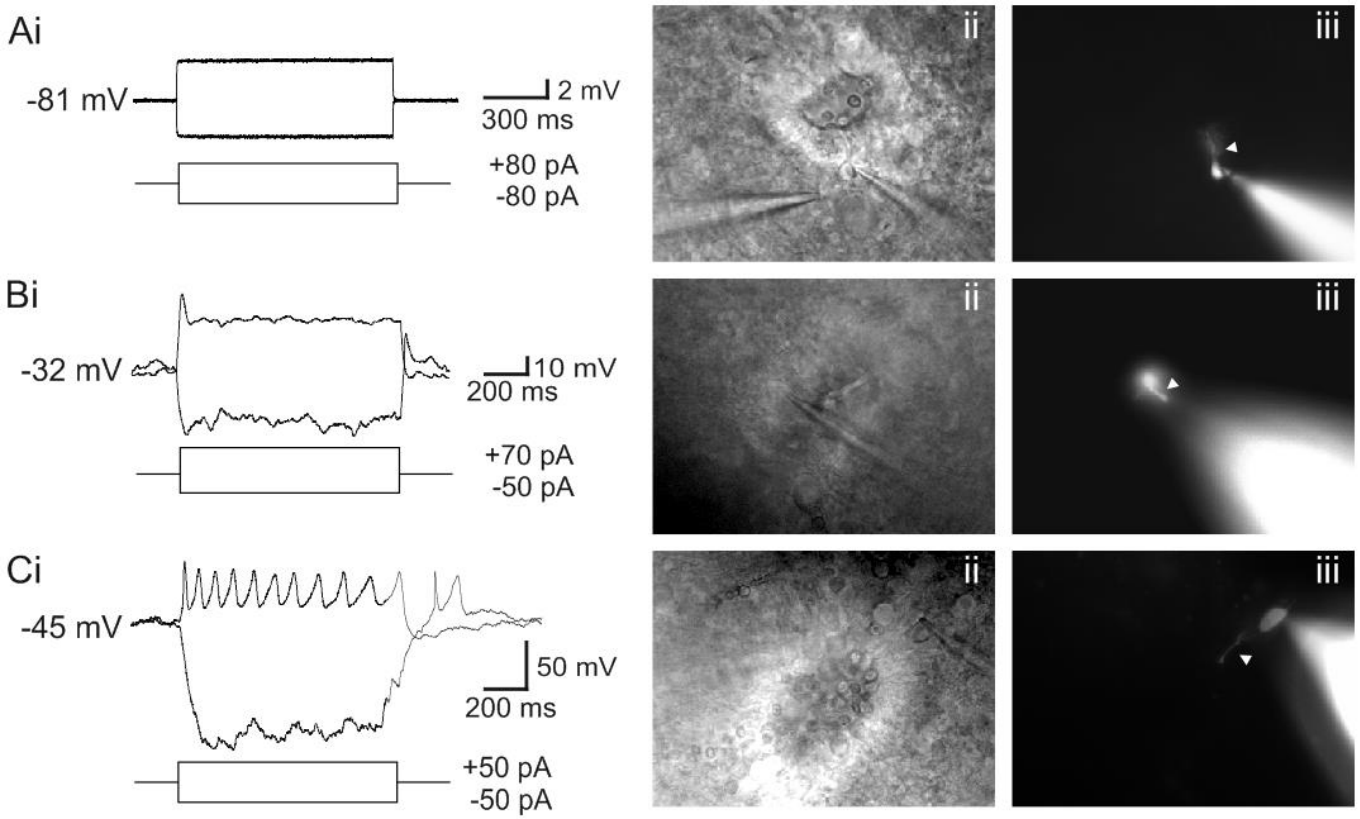


Figure 3.1 Characteristics of CSFcC 1s, CSFcC 2s and CSFcC 3s

Ai, Bi and Ci) Whole cell patch clamp traces in current clamp of a CSFcC 1, CSFcC 2 and CSFcC 3 respectively, responding to a negative and positive current pulse. Aii, Bii and Cii) The DIC image of the recorded cell showing location around the CC. Aiii, Biii and Ciii) Post recording excitation of rhodamine produces shows fluorescent image of recorded cell, closed arrowheads show the CSFcC processes protruding into the CC with bouton on the end.

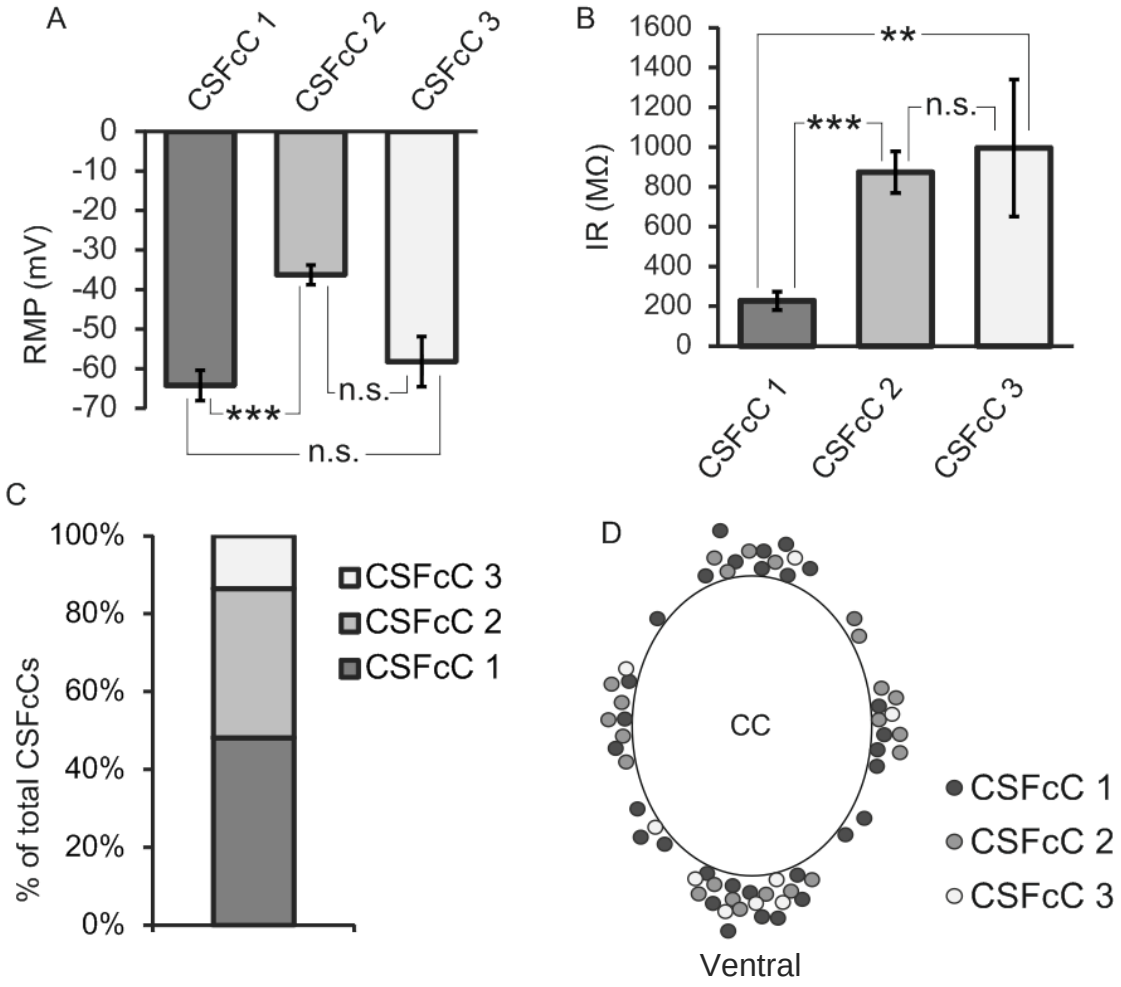


Figure 3.2 Comparisons of CSFcC subtypes

A-B) Comparison of the mean resting membrane potential (RMP) ± S.E. (A) and the mean input resistance (IR) ± S.E. (B) for the 3 CSFcC subtypes by Kruskal-Wallis test as data previously determined not to be normally distributed by Shapiro-Wilks tests. ** = $p < 0.01$. *** = $p < 0.001$. C) Proportion of recorded CSFcCs by subtype. D) Location of cells of each CSFcC subtype.

Despite having a significantly lower mean IR than that for CSFcC 2s and CSFcC 3s, the IRs of some CSFcC 1s overlap with some of those of the spiking subtypes. This can be seen when the data points are plotted on a frequency histogram. A combined histogram for CSFcC 2 and CSFcC 3 IR resembles a typical right skewed distribution as would be expected when the mean is closer to 0 MΩ than the extent of the spread of the distribution (Figure 3.3 B). However, a histogram for CSFcC 1 IR data did not fit a similar distribution and instead looked like it may be a bimodal distribution (Figure 3.3 B). Within this distribution, there is a population of IR measurements that is in line with the distribution of the IR for the CSFcC 2s and CSFcC 3s, although increasing the skewedness of the distribution. The other modal peak of the CSFcC 1 IR distribution is considerably lower than any of the recordings for IR from the other CSFcC subtypes.

One explanation for this is the difference within the CSFcC-identified population, particularly within the CSFcC 1 population, in levels of gap junction coupling. Most CSFcCs, including all spiking CSFcCs, do not show any gap junction coupling. During electrophysiological recording, the cell is filled with Neurobiotin, which is included in the intracellular solution in the electrode. Neurobiotin can travel through gap junctions and allows for gap junction connectivity to be viewed post-fixation and staining of the Neurobiotin with DAB staining. Most CSFcCs appear as a single cell with a visible process (Figure 3.3 C). However some CSFcC 1s are gap junction coupled. Therefore these appear as a spread of dye throughout part of the ependymal cell layer (Figure 3.3 D). Some processes heading dorsally and ventrally can also be observed in the post-fix images. The cell exemplified could clearly be identified as CSFcC by the rhodamine-enabled fluorescent imaging.

The reason why the differential levels of gap junctions in some CSFcC 1s could account for the separate population in terms of IR distribution is that the higher gap junction coupled a cell is the lower the input resistance. The IR of a cell is lower in cells with more open ion channels and/or with more gap junctions. This has been demonstrated in other cells types by use of gap junction blockers to increase IR (Corns et al., 2013).

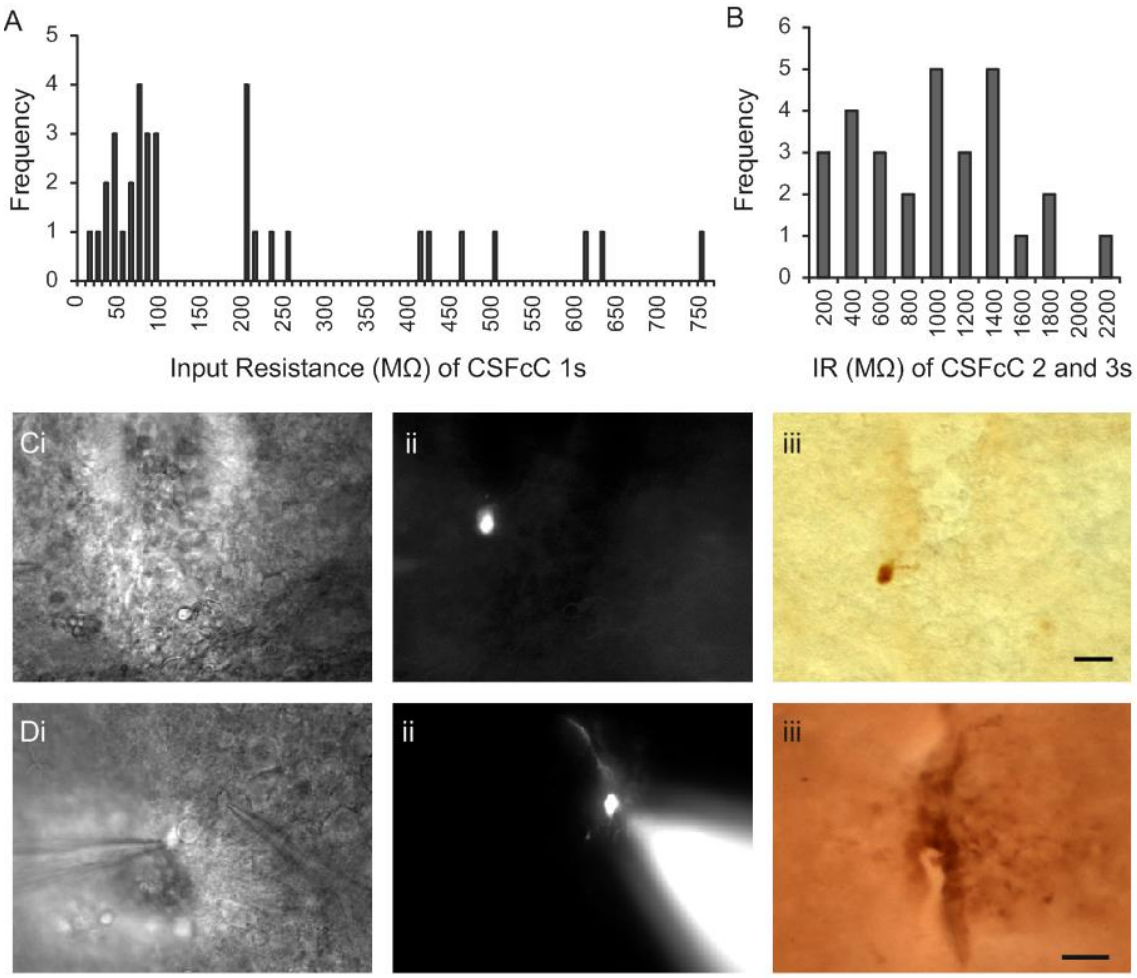


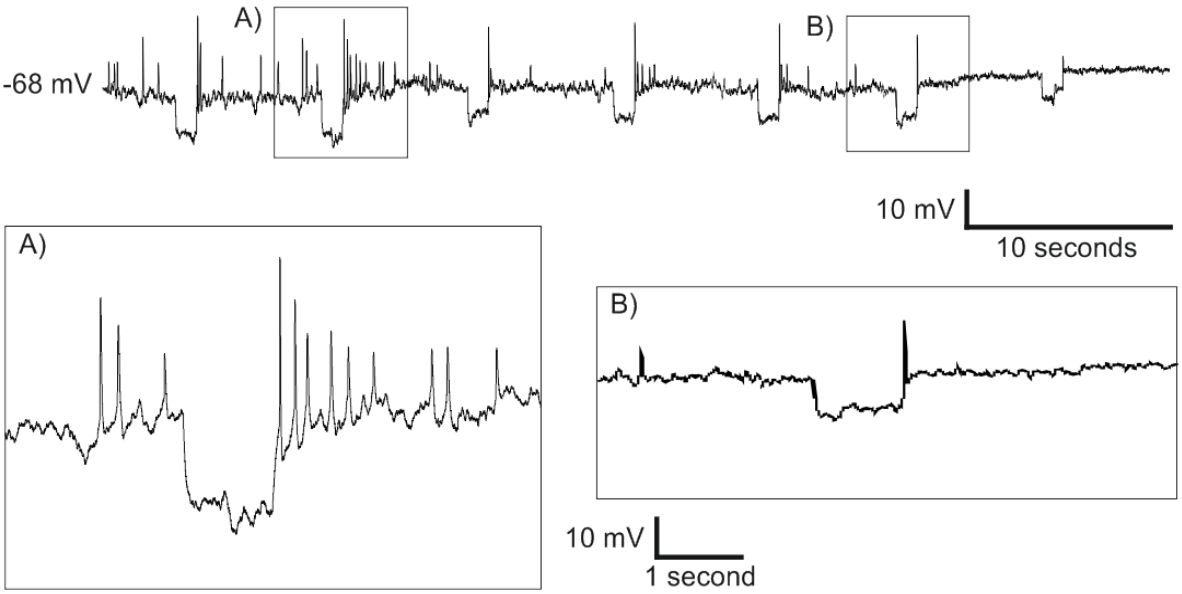
Figure 3.3 There are two populations of CSFcC 1s based on input resistance

A) Frequency histogram for input resistances for CSFcC 1s showed a bimodal distribution. B) Frequency histogram for the input resistances of the combined population of spiking CSFcCs (CSFcC 2 and CSFcC 3). C and D) Visualisation of a CSFcC that is not gap junction-coupled (C) and a CSFcC that is gap-junction coupled (D) with irDIC image showing location of the cell around the central canal (Ci and Di), the process demonstrated by fluorescence imaging (Cii and Dii) and post-fix DAB staining with dye-coupling indicating gap junction coupling if present (Ciii and Diii). Scale bar = 20 μm (Ciii) and 50 μm (Diii).

3.2.2 Some CSFcCs show loss of spiking throughout recordings

Cells were classified into subtypes during the initial characterisation period of the recording. In the vast majority of recordings, the cell may have drifted somewhat in its membrane potential, which was corrected for by adjusting the holding potential, however they stayed within the classification criteria for the same CSFcC subtype. A proportion of the cells with clearly defined spikes held for > 20 minutes displayed changes in phenotype with a reduction or loss of spikes (n = 8 of 15). These changes were more often a change from a cell with high levels of spiking (CSFcC 3) to a cell with less spiking (CSFcC 2) (Figure 3.4). They were frequently accompanied by a change in membrane potential, however even when corrected for by adjustment of holding potential, the initial responses could not be resurrected (data not shown).

Figure 3.4 CSFcC 3s can lose spontaneous spiking and become CSFcC 2s



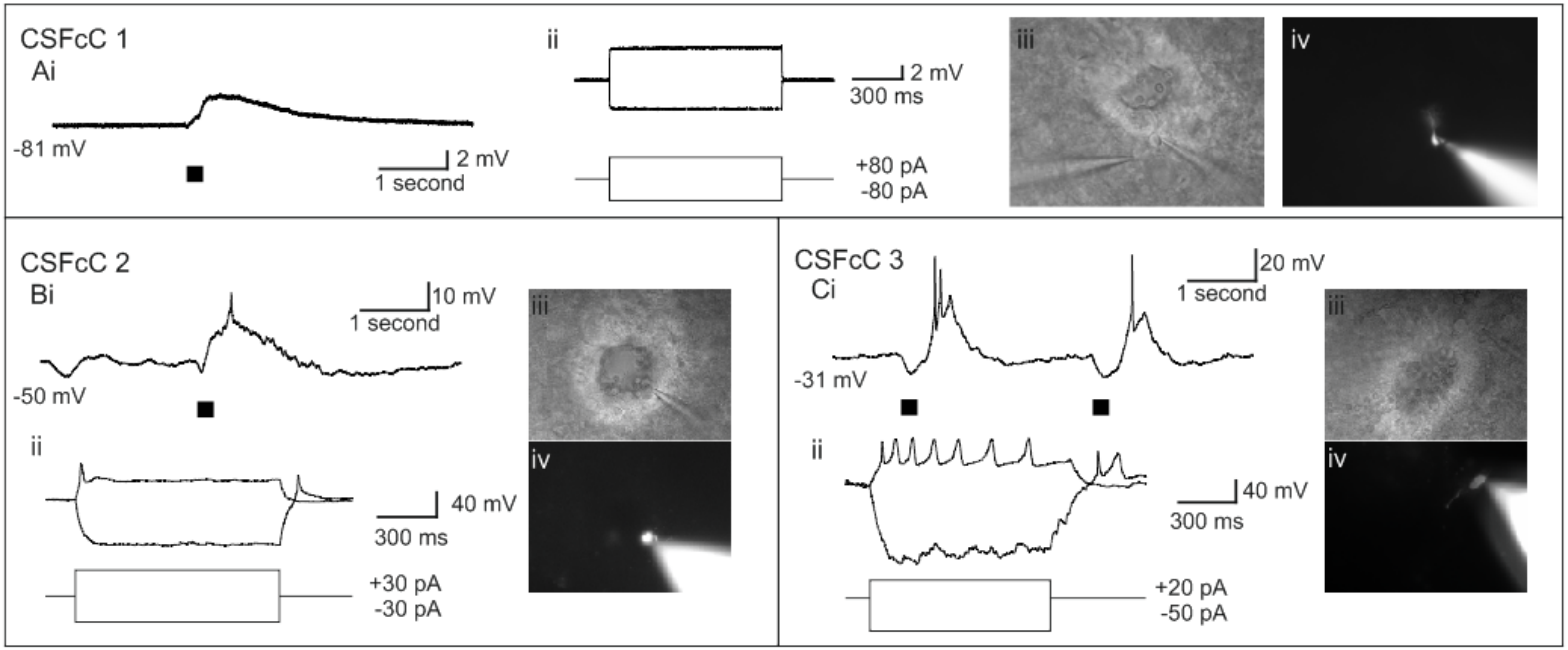
Whole cell patch clamp continuous recording of a CSFcC changing from a subtype 3 phenotype (A) to a subtype 2 phenotype (B) as shown by the shape of the voltage responses to hyperpolarising current pulses (-50 pA).

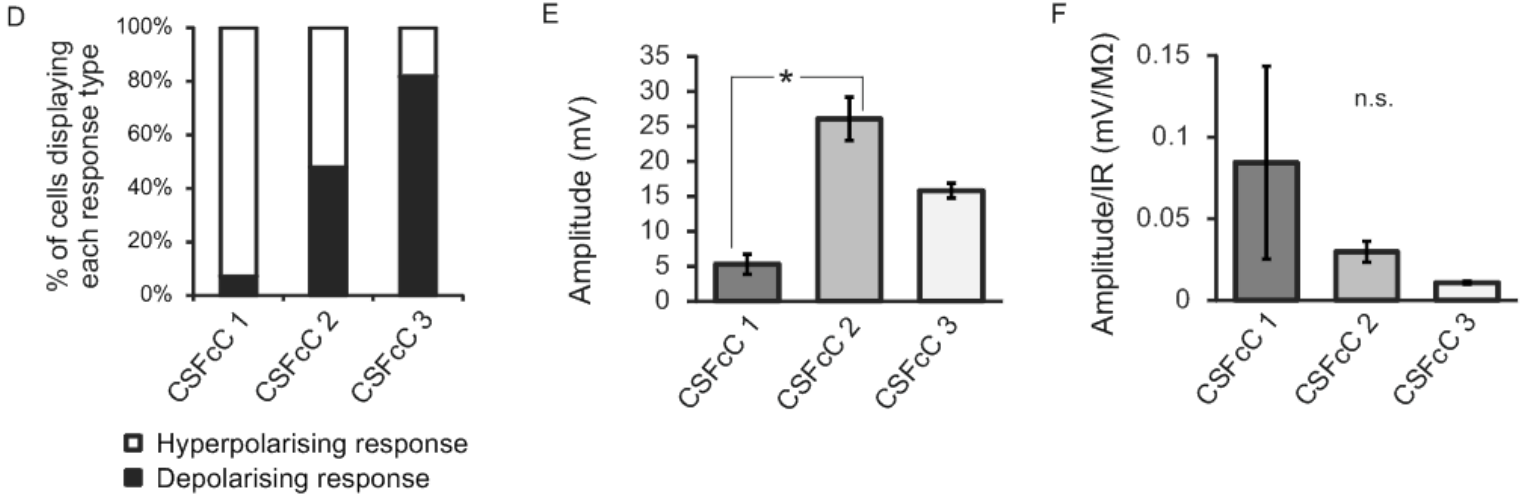
3.2.3 A population of CSFcCs responded to ATP with a depolarising response

This study applied 300 μM ATP locally, using pressure ejection, to CSFcCs. All CSFcCs recorded showed a response to the pressure ejection of ATP. One population of CSFcCs responded with a depolarisation. Depolarising responses were observed in CSFcCs from subtypes 1, 2 and 3 (Figure 3.5 Ai, Bi, Ci respectively) with the range of subtypes demonstrated by the voltage responses to depolarising and hyperpolarising current pulses (Figure 3.5 Aii, Bii and Cii) in addition to the visualisation of the process (Figure 3.5 Aiv, Biv and Civ) through the ependymal cell layer (Figure 3.5 Aiii, Biii and Ciii). The proportion of cells within each subtype that showed the depolarising response to local application of ATP was not consistent. Amongst CSFcC 1s, the majority of cells did not show a depolarising response (2 of 28 cells depolarised; 7.14 %) (Figure 3.5 D). Approximately half of CSFcC 2s responded to ATP with a depolarisation (12 of 23 CSFcC 2s; 47.8 %) and the majority of CSFcC type 3s responded to local application of ATP with a depolarising response (9 of 11 CSFcC 3s depolarised; 81.8 %). There was a correlation between the response to ATP (as measured by the amplitude change upon application of ATP) and the CSFcC subtype (Pearson correlation value 0.314, P = 0.005). CSFcC 2s produced significantly larger depolarisations than CSFcC 1s to ATP, however there was no significant difference between the amplitude of depolarisations of CSFcC 3s and those of any either of the other subtypes (Figure 3.5 E). The average size of depolarisation to ATP was 5.30 ± 2.04 mV for CSFcC 1 (n = 2; N = 2), 26.08 ± 9.80 mV for CSFcC 2 (n = 10; N = 10) and 15.81 ± 1.51 mV for CSFcC 3 (n = 2; N = 2). The size of a voltage change is affected by the input resistance of the cell, therefore the data was then analysed as voltage change (mV)/ input resistance (MΩ). The data analysed in this format did not show any significant differences between the depolarisations produced by the CSFcC subtypes (Figure 3.5 F).

Figure 3.5 Subpopulations of all CSFcC subtypes respond to local application of ATP with a depolarisation

Ai, Bi, Ci) Example of whole cell patch clamp recordings of a CSFcC 1 (Ai), CSFcC 2 (Bi) and CSFcC 3 (Ci) depolarising to a 200 ms pressure ejection puff of 300 μM ATP (black bar). Aii, Bii and Cii) corresponding recordings of the cells exemplified in Ai, Bi and Ci of the responses to positive and negative current pulses, indicating CSFcC subtype. Aiii, Biii, Ciii) DIC images of the cells recorded from in i and ii, showing location around the CC. Aiv, Biv and Civ) Rhodamine-excited fluorescent images showing CSFcC processes. D) Proportion of cells in each subtype which responded to 300 μM ATP responding with a depolarisation, with the remainder responding with a hyperpolarisation. E) Comparison of amplitude of depolarising responses between CSFcC subtypes; * = $p < 0.05$. F) Comparison of amplitude by IR between subtypes in mV/MΩ.


CSFcC 1
Ai
-81 mV
2 mV
1 second
ii
2 mV
300 ms
+80 pA
-80 pA
iii
iv
CSFcC 2
Bi
10 mV
1 second
-50 mV
ii
40 mV
300 ms
+30 pA
-30 pA
iii
iv
CSFcC 3
Ci
20 mV
1 second
-31 mV
ii
40 mV
300 ms
+20 pA
-50 pA
iii
iv


D
% of cells displaying each response type
100%
80%
60%
40%
20%
0%
CSFcC 1
CSFcC 2
CSFcC 3
Hyperpolarising response
Depolarising response
E
Amplitude (mV)
35
30
25
20
15
10
5
0
*
CSFcC 1
CSFcC 2
CSFcC 3
F
Amplitude/IR (mV/MΩ)
0.15
0.1
0.05
0
n.s.
CSFcC 1
CSFcC 2
CSFcC 3

3.2.4 Depolarisations to ATP were mediated by P2X receptors

The depolarising response to 300 μM ATP in CSFcCs of all subtypes was partially antagonised by 50 μM suramin from a depolarisation magnitude of 29.95 ± 2.20 mV (n = 31; N = 24) in response to ATP prior to antagonist application, to 10.70 ± 3.18 mV (n = 14; N = 12) depolarisation to ATP in the presence of suramin (Figure 3.6 C). There was a partial return to control levels after antagonist washout, with amplitudes to ATP application increasing to 18.91 ± 4.24 mV (n = 4; N = 4). The amplitude of the depolarising response to ATP with suramin was significantly different from the response sizes before and after suramin application (P = 0.003), whilst there was no significant difference between the amplitudes of response after washout and control values (P = 0.06). This antagonism by suramin was seen in CSFcCs of different subtypes, including CSFcC 1s (Figure 3.6 Ai) which were shown to be CSFcCs by DIC and fluorescent imaging (Figure 3.6 Aii and Aiii, respectively) and spiking CSFcCs (Figure 3.6 B).

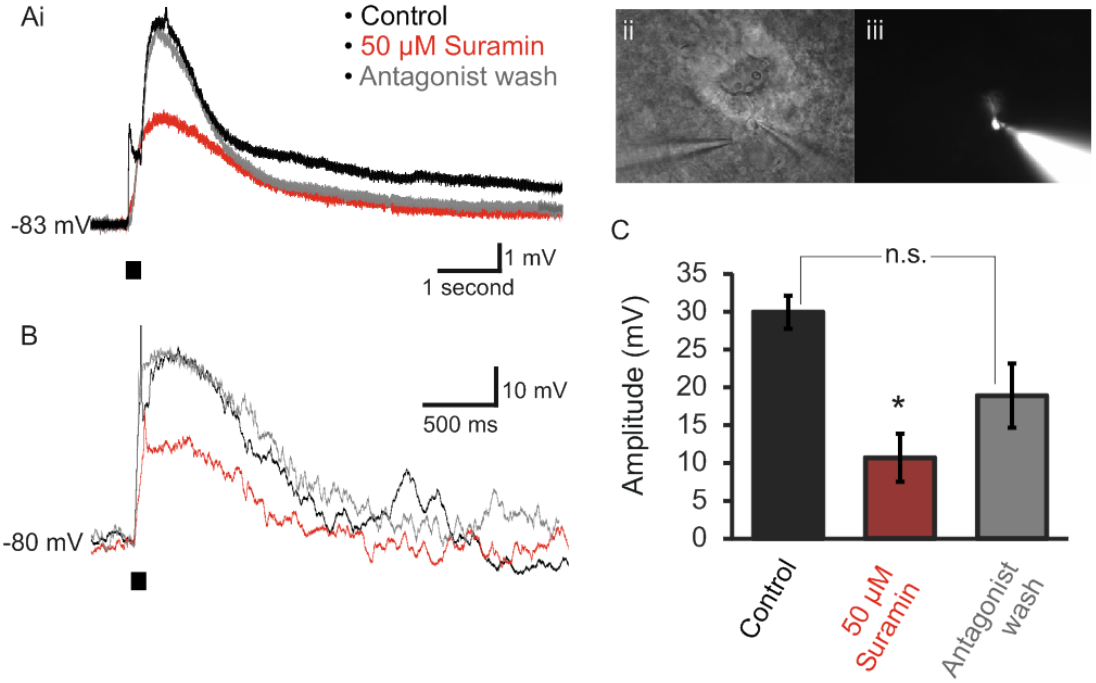


Figure 3.6 CSFcC depolarisations to local application of ATP were inhibited by bath application of suramin

Ai and B) Whole cell patch clamp recording of a CSFcC 1 (A) and a CSFcC 2 (B) depolarisation to 300 µM ATP (black bars), before (black traces), during (red traces), and after (grey traces) application of 50 µM suramin; CSFcC 1 imaged by DIC (ii) and fluorescent imaging (iii) showing process into the central canal. C) Bar chart showing average responses to 300 µM ATP in the presence of 50 µM suramin and after suramin washout. * = $p < 0.05$.

Amplitudes of depolarisations to ATP were measured in CSFcCs at a range of holding membrane potentials to allow reversal potential to be estimated for this response. As the membrane potential was raised manually by applying depolarising current, the amplitude of the response to ATP decreased (Figure 3.7 A). The amplitude of the depolarisation was plotted against the holding membrane potential allowing the reversal potential to be estimated (Figure 3.7 B). The reversal potentials were estimated by extrapolation of the line of best fit producing an estimate of -51.44 ± 4.329 mV (n = 4).

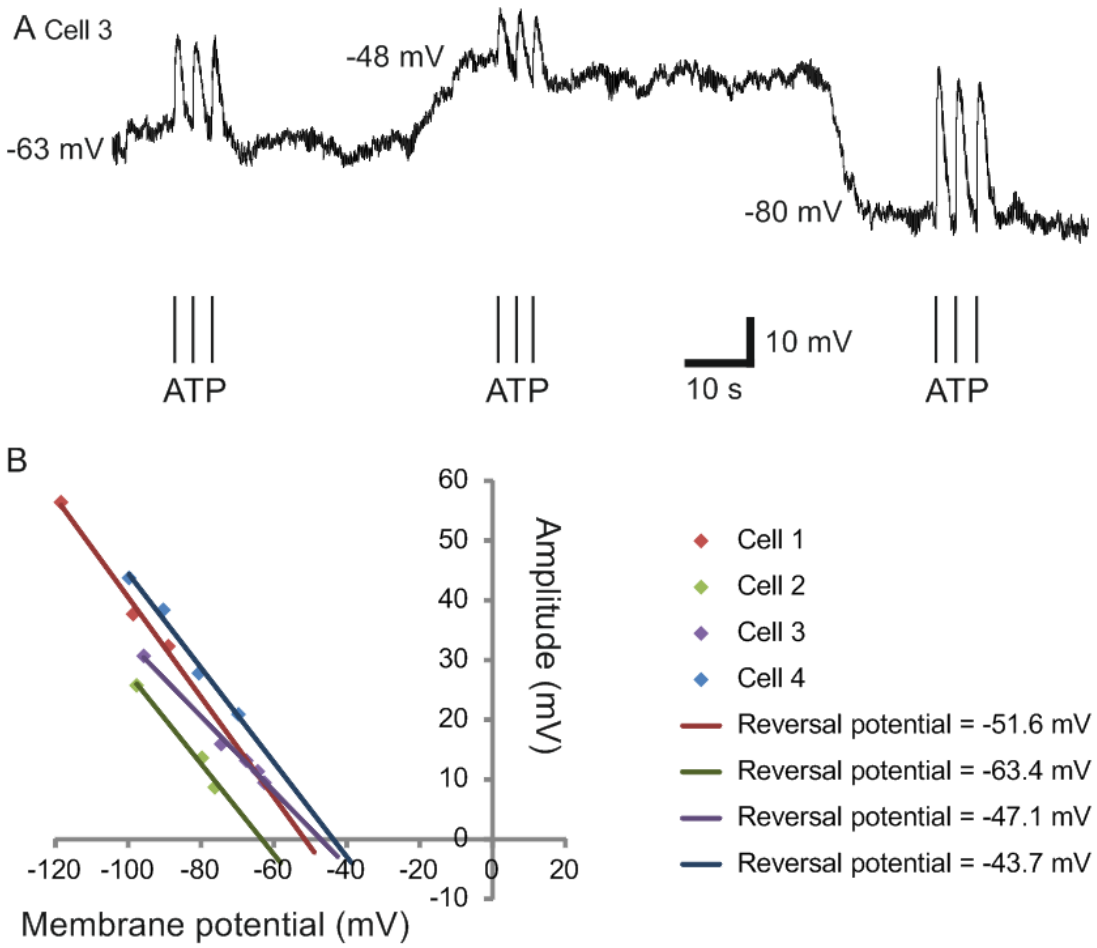


Figure 3.7 Reversal potentials of the depolarising response to ATP in CSFcCs

A) Whole cell patch clamp continuous recording of a CSFcC responding to application of 300 µM ATP (black bars) with depolarisations at 3 membrane potentials (-63 mV, -48 mV and -80 mV); membrane potentials changed manually. B) Lines of best fit on chart of amplitude of depolarisation to ATP against membrane potential extrapolated to estimate reversal potential of response and adjusted for the liquid junction potential.

3.2.5 A population of CSFcCs responded to ATP with a hyperpolarising response

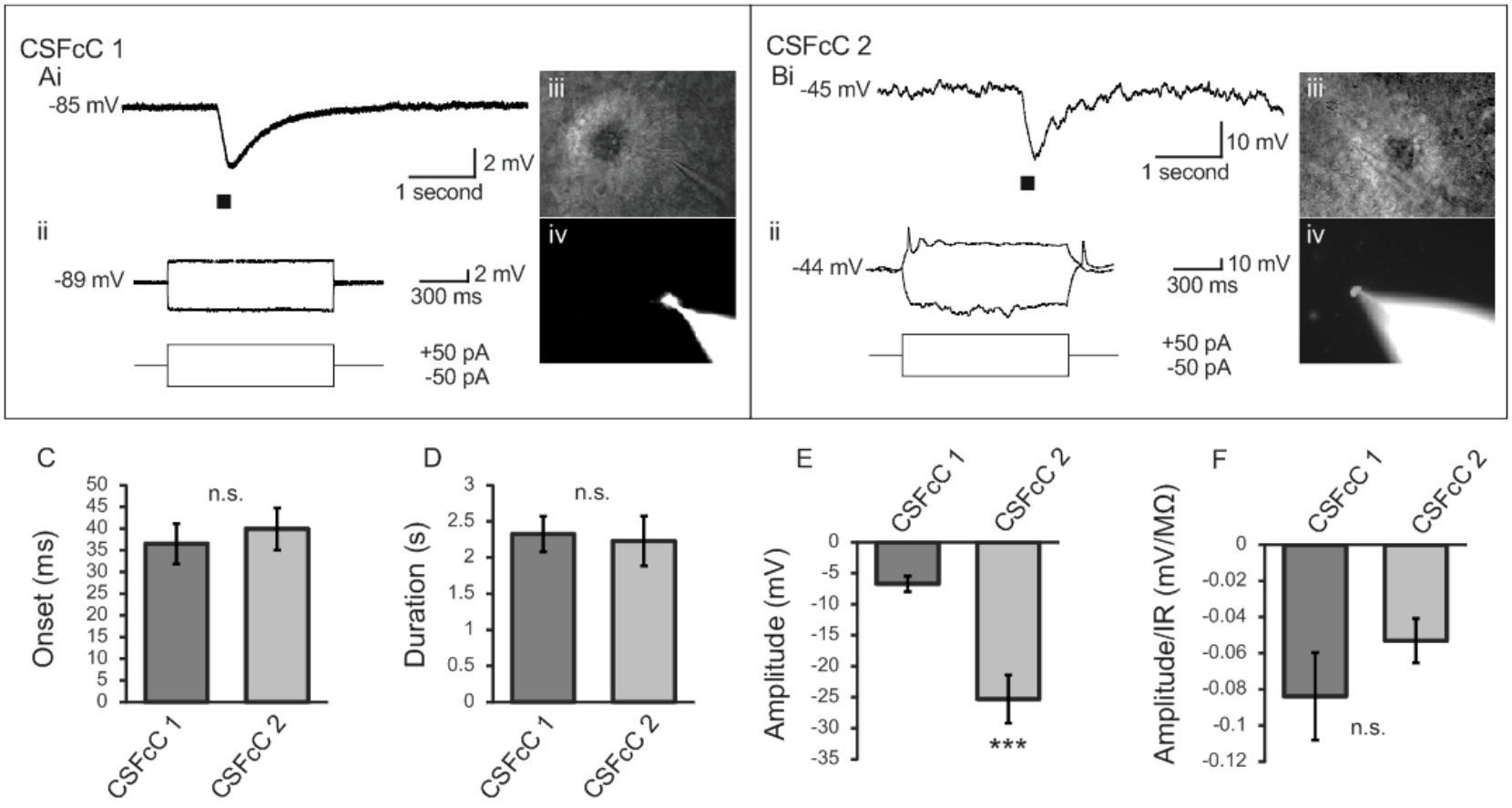
As shown by Figure 3.4 D, a proportion of CSFcC 1s (26 out of 28 cells), CSFcC 2s (11 out of 23 cells) and CSFcC 3s (2 out of 11 cells) showed hyperpolarising responses to local application of ATP. The hyperpolarising response to ATP was seen in both non-spiking (Figure 3.8 A) and spiking CSFcCs (Figure 3.8 B). The hyperpolarising responses in CSFcC 3s were of large amplitude (-26.4 mV and -33.8 mV) similar to those seen in CSFcC 2s, however the responses of CSFcCs are not analysed further as there were only 2 examples recorded with a large difference in

their amplitudes. The data from the responses in CSFcC 1s and CSFcC 2s were analysed to determine if there were any differences in the characteristics of the response between the cell subtypes (n = 22; N = 22 for CSFcC 1 and n = 11; N = 10 for CSFcC 2). The onset of response was not significantly different between the subtypes (36.50 ± 4.64 ms for CSFcC 1 and 39.91 ± 4.85 ms for CSFcC 2; P = 0.475; Figure 3.8 C). The duration of the response was also not significantly different (2.33 ± 0.25 s for CSFcC 1 and 2.23 ± 0.35 s for CSFcC 2; P = 0.827; Figure 3.8 D). When analysed in terms of amplitude of response, there was a significant difference between CSFcC 1 and CSFcC 2 (-6.71 ± 1.26 mV and -25.30 ± 3.90 mV respectively; P < 0.001; Figure 3.8 E). However magnitude of a voltage response is affected by the input resistance of a cell and my data have shown that there is a significant difference in the IRs of CSFcC 1 and CSFcC 2s. Therefore, further analyses were done on the amplitude of change (mV)/ IR (MΩ) and this showed no significant difference between CSFcC 1 and CSFcC 2s (-0.084 ± 0.024 mV/MΩ for CSFcC 1 and -0.053 ± 0.012 mV/MΩ for CSFcC 2, P = 0.593; Figure 3.8 F).

The results were analysed to assess whether there was any correlation between the responses to ATP recorded from different cells but on the same day. The number of days 2 or more CSFcC 2s or 3s were recorded from on the same day was counted and the numbers of days the same or different responses observed were compared to the expected likelihood of receiving those outcomes. The likelihood of getting 2 CSFcC 2s or 3s that produced the same response to ATP was higher than would have been expected if there was no correlation of the response type of cells recorded on the same day (6 days rather than the 3.56 days predicted by chance alone), with fewer than expected with 1 hyperpolarising and 1 depolarising response (1 day compared to the 3.44 days predicted).

Figure 3.8 A subset of CSFcC 1s and CSFcC 2s respond to local application of ATP with a hyperpolarisation

Ai and Bi) Whole cell patch clamp recordings of CSFcC 1 (Ai) and CSFcC 2 (Bi) which hyperpolarised to 300 μM ATP (black bars). Aii and Bii) Voltage responses to negative and positive current pulses indicating CSFcC subtype. Aiii-iv and Biii-iv) DIC and fluorescent images showing the location of the recorded cells and their processes. C-E) Comparison of the hyperpolarisations of CSFcC 1 and CSFcC 2 in terms of onset (C), duration (D), amplitude (E) and amplitude/IR (E). *** = $p < 0.001$; comparisons used Mann-Whitney U test as data not normally distributed


CSFcC 1
Ai
-85 mV
2 mV
1 second
iii
ii
-89 mV
2 mV
300 ms
iv
+50 pA
-50 pA
CSFcC 2
Bi
-45 mV
10 mV
1 second
iii
ii
-44 mV
10 mV
300 ms
iv
+50 pA
-50 pA
C
Onset (ms)
n.s.
CSFcC 1
CSFcC 2
D
Duration (s)
n.s.
CSFcC 1
CSFcC 2
E
Amplitude (mV)
CSFcC 1
CSFcC 2
***
F
Amplitude/IR (mV/MΩ)
CSFcC 1
CSFcC 2
n.s.

.

Unlike the depolarising response to ATP, the hyperpolarising response was not antagonised by 50 μM suramin. This lack of antagonism by suramin was seen in all CSFcC subtypes, including CSFcC 1s (Figure 3.9 Ai) which constitute the majority of the hyperpolarising responses (cell identification demonstrated by the voltage responses to current pulses (Figure 3.9 Aii) and the visualisation of the process (Figure 3.9 Aiii)). There was no significant difference between the amplitudes of the hyperpolarisation to ATP, before, during or post-suramin application (pre-suramin: -4.91 ± 1.29 mV (n = 8; N = 8), with suramin application: -5.64 ± 1.98 mV (n = 8; N = 8), P = 0.459; Figure 3.8 B).

CSFcC 2s produce either depolarising or hyperpolarising responses to ATP application in reasonably similar proportions (Figure 3.5 D), and as such, this is a good population to examine any relationship between cell characteristics and type of response. CSFcC 2s that depolarised to ATP were compared with those that hyperpolarised in terms of RMP and IR (Figure 3.9 C and D respectively). In both of these comparisons, there were no significant differences between the depolarising and hyperpolarising CSFcC 2 groups. The depolarising group had a RMP which was -37.16 ± 4.51 mV (n = 12; N = 11), whilst that for the hyperpolarising group was -34.85 ± 3.75 mV (n = 11; N = 9; P = 0.702). The IR for the depolarising CSFcC 2 was 1169.39 ± 256.18 MΩ (n = 12; N = 11), and 712.13 ± 110.42 MΩ (n = 11; N = 9) for the hyperpolarising group (P = 0.127).

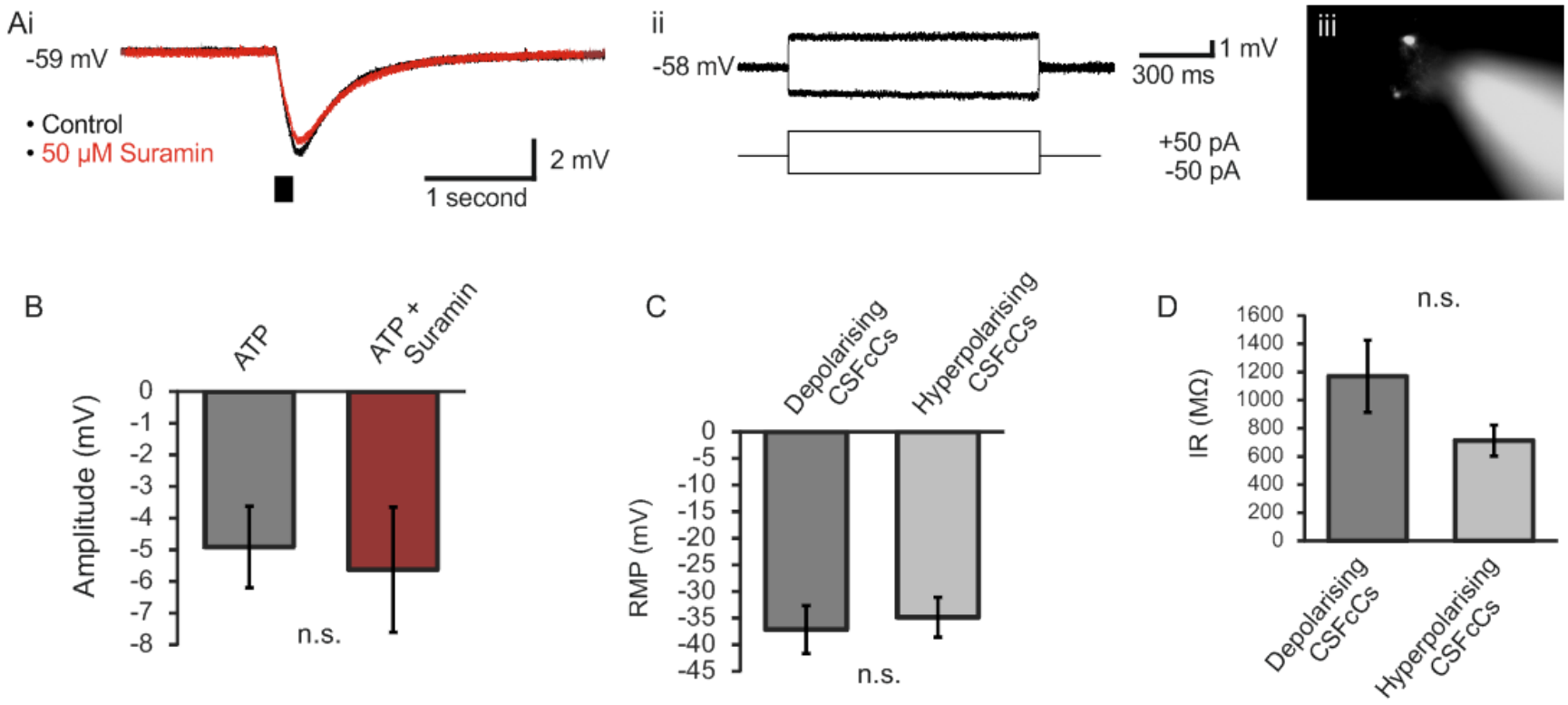


Figure 3.9 The hyperpolarisations of CSFcCs to ATP were not antagonised by suramin

Ai) Hyperpolarisations recorded in whole cell patch clamp recordings in response to application of 300 µM ATP (black bar) before 50 µM suramin application (black trace) and during suramin application (red trace). Aii) Voltage responses to positive and negative current pulses indicated the recorded cell was CSFcC 1. Aiii) Rhodamine-excited fluorescent image shows CSFcC process. B) Amplitude of hyperpolarisations not reduced by application of 50 µM suramin as determined by paired t-test. C-D) Comparison of CSFcC 2 that depolarise to ATP to those that hyperpolarise to ATP in terms of RMP (C), and IR (D) with comparisons by independent-samples t-test.

3.2.6 A population of cells that initially depolarised to ATP, later showed hyperpolarisations.

Similarly to how there were CSFcCs which were observed to be changing from subtype 3 to subtype 2 (Figure 3.2), there were also cells which changed their response type to ATP (n = 5; N = 5). This was sometimes in line with changes in subtype (n = 3; N = 3). For example, one CSFcC changed from a CSFcC 3 which depolarised to local application of ATP (Figure 3.10 Ai), to a CSFcC 2 which hyperpolarised to ATP (Figure 3.10 Aii). These changes were accompanied by a reduction in IR and a decrease in spontaneous spiking. The change that was observed in membrane potential was not necessary for the change in response to ATP. This is demonstrated by an alternative recording which was accompanied by a hyperpolarisation of membrane potential (Figure 3.10 B), and also shows that when the holding membrane potential was raised back up to the starting membrane potential, the response to ATP does not return to the initial depolarising response.

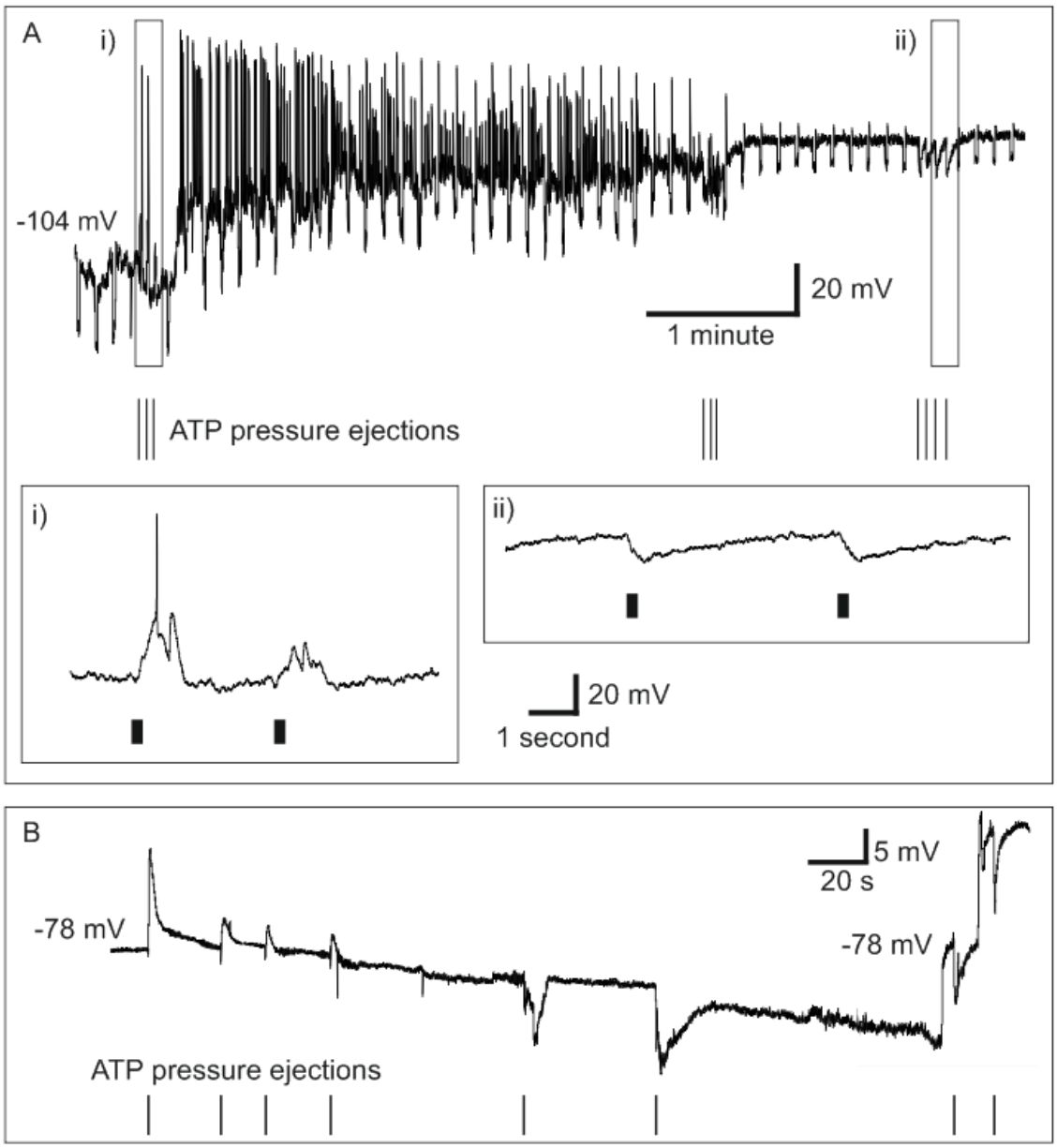


Figure 3.10 Changes in spiking can be associated with a change in response to ATP

A) Whole cell patch clamp continuous recording showing a CSFcC changing both response type to 300 μM ATP (black bars) and subtype phenotype over time with phenotype demonstrated by voltage responses to hyperpolarising current pulses. At Ai) the cell shows a CSFcC 3 phenotype and depolarises to ATP, at Aii) a CSFcC 2 phenotype and robust hyperpolarisations are displayed. B) Whole cell patch clamp recording showing a cell changing from a depolarising to a hyperpolarising response to 300 μM ATP (black bars) accompanied by a hyperpolarisation of membrane potential, which was then raised back to starting membrane potential and higher.

In addition to changes in response type to ATP over a period of minutes, there were also sometimes changes in response over a matter of seconds. These recordings indicated a desensitisation of the depolarising response to application of ATP (Figure 3.11). In some cases this desensitisation reduced the amplitude of the depolarising response to ATP (Figure 3.11 A), whilst in others the depolarising response to ATP gave way to a hyperpolarising response (Figure 3.11 B). Additionally there were examples where there were elements of both a depolarising and hyperpolarising response to ATP, with the hyperpolarisation occurring first (Figure 3.11 C). In these cases the depolarising response reduced in amplitude, as the hyperpolarising response increased. To minimise the impact of this desensitisation on the statistical analyses, measurements were taken from the first response from a group of 3, with an interval of >2 minutes between the sets of ATP pulses as the response size had returned to control levels after this time period without ATP application.

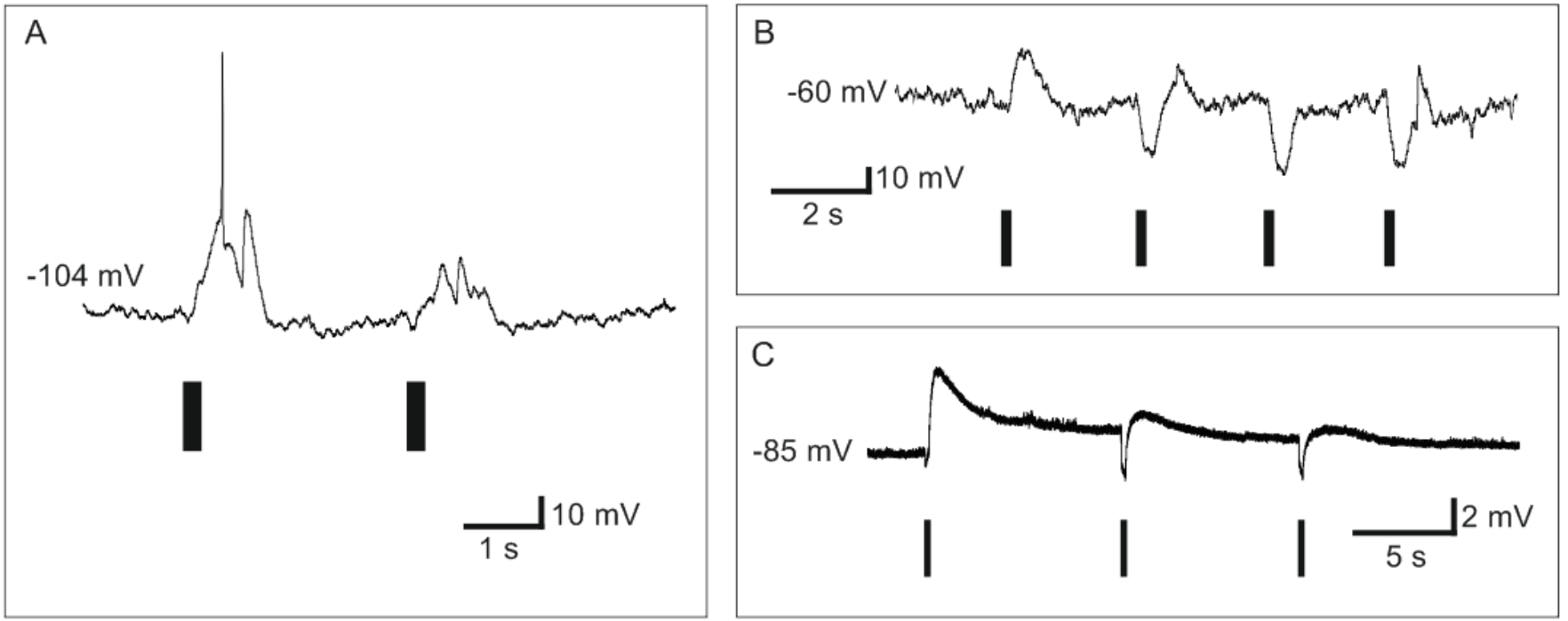


Figure 3.11 Depolarising responses to ATP show desensitisation

A, B and C) Whole cell patch clamp recording of CSFcCs responding to 300 μM ATP (black bars) showing desensitisation of the depolarising response to repeated application of ATP.

3.2.7 $P2X_3$ is involved in the depolarising response to ATP in a subset of CSFcCs

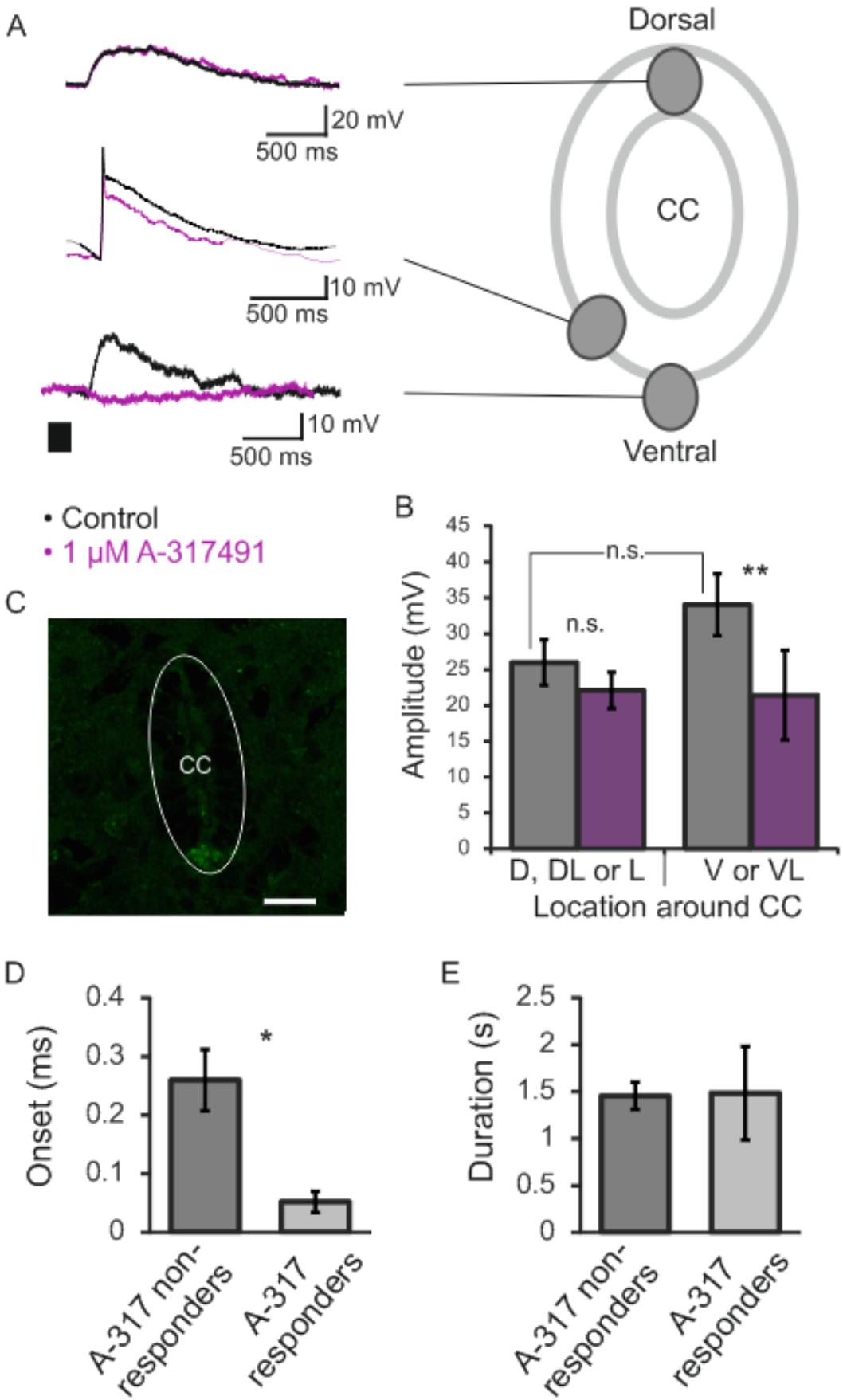
A subset of CSFcCs that responded to application of ATP with a depolarisation showed either reduction in magnitude or complete elimination of the depolarisation with the $P2X_3$/$P2X_{2/3}$ specific antagonist A-317491 (1 μM; Figure 3.12 A). These cells were located predominantly in the ventral aspect of the central canal region. Cells that were located dorsal, dorsolateral or lateral (n = 5; N = 4) were not affected by application of A-317491 (25.96 ± 3.17 mV versus 22.08 ± 2.52 mV with A-317491, P = 0.366, Figure 3.12 B). However cells that were located either ventral or ventrolateral (n = 11; N = 10) had the amplitude of the depolarisation to ATP significantly reduced in the presence of A-317491 (34.02 ± 4.32 mV to 21.42 ± 6.26 mV with A-317491, P = 0.005).

The responses to ATP of the CSFcCs were then divided by presence or absence of $P2X_3$-involvement as assessed by whether or not their response to ATP was antagonised by A-317491. When comparing the responses to ATP which were elicited in part or full by $P2X_3$-containing receptors to those without $P2X_3$ involvement, there was no significant difference between the amplitudes of the depolarisations to ATP prior to antagonist application (P = 0.25). The A-317491 responders (n = 9 of 19) had a significantly shorter onset in their depolarising response to ATP than the A-317491 non-responders (0.052 ± 0.018 ms compared to 0.260 ± 0.052 ms; p = 0.020; Figure 3.12 D). There was, however, no significant difference in the duration of the response to ATP between the A-317491 responders and the A-317491 non-responders (1.48 ± 0.50 s for responders and 1.45 ± 0.14 s for non-responders; p = 0.70; Figure 3.12 E).

The presence of the $P2X_3$ receptor subunit around the ventral extent of the central canal region was demonstrated using immunohistochemistry with an antibody raised against the $P2X_3$ subunit (Figure 3.11 C). The $P2X_3$ subunit appeared not to be expressed strongly in cells, instead with the most intense staining being in small, circular points at the ventral end of the central canal. This staining suggested that the $P2X_3$ was possibly present in fibres sitting between, and immediately ventral of, the cells of the ependymal cell layer and possibly in limited regions of the ventral CSFcCs.

Figure 3.12 Involvement of $P2X_3$ in the depolarising response to ATP

A) Whole cell patch clamp recordings of CSFcCs depolarising to 300 μM ATP (black bar) before (black trace) and during application of 1 μM A-317491 (purple trace) with a graphical representation of the location of each cell around the central canal. B) Comparison of the effects of A-317491 on depolarisation amplitude to ATP with cells group by location around central canal. ** = $p < 0.01$; data assessed by paired or independent-samples t-tests as appropriate. C) Immunohistochemistry for the $P2X_3$ subunit (green) around the CC. Scale bar = 50 μm. Immunohistochemistry completed by Lucy Peers. D and E) Bar charts showing averages of the onset (D) and duration (E) of A-317491 responders and non-responders, * = $p < 0.05$.


A
Dorsal
20 mV
500 ms
CC
10 mV
500 ms
10 mV
500 ms
Ventral
• Control
• 1 μM A-317491
B
Amplitude (mV)
45
40
35
30
25
20
15
10
5
0
n.s.
n.s.
**
D, DL or L
V or VL
Location around CC
C
CC
D
Onset (ms)
0.4
0.3
0.2
0.1
0
*
A-317 non-responders
A-317 responders
E
Duration (s)
2.5
2
1.5
1
0.5
0
A-317 non-responders
A-317 responders

3.3 Discussion

This chapter has characterised the basic characteristics of CSFcCs. These electrophysiological characteristics were then used to subcategorise CSFcCs into subtypes albeit with a continuum between subtypes and occasional changes of subtype-defining characteristics within a recording. The subtypes were defined by the ability of the cells to produce spikes in response to depolarising current pulses, with type 1 unable to produce spikes, type 2 able to produce a single spike and type 3 producing a train of spikes. CSFcCs were shown to respond to locally applied ATP with either a depolarisation or a hyperpolarisation. There was involvement of $P2X_3$ receptors in a spatially-dependent subset of depolarising responses to ATP.

3.3.1 CSFcC subtypes may represent temporary sub-states

The differences observed in electrophysiological characteristics of CSFcCs cover a considerable range. This range is continuous between the spiking behaviours, with no discrete boundaries between electrophysiological profiles of CSFcC 2 and CSFcC 3s. All cells that could be confirmed to be CSFcC by visualisation of the process were assigned a subtype, however there was no definite boundary about what constituted a spike and particularly, or what constituted multiple spikes after the first as there are often membrane potential oscillations. The assessment was done on what behaviour the cell typically showed to a +50 pA current pulse with spikes taken to be depolarisations with a rapid repolarisation. Previously, CSFcC type 2 and 3 have been described (Marichal et al., 2009) with some research suggesting non-spiking CSFcCs are a different classification of cells.

The distribution of the input resistances of CSFcC 1s suggests that there may be a long-term difference between subgroups of CSFcC (subtypes rather than sub-states). CSFcC 2 and 3s have a typical right skewed distribution, which would be what would be expected of a population with a mean relatively close to zero, a large standard deviation and no negative values. However the histogram for CSFcC 1s less clearly resembles this distribution and looks to be bimodal. This is supported by previous data which shows that one subpopulation of CSFcC 1s, but none of the spiking CSFcCs, have gap junction connections similarly to ependymal cells and therefore would be likely to have a much lower IR (Corns, 2012). As gap junctions are unlikely to change readily, this fits with the fact that the changes in subtype observed were

from a CSFcC 3 profile with high levels of spontaneous and evoked activity, to a CSFcC 2 profile with far lower frequency and amplitude of spiking, but did not change to a CSFcC 1 profile. Additionally to this, the different electrophysiological phenotypes have been shown to correlate with cell markers in turtle spinal cord (Russo et al., 2004). Cells which passively responded to current pulses and correlated with CSFcC 1s, expressed S100, as did cells with delayed rectifying currents, however spiking cells did not express S100, but did express HuC/D.

Despite there being many similarities in the CSFcC subtype data between that found in this study and previous research, there were differences in the proportions of each CSFcC subtype recorded. Previous research observed a far higher proportion of CSFcCs recorded to be CSFcC 3s, 27 % (Corns et al., 2015) compared to 13.6 % in this study. As the spatial distribution of CSFcC 3s was not equal around the central canal, this difference in the observed proportion of CSFcC subtypes could have been due to differences in the rate at which the different locations around the central canal were targeted for cell recording. It has been reported that PKD2L1-positive CSFcCs are derived from 2 distinct developmental locations, with one smaller population derived from the ventral pole of the neural tube, which produced the majority of the ventral CSFcCs, and the other the larger population originated from the late-P2 and pOL domains, being more widely spread throughout the central canal region (Petracca et al., 2016). Petracca et al. (2016) also observed spatial differences in the spiking behaviours however this was opposite to what was observed presented here. They reported that cells derived from the floor plate sat ventrally and showed single spiking profiles, while cells from the late-P2 and pOL domains were found laterally and the majority could produce trains of spikes, whereas our findings show a greater proportion of multiple spiking CSFcC 3s ventrally. There are a number of explanations for these differences. Firstly Petracca et al. (2016) used considerably younger mice (P1-2) compared to the P11 rats used here. Another difference could be the population of CSFcCs examined, as their pool was limited to those expressing PKD2L1.

There is very little synchrony in central canal literature as to the cell types found with almost no consensus on names for cells. One possibility is that CSFcC 1s represent 2 distinct groups of cells as differentiated by the presence and absence of gap junction coupling, which therefore caused differences in input resistance. It could be that the low IR CSFcC 1s represent the glial fibrillary acidic protein (GFAP) positive

central canal-contacting astrocytes identified by Alfaro-Cervello et al. (2012). They used electron microscopy to identify central canal contacting astrocytes with a process through the ependymal cell layer with a single primary cilium, while central canal contacting neurons (which correspond to CSFcCs) had a process that ended with a mitochondria-rich globular expansion, with multiple other ultrastructural differences.

Connexin gap junctions have been observed between astrocytic processes and ependymal cells and also between ependymal cells and unidentified inter-digitating processes (Ochalski et al., 1996). Whist zonulae adherens have been observed between CSFcCs and ependymal cells, no gap junctions have been observed in electromagnetic imaging studies (Stoeckel et al., 2003). The fact gap junctions have been observed between cells identified as CSFcCs both in this study and by previous work in our lab (Corns, 2012) suggests that these cells may not be the same as the CSF-contacting neurons identified in other studies.

The fact that hyperpolarising and depolarising responses are seen in both non spiking and spiking CSFcCs, although to differing degrees, would suggest that although there may be considerable differences between the cells sampled, these differences don't align with a split in cells identified as CSFcC 1 and with a homogenous sample of spiking CSFcCs. It is however possible that within a population of CSFcC-identified cells with a range of responses, there is a population of astrocytes that happen to respond in the same manner as one subpopulation of CSFcCs. Whilst there was no significant difference between the IRs of CSFcC 1s located dorsal or dorsolateral (D/DL) and those located ventral or ventrolateral (V/VL) the former group had 71% with IR in the first peak (<100 MΩ) whilst the second group had only 33%. This has significance because astrocytes have been reported predominantly dorsal to the central canal (Sabourin et al., 2009) and proliferation also occurs predominantly in the dorsal region (Hamilton et al., 2009).

Marichal et al. (2009) did not observe CSFcCs with no spontaneous or evoked activity in turtle spinal cord. Furthermore all GFP-positive cells in a $Gata2^{GFP}$ mouse model produced either a train of spikes or a single spike (Petracca et al., 2016). The transcription factor Gata2 colocalised with PKD2L1, which is known to be expressed in CSFcCs (Djenoune et al., 2014), does not appear to be a transcription factor in CSFcC 1s. Therefore, it could be that all CSFcC 1s are actually GFAP-positive

astrocytes, with separate populations of gap-junction coupled and non-gap junction coupled astrocytes. However the fact that 2 CSFcC 1s were observed to respond to ATP with a depolarisation, similarly to CSFcC 2 and 3 would require these astrocytes to express the same purinergic receptors. Whilst similarity of purinergic receptor expression makes the possibility of all CSFcC 1s being central canal-contacting astrocytes less likely, it has been reported that ATP can cause proliferation and gliosis in cultured astrocytes via $P2X_2$ receptors (Neary et al., 2003) suggesting the presence of $P2X_2$ receptors on astrocytes could occur in some cases. Immunohistochemistry for GFAP on a slice with a Neurobiotin filled-CSFcC 1 could check for co-localisation to confirm this possibility. This could provide greater insight into the characteristics and identity of CSFcC 1s, particularly those with gap junction coupling.

There is also debate as to whether the astrocytes in this area are capable of stem cell behaviour and the extent of any contribution to the increase in proliferation seen with spinal cord injury (Kriegstein and Alvarez-Buylla, 2009; Moore and Oglesbee, 2015; Zhou et al., 2015). This is largely due to the fact that astrocytes are regularly identified by the expression of GFAP or the intermediate filament protein, vimentin. However subpopulations of ependymal cells express one or both of these molecules. GFAP and vimentin positive cells have been identified in the central canal region as having multi-potency (Moore and Oglesbee, 2015) whilst others report the stem cells to be GFAP negative (Meletis et al., 2008), and some report some level of stem activity in both populations (Sabourin et al., 2009). Further evidence suggests that the GFAP positive cells are the stem cells as GFAP has been shown to co-localised with the carbohydrate adhesion molecule (CD15) and brain lipid-binding protein (BLBP) (Sabourin et al., 2009), which are both stem cell markers (Keilani and Sugaya, 2008; Abranches et al., 2009; Lukenda et al., 2016). In humans, CD15 staining has been shown to be present in ependymal cells except for those at the ventral aspect of the central canal (Dromard et al., 2008). BLBP is, however, only expressed in a subset of ependymal cells (Sabourin et al., 2009). Additionally, it seems unlikely that astrocytes play a key stem cell role in the central canal region, since while astrocytes have been positively reported in this area, they have been observed not to be proliferating under normal circumstances (Alfaro-Cervello et al., 2012).

It may be that some of the cells represented here as CSFcCs are in fact astrocytes, as suggested by the similarities in observed morphology (Figure 3.12), however the

current research would suggest that they are not the main stem cell in this area. The techniques utilised here do not always allow for conclusive identification as the most significant differences are ultrastructural (Alfaro-Cervello et al., 2012). As the responses to purinergic signalling presented here do not show a pattern matching to the bimodal pattern seen in gap junction coupling or CSFcC subtype, these hyperpolarising and depolarising response groups have not been split according to any of these other factors.

3.3.2 Understanding the different responses within the CSFcC population

Even aside for the possible inclusion of astrocytes within the CSFcC-identified population, there is a range of responses produced to local application of 300 μM ATP. There are several possibilities as to the cause of these different responses.

There could be differences in the cellular mechanisms or molecular expression profile of the cells. There is a correlation between the response type to ATP application and the spiking behaviour suggesting that some cellular differences that cause the differential spiking behaviour could be linked to the different ATP responses. However, there were CSFcC 1s that depolarised to ATP despite showing no spiking activity, meaning that the ATP-response differences cannot be solely due to the same molecular or expression differences that cause the spiking behaviour in some CSFcCs. There could be an impact of differential expression of purinergic receptors, as whilst $P2X_2$ has been shown to be expressed in CSFcCs, there was a subpopulation which did not show $P2X_2$ immunofluorescence (Stoeckel et al., 2003). It is therefore possible that the hyperpolarising responses come from this $P2X_2$-negative subpopulation. However, there was a correlation between the ATP response and CSFcC subtype and both of these were observed changing, sometimes in parallel.

The cause of the expression differences of purinergic receptors (Stoeckel et al., 2003) and channels involved in spiking is not known. It has been shown that the likelihood of cells producing spikes or multiple spikes is not correlated with the age of the cell, with CSFcCs born at E14.5 (Marichal et al., 2009; Petracca et al., 2016). An alternative could be differences in the age of the animals, perhaps with hyperpolarising responses being immature responses; however both depolarising and hyperpolarising responses were recorded from animals ages P7 to P13. This

covers the extent of the age range used for these experiments indicating that neither response type was restricted to either extent of the age range.

There could have instead been a difference in the experimental set up that caused the differences in response type. This could have been involvement of factors such as the pH of the aCSF, the pH of the intracellular solution, the temperature of the recordings, the position of the electrode or the flow rate. With most of these factors we would expect some correlation between recordings made on the same day. When analysis was done to investigate if the responses recorded from different cells on the same day were independent of each other, the data showed that there was a higher chance of observing 2 CSFcC 2s or 3s producing the same response on the same day than would be expected by chance alone. This would suggest that there may be a factor which increases the chance of recording the same response in a day, but as different responses were recorded in a day, it is not absolute. In one case, the first cell of the day depolarised to ATP, the next hyperpolarised and the third depolarised, whilst other cells changed their response to ATP within the course of a recording. Additionally, desensitisation was observed frequently within the recordings, in some cases changing the response type from depolarisation to hyperpolarisation. This desensitisation could occur via different cellular mechanisms than the longer time-period changes. However, the same factor that causes the desensitisation could also be involved in the longer term changes and also the initial response type. A possible factor in this situation could be ion balance; however the intracellular ion concentrations should have been set by their concentration in the intracellular solution.

One factor of experimental set up that differed between cells is the relative location of the puff electrode with respect to the cell being recorded from, as both the distance between release of drugs and the cell and the composition of the tissue in between the two is likely to affect the distribution of the drug. This is unlikely to affect the type of response but is likely to affect the time course and concentration of drug delivery. The distance between the cell body and puff electrode was kept consistent throughout the electrophysiological experiments to minimise the differences in drug delivery, however proximity of processes and composition of tissue in between could not be controlled and may contribute to some of the differences in response size and shape to agonist application.

Another factor that could be involved in differences in the responses of CSFcCs to ATP could be changes in permeability of the channel to those ions. Whilst permeability of an ion channel protein to its namesake ion is a typically stable property of membrane proteins, $P2X_2$ is thought to initially open a small pore which only allows small ions such as Na^+, K^+ and Ca^{2+} with prolonged activation further dilating the pore (Li et al., 2015). The larger pore allows through larger cations such as $NMDG^+$ and Yo-Pro (Virginio et al., 1999). This is a feature that only occurs with high expression levels of $P2X_2$ (Fujiwara and Kubo, 2004; Li et al., 2015) so could have differential levels of effects in cells with different $P2X_2$ expression levels. Rate of dilation is dependent on the time and concentration of ATP application (Virginio et al., 1999), which is not easy to assess with local puff application as the concentration is not constant, but with the concentration threshold for induction of permeability increases being 10-20 μM (Virginio et al., 1999), it is likely that the puff applications passed that threshold, particularly as there may be some baseline level of extracellular ATP due to the damage caused to the slice during the sectioning. This however is unlikely to account for the hyperpolarisations seen in CSFcCs as P2X receptors are non-selective cation channels and due to the hyperpolarised membrane potential of the cells, the net flow of cations, of any size is from outside to inside, acting to depolarise the cell.

This damage to cells on the top and bottom of the slices is likely to have caused release of multiple factors of damage (Paemeleire and Leybaert, 2000; Lahne and Gale, 2010). While these factors released may have been washed away by the constant flow of aCSF through the bath in which the slices are held, there may be some remaining and perhaps depth into the slice or proximity of the cell to the top or bottom of the slice could affect the presence of these factors. In addition to ATP, these damage-related factors present in the spinal cord slices may include growth factors (PDGF, VEGF) (Khallaf et al., 2016) and cytokines (IL-1β, IL-6) (Coronel et al., 2016) which are also released at higher concentrations after spinal cord injury. These could have caused changes to the CSFcCs, potentially in receptor expression. Variations in levels of these factors could cause variation in CSFcCs which might account for the variation in the responses of CSFcCs to ATP. However the link between response type to ATP and CSFcC subtype mean that these changes in response to spinal cord injury factors would have to be very broad, making this explanation less likely.

Another possible characteristic of $P2X_2$ receptors which may be involved in the responses of the ependymal cells to extracellular ATP is the coupling of $P2X_2$ receptors and $GABA_A$ receptors (Boué-Grabot et al., 2004b). This coupling leads to cross-inhibition dependent on $GABA_A$ subunit composition (Boué-Grabot et al., 2004a). There is also inhibitory cross-talk and physical clustering of 5-HT_3 and $P2X_2$ receptors (Boué-Grabot et al., 2003) and of 5-HT_3 and $P2X_2$ and/or $P2X_3$ receptors in pelvic afferent neurons (Ma et al., 2006). These indicate that $P2X_2$ receptors can interact with multiple other neurotransmitter-gated channels, producing current modulation and plasticity that could be involved in the range of responses seen in CSFcCs in response to local application of ATP.

3.3.4 The role of $P2X_3$ subunits in the depolarising response to ATP

The selective involvement of $P2X_3$-containing receptors is of significance because these receptors have different characteristics compared to $P2X_2$-containing receptors. It is not known if the $P2X_3$ containing receptors were in the form of homomeric or heteromeric assemblies, so the implications of both will be considered. The homomeric $P2X_3$ receptor has a far more rapid desensitisation than the $P2X_2$ receptor (North, 2002). The desensitisation of the response of $P2X_3$–receptors is seen predominantly at higher concentrations of ATP, whilst at lower concentrations of ATP (<300 nM), the response is sustained for much longer. As mentioned previously, it is not possible to know exactly what concentration of ATP is in the extracellular space around the cell with puff application of ATP, but as we are applying 300 μM ATP, it is likely that the concentration around the cell at peak concentration is well above 300 nM. $P2X_2$ receptors do exhibit calcium-independent desensitisation however this desensitisation opposes a dilation of the pore, allowing larger cations to flow through, meaning desensitisation is not necessarily observable (Khadra et al., 2012). This fits with the fact that the majority of cells did not show desensitisations in the depolarisations in response to ATP in this study.

Once the $P2X_3$ receptor has desensitised, its recovery to a closed state takes a long time, with gaps of 15 minutes necessary to produce similar, non-desensitised responses, however high calcium can reduce this time considerably (Cook et al., 1998). The heteromeric $P2X_{2/3}$ receptors have desensitisation properties similar to homomeric $P2X_2$ receptors (North, 2002). As the duration of response to ATP in cells that had showed a reduction in amplitude of response to ATP with A-317491 was not

different from those unaffected by A-317491 application, this would suggest that either the desensitisation was not taking effect until after the ATP had diffused away or that the $P2X_3$ receptors exist in $P2X_{2/3}$ heteromers.

$P2X_2$ and $P2X_3$ receptors and the heteromeric $P2X_{2/3}$ receptor are permeable to calcium, although $P2X_2$ receptors are more permeable than both $P2X_3$-containing receptors (Virginio et al., 1998). $P2X_2$ receptors are also known to be strongly inhibited as the levels of external calcium approach 10 mM (Evans et al., 1996). Homomeric $P2X_3$ receptors were relatively insensitive to this calcium-induced ATP response blockade, with $P2X_{2/3}$, displaying an intermediary profile with a slight blockade at very high extracellular calcium levels (>20 mM Ca^{2+}; (Virginio et al., 1998)). As the extracellular calcium concentration in this study was 2 mM (North, 2002), there is unlikely to be a significant effect of this on our data, however it could provide some insight on the differential functioning of the CSFcCs with different purinergic receptor subtypes in physiological situations.

These spatial differences in the receptor type and involved in producing the response to ATP could have implications in spinal cord injury. $P2X_3$-containing receptors have been implicated in multiple forms of pain (de Oliveira et al., 2010; Hansen et al., 2012; Xu et al., 2012; Li et al., 2013). One difference between $P2X_2$ receptors and $P2X_3$-containing receptors is the differential response to calcium. Whist $P2X_2$ receptors are inhibited by high levels of external calcium (Evans et al., 1996), whilst $P2X_3$-containing receptors have their desensitisation of response attenuated by increased calcium (Cook et al., 1998). This means that the functioning of these receptors will alter over time after spinal cord injury as the levels of extracellular calcium vary. Initially, extracellular calcium levels fall, the levels then rose such that by after 2 hours calcium levels had risen significantly higher than sham-operated control levels and also significantly higher than neighbouring spinal cord segments (Moriya et al., 1994). The implications of this are that the $P2X_2$ receptors have a larger response immediately after spinal cord injury, then during the following couple of hours $P2X_3$ receptors have a less desensitised response to the still increased levels of extracellular ATP (Wang et al., 2004).

Unlike staining observed with $P2X_2$ antibodies (Studeny et al., 2005), the $P2X_3$ expression does not appear to be intense within the CSFcCs themselves, rather in fibres running up rostro-caudally along the ventral aspect of the ependymal region.

These fibres are contacted by processes from the cells in the ependymal layer (Bruni and Reddy, 1987). It could be that these processes come from CSFcCs and are the means by which receptors on the fibres are able to cause a response within the CSFcCs. Many of the fibres in this area are axons of autonomic visceral afferents (Morgan et al., 1981; Neuhuber, 1982) and observed penetrating between the ependymal cells (Neuhuber, 1982). Significantly, synapses have been reported on these longitudinally-running sympathetic afferents at the ventral end of the central canal (Neuhuber, 1982). Fibres in this location have also express substance P (Gibson et al., 1981; Probert and Hanley, 1987). The presence of Substance P degrading enzyme in ependymal cells (Probert and Hanley, 1987), suggests that these fibres may interact with ependymal cells as well as CSFcCs. It is also worth noting that $P2X_2$ staining has also been observed in transversely sectioned axons, predominantly at the ventral end but also at the dorsal end of the central canal (Stoeckel et al., 2003). However the onset for responses in CSFcCs sensitive to A-317 (and therefore with $P2X_3$ involvement) had a quicker onset than those that were non-sensitive to A-317491, therefore making it less likely for the response to be mediated by multiple steps. This indicates that there may be faint $P2X_3$ staining on the CSFcCs made hard to distinguish by that in the fibres but nevertheless, the receptors in the fibres will respond to the ATP applied and may well have secondary effects on the ependymal region.

3.3.5 Conclusions

There is considerable diversity in the responses of the CSFcCs to ATP, as well as in the CSFcCs themselves. CSFcCs can be differentiated by their level of spontaneous and evoked activity, with CSFcC 1s not showing any spontaneous or evoked activity in response to depolarising current pulses, and CSFcC 2s and 3s showing a single spike and a train of spikes respectively, with a continuum of responses between the two. The CSFcCs could also be divided by the presence or absence of gap-junction coupling. Whilst the majority, including all the spiking CSFcCs had no gap-junction coupling, a subset of CSFcC 1s displayed gap-junction coupling to the surrounding CSFcCs, as shown by lower input resistances and dye-coupling due to movement of Neurobiotin through the gap junctions. The third variation in the CSFcC population was their response to ATP. A proportion of predominantly spiking CSFcCs showed a suramin-sensitive depolarisation to ATP, whilst the remaining CSFcCs produced a

suramin-insensitive hyperpolarisation. Of the depolarising responses, the majority of these were likely to have been mediated by the $P2X_2$ receptors that have been shown to be strongly expressed in CSFcCs (Marichal et al., 2009). In a ventral subset of CSFcCs, the depolarising responses to ATP were sensitive to the $P2X_{3,\ 2/3}$-specific antagonist A-317491 indicating $P2X_3$ involvement in these responses. The fact that the depolarising responses with $P2X_2$ and $P2X_3$ involvement but not the hyperpolarisations were sensitive to suramin will allow further investigations using suramin as the antagonist to look at the effect of the depolarising responses in CSFcCs. The range of CSFcC responses to ATP and the variety of receptors involved in mediating these responses suggest that it is likely that spinal cord CSFcCs are involved in a range of functions related to ATP signalling.

Chapter 4 - Purinergic activation produces hyperpolarisations in ependymal cells

4.1 Introduction

While originally proposed as passive cuboidal ependymal cells (Bjugn et al., 1988), ependymal cells have been shown to respond to a number of neurotransmitters, and in some of these cases, similarly to some CSFcCs. Ependymal cells respond to GABA predominantly via $GABA_A$ receptors (Corns et al., 2013), which may be released from GABAergic terminals on varicosities near the central canal (Magoul et al., 1987). Ependymal cells also depolarise in response to acetylcholine, with a receptor composition that is similar to that seen in the non-spiking CSFcC 1s (Corns et al., 2015). Both these cell types, unlike spiking CSFcC 2 and 3s, have depolarisations that are mediated by α7-containing nicotinic ACh receptors (α7*nAChrs) and non-α7*nAChrs in similar proportions, while CSFcC 2s and 3s have only a very small or negligible proportion of their responses to ACh mediated by α7*nAChrs. This indicates that there are some aspects in which ependymal cells behave similarly to CSFcCs, particularly CSFcC 1s.

Ependymal cells differ from CSFcCs in the presence and extent of gap junction coupling in ependymal cells (Ochalski et al., 1996; Corns et al., 2013; Rodriguez-Jimenez et al., 2016) as demonstrated by the presence of lack of dye-coupling seen with cells filled with Neurobiotin during patch clamp recordings (in this work and previously (Corns et al., 2015). Whilst a subset of CSFcCs have been observed to show gap junction coupling (Corns, 2012), this was only present in a small number and was not observed in any of the spiking CSFcCs. In contrast all ependymal cells show gap junction coupling. Gap junctions can be modulated by a number of neurotransmitters in other cell types such as retinal ganglion cells where permeability modulation occurs via dopamine signalling (Bu et al., 2014), and in photoreceptors, where levels of phosphorylation are modulated by adenosine (Li et al., 2014). Within the spinal cord, ependymal cells have downregulated connexin-50 (Cx-50) after severe contusion spinal cord injury in rats as shown by western blotting of epSPCis compared to epSPCs (Rodriguez-Jimenez et al., 2015). This downregulation of the Cx-50 resulted in a change in the fate of the newly-proliferated cells after spinal cord injury away from a glial fate. This was shown by upregulation of Cx-50 which resulted in more GFAP+ cells (an astrocytic marker) and few Tuj1+ cells (a neuronal marker)(Rodriguez-Jimenez et al., 2015). This means that ependymal cells modulate their expression of ion channels, in addition to a range of other proteins such as Sox2

(Lee et al., 2013), in response to detection of damage. It is possible that the raised levels of ATP after spinal cord injury are part of the signals that trigger this upregulation and therefore that purinergic signalling may affect the gap junctions present in the ependymal cells.

$P2X_2$ staining has been hypothesised to be expressed in a subpopulation of ependymal cells based on the expression of $P2X_2$ around the central canal (Studeny et al., 2005), however other studies show co-localisation of $P2X_2$ with known markers for CSFcCs (Stoeckel et al., 2003). $P2X_7$ has been shown to be expressed in ependymal cells lining the ventricles (Yu et al., 2008) and around the central canal in the spinal cord (Genzen et al., 2009; Marichal et al., 2016), with $P2X_7$ functionally active as shown by electrophysiological responses to the agonist BzATP, which is far more potent at $P2X_7$ receptors than ATP. The responses caused by $P2X_7$ receptors can be differentiated from responses caused by other P2X receptors by the fact that $P2X_7$ receptors have low affinity towards ATP (EC_{50} > 100 μM) (Surprenant et al., 1996). This was demonstrated in puff application experiments with ependymal cells around the central canal by Marichal et al. (2016) who saw no current when 50 ms of 1 μM ATP was applied and almost none when 500 ms was applied, only seeing a significant response when 1 s of 1 μM ATP was applied. Whilst it is known that ATP levels are raised after spinal cord injury (Wang et al., 2004), the concentrations of ATP in different regions of the spinal cord and at different time points after injury are not known. Therefore it is important to understand what the responses of the cells in the central canal area are to lower levels of extracellular ATP, as these levels are likely to occur at a larger distance from the injury and be sustained for longer than the high levels of ATP necessary to activate $P2X_7$ receptors.

4.1.1 Hypothesis

The hypotheses of this chapter are the ependymal cells around the central canal of the mammalian spinal cord:

1. Have passive electrophysiological profiles which do not distinguish them into subcategories
2. Do not respond acutely to local application of ATP via $P2X_2$ receptors.

4.1.2 Aims

The aims of this chapter were to utilise patch clamp electrophysiology and intracellular dye loading to investigate:

1. The differences and similarities between ependymal cells and CSFcCs around the central canal
2. The presence of any effect of local application of ATP on the ependymal cells
3. The mechanisms involved in any response to ATP seen
4. The effect of local application of other nucleotides.

4.2 Results

Data for this chapter came from experiments using P7-13 Wistar rats.

4.2.1 Ependymal cells show passive electrophysiological characteristics

Electrophysiological characterisation showed that ependymal cells show passive recordings, where the cells do not show any spiking activity. This was shown by a complete absence of spikes or action potentials in response to depolarising current pulses (Figure 4.1 Ai). In terms of visualisation, ependymal cells were positioned within the ependymal cell layer, with a less distinct cell outline when viewed with irDIC microscopy (Figure 4.1 Aii), particularly when compared to CSFcCs or neurones. At post-recording visualisation via rhodamine-enabled fluorescence, ependymal cells are small and display no apical process into the central canal (Figure 4.1 Aiii). The cilia are not visible on any visualisation methods used in this study. Some cells with positioning within the ependymal cell layer and ependymal cell-like electrophysiological properties display a single thin basal process. These cells have been described as tanycytes (Rafols and Goshgarian, 1985; Meletis et al., 2008) and are detailed in the section 4.2.4. Due to similarities in responses shown in section 4.2.4, tanycytes will be treated as a subset of ependymal cells and included in ependymal cell data unless otherwise specified. Ependymal cells had a hyperpolarised resting membrane potentials (RMP = -75.38 ± 6.23 mV), which was significantly more negative than CSFcC 2s ($P < 0.001$) and CSFcC 3s ($P = 0.019$), but not significantly different from CSFcC 1s ($P = 0.121$) (Figure 4.1 B). Ependymal cells had a low input resistance (IR = 74.11 ± 17.24 MΩ), which was significantly

lower than that of both CSFcC 2s and CSFcC 3s (P < 0.001 and P = 0.001 respectively), however not significantly different from CSFcC 1s (P = 1.000; Figure 4.1 C). These low input resistance values are likely to be due to the high levels of gap junction coupling, as indicated by the post-fixation DAB visualisation of dye-coupled cells (Figure 4.1 Aiv). Ependymal cells do not show a significantly different IR to CSFcC 1s, likely due to a subpopulation of the latter also show gap junction coupling. Due to the similarities of this passive electrophysiological profile to that of CSFcC 1s the visualisations were used for cell identification, with an absence of visible processes used to identify ependymal cells.

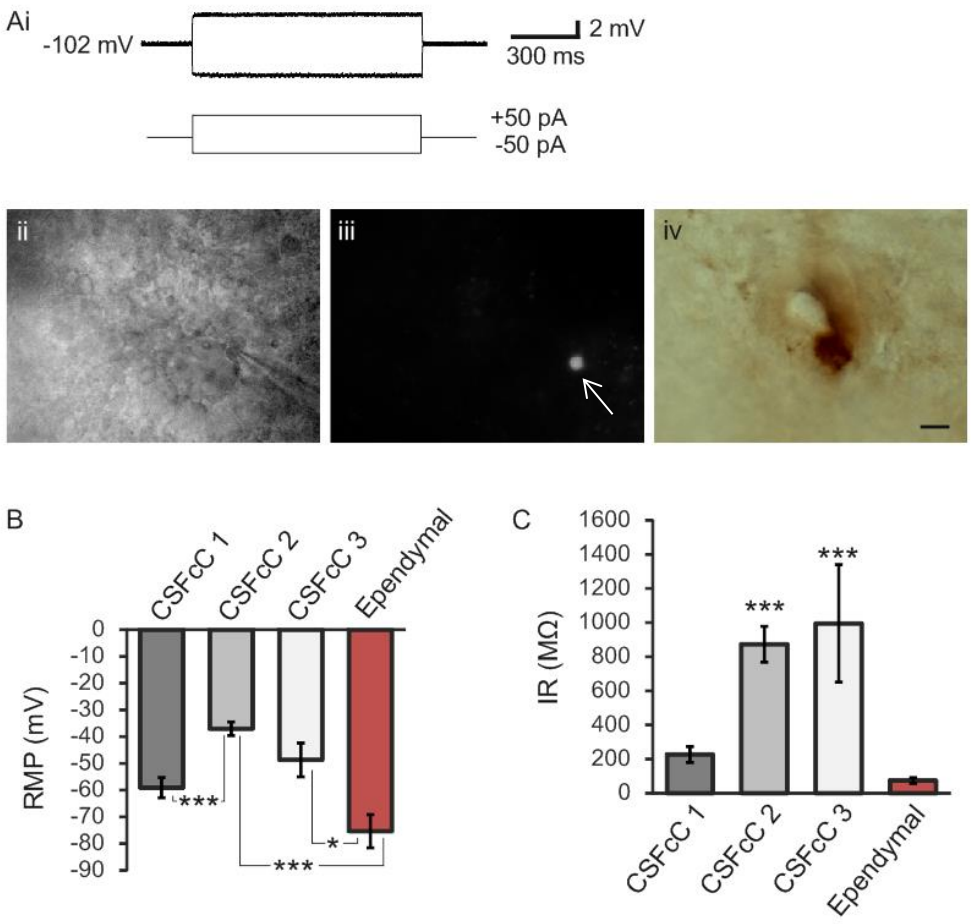


Figure 4.1 The characteristics of ependymal cells

Ai) Whole-cell patch clamp recording of an ependymal cell showing passive voltage responses to depolarising and hyperpolarising current pulses. Aii-iv) The DiC (ii), fluorescence visualisation of recorded cell (arrow) filled with rhodamine (iii) and dye coupling of the neurobiotin-filled ependymal cell detected by post-fixation DAB images, indicating gap junction coupling (scale bars: 20 µm for Aiv). B-C) Comparison of the resting membrane potential (RMP; B) and input resistance (IR; C) of ependymal cells with the subtypes of CSFcCs assessed by Kruskal-Wallis test as prior Shapiro-Wilk determined the data to be not normally distributed.

4.2.2 Characterisation of ependymal hyperpolarising responses to ATP

All ependymal cells responded to local application of ATP with a hyperpolarisation (n=9/9; Figure 4.2 Ai). This response was similar to those seen CSFcC sub-population which produced a hyperpolarising in response to ATP application, composed of mainly CSFcC 1s with lower numbers of CSFcC 2s and 3s (Figure 4.2 Bi). When these responses of ependymal cells were compared those of hyperpolarising CSFcCs, there were multiple similarities between the characteristics of the responses. There was no significant difference between either the onset of response or the duration of the response (Figures 4.2 F and 4.2 G respectively). The onset of the ependymal response (38.80 ± 12.53 ms) did not differ from that of CSFcCs (39.20 ± 3.25 ms) ($p = 0.473$). Similarly, there was no significant difference between the duration of ependymal cell responses (2.64 ± 0.47 s; $n = 9$; $N = 9$) and the duration of CSFcC responses (2.27 ± 0.18 s; $n = 49$; $N = 42$) ($p = 0.975$).

Ependymal cells and CSFcCs were also compared with respect to the amplitude of the hyperpolarising response to locally applied ATP. In this characteristic of the response, there was a significant difference between the cell types. Ependymal cells had significantly smaller amplitude of response (-3.69 ± 0.68 mV voltage change) compared to CSFcCs (-12.45 ± 1.56 mV voltage change; $p = 0.04$). However the extent of voltage change is affected by the input resistances and as there are CSFcC 2s and 3s included in the hyperpolarising CSFcC group, there is a difference in IR levels between the groups ($p = 0.049$). Therefore the responses were analysed as amplitude of response (mV) / IR (MΩ). When these differences in IR were controlled for, there was no significant difference between the amplitudes of the responses for ependymal cells (-0.0746 ± 0.0214 mV/MΩ) and CSFcCs (-0.0834 ± 0.0169 mV/MΩ; $p = 0.834$). This suggests that the differences in amplitude were due to the differences in input resistance.

When a greater amount of ATP was applied, by a longer pulse (1 s compared to 300 ms) of higher concentration (1 mM as opposed to 300 µM), ependymal cells produced a depolarisation (Figure 4.2H). This depolarisation was not seen when lower concentrations and volumes of ATP were applied to ependymal cells.

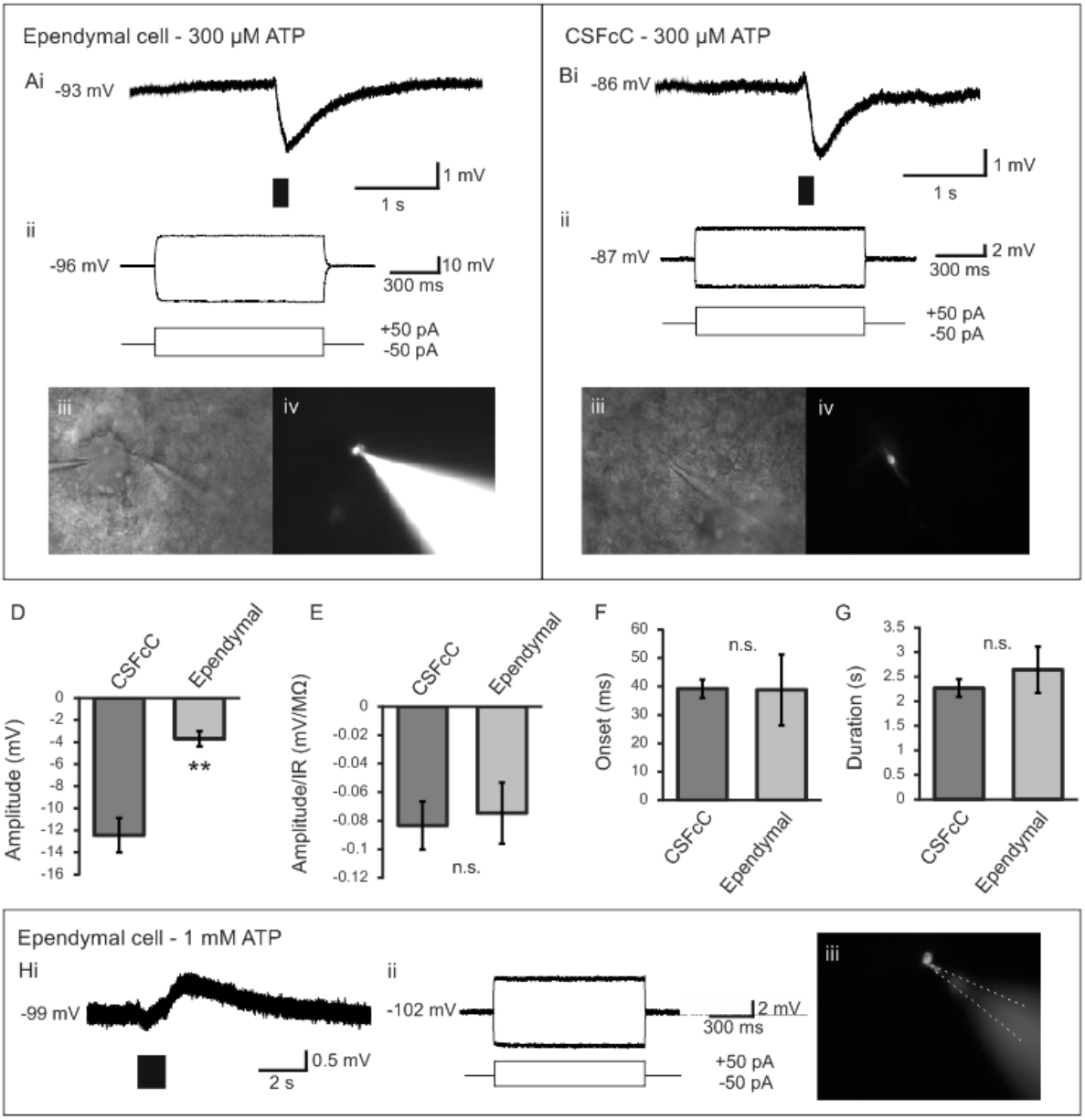


Figure 4.2 Ependymal cells hyperpolarise to 300 µM ATP and depolarise to 1 mM ATP

Ai and Bi) Example whole-cell patch clamp traces of hyperpolarisations in response to local application of 300 µM ATP (black bar) in an ependymal cell and a CSFcC respectively. D-G) Comparisons of the ATP-induced hyperpolarisations in ependymal cells to those produced by all hyperpolarising CSFcCs in terms of amplitude (D), amplitude/IR (E), onset (F) and duration (G). D-E) Significance assessed by Mann-Whitney test as data not normally distributed as determined by Shapiro-Wilks test (** = $p < 0.01$. F-G) Data normally distributed so significance determined by independent samples t-test. Hi) 1s pulse of 1 mM ATP elicits a depolarising response when applied to ependymal cells.

4.2.3 Large variation in estimated reversal potential of hyperpolarisations

The amplitude of the hyperpolarisations decreased as the holding membrane potential depolarised. This was seen for all hyperpolarising responses in ependymal cells and CSFcCs. In some examples, the response was seen to pass the reversal potential for that cell and show a depolarisation (Figure 4.3 Cell 8). However, unlike with the depolarising responses there was little correlation between the reversal potentials for the different cells (Figure 4.3), with reversal potentials ranging from -78.9 mV to + 106.3 mV. This value does represent reversal potentials of hyperpolarising responses from all cell types which hyperpolarise to ATP, however there was no observable correlation between the cell type producing the hyperpolarisation and the reversal potential.

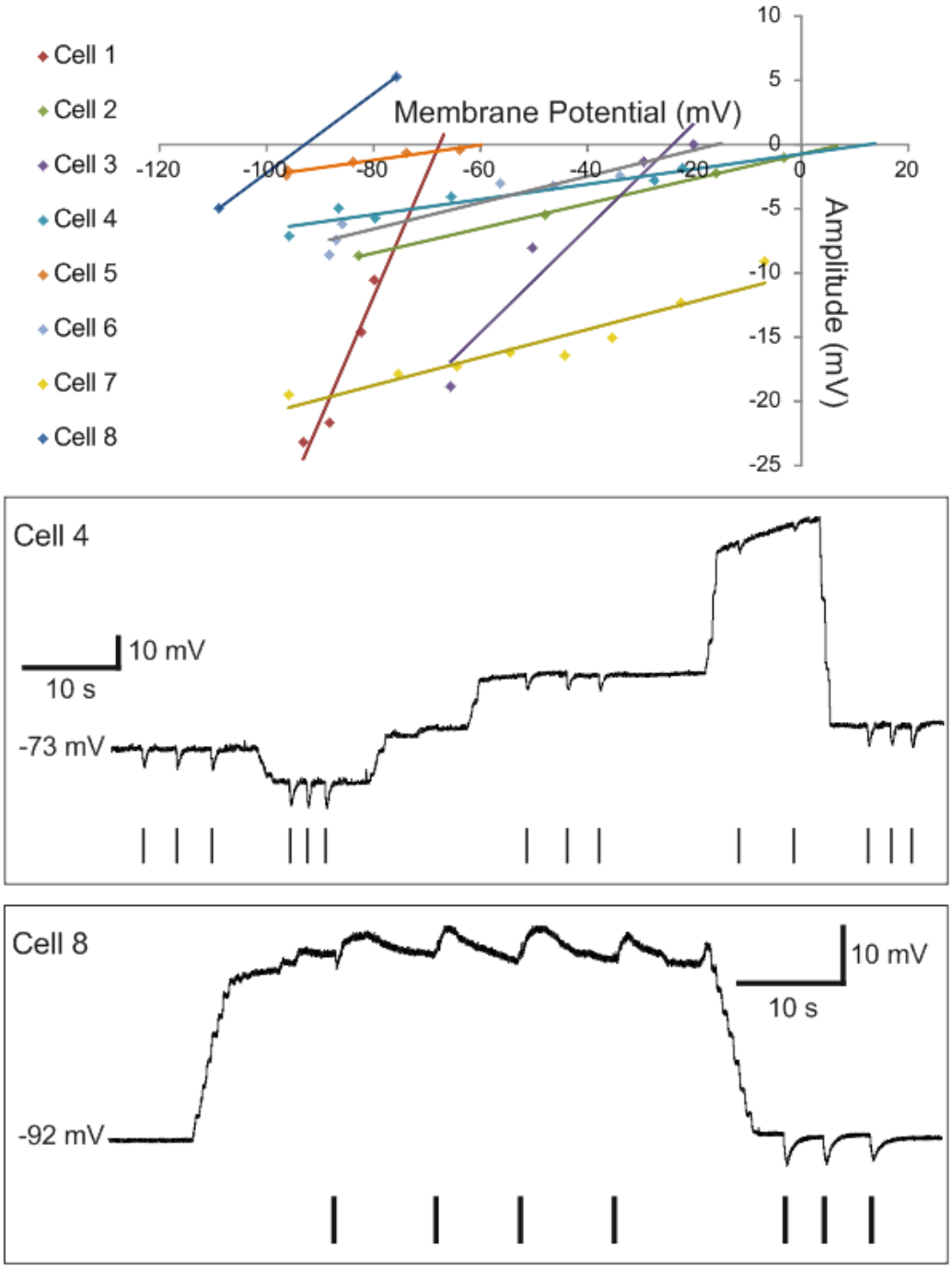


Figure 4.3 Reversal potential of hyperpolarising response to ATP

Reversal potential for a number of cells including ependymal cells adjusted for the liquid junction potential (cell 6), CSFcCs (cells 2 and 7) and tanycytes (cells 4, 5 and 8) and cells that couldn't be identified (cells 1 and 3) are estimated based on the amplitude of the hyperpolarisation to 300 μM ATP (black bars) at a range of holding membrane potentials. Beneath 2 example traces are shown with responses reducing in amplitude as the membrane potential of the cells is depolarised and in the second example, this passes the reversal potential and a hyperpolarisation is produced in response to ATP application.

4.2.4 Responses of Tanycyte-like cells to ATP

A population of cells (n = 3) were identified which bore similarity to cells previously described as tanycytes (Rafols and Goshgarian, 1985; Meletis et al., 2008), generally regarded as a subpopulation of ependymal cells (Bruni and Reddy, 1987). These cells had ependymal cell-like electrophysiological profiles but possessed a basal process leading away from the central canal (Figure 4.4 A, B and C). These processes were clearly visible in the fluorescent imaging of the rhodamine filled cells (Figures 4.4 Aii, Bii and Cii), whilst no apical processes were visible. Tanycyte-like cells responded to local application of ATP with a hyperpolarisation, similar to those seen in both ependymal cells and hyperpolarising CSFcCs. The hyperpolarisations of ependymal cells and tanycyte-like cells were compared to examine if the responses to ATP were likely to be caused by the same mechanisms. The amplitude of responses for tanycyte-like cells was -2.84 ± 0.43 mV compared to -4.23 ± 0.91 mV for ependymal cells ($p = 0.493$; Figure 4.4 D) and was not significantly different. Neither was the duration of the response different (3.43 ± 0.39 s for tanycyte-like cells compared to 2.65 ± 0.51 s for ependymal cells; $p = 0.262$; Figure 4.4 E) nor the onset of the response to ATP (69.0 ± 31 ms for tanycyte-like cells and 31.0 ± 5.9 ms for ependymal cells; $p = 0.342$; Figure 4.4 F).

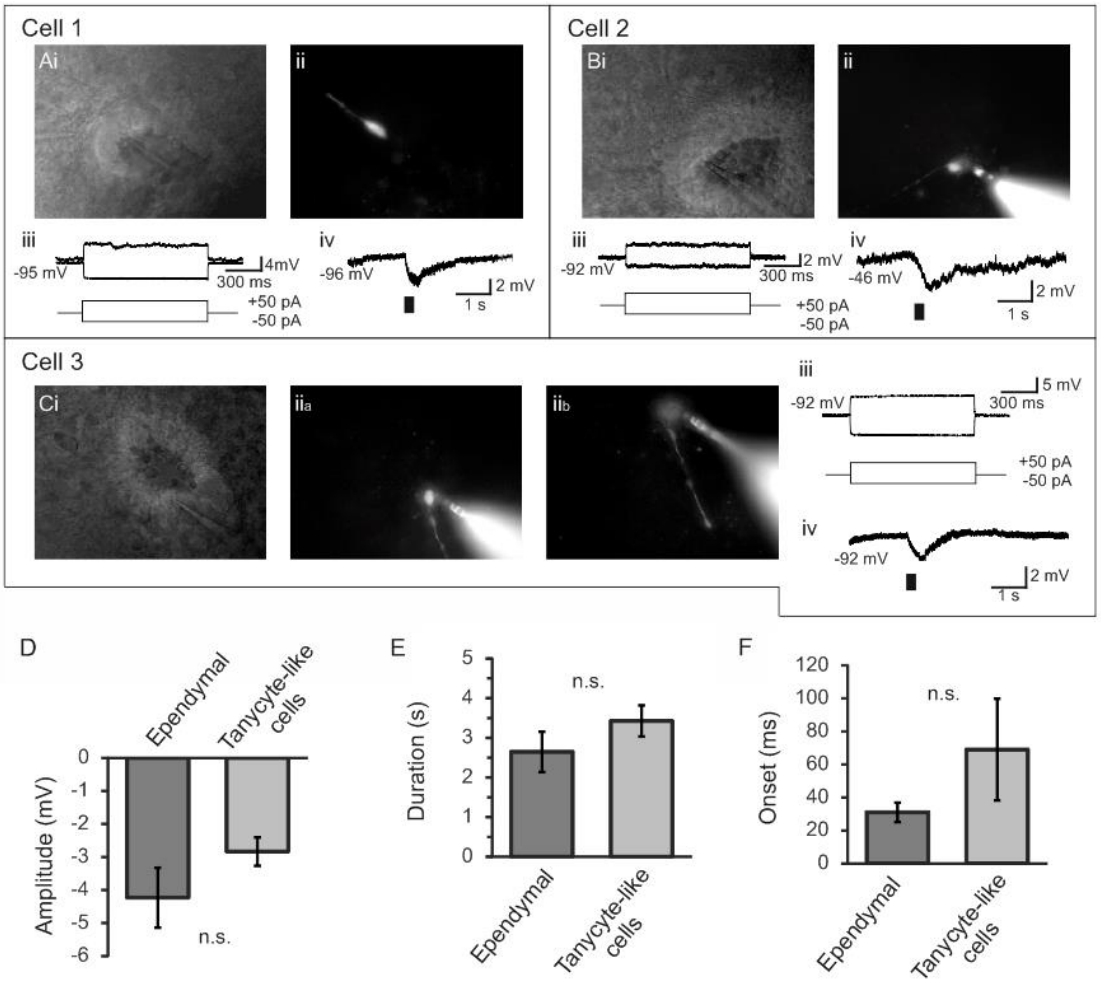


Figure 4.4 Characteristics and responses to ATP of tanycyte-like cells

Ai, ii-Ci, ii) Examples of the visualisations and fluorescent imaging showing processes leading away from the central canal but an absence of processes leading into the central canal and a location within the ependymal cell layer. Aiii-Ciii) Whole cell patch clamp recordings showing passive voltage responses to depolarising and hyperpolarising current pulses. Aiv-Civ) Responses of tanycyte-like cells to local application of 300 μM ATP (black bars). D-F) Comparisons of responses to ATP application between tanycyte-like cells and ependymal cells in terms of amplitude (D), duration (E) and onset (F) (amplitude data were not normally distributed as determined by Shapiro-Wilk test, so Mann-Whitney test was used to determine significance of difference, data for duration and onset were normally distributed so an independent measures t-test was used for these parameters).

4.2.3 Hyperpolarisations not affected by a range of antagonists

The similarities in characteristics of the hyperpolarising responses to ATP have been demonstrated between ependymal cells (including tanycyte-like cells) and CSFcCs (see section 4.2.2). Due to this similarity, the hyperpolarising responses have been considered as a similar population for the purposes of antagonist assessment. No significant effect was observed with any of the antagonists applied to the hyperpolarising responses (Figure 4.5 A). To ensure that there was no effect limited to the responses of one cell type which might be masked in statistical analysis by examples from the other cell types, individual recordings were examined for a reduction of hyperpolarisation amplitude followed by a recovery of response amplitude, however none of these responses were seen in any of the cell types with any of the antagonists used.

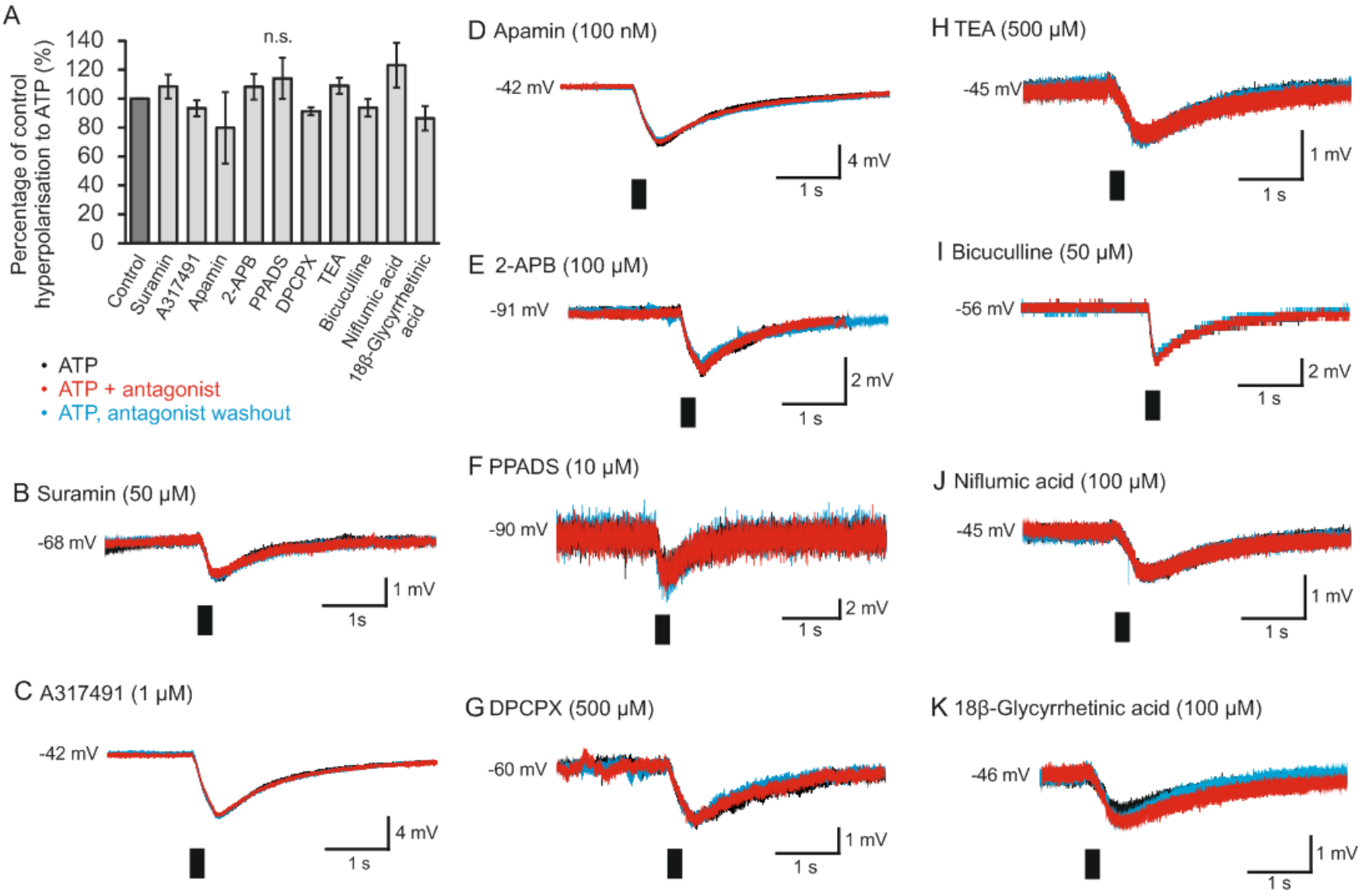
Suramin (Figure 4.5 B), the $P2X_3$ and $P2X_{2/3}$ antagonist A-317491 (Figure 4.5 C), apamin (Figure 4.5 D), 2-APB (Figure 4.5 E), PPADS (Figure 4.5 F), DPCPX (Figure 4.5 G), TEA (Figure 4.5 H), bicuculline (Figure 4.5 I), niflumic acid (Figure 4.5 J), the gap junction blocker, 18β-glycyrrhetinic acid (Figure 4.5 K) all did not have any significant effect on the amplitude of the hyperpolarisations to ATP seen in ependymal cells and CSFcCs of the central canal.

Antagonist name	Conce-ntration	Action	Control hyp amplitude (mV)	Antagonist hyp amplitude (MV)	n number	N number	P value
Suramin	50 μM	Broad spectrum antagonist of P2X and P2Y receptors at different potencies (North and Jarvis, 2013)	-0.79 ± 2.71	-2.87 ± 2.49	15	15	0.842
A-317491	1 μM	Selective $P2X_3$ and $P2X_{2/3}$ antagonist	-5.21 ± 2.14	-4.68 ± 2.19	23	20	0.131
Apamin	100 nM	Ca^{2+}-activated K^+ channel (SK channel) blocker	-6.45 ± 1.78	-7.11 ± 1.82	16	16	0.605
2-APB	100 μM	TRP channel antagonist at TRP 1,3,5,6,V6,M3,M7,M8 and P2 and SOC antagonist	-3.58 ± 0.83	-4.47 ± 1.09	10	10	0.799
PPADS	10 μM	P2X antagonist	-5.80 ± 2.42	-6.39 ± 2.77	5	4	0.500
DPCPX	500 nM	Adenosine A1 antagonist	-4.61 ± 0.89	-4.23 ± 0.85	15	11	0.088
TEA	500 μM	K^+ channel blocker (and nAChr antagonist)	-6.94 ± 1.93	-7.28 ± 2.00	13	12	0.157
Bicuculline	50 μM	$GABA_A$ antagonist (and Ca^{2+}-activated K^+ channel blocker)	-6.15 ± 2.44	-5.94 ± 2.44	5	4	0.345
Niflumic Acid	100 μM	Chloride channel antagonist, COX 2 antagonist and TRPA1 activator	-9.00 ± 1.54	-11.2 ± 2.35	13	11	0.167
18β-Glycyrrhetinic Acid	100 μM	Gap junction inhibiter	-15.98 ± 4.01	-12.34 ± 3.62	15	12	0.280

Table 4.1 Antagonists applied to hyperpolarising responses elicited by 300 μM ATP; further information regarding source and evidence for concentration used of these drugs can be found in Table 2.2.

Figure 4.5 Antagonists applied to hyperpolarisations to ATP did not reduce the amplitude of the response

A) Bar chart showing the average amplitude of hyperpolarisations to 300 μM ATP in ependymal cells and hyperpolarising CSFcCs after application of antagonists compared to the amplitude of hyperpolarisation prior to antagonist application. B-K) 50 μM suramin (B), 1 μM A-317491 (C), 100 nM apamin (D), 100 μM 2-ABP (E), 10 μM PPADS (F), 500 μM DPCPX (G), 500 μM TEA (H), 50 μM bicuculline (I), 100 μM niflumic acid (J) and 100 μM 18β-glycyrrhetinic acid were bath applied (K) were applied to hyperpolarisations induced by 300 μM ATP (black bars); control trace (black), trace with antagonist (red), trace after antagonist washout (blue) (all exemplified applied to an ependymal cell). n.s. = $P > 0.05$


A
Percentage of control hyperpolarisation to ATP (%)
140
120
100
80
60
40
20
0
n.s.
Control
Suramin
A317491
Apamin
2-APB
PPADS
DPCPX
TEA
Bicuculline
Niflumic acid
18β-Glycyrrhetinic acid
• ATP
• ATP + antagonist
• ATP, antagonist washout
B Suramin (50 μM)
-68 mV
1 mV
1s
C A317491 (1 μM)
-42 mV
4 mV
1 s
D Apamin (100 nM)
-42 mV
4 mV
1 s
E 2-APB (100 μM)
-91 mV
2 mV
1 s
F PPADS (10 μM)
-90 mV
2 mV
1 s
G DPCPX (500 μM)
-60 mV
1 mV
1 s
H TEA (500 μM)
-45 mV
1 mV
1 s
I Bicuculline (50 μM)
-56 mV
2 mV
1 s
J Niflumic acid (100 μM)
-45 mV
1 mV
1 s
K 18β-Glycyrrhetinic acid (100 μM)
-46 mV
1 mV
1 s

4.2.4 UTP application, but not ADP or aCSF produces similar hyperpolarisations

To determine whether the hyperpolarisations are due to just flow of the solution from the electrode, rather than activation of a specific receptor, the solution used to dissolve the drugs, artificial cerebrospinal fluid (aCSF), was locally applied using the same Picopump apparatus and settings as those used when the ATP had been applied previously. This produced no clear and consistent response in ependymal cells or CSFcC 1s or 2s (-0.0817 ± 0.833 mV; n = 6; N = 5) (Figure 4.6 A). Other nucleotides, UTP and ADP were also applied using the Picopump apparatus. UTP, but not ADP, was able to replicate the hyperpolarisations seen with ATP. 300 μM ADP did not produce any clear response in ependymal cells and CSFcC 1s (-0.503 ± 0.633 mV; n = 4; N = 2) (Figure 4.6 B).

Conversely, UTP was able to produce a replicable hyperpolarisation in all the cells types that ATP is frequently able to produce a hyperpolarisation, namely, CSFcC 1s (Figure 4.6 C), ependymal cells (Figure 4.6 D) and tanycyte-like cells (Figure 4.6 E). However when UTP was applied to spiking CSFcCs which are likely to depolarise to ATP, UTP did not produce a response (Figure 4.6 F). The proportion of cells of each cell type that hyperpolarised to UTP is similar to the proportions of cells that hyperpolarise to ATP, particularly in that a smaller proportion of CSFcCs hyperpolarise to UTP than the proportion of ependymal cells that hyperpolarise to UTP (Figure 4.7 E). This was a similar pattern to that seen with ATP hyperpolarisations, however unlike with ATP application, no depolarisation was seen in the cells that did not hyperpolarise to UTP (Figure 4.6 F).

The characteristics of the hyperpolarisations caused by local application of UTP were compared with those of hyperpolarisations caused by ATP across all the cell types that show hyperpolarisations to these agonists. With respect to the onset of response, there was no significant difference between those caused by UTP and those caused by ATP (25.6 ± 4.27 ms (n = 8; N = 5) and 36.1 ± 4.71 ms (n = 38; N = 36) respectively; p = 0.183; Figure 4.7 A). Similarly with duration, there was no significant difference between the responses (2.21 ± 0.24 s for UTP and 2.32 ± 0.21 for ATP; p = 0.483; Figure 4.7 B). There was however a difference in the amplitude of the response, with the hyperpolarisations to local application of UTP having a significantly smaller amplitude compared to those caused by local application of UTP

(-1.88 ± 0.37 mV for UTP and -6.43 ± 0.94 mV for ATP; p = 0.004; Figure 4.7 C). Unlike with the difference in hyperpolarisation amplitude between CSFcCs and ependymal cells, the difference between hyperpolarisations caused by ATP and those caused by UTP did not disappear when amplitudes were considered as amplitude/IR (-0.022 ± 0.007 mV/MΩ for UTP and -0.091 ± 0.018 mV/MΩ for ATP-initiated hyperpolarisations; p = 0.012; Figure 4.7 D).

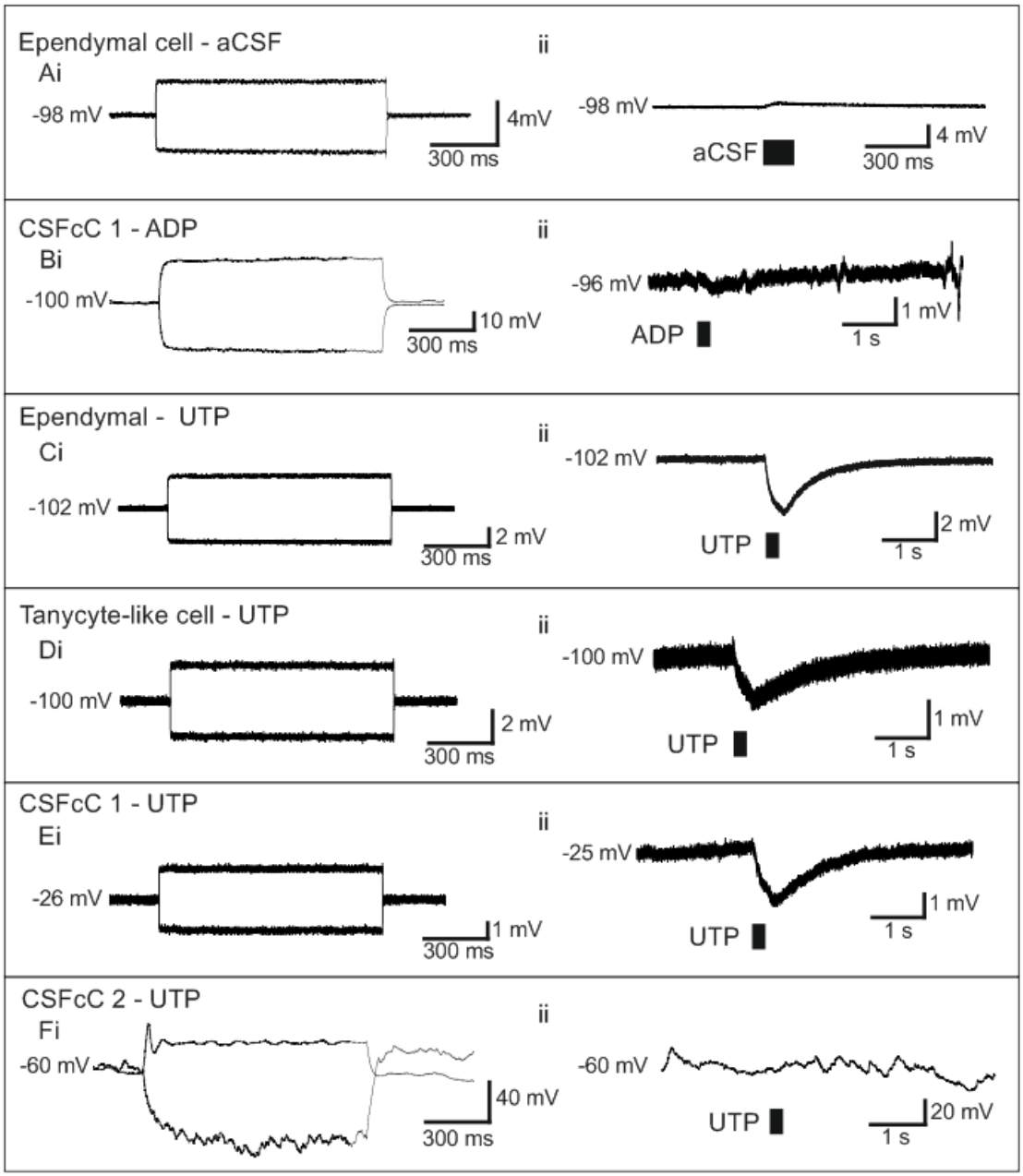


Figure 4.6 UTP but not ADP or aCSF produces hyperpolarisations in ependymal cells and CSFcCs

Ai - Fi) Cells characterised by voltage responses to current injection of +50 and -50 pA. Aii) Local puff application of aCSF (black bar) did not produce a hyperpolarisation, shown in an ependymal cell. Bii) 300 μM ADP (black bar) did not produce any change in membrane potential exemplified in a CSFcC 1. Cii, Dii, Eii) 300 μM UTP (black bars) produced hyperpolarisations in some CSFcCs (C), ependymal cells (D) and tanycyte-like cells (E). Fii) 300 μM UTP (black bar) did not produce a hyperpolarisation in a CSFcC 2.

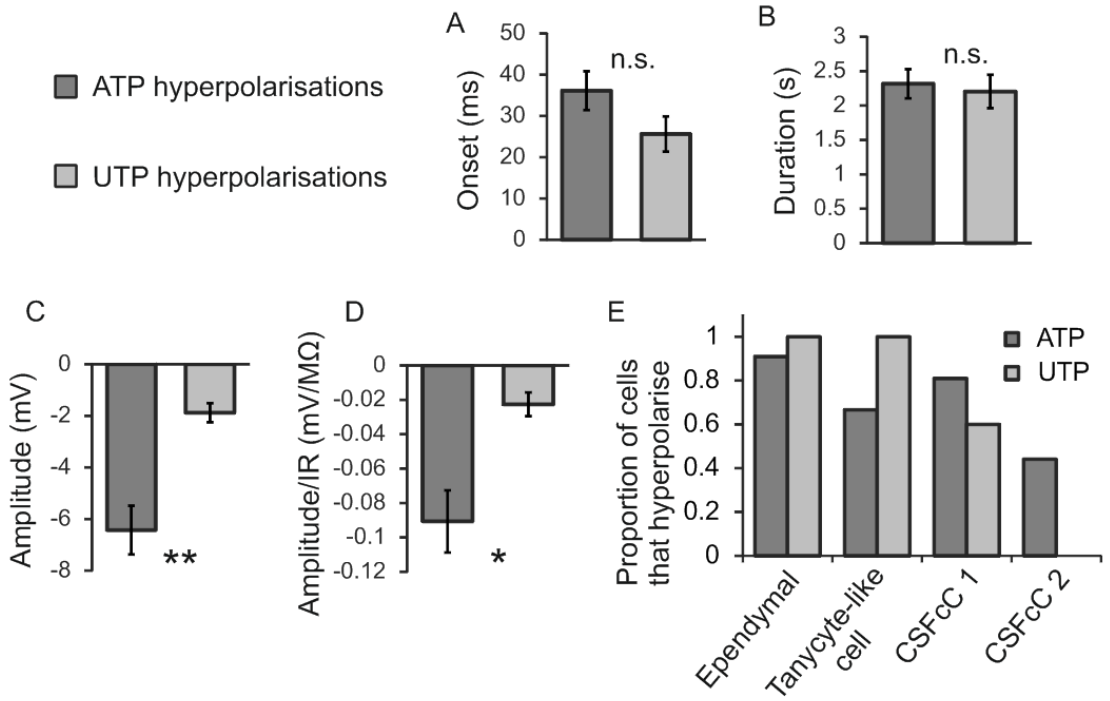


Figure 4.7 Comparison of the hyperpolarisations produced by ATP and UTP

A-D) Comparisons of the hyperpolarisations produced by UTP to those produced by ATP in terms of onset (A), duration (B), amplitude (C), and amplitude/IR (D). Duration data were normally distributed (as determined by Shapiro-Wilk test) so were assessed by independent samples t-test, all other data were not normally distributed so were assessed by Mann-Whitney tests; ** = $p < 0.01$, * = $p < 0.05$. E) Proportion of each cell type which responded to local application of either ATP (darker bars) or UTP (lighter bars) with a hyperpolarisation.

4.2.5 Ap4A produces depolarisations in cells that hyperpolarise to ATP

In addition to local application of ATP, a stable analogue of ATP was also applied Ap4A could be applied in the bath solution as it was not as readily broken down by ecto-ATPases present in the extracellular solution. This allowed Ap4A to be applied to the same cells to which ATP was applied. Ap4A (100 μM) produced a small depolarisation in ependymal cells, tanycyte-like cells and CSFcCs (2.012 ± 0.47 mV; n = 15; N = 11) (Figures 4.8 A, B and C respectively). These cells which depolarised to Ap4A responded to local application of ATP with a hyperpolarisation. Although the depolarisation to Ap4A is small in magnitude it is a significant depolarisation which returns to baseline levels after washout of Ap4A (Figure 4.8 D; $p < 0.001$). The depolarisation caused by Ap4A application is eliminated by the application of 50 μM suramin, even with longer Ap4A application used to ensure that a longer application couldn't overcome suramin antagonism (n = 11; Figure 4.8 E).

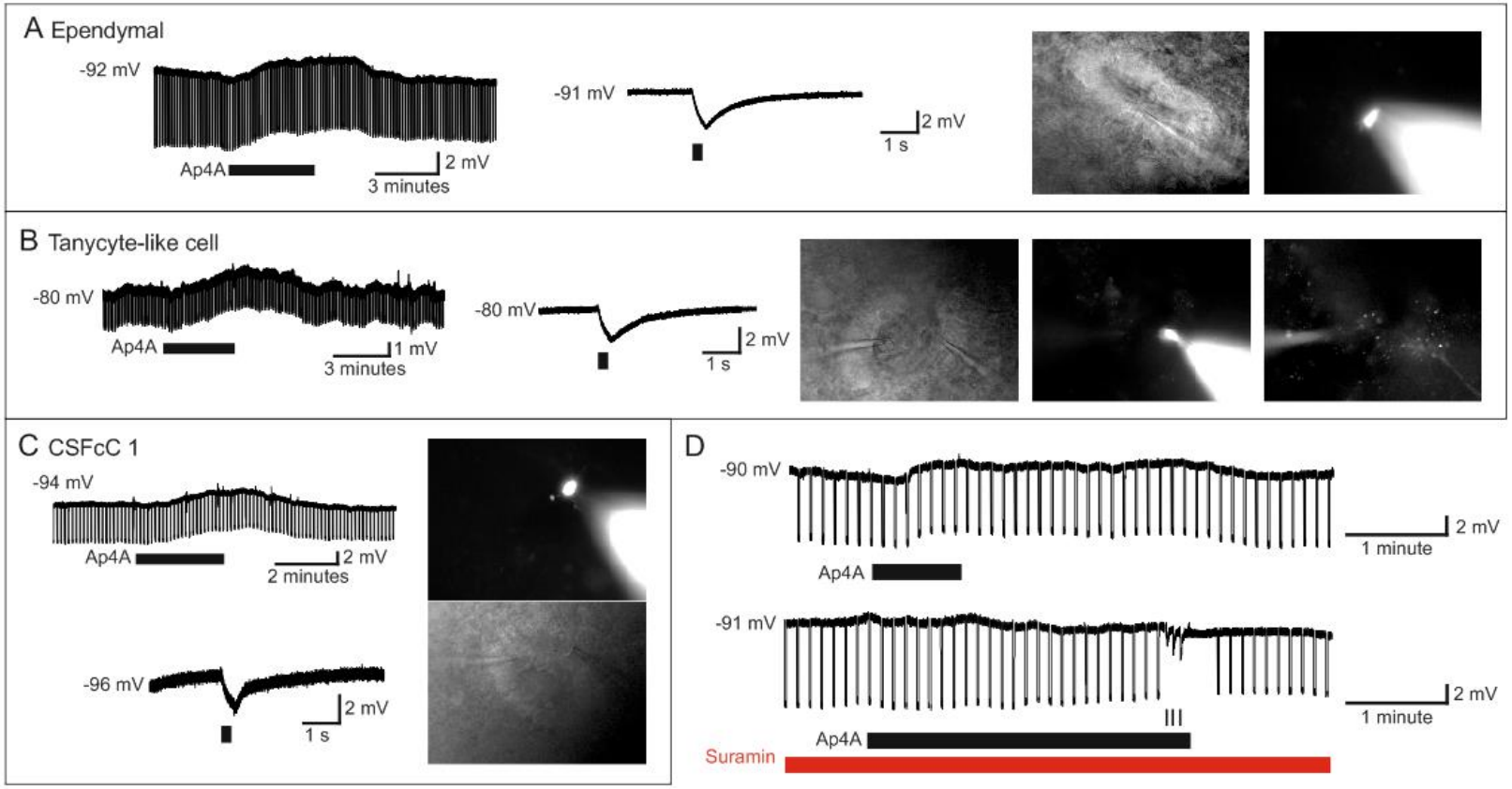


Figure 4.8 Bath applied Ap4A produced small depolarisations in cells that hyperpolarised to local application of ATP

A-C) 100 μM Ap4A produced small depolarisations in ependymal cells (A), tanycyte-like cells (B; tanycytic process indicated by the arrow) and CSFcCs (C) which produced a hyperpolarisation in response to local application of 300 μM ATP (unlabelled black bars). D) This depolarisation was small but was a significant as assessed by repeated measures ANOVA; *** = $p < 0.001$.

4.3 Discussion

4.3.1 The response of ependymal cells to local application of ATP

ATP produces hyperpolarising responses in ependymal cells, which are similar to those produced by a subset of CSFcCs with respect to onset, duration and also amplitude once the differences in IR are accounted for. The CSFcCs that are more likely to produce a similar response to ATP as ependymal cells are the non-spiking CSFcC 1s. This is not the only case where the response to neurotransmitters of CSFcC 1s bears more similarity to those of ependymal cells than those of spiking CSFcCs. In the response of these cell groups to acetylcholine, CSFcC 1s bear more similarity to ependymal cells than to CSFcC 2s and 3s. CSFcC 1s and ependymal cells have considerable contribution from α7*nAChRs while CSFcC 2s and 3s have very minimal α7*nAChRs contribution (Corns et al., 2015).

The hyperpolarisations caused by ATP in ependymal cells and the hyperpolarising population of CSFcCs were not affected by a range of antagonists. The fact that they were not antagonised by either suramin or PPADS makes it unlikely that the ATP is acting on P2X receptors as although these antagonists are variably potent on different receptor subtypes, only the $P2X_4$ receptor is not strongly antagonised by at least one of these antagonists (Charlton et al., 1996; Lambrecht, 2000; Kennedy, 2005). $P2X_4$ receptors have been observed in the progeny of spinal cord ependymal cells (Gómez-Villafuertes et al., 2015), although expression *in vivo* has not been determined. After spinal cord injury $P2X_4$ is present in microglia but not in sham-operated animals (Lu et al., 2013) and has a role in the development of tactile allodynia (Tsuda et al., 2003). Whilst it is not possible to rule out involvement of $P2X_4$ in the production of hyperpolarising responses to ATP it is an unlikely channel to cause hyperpolarisations as, similar to $P2X_2$ receptors, $P2X_4$ is a non-selective cation channel (Shieh et al., 2006) and is therefore most likely to cause a depolarisation.

The other P2X receptor that has been identified and been shown to be functionally active in ependymal cells is $P2X_7$ (Marichal et al., 2016). It is unlikely that $P2X_7$ are the receptors responsible for the hyperpolarisations seen in ependymal cells with application of ATP for two reasons. Firstly $P2X_7$ is a non-selective cation channel (Rassendren et al., 1997; Chessell et al., 1998) similar to the other P2X receptors and is therefore most likely to cause a depolarisation upon channel opening.

Secondly, the $P2X_7$ receptor has relatively low affinity for ATP compared to the other P2X receptors (Bartlett et al., 2014), such that previous experiments which puff applied ATP used 1s applications of 1 mM ATP to activate $P2X_7$ receptors (Marichal et al., 2016). These indications that the hyperpolarisations were independent of $P2X_7$ were supported by the fact that the depolarising responses mediated by $P2X_7$ observed previously could be replicated by used of the same puffing parameters used in that study.

ATP is readily broken down by ecto-ATPases, which dephosphorylate ATP to ADP to AMP and then to adenosine (Fuentes and Palomo, 2015). It is therefore a possibility that the responses seen with application of ATP did not actually use ATP as the agonist and instead, ATP was dephosphorylated and one of the products of this acted as the agonist. However, the rapid onset of response and time to peak response of the hyperpolarisations make this an unlikely method of action. Additionally, when ADP was applied no hyperpolarisations were seen. As ADP is a step on the dephosphorylation pathway of ATP, the lack of response with ADP indicates that neither ADP nor any of the downstream produces, namely AMP or adenosine, were the mediators of the hyperpolarising response. This was supported by the lack of effect of the adenosine A_1 receptor antagonist DPCPX on the hyperpolarisations elicited by ATP.

Another possible cause of the hyperpolarisations to local application of ATP could be the pressure wave used to release the ATP locally, rather than the ATP itself. There are a number of pressure and stretch sensitive channels which respond to changes in flow and pressure within a tissue or fluid such as the CSF. One such channel is TMEM16A/anoctamin 1. This is a calcium-activated chloride channel which, whilst it has not been identified around the central canal, has been shown to detect stretch within the cerebral arteries (Bulley et al., 2012). However, the response produced by this stimulation is a depolarisation that is sensitive to 2-APB (Viitanen et al., 2013; Zhu et al., 2015), making it unlikely to be the cause of the 2-APB-insensitive hyperpolarisations seen with ATP. This conclusion is supported by lack of response when aCSF was locally applied in the same manner to ATP to replicate the pressure wave without any changes to the composition of the solution around the cells.

2-APB is also an antagonist of the intracellular signalling molecule IP_3, which triggers release of calcium from intracellular stores (Saleem et al., 2014). IP_3 is a second

messenger in the $G_{q/11}$ signalling pathway via the G_{α} protein. The lack of inhibition of the hyperpolarisations with 2-APB indicates that IP_3 and therefore the $G_{\alpha q/11}$ is not involved in this response. It is worth noting that the $G_{q/11}$ signalling pathway could still be involved, via the $G_{\beta\gamma}$ proteins, which are often play a role in interactions with ion channels and other intracellular proteins (Kahanovitch et al., 2014; Shymanets et al., 2015).

Other antagonists that did not affect the amplitude of the hyperpolarisations to ATP include the non-selective potassium channel blocker TEA (Liu et al., 2014), which also acts as a competitive inhibitor at nicotinic acetylcholine receptors (Akk and Steinbach, 2003). The $GABA_A$ antagonist bicuculline (Braden et al., 2015) and blocker of calcium-activated potassium channel (Khawaled et al., 1999) also did not affect the hyperpolarisations. Similarly, the calcium-activated chloride channel blocker niflumic acid (Criddle et al., 1996) did not antagonise the receptors responsible for the hyperpolarising response to ATP. Niflumic acid also acts as a blocker of $GABA_A$ receptors (Sinkkonen et al., 2003), NMDA channels (Lerma and Martín del Río, 1992) and T-type calcium channels (Balderas et al., 2012). These results suggest that none of these channels are involved in the production of the hyperpolarising response to ATP by ependymal cells and CSFcCs. Furthermore, the lack of change in the amplitude of the hyperpolarisations with application of the gap junction blocker 18β-glycyrrhetinic acid indicates that the hyperpolarisation is not reliant on the gap junction-coupling of the ependymal cells. This is also supported by the fact that hyperpolarisations are also seen in some CSFcC 2s and 3s, whereas no gap junction-coupling is observed in these cell types (see chapter 3 for discussion of this).

4.3.2 The response of ependymal cells to local application of UTP

The signalling of UTP in addition to ATP has been investigated for a number of reasons. The first is that UTP has been hypothesised to be released at sites of injury in a similar way to that which ATP is released (Wang et al., 2004; Ceruti et al., 2009). The second reason is that metabotropic P2Y receptors have been identified on ependymal cells, some of which respond to UTP. The progeny of ependymal cells which have been grown in culture are termed ependymal spinal cord-derived stem/progenitor cells (epSPCs). Within these cells, metabotropic P2Y receptors have been identified, in addition to ionotropic P2X receptors (Gómez-Villafuertes et al., 2015). Both $P2Y_1$ and $P2Y_4$ have been identified in epSPCs and they are differentially

affected by spinal cord injury, with epSPCs from an injured animal (epSPCis) showing a downregulation of $P2Y_1$ receptors and $P2Y_4$ receptors. Whilst some P2Y receptors are coupled to inhibitory $G_{\alpha i}$ proteins, both $P2Y_1$ and $P2Y_4$ usually couple to $G_{q/11}$ proteins which release calcium from internal stores and would therefore depolarise the cell (Gómez-Villafuertes et al., 2015).

Local application of UTP produced hyperpolarisations in ependymal cells and CSFcCs that were similar to those produced by application of ATP, except with lower amplitude of response. Despite the presence of ectonucleoside-diphosphokinases on some cells which can act to convert dinucleotides to trinucleotides (Harden et al., 1998), the application of ADP did not produce any response. The short time course of the local application puff of ADP was likely to have helped ensure that there was not significant phosphorylation of ADP.

P2X receptors are not activated by UTP or ADP (Puchałowicz et al., 2014; Jacobson and Müller, 2016), further suggesting that P2X receptors are not responsible for the hyperpolarisations in response to either ATP or UTP. Unlike P2X receptors, metabotropic P2Y receptors are activated by a wider range of native agonists including ATP, ADP, UTP, UDP, and UDP-glucose (Jacobson and Müller, 2016). Whilst not all P2Y receptors respond to all of these native agonists, $P2Y_2$ and $P2Y_4$ respond to both ATP and UTP (Wildman et al., 2003; Lechner and Boehm, 2004).

$P2Y_2$ and $P2Y_4$ mainly couple to pertussis toxin resistant $G_{q/11}$ proteins (Köles et al., 2008; von Kügelgen and Hoffmann, 2016) but there are reports of individual receptors also coupling to pertussis toxin resistant $G_{i/o}$ proteins (Erb et al., 2006; Rossi et al., 2007). However in none of these examples does the combined coupling produce a hyperpolarisation, instead using the G_o protein to increase intracellular calcium levels (Mosbacher et al., 1998; Bagchi et al., 2005).

P2Y receptors interact with a number of intracellular proteins including the MAPK/extracellular signal-regulated kinase pathway (Soltoff et al., 1998; Sellers et al., 2001), integrins in platelets (Erb et al., 2001; Hollopeter et al., 2001; Bagchi et al., 2005) and tyrosine kinases (Liu et al., 2004; Seye et al., 2004). However the results from this study indicated that the latency to onset was shorter for the hyperpolarising response than it was for the depolarising responses. This makes it unlikely that interactions with these or other cellular macromolecules are responsible for the rapid hyperpolarisations seen in ependymal cells and predominantly non-spiking CSFcCs.

P2Y receptors can also interact with ion channels. They have been observed to interact with voltage-sensitive sodium channels (Baker, 2005), voltage-activated calcium channels (Filippov et al., 2003) and glutamate receptors (Wirkner et al., 2007). Most significantly for the data reported here, $P2Y_2$ and $P2Y_4$ have been reported to interact with potassium channels (Lechner and Boehm, 2004; Köles et al., 2008). Specifically, both ATP (Lee et al., 2014b) and UTP (Lee et al., 2015) have been shown to produce outward currents and hyperpolarisation in PDGFRα-positive interstitial cells due to the activation of small-conductance Ca^{2+}-activated K^+ channels via $P2Y_2$ or $P2Y_4$ receptors. In contrast to the hyperpolarisations presented in this study, the hyperpolarisations in PDGFRα-positive cells were inhibited by the SK_2 channel blocker apamin (300 nM) and the broad-spectrum purinergic antagonist suramin (30 μM). A lower concentration of apamin (100 nM) but a comparable concentration of suramin (50 μM) failed to reduce the amplitude of the hyperpolarisations seen in ependymal cells and CSFcCs. However, it is worth noting that $P2Y_4$ is the only P2Y receptor not antagonised by suramin (Köles et al., 2008), which would suggest that the channels activated in the PDGFRα-positive interstitial cells were $P2Y_2$ channels. The inward currents induced in PDGFRα-positive cells reversed at approximately 0 mV, which is within, the large range of reversal potentials estimated for the hyperpolarising responses of cells around the central canal. It is therefore a possibility that the hyperpolarisations seen in ependymal cells and CSFcCs with application of ATP or UTP are mediated through $P2Y_4$ receptors, using similar intracellular mechanisms as observed with $P2Y_2$ in PDGFRα-positive interstitial cells, potentially via SK_1 channels, although as SK channels are responsible for the slow after-hyperpolarisation a slower onset to response might be expected.

There are other UTP-sensitive receptors within the spinal cord ependymal cells, such as GPR17 (Ceruti et al., 2009). GPR17 is an orphan receptor highly expressed in organs during periods of ischemic damage (Köles et al., 2008). It is $G_{i/o}$ coupled (Simon et al., 2016) but has not been shown to respond to ATP so is unlikely to be responsible for the hyperpolarisations seen in response to local application of ATP. Due to the complexity of P2Y channels the exact conformation and identity would need further investigation, however the sensitivity to ATP and UTP and insensitivity to suramin suggest the involvement of $P2Y_4$ in the hyperpolarisations.

4.3.3 The response of ependymal cells to bath application of Ap4A

Ependymal cells and CSFcCs that hyperpolarised in response to local application of ATP were shown to produce a small depolarisation in response to bath application of Ap4A. Diadenosine polyphosphates, such as Ap4A, are naturally occurring nucleotidic compounds that are linked to the proliferative state of the cell (Rapaport and Zamecnik, 1976; Pintor et al., 2000) as it is synthesised in response to DNA damage and blocks further DNA replication (Marriott et al., 2015). Ap4A is able to be bath applied as it is not broken down by extracellular nucleotidase enzymes and there are fewer, more localised enzymes that do degrade it (Andersson, 1989). However Ap4A does not act on all purinergic receptors with the same potency as ATP. Ap4A can activate $P2Y_1$ (Schachter et al., 1996), $P2Y_2$ (Lazarowski et al., 1995), $P2Y_4$ (Communi et al., 1996) and $P2X_1$ (Wildman et al., 1999), $P2X_2$ (Pintor et al., 1996), $P2X_3$ (Wildman et al., 1999), $P2X_4$ (Wildman et al., 1999). Of interest is the fact that at the $P2Y_4$ receptor, Ap4A has an EC_{50} value of ~5 μM but the maximal response size is 25 % of the maximal response size to UTP activation (Communi et al., 1996). Despite both ATP and Ap4A producing responses in ependymal cells, the responses they produce are very different. ATP produces a rapid hyperpolarisation, whilst Ap4A produces a slow onset depolarisation. It may be that these agonists are activating different receptors, or it could be that they are activating the same receptor, possibly $P2Y_4$, but activating different mechanisms internal mechanisms: outwards currents via potassium channels for the ATP and UTP responses and release of calcium from intracellular stores for the Ap4A response.

4.3.4 Tanycyte-like cells

In this study, tanycyte-like cells were differentiated on the basis of basal processes leading away from the cell and presumed to be terminating on blood vessels as previously reported (Rafols and Goshgarian, 1985). The reporting of tanycyte-like cells in studies of this area is very variable, suggesting that they may be identified as other cell types in some studies. While some papers looking at rat spinal cord tissue discuss ependymal cells and tanycyte-like cells (Mothe and Tator, 2005), other studies in murine spinal cord, discuss cuboidal ependymal cells, with no processes, radial ependymal cells, which are described as having a long basal process and

reside in the dorsal and ventral poles, and tanycyte-like cells, which were differentiated by their darker cytoplasm (Meletis et al., 2008). Radial ependymal cells had intermediary characteristics between ependymal cells and tanycyte-like cells in aspects such as the shape of the nucleus under electron microscopy. In other studies of murine spinal cord, astrocytes and some ependymal cells were identified to have processes, without separation of ependymal subgroups on the basis of processes (Hamilton et al., 2009), and in other studies GFAP-positive cells with radial processes have been identified as radial glia (Sabourin et al., 2009). It has also been suggested that GFAP-positive dorsal cells with basal processes which have previously been identified as astrocytes (Alfaro-Cervello et al., 2012) may be a subtype of tanycyte (Hugnot and Franzen, 2011). In this study, there were no differences in the responses to ATP, UTP or Ap4A of ependymal cells without any basal processes, and cells with basal processes and ependymal electrophysiological characteristics (identified as tanycyte-like cells). Therefore they were grouped as ependymal cells for comparisons between ependymal cells and CSFcCs and this lack of differences suggests that tanycyte-like cells may be a subtype of ependymal cells.

4.3.4 Conclusions

Ependymal cells and tanycyte-like cells were identified by their lack of apical processes and square, passive voltage responses to current injection. These cells, collectively referred to as ependymal cells, responded to 300 μM ATP in a similar way to the hyperpolarising population of CSFcCs, however with smaller amplitude due to their smaller input resistance. Once this difference in IR was accounted for, the responses to 300 μM ATP were identical. This response was separate from the $P2X_7$-mediated response elicited by higher concentration ATP application for a longer time period, as observed previously (Marichal et al., 2016). The hyperpolarisations to ATP could be replicated by application of UTP, but not ADP or aCSF, indicating that they may be mediated by a P2Y receptor sensitive to both ATP and UTP, but are unlikely to be mediated by a dephosphorylation product of ATP or the pressure wave of application. Ap4A produces a small depolarisation in the cells that produce a hyperpolarisation to ATP and UTP, either via alternative receptors or via the same receptor but triggering different intracellular mechanisms.

Chapter 5 - Purinergic signalling effects on proliferation and culture preparations

5.1 Introduction

In a physiological state, the ependymal stem cells show very low levels of proliferation, predominantly self-renewing to produce more ependymal cells (Barnabé-Heider et al., 2010). Traumatic spinal cord injuries, but not demyelinating lesions, dramatically increase their proliferation rate (Cizkova et al., 2009; Moreno-Manzano et al., 2009; Lacroix et al., 2014). The proliferation seen after SCI is predominantly asymmetric division as shown by the plane of cleavage relative to the luminal surface of the cell (Johansson et al., 1999). The proliferation rate of ependymal cells can also be increased by stimulation with growth factors such as epidermal growth factor (EGF) + fibroblast growth factor 2 (FGF2) (Kojima and Tator, 2000) and EGF+FGF2+heparin, even when injected into the 4th ventricle (Martens et al., 2002). Levels of proliferation can also be affected by altering neurotransmitter signalling, with potentiation of cholinergic signalling increasing cell proliferation with both in organotypic slice culture and *in vivo* (Corns et al., 2015). This increase in proliferation with increased cholinergic signalling was seen in the central canal region, where ependymal cells were the dividing cells, and in the rest of the grey and white matter, where the cells replicating expressed markers for oligodendrocyte precursors. The increase in proliferation is accompanied by a number of other changes after spinal cord injury, including an increase in nestin expression in the ependymal cells with processes leading dorsal and away from the cc, and later predominantly in the white matter, where it was accompanied by GFAP staining (Shibuya et al., 2002; Mothe and Tator, 2005).

Once the cells in the ependymal region have proliferated, they then migrate out away from the central canal. By 3 days after a minimal spinal cord injury, the newly-proliferated cells from the central canal region have migrated 70 μm away from the central canal (Mothe and Tator, 2005). These cells migrate in dorsal and ventral directions, showing internalisation of the ATF3 protein into the nucleus during migration (Mladinic et al., 2014), and migrate to the site of injury (Johansson et al., 1999; Meletis et al., 2008). Despite increases in proliferation in astrocytes and oligodendrocyte precursors, oligodendrocyte precursors do not migrate to the injury site, whilst astrocytes migrate to the periphery of the injury, and ependymal cells migrate to the centre of the injury site (Barnabé-Heider et al., 2010). This migration to the injury site was not seen when a minimal injury model was used (Mothe and Tator, 2005). In the minimal injury model, very limited cell death was observed from the

injury site, with only a few apoptotic cells in the white matter. It may be that not enough of the attractant chemicals were released for the cells to migrate towards.

After spinal cord injury, ATP is released initially by the cells undergoing cell death and then by perilesional cells extending the raised extracellular ATP levels for over 6 h (Wang et al., 2004). It is therefore hypothesised that the increased levels of purinergic signalling induced by this increased levels of extracellular ATP could play a role in the modulation of ependymal cell proliferation or the directional migration of newly-proliferated ependymal cells following spinal cord injury.

5.1.1 Hypothesis

The hypotheses tested in this chapter are that:

1. Modulation of purinergic signalling will have an effect on proliferation
2. Modulation of purinergic signalling will have an effect on the migration of newly proliferated cells.

5.1.2 Aims

The aims of this chapter are to determine the longer term effects of purinergic signalling. This will involve the use of proliferation preparations and culture preparations to investigate the effects of modulation of purinergic signalling on the number, location and characteristics of the newly proliferated cells as marked by EdU.

5.2 Results

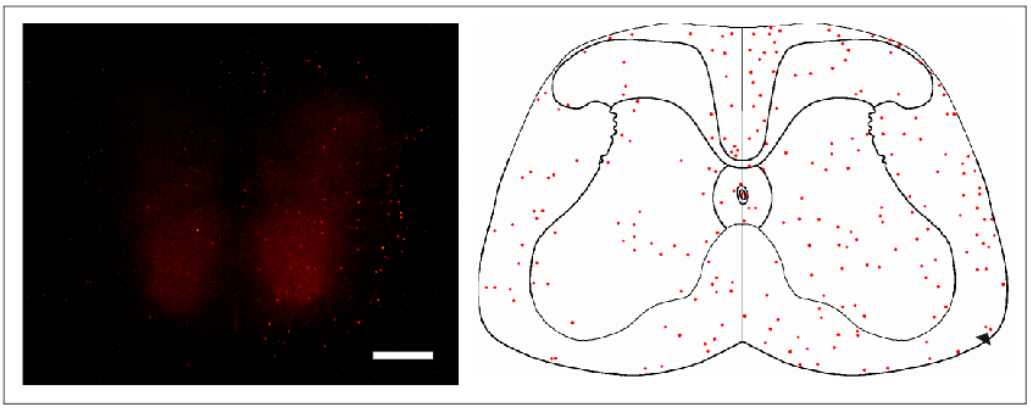
5.2.1 Modulation of purinergic signalling does not alter number of newly-proliferated cells within each slice

The effect of modulation of purinergic signalling on proliferation rate was assessed using a preparation where slices were held in aCSF in the presence of EdU for 4 hours. After fixation and immunohistochemistry for EdU, the slices were visualised and schematic representations made of each slice as the EdU positive cells were on a number of visual planes within the 300 µm slices. As such, the slices couldn't be imaged in a way that either captured all the EdU positive cells in one image or in a way that took multiple images without capturing the same cell multiple times. An image taken at one plane of depth missed many cells which could be accurately marked on the corresponding schematic representation (Figure 5.1 A). There were quite variable numbers of EdU positive cells between the slices, with EdU positive cells present throughout the slice (Figure 5.1 B-D). Despite the widespread nature of the newly proliferating cells, there were several trends observed. There were relatively high levels of newly proliferated cells within the white matter in all experimental conditions. There were also many examples of paired cells where they were positioned very close to each other and sometimes distant from other cells (Figures 5.1 B-D; arrowheads on example schematic representations of slices from each condition).

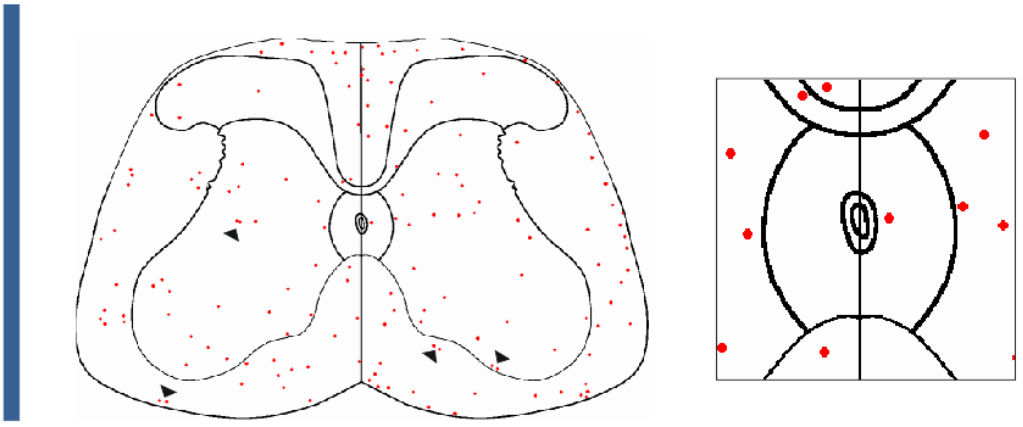
Figure 5.1 Schematic representations of EdU+ cells after 4 hours in EdU

A) Example of image from which the schematic representation adjacent was produced; scale bar 300 µm. B-D) Example schematic representations of the EdU+ cells within a slice, from each of the three conditions: control condition where EdU alone was applied (C), condition where 50 µM ARL 67125 (ecto-ATPase inhibitor) was applied with EdU (B) and the condition with 200 µM suramin (purinergic antagonist) + EdU application (D); arrowheads indicate examples of paired EdU+ cells.

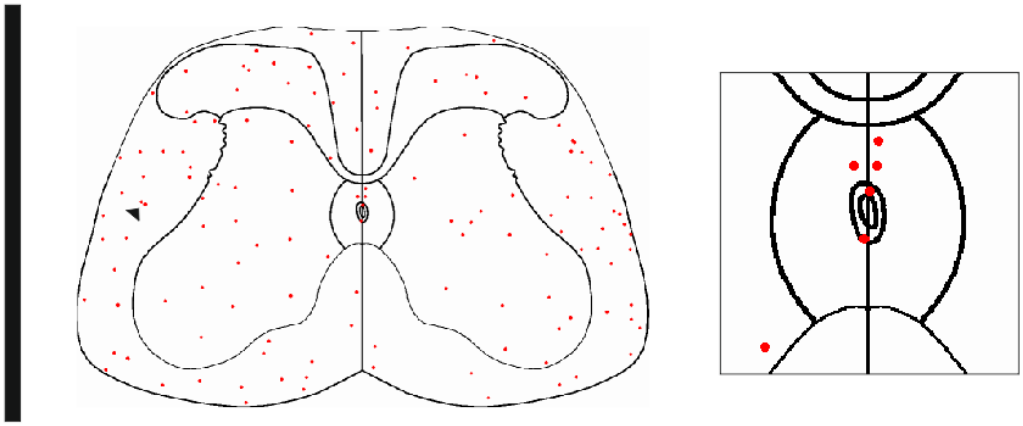
A



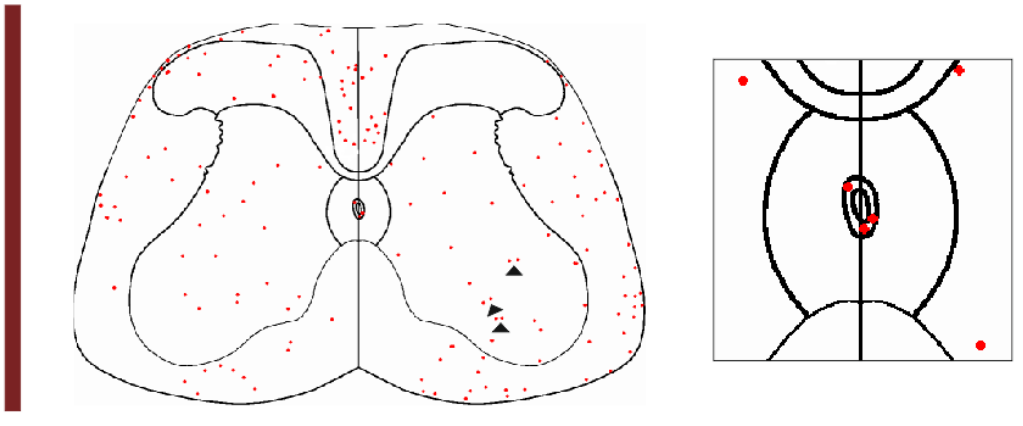
B 50 µM ARL 67125



C Control



D 200 µM suramin



The number of EdU positive cells within each region of the spinal cord were then manually counted using the schematic representations. When the whole slice was counted, there was no significant difference between the control condition (n = 36; N = 6) and either the condition where purinergic signalling was increased by increasing the amount of extracellular ATP (by inhibition of its breakdown using ARL 67125; n = 21; N = 5) or where the levels of purinergic signalling were decreased by use of the antagonist suramin (n = 26; N = 6) (ARL: 176.2 ± 25.0 cells, control: 176.0 ± 16.2 cells, suramin: 177.4 ± 19.0 cells; p = 0.999; Figure 5.2 A). This was the same when the grey matter including lamina X and the central canal region was counted (ARL: 53.3 ± 8.9 cells, control: 56.1 ± 6.0 cells, suramin: 54.3 ± 6.7 cells; p = 0.863; Figure 5.2 B). The numbers of newly-proliferated cells in the central canal region and lamina X were counted and again there was no significant difference between the conditions (ARL: 2.95 ± 0.51 cells, control: 2.58 ± 0.39 cells, suramin: 3.96 ± 0.71 cells; p = 0.161; Figure 5.2 C). However, when only cells within the central canal region were counted, the number of cells in the suramin condition was significantly higher than in the control and the ARL condition (ARL: 0.76 ± 0.2 cells, control: 0.53 ± 0.13 cells, suramin: 1.54 ± 0.33 cells; p = 0.07; Figure 5.2 D). When the EdU positive cells in the white matter alone were counted, there was once again no effect of purinergic modulation (ARL: 66.7 ± 7.0 cells, control: 61.22 ± 4.3 cells, suramin: 64.8 ± 5.6 cells; p = 0.840; Figure 5.2 E).

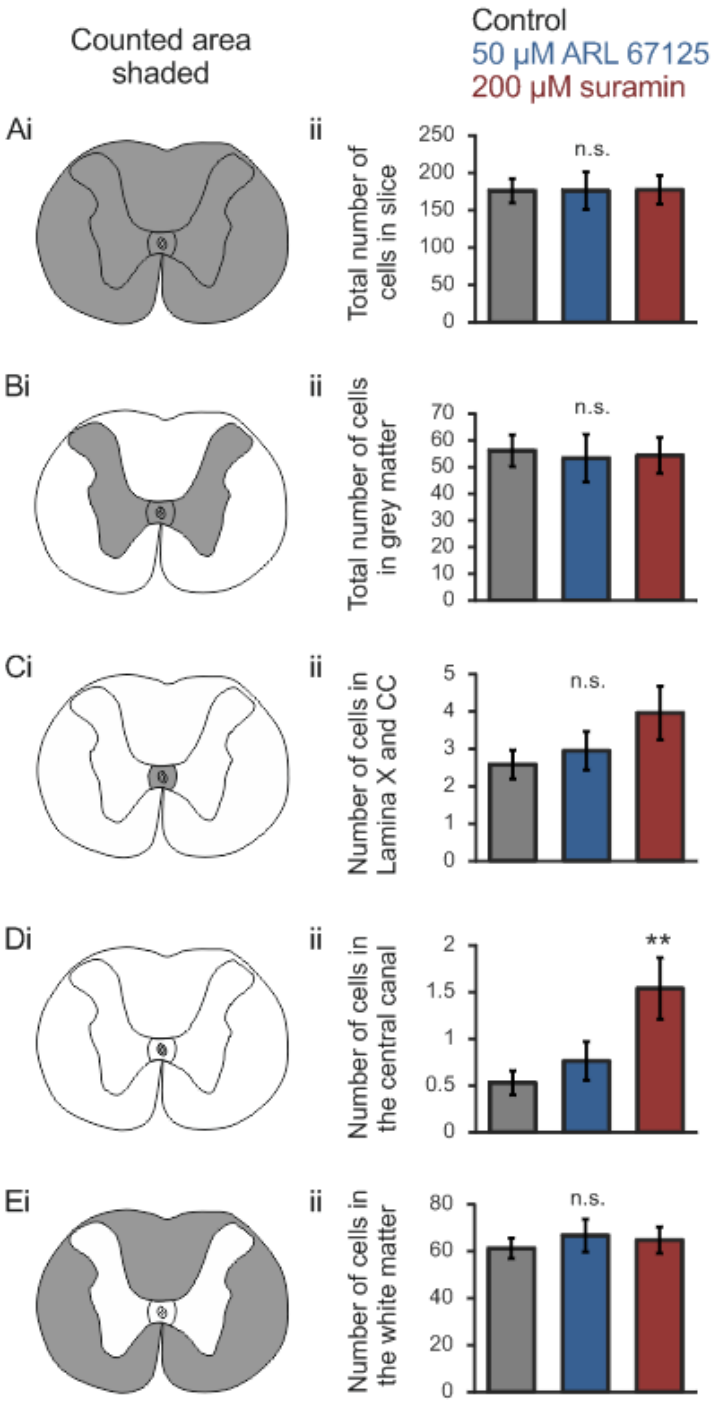


Figure 5.2 Suramin affects number of EdU+ cells near CC

Aii-Eii) Comparison of numbers of proliferated cells as determined by detection of EdU-incorporation into the DNA between the control condition and the conditions with altered purinergic signalling. Ai-Ei) Comparisons split by spinal cord region counted, with counted regions indicated by shading on schematic representation of the spinal cord. Areas counted were the whole slice (A), the grey matter including lamina X and CC (B), lamina X and CC (C), CC (D) and the white matter (E). For all spinal cord regions, counts were not normally distributed (as determined by Shapiro-Wilk) so comparisons used Kurskal-Wallis test to determine significance of difference.

5.2.2 Application of suramin or ARL 67125 affects the location of the newly-proliferated cells

To further investigate the difference seen in number of newly proliferated cells around the central canal with decreased purinergic signalling, the next experiment used 5 day organotypic spinal cord slice cultures, with the same modulators of purinergic signalling. In these experiments, the EdU positive cells could not be counted, as there were considerably more EdU positive cells due to the fact that the slices were in the presence of EdU for 3 days. This higher amount of EdU+ cells meant that there was frequent overlap between cells (Figure 5.4 B), especially around the central canal, making counting particularly difficult, either by hand or using image analysis packages. Therefore, the slices were imaged and the images processed to remove background fluorescence (Figure 5.3 Ai, Bi and Ci). The images were then analysed as a function of fluorescence intensity (in arbitrary units (A.U.)) laterally from the midpoint of the central canal out to the edge of the slice, for a 300 pixel (256 μm) strip at the level of the central canal (Figure 5.3 Aii, Bii and Cii). The value of the fluorescence intensity was taken as a representation of the number of EdU+ cells at that position in the slice. Within each condition, the data for each distance point away from the midpoint of the central canal was averaged, normalised to total to the same overall fluorescence (as equal levels of proliferation were demonstrated in both the proliferation preparation (section 5.2.1) and in culture experiments (section 5.2.3)) and then used to produce a running average (Figure 5.3 D). This showed that when the level of purinergic signalling was reduced by use of the purinergic antagonist suramin (n = 26; N = 10), there was a higher level of fluorescence intensity, and therefore likely more EdU+ cells, closer to the central canal relative to the rest of the slice, compared with control (n = 26; N = 11;Figure 5.4 B and C). This was a similar pattern to that observed with suramin in the proliferation experiments (see section 5.2.1), with more cells in the central canal area. Additionally, the running averages indicate that the opposite occurred with the ecto-ATPase ARL 67156 (n = 23; N = 7), where there were fewer cells in the central canal, relative to the rest of the slice, compared to both the control condition and the suramin condition (Figure 5.4 A).

One pattern in the locations of the newly proliferated cells that was observed often in the ARL and control conditions was a star shape pattern around the central canal (n = 5), with cells appearing to be placed on radial lines coming away from the central canal (Figure 5.4 D). Along these radial lines cells were elongated along the axis of

each radial line. This pattern was not seen in suramin conditions, where the central canal was often quite clearly defined, with a large proportion of cells contained within the CC (Figure 5.4 C). Another pattern occasionally observed in every condition was a concentration of EdU+ cells along the midline (n = 3) (Figure 5.4E).

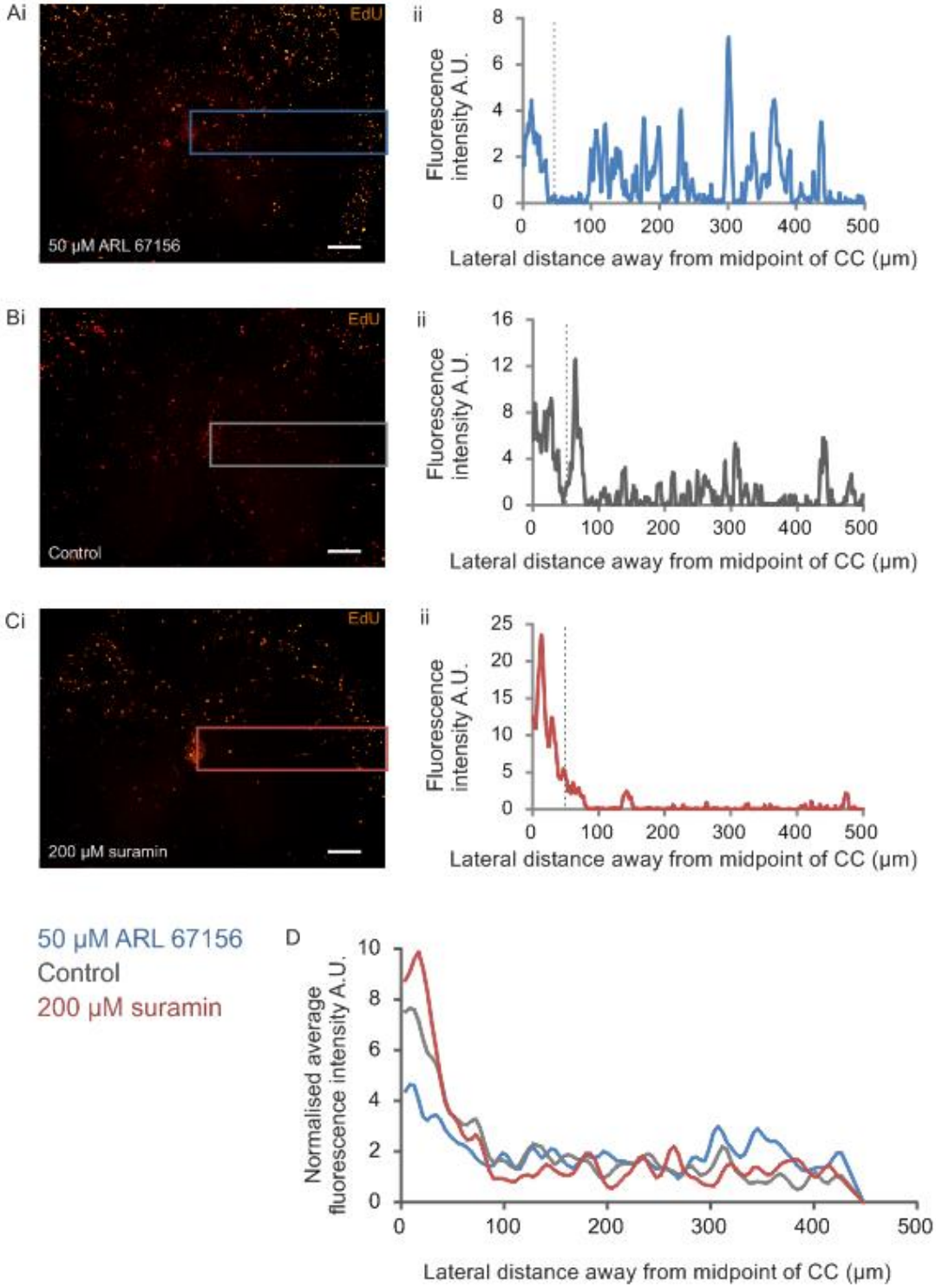


Figure 5.3 Application of suramin or ARL 67125 location of newly proliferated cells

Ai, Bi and Ci) Example images of cultures slices from each of the 3 conditions, ARL (A), control (B) and suramin (C) with the area analysed in Aii, Bii and Cii represented by the coloured box; EdU in red; scale bars = 200 µm. Aii, Bii and Cii) Fluorescence intensity as a function of lateral distance away from the midpoint of the central canal with dashed line representing the average edge of the central canal ependymal cell layer (40.85 ± 2.56 µm). D) Normalised running average for each of the conditions.

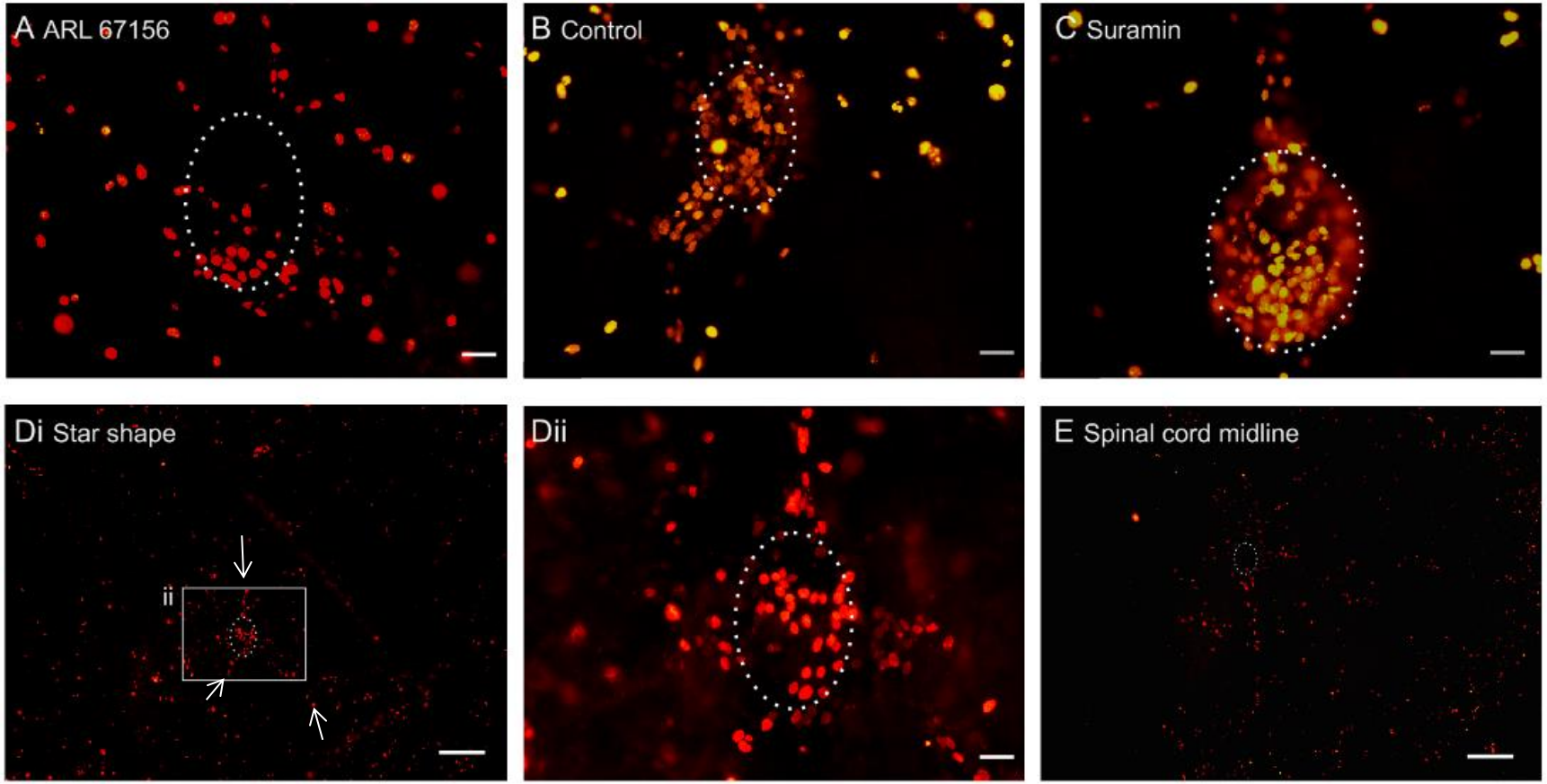


Figure 5.4 Patterns observed in 5 day culture experiments

A-C) Location of EdU+ cells around the central canal in ARL 67156 condition (incubated with 50 μM ARL 67156) (A), control condition (B) and suramin condition (incubated with 200 μM suramin) (C). D) A star-shaped pattern of EdU+ cells observed in a slice incubated with 50 μM ARL 67156 (arrows indicate prongs of starring). E) EdU+ cells concentrated along the midline of the spinal cord. Scale bars: 30 μm in A, B, C and Dii; 200 μm in Di and E.

5.2.3 Newly-proliferated cells migrate away from the central canal

Organotypic slice cultures fixed at different time points after EdU application were used to assess movement of the newly proliferated cells. After a 4 hour incubation with EdU on day 3 of culturing, one set of control and ARL slices were fixed (Control (3) (n = 24; N = 5) and ARL (3) (n = 22; N = 4) respectively) with the other set returned to EdU-free media, for a further 2 days to be fixed on day 5 (Control (5) (n = 34; N = 7) and ARL (5) (n = 43; N = 7); Figure 5.5). After fixation the EdU containing cells were visualised using immunofluorescence and imaged (Figure 5.5 Ai, Bi, Ci and Di). As with the previous experiment, fluorescence intensity images were produced for a 300 pixel (256 μm) strip (Figure 5.5 Aii, Bii, Cii and Dii).

These data were then used to produce normalised running averages as done previously for the 5 day culture experiments (Figure 5.6 Ai). When the section of the data nearest the central canal was analysed (Figure 5.6 Aii), the relative location of the peaks of fluorescence intensity indicating the EdU-positive cells for each condition could be identified. Both conditions fixed on day 3 (Control (3) and ARL (3)) showed a higher proportion of the fluorescence intensity near the central canal than in conditions fixed on day 5 (Control (5) and ARL (5)). When the ARL conditions were compared to the corresponding control condition, of those fixed in day 3, immediately after EdU incubation, the slices also incubated in 50 μM ARL 67156 had a higher proportion of fluorescence intensity in the central canal region compared to the control condition. However, after the slices had been returned to an non-EdU-containing medium for a further 2 days and fixed on day 5, the ARL (5) condition had a lower proportion of the fluorescence intensity and indicating a lower proportion of EdU-positive cells in the central canal region, compared to the control (5) condition.

The numbers of cells were also counted for the lateral half of slice on the side of which the fluorescence analysis was done but the entire half slice not a strip as was used for the fluorescence analysis. This showed that there were significantly more cells in both the 5 day conditions, compared to the 3 day conditions ($p = 0.03$ for ARL (3) and ARL (5); and $p = 0.17$ for Control (3) and Control (5); Figure 5.6 B), but that there was no significant difference between either of the ARL conditions and their corresponding control condition ($p = 0.524$ for ARL (3) and Control (3); and $p = 0.559$ for ARL (5) and Control (5).

Figure 5.5 Example fluorescence intensity traces from control and ARL 67156 conditions at 3 and 5 day time points

Schematic protocol for the migration experiments and examples from each conditions of the experiment: Control 3 (A), ARL 3 (B), Control 5 (C) and ARL 5 (D). Ai, Bi, Ci and Di) Example images from each of the 4 conditions indicating the area assessed in the fluorescence analysis; red fluorescence indicates EdU; scale bars = 200 μm. Aii, Bii, Cii and Dii) Fluorescence intensity (in arbitrary units (A.U.) as a function of the distance from the midpoint of the central canal for the 300 pixel strip of the image exemplified in Ai, Bi, Ci and Di.

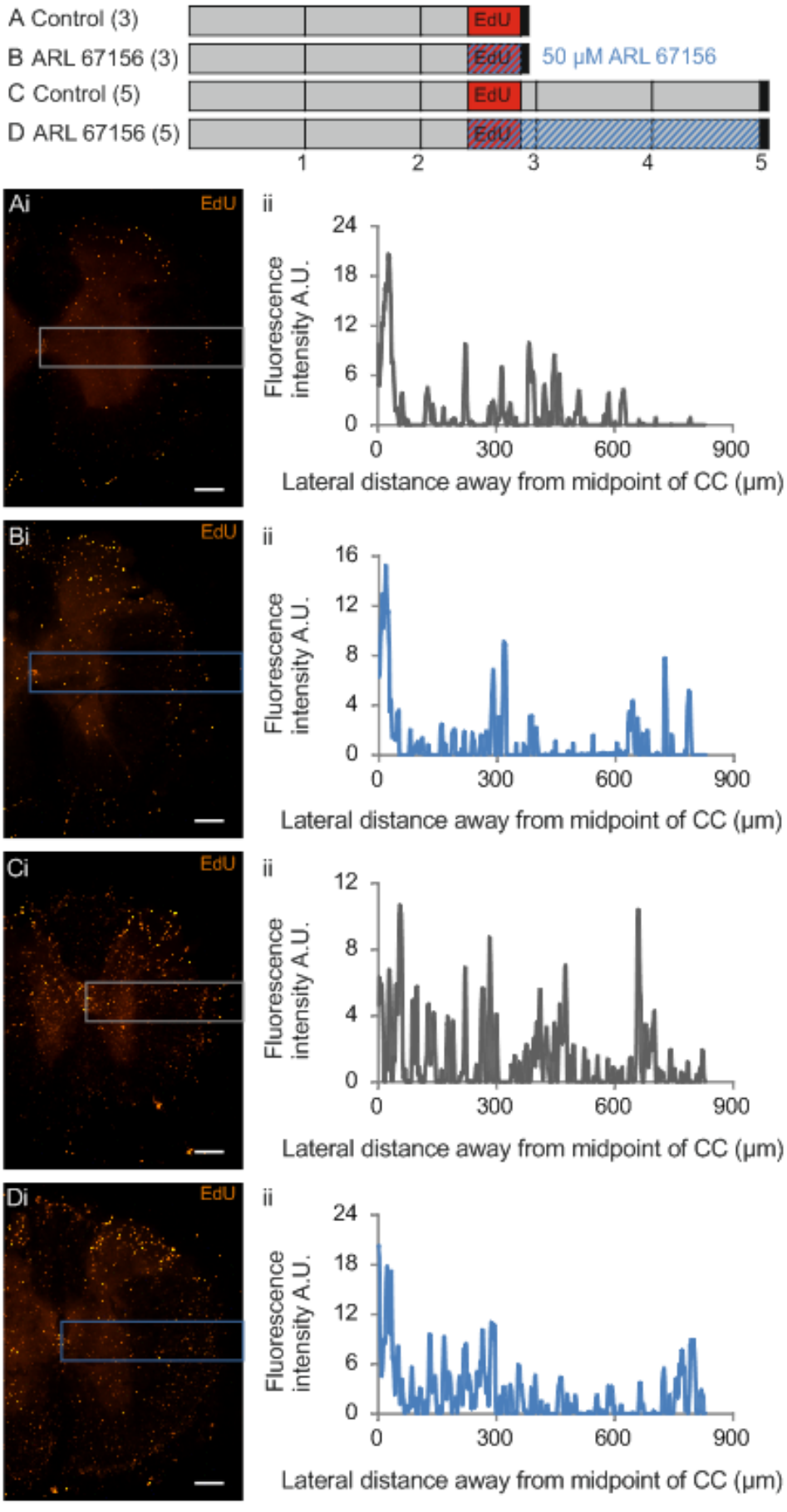
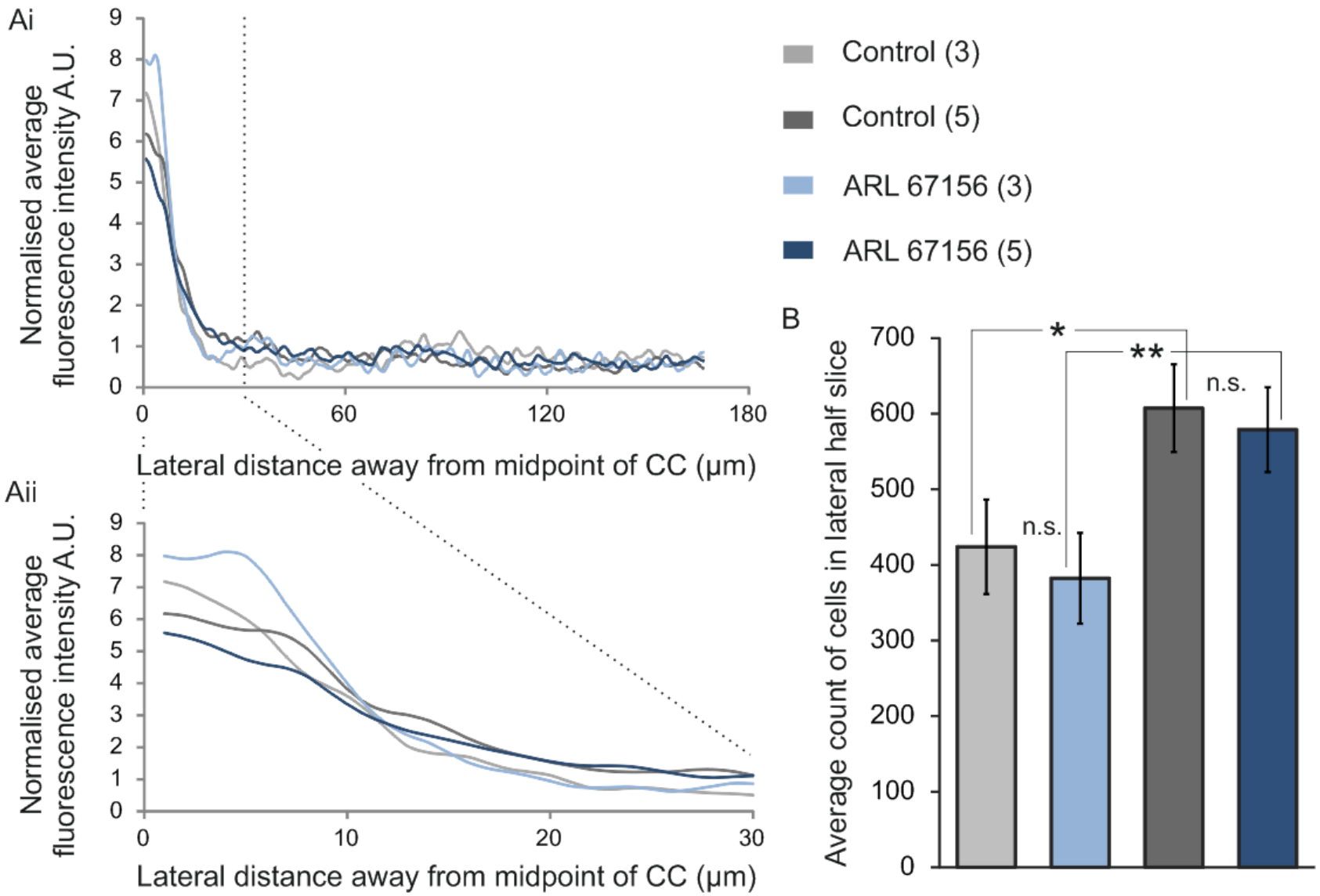

A Control (3)
B ARL 67156 (3)
C Control (5)
D ARL 67156 (5)
EdU
50 μM ARL 67156
1
2
3
4
5
Ai
ii
Bi
Ci
Di
Fluorescence intensity A.U.
Lateral distance away from midpoint of CC (μm)
0
300
600
900

Figure 5.6 Time point culture studies showed that application of ARL 67125 facilitates migration of EdU+ cells away from the CC without affecting number of EdU+ cells

Ai) Normalised running averages for each of the 4 conditions exemplified in Figure 5.4 A-D of the fluorescence intensity over a 300 pixel strip as a function of the distance from the midpoint of the central canal. Aii) A zoomed in version of the innermost 30 μm of Ai. B) Average counts of EdU positive cells in a lateral half of a slice for each of the conditions; ** = $p < 0.01$; * = $p < 0.05$; n.s. = $p > 0.05$.


Ai
Normalised average fluorescence intensity A.U.
Lateral distance away from midpoint of CC (μm)
Aii
Normalised average fluorescence intensity A.U.
Lateral distance away from midpoint of CC (μm)
Control (3)
Control (5)
ARL 67156 (3)
ARL 67156 (5)
B
Average count of cells in lateral half slice
*
**
n.s.
n.s.

5.3 Discussion

In the experiments presented in this chapter suramin has been used for its action as a broad-spectrum purinergic antagonist and ARL 67125 used for its action as ecto-ATPase inhibitor, to increase the concentration of extracellular ATP. Whilst suramin is known to have actions outside the antagonistic effects on purinergic receptors, including effects on cell migration via growth factor binding (Takano et al., 1994), the functions of ARL 67125 are less varied. As the effects seen with suramin are opposed by the effects seen with ARL 67125, this provides support for the function of suramin as a purinergic antagonist been significant in these responses. The results presented above are discussed below based on this argument for the role of purinergic signalling in these responses.

5.3.1 Purinergic signalling did not affect proliferation rate of stem cells but does affect the location of the newly proliferated cells

The effect of modulation of purinergic signalling on proliferation was measured over a relatively short period such that the extent of cell death on the number of EdU-positive cells was minimised. This method did not replicate the conditions of non-injured, intact spinal cord as the numbers of newly proliferated cells after 4 hours in EdU were far higher than in previous research where EdU has been injected intraperitoneally daily for 4 days prior to fixation (Corns et al., 2015). Given that damage to the spinal cord increases the amount of cell proliferation (Barnabé-Heider et al., 2010), this suggests this slice preparation model causes some outcomes of damage to be present in the tissue, likely to be due to the sectioning process, which then causes the higher levels of proliferation seen.

The results from the proliferation preparation indicated that there is no effect of suramin-sensitive purinergic signalling on the proliferation rate of the cells of the spinal cord. An important point to consider is that all the proliferation and culture experiments used suramin as the antagonist. This is significant because of the two response types observed in ependymal cells and CSFcCs, only the depolarising response in CSFcCs was sensitive to suramin, with the hyperpolarising response to ATP seen in ependymal cells and some CSFcCs insensitive to suramin. However the compound used to increase purinergic signalling was an ecto-ATPase inhibitor, the effect of which was to reduce the breakdown of ATP, thereby increasing the amount of extracellular ATP and therefore the amount of purinergic signalling. It is also worth

noting that this will have the impact of reducing the amount of the dephosphorylation products of ATP such as ADP, AMP and adenosine. The differing nature of the drugs applied allowed differentiation of the cause of any effects observed. Any differences seen with suramin will be due to suramin-sensitive signalling, including the depolarising responses of CSFcCs, whereas, any differences seen just with ARL could be due to a wider range of purinergic signalling pathways.

Whilst EdU (ethynyldeoxyuridine) and another commonly used thymidine analogue BrdU (bromodeoxyuridine) are frequently used as proliferation assays (Taupin, 2007; Mead and Lefebvre, 2014), they work by replacing the thymidine nucleoside during DNA replication. In addition to DNA replication, nucleosides have also been shown to be incorporated into DNA during DNA repair (Kao et al., 2001; Lee et al., 2011). BrdU has been shown to be incorporated into DNA during DNA repair (Yoshii et al., 2016) as shown by comparison with phospho-histone H3, a marker of cell mitosis. This research has looked at BrdU but it is highly likely that these mechanisms occur with EdU too as the differences between EdU and BrdU are in the detection methods, rather than the incorporation methods (Mead and Lefebvre, 2014). However, the assessment of EdU labelling in this study did not quantify total EdU, instead it looked at the cells in which it was expressed. It is unlikely that the amount of EdU incorporated for DNA repair would come close to the amount incorporated during DNA replication, so EdU incorporated due to DNA is likely to appear much fainter than that for replication. As only clear cells were counted, it is unlikely to have affected the counts significantly. In the fluorescence analysis, it could have some effect as faint cells were not ruled out, however most cells appeared brightly stained, and thus will have had a far larger effect on the overall analysis. It is also worth noting that EdU has been observed to decrease cell survival and increase cell volume of surviving cells in transplanted myogenic precursor cells (Andersen et al., 2013). This is an effect that must be considered, although, as all conditions included EdU application, this should not create any confounding effects for between condition comparisons.

For these experiments all tissue used was taken from thoracic or lumbar levels. This was due to requirements to balance consistency of results and to maximise the use of animal tissue and the numbers of slices needed to populate the conditions. The proliferation under physiological levels is not the same along the rostro-caudal axis of the spinal cord (Blasko et al., 2012). Under physiological, non-injured conditions,

proliferation is very low within the ependymal region with sacral/coccygeal regions showing the highest levels of proliferation and cervical showing the lowest. Thoracic and lumbar regions have comparable levels of proliferation and it is for these reasons that these levels were used for these experiments. Also significant for these experiments was the numbers of CSFcCs along the rostro-caudal axis of the spinal cord. Unpublished work from our group has shown that there are higher levels of CSFcCs in the thoracic and lumbar regions compared to the cervical and sacral regions, and that the levels are not different between the thoracic and lumbar regions (Daniel, 2015).

Paired cells were frequently observed in all conditions, which could be indicative of recent division. Immunohistochemistry for other markers could give indication if symmetric or asymmetric division is occurring but it is highly likely that some of these occurrences of cells near each other is merely co-incidence and as such, the type of division happening could not be stated with certainty using these experimental protocols.

Despite there being no difference in the total number of newly proliferated cells in the slices between the conditions when the entire slices were counted, there was a difference when only the central canal region was counted. The slices where the suramin-sensitive currents had been inhibited by application of suramin had significantly more cells in the central canal region than in the control and ARL condition where the level of extracellular ATP had been raised.

This pattern of greater numbers of cells in the central canal region relative to the rest of the slice in slices with inhibited suramin-sensitive currents was also seen in the organotypic slices culture experiments where the slices were in the presence of EdU and purinergic modulators for 3 days. In this experiment the converse was also seen, with fewer EdU-positive cells around the central canal, and more in the grey and white matter in the ARL condition. In this experiment EdU-positive cells were not counted as there were too many to distinguish, however the proliferation experiment demonstrated that there was not a difference in number of newly proliferated cells between the three conditions and this same lack of difference was shown between control and ARL conditions in 3 and 5 day cultures. This still leaves the question of whether these longer culture experiments were differentially affected by cell death. In the time-point experiments, EdU-positive cells were counted and there was no

significant difference between the numbers of EdU-positive cells in control or ARL conditions either immediately after EdU application or when kept for the same duration of time as for these culture experiments (note there was no suramin condition in these time-point experiments). This suggests that the changes in relative proportion of cells in certain slice regions with purinergic modulation were not due differential rates of cell survival of the newly-proliferated cells.

It is important to note that ependymal cells are not the only cells proliferating in these slices. There are also astrocytes and oligodendrocyte precursors that proliferate both under intact and injured conditions (Barnabé-Heider et al., 2010). Of the three cell types, ependymal cells undergo the lowest rate of proliferation in uninjured tissue, but as shown by the higher rate of proliferation in the slices from these experiments compared to *in vivo* experiments, this experimental model involves injury to the cells and produces a response in line with the increases in proliferation rate seen after injury. Despite there being increases in the proliferation rate of all these cell types with a transverse cut in the dorsal funiculus at the injured segment compared to the adjacent segment, the greatest increase was in the ependymal cells as determined by genetic fate mapping (Barnabé-Heider et al., 2010).

There are two different possibilities for the differences in location of newly proliferated cells within the 5 day cultured slices with different levels of purinergic signalling. The first is that increased purinergic signalling is increasing the proliferation rate of one of the cell types dispersed around the spinal cord slice and decreasing the proliferation rate of the ependymal cells within the central canal region by an equal amount (working on the assumption of equal levels of proliferation as demonstrated by the proliferation experiment). The second is that the increased purinergic signalling is not altering the proliferation rate of any of the cell types but is halting the migration of the ependymal cells away from the central canal region. The relation of these findings to previous research is discussed in the following section.

5.3.2 Location of cells following spinal cord injury

Ependymal cells have been shown to be located exclusively within the central canal region in non-injured animals, however after spinal cord injury, the majority of the newly-proliferated progeny of ependymal cells were found to be in the core of the scar tissue within the injury site, in this case the dorsal funiculus (Barnabé-Heider et

al., 2010). Some ependymal cell progeny have also been identified dispersed around the grey and white matter (Meletis et al., 2008). However cells of ependymal lineage were not the only cell type of the newly-proliferated cells to change the location. Newly-proliferated cells from astrocytic lineage were also observed to be located in the region of the injury, however in their case they appeared to be located on the perimeter of the scar tissue (Meletis et al., 2008; Barnabé-Heider et al., 2010).

Other studies have used time-points to observe migration of the ependymal cells away from the central canal towards the site of a minimal needle injury (Mothe and Tator, 2005). This study utilised DiI labelling to trace only the cells originating from the central canal region, as DiI is a lipophilic tracer that is irreversibly incorporated into the cell membrane. They showed that with a minimal injury, GFAP negative ependymal cells migrated away from the central canal by about 70 μm between 3 and 14 days post injury. In this minimal injury model, no ependymal progeny were observed in the injury site but given the data above, it is likely that the stimulus from the minimal injury wasn't large enough.

The data from the time-point organotypic slice culture experiment supports these findings that the ependymal cells are migrating away from the central canal region and provides evidence that purinergic signalling is involved in the facilitation of this migration. Other studies have reported ependymal cells migrating towards the site of the injury, however in the preparation used for these culture experiments, there is no specific injury site, with the injury factors most likely being released from the damaged layer of cells from the top and bottom of the slice. For this reason, despite the fact that purinergic signalling was not increased by adding ATP but by inhibiting the breakdown wherever the extracellular ATP was in the slice, migration towards any damage mediators would likely be towards the surfaces of the entire slice. This could be assessed in future experiments by use of confocal imaging at different z levels.

The time-point experiments showed that immediately after incubation with ATP there is a greater proportion of the newly proliferated cells within the centre of the slice, near the central canal region, however after a further 2 days in a non-EdU-containing medium, a greater proportion of the newly proliferated cells was located further out into the grey and white matter of the slice. There were significantly more cells in the condition where the slices had a further 2 days in non-EdU-containing medium indicating that further divisions occurred between EdU containing cells, marking both

progeny of those divisions, however the numbers of EdU containing cells did not even double, suggesting that most did not proliferate more times after that period.

This study also looked at the 2 time points in slices incubated with ARL to increase purinergic signalling. At the first time point, it appears that there is a greater proportion of cells in the central canal area in the ARL condition, compared to the control condition. The hypothesis for this experiment was that the cells would migrate away from the central canal at the later time point and that increased purinergic signalling by ARL would enhance this migration. It would have been expected that the ARL would have minimal effect at the first time point as there had not been time for much migration of newly-proliferated ependymal cells to occur. It must be remembered that by the time that the EdU and ARL is applied, there are already ependymal cells, unlabelled by EdU, that are differentiating and migrating away from the central canal as there is already endogenous ATP released by the damage caused by slicing. It is also likely that the ARL has a lower effect earlier in the experiment, when there is likely to be more extracellular ATP anyway such that there would still be purinergic signalling occurring even without inhibition of the ATPase enzymes. When the later time point ARL condition was compared to the later time point control condition, there was a smaller proportion of ependymal cells in the central canal area compared to the entire slice, similar to what was seen in the previous culture experiments. These experiments don't tag the cells, so we cannot prove that the greater proportion of cells away from the central canal region originate from the ependymal cell layer but there are factors which when considered with the previous research that has tagged the cells, support this conclusion.

5.3.3 The migration of spinal cord ependymal cells

One factor supporting the role for purinergic signalling in the migration of ependymal cells away from the central canal is the position of the cells around the central canal in the slice culture experiments. In some of the ARL cultured slices, groups of ependymal cells appear to be placed along strands with cells on these strands having an elongated shape, the long axis of which is perpendicular to the ependymal cell layer. This pattern was not observed in slices incubated in suramin, where there was sometimes a very clear outline of the central canal region, with a large proportion of the EdU-positive cells contained within this region.

It is likely that these patterns around the central canal and further out into the spinal cord are similar to the patterns of nestin expression. Nestin is an intermediate filament protein which is expressed during development in immature CNS cells and in multiple glioma cell lines (Tohyama et al., 1992). The expression during development is not maintained and by P6 there is no immunoreactivity for nestin around the central canal (Hockfield and McKay (1985) where nestin was identified by Rat-401). However nestin is expressed in stem cells within the subventricular zone (Morshead et al., 1994) and is re-expressed in the ependymal cells around the central canal after spinal cord injury (Frisén et al., 1995; Shibuya et al., 2002; Mothe and Tator, 2005). After spinal cord injury nestin expression is present in processes extending away from the ependymal cell layer, at the injury site and is also found in the white matter of the spinal cord, with processes extending into the grey matter (Shibuya et al., 2002). The nestin expression at the white matter was co-localised with GFAP suggesting that it was due to precursor sub-pial astrocytes. In injury models the patterns of newly-proliferated cells from the central canal (as traced by DiI labelling) mimic the patterns of nestin labelling as well as the cells themselves being nestin positive (Johansson et al., 1999; Mothe and Tator, 2005). This matched the pattern of EdU-positive cells seen in the culture experiments presented here where the greatest concentration of EdU-positive cells was around the central canal and in the white matter. In another neural stem cell, population nestin was shown to be necessary for the migration of the neural stem cells (Yan et al., 2016). It would be of interest for future experiments to investigate the effect of inhibition of suramin-sensitive purinergic signalling on nestin expression to investigate if purinergic signalling is involved in the re-expression of nestin fibres or the ability of cells to migrate along these fibres.

Another protein that is involved in migration of ependymal cells that may be affected by purinergic signalling is the transcription factor ATF3 which co-localises with nestin following spinal cord injury (Mladinic et al., 2014). These also co-localise with vimentin and Sox2. Nestin and AFT3 staining was observed around the central canal and in processes extending away from the central canal, in the white matter and also along the ventral and dorsal midline away from the central canal. This is of interest as in some of the slices from the 5 day culture experiments, a concentration of EdU positive cells was observed along this midline. There appear to be species differences in ATF3 expression with this matched expression with nestin being seen

in rats, but while nestin expression is present in mice (Krityakiarana et al., 2016), ATF3 expression was not observed (Mladinic et al., 2014). This suggests that nestin expression may be more significant in the culture experiments as the slices used were from mouse spinal cord.

Another factor involved in the migration of ependymal cells is the chemotactic cytokine CXCL12 (or SDF1), which has effects on a range of cell types (Jaerve et al., 2012). Levels of CXCL12 were increased for 2-42 days after moderate and severe spinal cord injury (Friederike Knerlich-Lukoschus et al., 2011). The raised levels of CXCL12 promote migration of oligodendrocyte precursors (Dziembowska et al., 2005), CXCR4-positive macrophages and activated microglia (Tysseling et al., 2011) and transplanted neural stem cells (Takeuchi et al., 2007) towards the site of injury. Ependymal cells have been shown to express CXCR4 (Tysseling et al., 2011) levels of which are also elevated after spinal cord injury, peaking after 7 days (Bai et al., 2016) and are therefore also likely to migrate towards concentrations of CXCL12. Other responses to increased levels of CXCL12 include stimulation of axonal sprouting (Jaerve et al., 2011) and recruitment pericytes for revascularisation (Graumann et al., 2010).

Purinergic signalling via both P2X and P2Y receptors has been shown to interact with chemokine-based chemotaxis in a range of cell types. In human mesenchymal stem cells (hMSCs), there has been a connection shown between purinergic signalling and the chemotactic response to CXCL12 (Ferrari et al., 2011). Application of ATP, UTP, ADP, UDP and BzATP were all able to elicit increases in internal Ca^{2+} concentration which were reduced in magnitude when the extracellular solution was depleted of Ca^{2+} indicating a significant role for ionotropic P2X receptors in the intracellular Ca^{2+} increases seen. Pre-incubation with ATP to hMSCs pushed the cells from a proliferative state to a state sensitive to CXCL12-triggered chemotaxis as shown by an upregulation of genes involved with migration. While application of ATP alone did not increase migration in these cells, application of ATP but not UTP significantly increased the chemotactic response to the chemokine attractant CXCL12 (Ferrari et al., 2011).

In other cell types there is involvement of P2Y receptors in the chemotactic response to cytokines. In THP-1 cells (a human monocyte cell line) and human peripheral mononuclear cells (PBMCs) the Ca^{2+} response to application of the cytokine CCL2 is

modulated by the levels of extracellular nucleotides (Campwala et al., 2014). Scavenging of extracellular nucleotides by apyrase reduced the Ca^{2+} response to CCL2 by up to 80% and strongly reduced the chemotactic response of THP-1 cells to CCL2, while intracellular Ca^{2+} levels increased further upon CCL2 application when nucleotide levels had been increased by application of ARL 67156. Unlike in hMSCs where P2X receptors where suspected to mediate the response, $P2Y_6$ receptors have been implicated in this interaction as application of a specific $P2Y_6$ antagonist reduced both the Ca^{2+} response and the chemotactic response to CCL2 application (Campwala et al., 2014).

Additionally, purinergic signalling was shown to be involved in the chemotactic response to other chemotactic peptides. The chemotactic response to application of fMLP was also inhibited by apyrase, as was the Ca^{2+} response but in this case, the $P2Y_6$-selective antagonist had no effect indicating a different purinergic signalling receptor was involved in the response to fMLP (Campwala et al., 2014).

A similar involvement of purinergic signalling in chemokine-mediated migratory responses has been seen in hematopoietic stem cells which migrate towards CXCL12 (Rossi et al., 2007). This response is sensitive to pertussis toxin, indicating the receptors involved are P2Y receptors coupled to the $G_{\alpha i}$ intracellular proteins. Expression analysis of these hematopoietic stem cells confirms that the response is elicited by $P2Y_2$ receptors as supported by the fact that ATP produced a similar response. As with THP-1 cells, increased levels of extracellular nucleotides, achieved by UTP application, increased the Ca^{2+} response to chemokine (CXCL12) application (Rossi et al., 2007). These studies show a tendency for involvement of purinergic signalling and concentration of extracellular nucleotides in increasing the migratory response towards other chemotactic factors.

Whilst it cannot be said for certain which cells and which receptors are responsible for eliciting the differences in levels of migration in newly-proliferated cells from the ependymal layer in the culture experiments detailed in this chapter, we can exclude certain receptors. Despite the hyperpolarisations being consistently shown in ependymal cells in response to local application of ATP, they were not sensitive to suramin, and suramin application produced a clear effect in the culture experiments. It has previously been suggested that the $P2X_7$ receptors expressed by ependymal cells could act to detect damage such as spinal cord injury (Marichal et al., 2016) as

its low affinity to ATP and increased permeability with sustained application of ATP could be useful characteristics. However $P2X_7$ is relatively insensitive to suramin, requiring at least 500 μM (Kennedy, 2005), which is significantly higher than the slices in these culture experiments received, suggesting that these responses here are not mediated by $P2X_7$. We cannot rule out involvement of other cell types not recorded from in the patch clamp experiments as some spinal neurons also express P2X receptors (Hugel and Schlichter, 2000; Aoyama et al., 2010). Although there are likely be other cells which detect the rise in ATP due to injury and extended by application of ARL, the proximity of the CSFcCs to the ependymal cells and their features suggesting sensory roles make them a viable candidate for involvement in the response of ependymal cells to extracellular ATP and to spinal cord injury.

5.3.4 Conclusions

The results presented in this chapter indicate that modulation of purinergic signalling has no effect on the proliferation rate of cells of the spinal cord in an organotypic slice model with features of spinal cord injury. There is also indication that it doesn't affect the survival rate of the newly-proliferated cells. However, the data does support a role for purinergic signalling, via the increases in extracellular ATP seen in spinal cord injury (Wang et al., 2004), in the migration of the newly-proliferated cells away from the central canal region.

This is not the first cellular system in which purinergic signalling has been implicated in migration. As mentioned above, ATP, UTP and nucleotide diphosphates have been implicated in roles promoting migration either acting directly or by enhancement of the action of other chemotactic factors. Extracellular ATP, but not UTP, was shown to induce migration in human mesenchymal stem cells, hypothesised to be via a P2X receptor (Ferrari et al., 2011). In hematopoietic stem cells UTP acts to enhance the migration of the cells to the chemoattractant CXCL12 in the bone marrow, via activation of $P2Y_2$ receptors (Rossi et al., 2007). Conversely in rat oligodendrocyte precursor cells, $P2Y_1$ receptors are responsible for the stimulation of migration and differentiation by either extracellular ATP or ADP (Agresti et al., 2005a). These indicate that purinergic signalling is frequently involved in the promotion of migration, often towards a concentration of CXCL12 or other chemotactic factors and that these responses are mediated by a range of purinergic receptors. The fact that the migratory effects were sensitive to suramin provide support for the suggestion that

currents due to $P2X_2$ and $P2X_3$-containing receptors in CSFcCs could act on ependymal cells and be responsible for the migratory response, however further experiments using more specific antagonists would be need to be done to confirm this.

Chapter 6 - General Discussion

6.1 Impact of this study

This study has demonstrated the complexity of the responses of ependymal cells and CSFcCs around the central canal to purinergic signalling and has demonstrated a role for this signalling in the migratory responses in an organotypic slice culture model that has characteristics of an injury situation. There are multiple aspects to consider with respect to these findings and the directions in which future studies could build on this work. Whilst levels of extracellular ATP will be affected by multiple factors both in the healthy and injured or diseased spinal cord, the discussion below focuses on the situations where ependymal cell proliferation and differentiation has been previously shown to be activated. The pathological conditions discussed are spinal cord injury, Amytrophic Lateral Sclerosis and Multiple Sclerosis, particularly spinal cord injury as ATP release has been demonstrated to occur and have functional effects (Wang et al., 2007).

6.1.1 Applicability of these findings to spinal cord injury

A role for ependymal cells in spinal cord injury

It is difficult to dissect specifically the role of ependymal cells in spinal cord injury and the effects of their modulation due to the multitude of responses of other cell types in SCI. Some studies have made use of animal strains with selective deletion of Ras genes involved in mitosis in only FoxJ1 expressing cells (ependymal cells) upon application of tamoxifen (Sabelström et al., 2013). This method allowed investigation of the effect of removing the proliferative abilities of ependymal cells but without affecting them during development. The removal of ependymal cell proliferation reduced the glial scar and increased the injury depth over the following 9 weeks, whereas the group with normally proliferating ependymal cells showed reduction in injury depth over this time period. This deeper injury site was caused by increased cell death, likely due to reduced release of various survival-promoting factors by ependymal cells due to their reduced numbers. This study indicates that ependymal cells play an important role in the response to spinal cord injury and that loss of them has negative effects on the survival of neurons.

The converse has also been demonstrated with enhancement of the numbers of ependymal cells via transplantation of ependymal-derived stem/progenitor cells (epSPCs), cultured from ependymal cells *in vitro*, correlating with increased recovery

from spinal cord injury. Transplantation into the injured spinal cord of cultured ependymal cells from intact spinal cord (epSPCs) and from injured spinal cord (epSPCis) have both been shown to be beneficial (Parr et al., 2008; Moreno-Manzano et al., 2009). There were differences in the proliferation rate and rate of formation of differentiated cells between epSPCs and epSPCis *in vitro* but these differences did not render either epSPCs or epSPCis ineffective after transplantation. It may be that the differences between epSPCs and epSPCis are reduced after transplantation into injured cord where the injury-induced activation that occurred to epSPCis prior to culturing happens to epSPCs after transplantation into the injured cord. While the culture process may have changed the capabilities of the cells, the fact that the cultured epSPCs still had the capability to respond to spinal cord injury might suggest that the effect of culturing does not change the cells significantly. This would indicate that the beneficial results seen with transplantation of epSPCs or epSPCis is due to increased presence and action of ependymal cells rather than a unique quality of the cultured cells. The neurogenic potential of epSPCs and particularly epSPCis is not maintained *in vivo*, with the majority of transplanted epSPCs maintaining an undifferentiated state (Moreno-Manzano et al., 2009). This suggests that the positive results from transplantation of epSPCs/epSPCis are largely due to the increased numbers of ependymal cells responding to the spinal cord injury, rather than extra capabilities due to the culturing process. These studies that have reduced and increased the number of ependymal cells in spinal cord injury models have provide support for the idea that despite the increases in potential cell fates of ependymal progeny, a significant part of their role in the response to spinal cord injury is a structural and supporting role, stabilising surviving tissue and minimising the damage caused by the secondary injury of spinal cord injury.

Applicability of organotypic slice cultures to spinal cord injury

The study presented here did not use a spinal cord injury model but there is evidence to indicate that this organotypic slice culture protocol retains some of the features of damage caused by the slicing procedure. One way that this is demonstrated is the far higher rate of proliferation both in the central canal region and more generally across the white and grey matter of the spinal cord in slices where EdU was applied during organotypic slice culture experiments compared to spinal cord slices with EdU applied *in vivo*. Previous work from our group has used the same animal strain and the same EdU detection technique to look at rate of proliferation both *in vivo* and in

organotypic slice cultures (Corns et al., 2015). This work is particularly relevant to consider as the organotypic slice cultures followed the same protocol as those used in this study, allowing the conclusions regarding the effects of that method protocol to be applied to the data presented here. In 50 μm sections from the *in vivo* application of EdU (daily injections for 4 days of 0.1 mL of 10 mM EdU) Corns et al. (2015) saw 1.2 ± 0.3 EdU positive cells in the central canal region. However, in 300 μm organotypic cultured slices which had received a single application of 1 μM EdU left on for 16 hours 76 ± 5 EdU positive cells were observed around the central canal region. These experiments cannot be directly correlated as different concentrations and timings of EdU were applied but given that EdU was applied 4 times *in vivo* it is unlikely that the *in vivo* tissue received less EdU than the cultured tissue. Therefore as there are far higher numbers in the cultured experiments, even after accounting for the difference in slice thickness, that would suggest that this is due to a higher proliferation rate. Within the *in vivo* spinal cord, a higher proliferation rate is associated with spinal cord injury (Cizkova et al., 2009; Barnabé-Heider et al., 2010; Lacroix et al., 2014), suggesting that the organotypic slice cultures retain some features of the injured state.

Supporting this is the cell fates observed in organotypic slice cultures which show more similarity to the cell fates observed in spinal cord injury than those that are observed in the intact spinal cord injury. Again, previous work from our group provides a good system to compare the fates of cells in organotypic slice cultures and intact *in vivo*, as, since the ependymal cells are not tagged, only the cells around the central canal could be assessed and use of the same study ensures the area assessed was consistent. In slices from intact spinal cords with *in vivo* EdU application, there was no co-localisation between EdU and the astrocytic marker GFAP, the oligodendrocytic marker PanQKI nor the neuronal marker NeuN around the central canal (Daniel, 2015). However when the same markers were assessed for co-localisation in the organotypic slice cultures around the central canal region there was some co-localisation seen with both GFAP and PanQKI but not NeuN. Approximately 2.5 % of EdU+ cells expressed the astrocytic marker GFAP and 7.3 % of EdU+ cells expressed the oligodendrocytic marker PanQKI. While we cannot draw exact connections between these percentages and those observed in spinal cord injury due to those results not presenting co-localisations specifically within the central canal region and using different markers for each cell type, there were

considerable similarities. After spinal cord injury, approximately 25 % of cells labelled by the FoxJ1 transcription factor, the marker for the ependymal cells, expressed GFAP and approximately 4 % express the oligodendrocyte marker Olig2 (Barnabé-Heider et al., 2010). These proportions, while not identical, are reasonably similar considering the different populations of cells assessed, especially as FoxJ1-positive GFAP-positive cells were found mainly in the core of the injury site (Barnabé-Heider et al., 2010), explaining the far higher rate of GFAP expression in all ependymal cells (in the SCI experiment), compared to those just around the central canal (in the cultured slices).

Whilst this does not indicate that organotypic slice cultures produce the exact same response as spinal cord injury, it clearly indicates that in multiple respects the behaviour of ependymal cells in organotypic slice cultures bears more similarity to ependymal cells after spinal cord injury than ependymal cells in the intact spinal cord.

It is likely that the injury aspect comes from the damage to the top and bottom of the slice caused by the slicing procedure. Studies that have looked at the location of the newly-proliferated cells and particularly those from the ependymal cells after spinal cord injury used a dorsal funiculus incision (Barnabé-Heider et al., 2010). In this study, cells of ependymal lineage are found in the centre of the lesion site and therefore must have migrated from the ependymal layer around the central canal to the injury site. In the organotypic slice cultures presented in this work the cells were observed migrating away from the central canal in all directions and due to the nature of the protocol, it is likely that the damage done to the spinal cord tissue during the slicing procedure is the damage being sensed by the cells of the spinal cord.

It is important to recognise that results from organotypic slice cultures will not necessarily translate to spinal cord injury studies. This may be due to the retention of some factors but not others, as there is inevitably washout of some damage related factors and inability of influx of cells from other regions of the spinal cord or from outside the spinal cord. Any lack of translation of results to spinal cord injury could also be due to considerable differences in the effect of different spinal cord injury models on the central canal region. While partial transection models do not injure the central canal region, compression and contusion models frequently used for their consistency and relevancy to the types of spinal cord injury seen most frequently in humans include damage to the central canal (Cheriyan et al., 2014). The study by

Wang et al. (2004) imaged ATP release after a contusion spinal cord injury, it is not known how this may differ in the dorsal funiculus incision protocol that is often used for cell migration experiments (Stenudd et al., 2015). Additionally the cells within the central canal region will likely be differently affected factors in contusion injury compared to incisions by a range of other such as hypoxia, change in pressures and other factors released after spinal cord injury.

Applicability of the work to adulthood

Another important point to consider when discussing the translatability of this work is the fact that this work was all done on immature spinal cord tissue. Both the electrophysiological recordings and the organotypic slice cultures used spinal cord slices from animals under 3 weeks of age, which is well before the spinal cord has reached its full adult length. In the mouse spinal cord the number of dividing cells around the central canal is affected by the age of the animal (Alfaro-Cervello et al., 2012), with the highest proliferation rate around the central canal observed at 1 week of age. The proliferation rate is slightly lower at 2 and 4 weeks and considerably lower by 13 weeks (Alfaro-Cervello et al., 2012). This very low baseline level is maintained through adulthood but is increased upon spinal cord injury or exercise (Cizkova et al., 2009; Barnabé-Heider et al., 2010; Lacroix et al., 2014). This means that these experiments were done at ages with higher baseline levels of proliferation in the ependymal cells, but the fact that the responses under study had aspects of injury situation means this is less significant, because the responses to injury are maintained in adulthood. There may however still be differential responses to different signalling pathways and factors so the exact responses in adulthood would have to be fully investigated to draw conclusive conclusions as to the effect of purinergic signalling in the adult spinal cord. Importantly, however, the maintenance of the response to injury in adult mammals suggests similar responses that are not altered by the fact that the spinal cord has finished its growth.

6.1.2 Applicability of these findings to humans

The human spinal cord central canal area and ependymal cells

Multiple studies have reported that the patency of the central canal in the adult human spinal cord is not as consistent as that of adult rat and mouse spinal cords most commonly used for investigations into the central canal region and spinal cord

injury. Autopsy investigations have suggested that there is closure or partial closure of the central canal in human spinal cord at increasing incidence rates with age (Milhorat et al., 1994; Yasui et al., 1999). These findings showed that there was at least partial closure in the majority of individuals by adolescence (13 – 29 years). This autopsy data has been supported by MRI scans showing a similar lack of patency of the central canal region of the adult humans spinal cord (Garcia-Ovejero et al., 2015). This study also looked at the morphology of the ependymal regions where the central canal displayed closure and saw a dense accumulation of cells without any clear lumen or ependymal layer. The ependymal region displayed considerable presence of glial cells as shown by expression of GFAP-positive processes spreading through this region. There were groups of vimentin-positive cells identified as ependymocytes which possessed intercellular junctions, cilia and expressed CD15, features of ependymal cells (Sabourin et al., 2009; Garcia-Ovejero et al., 2013). There were some ependymocytes observed surrounding pseudo-canals but also sometimes observed surrounding blood vessels, called perivascular pseudo-rosettes (Garcia-Ovejero et al., 2015). This latter feature was deemed of significance due to its presence in ependymomas (Wippold and Perry, 2006), although no Ki67-positive cells were observed indicating no proliferation was taking place in these samples.

Garcia-Ovejero et al. (2015) investigated the expression profile of cells in adult human central canal area and suggest that there should be caution in considering the transferability of therapeutic options developed animal models because of the ependymoma-like properties of the human ependyma. While the effect of the partial or complete closure of the central canal on response to spinal cord injury is not known, the link to ependymomas based on the expression profile is not convincing. This experiment looked at expression of 117 genes designated ependymoma-related and compared the number of these expressed in the human ependymal region to the number of the ependymoma-related genes expressed in the ventral horn. They observed 43 ependymoma-related genes expressed in the ventral horn and ependymal region, and a further 53 ependymoma-related genes expressed in the ependymal region. This does not look at the expression of ependymoma-related genes in either younger humans nor in animals of any age so does not provide any insight into the translatability of therapeutics effective in animal models. Additionally, the finding that there is greater gene expression in common between an area high in

ependymal cells and ependymomas compared to a neuron rich area with no ependymal cells and ependymomas is a rather unsurprising conclusion.

This study also looked at the expression of neurogenic niche-genes and concluded that their low expression also reduced the chances of translatability from animal models (Garcia-Ovejero et al., 2015). Again there are multiple points that do not support this conclusion. The set of genes assessed contains a mixture of genes related to neurogenesis (e.g. BMI1), immature neurons (e.g. Dcx) and stem-cells (e.g. sox2 and S100β). The ventral horn was similarly used as the control in this experiment and was shown to express 47 of the 93 neurogenic-niche related genes, a high proportion considering the non-neurogenic nature of the ventral horn. Cells of the ependymal region were shown to express a further 14 neurogenic-niche related gene additional to those expressed in the ventral horn. This relatively low number of additional neurogenic-niche related genes compared to the ventral horn was also suggested to indicate the lack of transferability from animal studies. However these gene have not all been shown to be expressed in high levels in rodent spinal cords, and crucially, are unlikely to be expressed at high levels in the uninjured spinal cord, where no stem cell or neurogenic activity is thought to occur in any mammalian species during adulthood.

While the impact of the reduced flow of CSF that is likely to occur with the closure or partial closure of the central canal in the adult human spinal cord has not been assessed and may alter the response of this area to spinal cord injury, the ependymal cells have been shown to express Sox2 and have similar neurosphere-forming capabilities *in vitro* as rodent ependymal cells (Dromard et al., 2008; Mothe et al., 2011). Sections of human ependymal cells were enzymatically dissociated and the cells cultured and showed that they share with non-human ependymal cells the ability to form passagable neurospheres (Dromard et al., 2008). The rate of neurosphere formation was lower for the human ependymal cells compared to mouse ependymal cells. Despite this lower rate of neurosphere formation, the differentiated cells from the neurospheres produced astrocytes and NeuN positive neurons. The oligodendrocytes that are observed produced by neurospheres from rodent epSPCs (Moreno-Manzano et al., 2009) were not observed with the human ependymal cell cultures, although some of these experiments used specialised oligodendrocyte differentiation protocols and Dromard et al. (2008) used a neuronal culture medium. A subset of neurons produced by the human ependymal-derived neurospheres showed

K^+-currents and TTX-sensitive Na^+-currents indicative of functional neurons. This indicates that despite the frequent closure of the central canal (Dromard et al. (2008) observed a lack of clear central canal in over half the samples used for their imaging experiment) the ependymal cells still show the *in vitro* stem cell and neurogenic potential of non-human ependymal cells.

The human spinal cord after spinal cord injury

The most significant difference identified between the human ependymal region and the rodent ependymal region is the degree of patency of the central canal. Therefore when considering the translatability of findings in animal models of spinal cord injury, both the effect of spinal cord injury on the patency and the effect of the lower patency on the response to the injury need to be considered. No effect of spinal cord injury on patency was observed (Garcia-Ovejero et al., 2015).

The effect of the differences in patency and any expression differences on the response to spinal cord injury cannot be investigated in as much depth. However there are a number of factors in which human ependymal cells have been observed to behave in a similar manner to in non-human mammalian spinal cords. Human ependymal cells have been shown to increase the expression of nestin after spinal cord injury (Cawsey et al., 2015) as in rat ependymal cells (Liu et al., 2002; Mao et al., 2016) and significantly there was a correlation between the percentage of ependymal cells expressing nestin and the survival time after injury. Another interesting point is that nestin levels were observed increased at multiple levels of the spinal cord as well as the level of the injury site (Cawsey et al., 2015). This communication of injury could have occurred via non-canal mediated factors, but if the CSF-flow through the canal is significant, this would indicate that there is still sufficient flow of factors despite the reduced patency in the human spinal cord.

Many current treatments are looking at promoting axonal sprouting to attempt to re-connect the severed axons. One aspect of this that must be considered before being transferred into human spinal cord injury is that a symptom suffered by a larger proportion of spinal cord injured patients is chronic neuropathic pain and is a key factor in reducing their quality of life (Siddall and Loeser, 2001). Transplantation of spinal cord-derived neural stem cells into an animal model of spinal cord injury improved motor outcome but also produced allodynia as shown by decreased withdrawal reflex (Hofstetter et al., 2005). They were able to reduce the sprouting of

sensory fibres associated with this development of allodynia by transduction of the neural stem cells to express neurogenin-2, which had the effect of reducing the amount of astrocytic differentiation of the neural stem cells, increasing the oligodendritic differentiation and enabling a small amount of neural differentiation.

6.1.3 Applicability of these findings to other pathological conditions

While in healthy spinal cords, ependymal cells do not migrate away from the central canal or differentiate into astrocytes of oligodendrocytes, there are pathological conditions other than spinal cord injury, which cause ependymal cells to demonstrate these behaviours. In a SOD1 transgenic mouse model of amytrophic lateral sclerosis (ALS), a nestin positive subset of ependymal cells increased their proliferation and migrated first to the dorsal horns and then to the ventral horns where the degeneration of motor neurons was occurring (Chi et al., 2006). The increase in proliferation of the nestin-positive ependymal cells was seen even before the disease symptoms were apparent. The SOD1-mutant mice had higher CXCR4 expression in the nestin-positive ependymal cells compared to the control animals, suggesting that CXCL12 may be involved in the migration of ependymal cells in this SOD1 MS mouse model, in a similar manner to its probable involvement in spinal cord injury. Interestingly, whilst application of CXCL12 improves locomotor recovery from spinal cord injury by promoting cell survival, increasing the numbers of microglia and astrocytes and the amount of cell proliferation (Zendedel et al., 2012), the same positive effect is not seen in ALS. In a SOD1-mutant mouse model of ALS, application of the CXCR4 antagonist AMD3100 reduced the levels of expression of microglial inflammatory markers, increased motor neuron survival, improved motor functioning and extended animal survival (Rabinovich-Nikitin et al., 2016). Rabinovich-Nikitin et al. (2016) suggested that this effect may be due to a reduction in the glutamate excitotoxicity caused by glutamate release from glial cells. It may be that enhancing the migratory response of ependymal cells in a manner that doesn't elicit the glutamate excitotoxicity can provide a beneficial outcome in ALS.

Another degenerating condition that activates ependymal cells is multiple sclerosis (MS), which triggers increases of proliferation and expression of the radial glial marker, RC1 (Talbott et al., 2005; Danilov et al., 2006). Many treatments have looked at promoting remyelination of the demyelinated axons and thereby inhibiting their degradation. Ependymal cells have been shown *in vitro* and *in vivo* to produce a

limited number of oligodendrocyte progenitors (Shihabuddin et al., 2000; Barnabé-Heider et al., 2010). It has previously been shown that ependymal cells can be modulated to produce a higher proportion of cells immunoreactive for the oligodendritic marker PanQKI by modulation of cholinergic signalling (Corns et al., 2015). In an experimental autoimmune encephalomyelitis (EAE) model of MS nicotine application increased the proliferation of ependymal cells and increased the numbers of mature oligodendrocytes present although these may not have derived from the ependymal cells (Gao et al., 2015). However multiple studies have indicated that astrocytic involvement is of great importance in supporting the remyelination (Talbott et al., 2005), and thus the astrocyte-forming capabilities may be useful in promoting the remyelination of axons in multiple sclerosis. Greater understanding of the roles of ependymal cells in these demyelinating disorders and the signalling in the spinal cord that elicits these roles could help maximise the beneficial potential of ependymal cells.

6.2 Future work

There are many interesting facets of this research that would benefit from further research to elucidate the roles that purinergic signalling plays in the stem cell niche of the spinal cord. Two potential avenues for continuation of the work are detailed below.

6.2.1 Elucidation of the roles of purinergic signalling on the cell fate of ependymal progeny

The processes of cell proliferation, migration and differentiation are often interlinked such that changes in one cause a corresponding decrease in another.

Studies in human mesenchymal stem cells (hMSCs) demonstrate one aspect of these linked changes caused by ATP signalling (Ferrari et al., 2011). In this cell population, application of ATP and UTP induced increases in intracellular calcium concentration. In cultured cells, the ATP, but not UTP application, was associated with a decrease in proliferation rate. This decrease in proliferation rate was accompanied by an increase in migration *in vitro.* ATP application caused an increase in the spontaneous migration of hMSCs and also enhanced the directional migration of hMSCs towards CXCL12, a chemokine they migrate towards at a lower

rate without ATP application (Ferrari et al., 2011). Significantly, the migration triggered directly by ATP was shown not to be directional migration but rather an increase in cell motility (chemokinesis) as the increase in migration was only seen when ATP was in the same chamber of the transwells to the hMSCs; when ATP was added to a different chamber to the cells, no effect was seen, suggesting that perhaps the concentration was not high enough to elicit spontaneous migration. However this concentration was enough to significantly enhance the directional migration (chemotaxis) of hMSCs towards CXCL12. UTP did not promote any migration either directly or by potentiating the chemotactic effect of CXCL12. This suggests that in hMSCs, ATP signalling may increase migration via two mechanisms, chemokinesis and chemotaxis. Pre-incubation with ATP increased the migration of transplanted hMSCs *in vivo.* This effect of pre-incubation of ATP was likely due to the changes in gene expression elicited by ATP. ATP signalling affects expression of genes in hMSCs, downregulating genes involved in proliferation and upregulating genes involved in migration (Ferrari et al., 2011).

Purinergic signalling also acts on oligodendrocyte progenitors (OPs) (Agresti et al., 2005a). ATP and ADP but not UTP promote additional directional migration of OPs *in vitro* towards the ATP or ADP concentration gradient over the amount of migration seen in OPs without purine application (Agresti et al., 2005b). This response was eliminated by application of the $P2Y_1$ antagonist MRS2179. Agresti et al. (2005b) also saw a small increase in migration of OPs *in vitro* with application of adenosine or the A1-specific agonist CPA, however Othman et al. (2003) observed a larger effect on migration with CPA application, perhaps due to the longer incubation time in this study. ATP and ADP were also shown to have a minor effect increasing the rate of proliferation in OPs (by 10-15 %) however they had a more considerable effect in the opposite direction when applied to OPs treated with the growth factor platelet-derived growth factor (PDGF) (Agresti et al., 2005b). ATP and ADP reduced the pro-proliferative effect of PDGF by half with this reduction in proliferation inhibited by the $P2Y_1$ antagonist MRS2179. In the PDGF-treated OPs, the anti-proliferative effect of ATP and ADP was accompanied by an increase by about 80 % in the percentage of cells expressing O1, a marker for differentiated oligodendrocytes. This indicates that in the PDGF-treated OPs, purinergic signalling switches cells from a proliferating to a differentiating phenotype, counteracting the proliferative effect of PDGF.

Modulation of cell fate within ependymal cells

Within ependymal cells, the results presented here suggest that modulation of purinergic signalling affects migration but does not affect the proliferation rate. It is not known if purinergic signalling is involved in the differentiation of the cells from stem cells to differentiated cells or if it favours any fates over others. As the ependymal cells migrate after spinal cord injury, they change their phenotype. They cease expressing an ependymal phenotype, for example through Sox2 expression and use of the FoxJ1 promoter, and begin expressing Sox9 and GFAP (Mothe and Tator, 2005; Meletis et al., 2008). After 4 months post-injury, there was also a population of mature oligodendrocytes descended from central canal ependymal cells, showing that ependymal cells increase their range of cell fates after spinal cord injury (Barnabé-Heider et al., 2010), compared to in uninjured spinal cords.

Signalling with neurotransmitters can modulate events in ependymal cells affecting both the rate of proliferation and the proportions of cells expressing markers of differentiated cells. Modulation of cholinergic signalling via application of PNU 120596 (a selective α7*nAChR potentiator) increases the amount of proliferation in ependymal cells in organotypic slice cultures (Corns et al., 2015). Application of PNU 120596 also increases the percentage of newly-proliferated cells labelled with the oligodendrocyte progenitor and oligodendrocyte marker PanQKI. This again shows duel effects on proliferation and differentiation, in this case increasing both proliferation and differentiation; while ependymal cells differentiate into multiple cell types no reduction in expression of other markers of other post-differentiated cell types was observed (Corns et al., 2015). This modulator was also applied to the intact spinal cord *in vivo* and whilst the increase in proliferation was still seen (Corns et al., 2015), there was no increase in proportion labelled with PanQKI . This is likely due to the fact that *in vivo* in an uninjured cord, no differentiation into oligodendrocytes (or astrocytes) is seen as ependymal cells just replace themselves in the intact cord. Therefore there was no oligodendrocytic differentiation pathway open for PNU 120596 signalling to upregulate, suggesting that these drugs do not push ependymal cells into differentiation pathways without prior activation by spinal cord injury. This may occur by altering expression of some of the cell fate determinants that have been identified as upregulated after spinal cord injury, such as Notch1, Numb, Pax6, BMP4, Shh and Msx2 (Yamamoto et al., 2001; Chen et al., 2005) once the situation of spinal cord injury has enabled their expression. Once the

full effects of modulation of ependymal signalling have been understood, it may well be that different signalling modulators need to be applied at different times to produce the most beneficial effect for recovery from spinal cord injury.

For purinergic signalling, this could be investigated using the organotypic slice culture protocol as the results with modulation of cholinergic signalling (Corns et al., 2015) have demonstrated that this protocol provides enough activation seen in spinal cord injury to produce differentiation responses into astrocytes and oligodendrocytes, similar to that seen *in vivo* after spinal cord injury.

6.2.2 Investigation of the functional implications of spatial differences in P2X receptors in CSFcCs

One particular unexpected result was the involvement of the $P2X_3$ subunit in mediating the responses to ATP of a spatially-determined subset of CSFcCs. $P2X_3$-containing receptors have very different properties to $P2X_2$ receptors and as such are likely to function differently in a range of situations, including producing different responses in raised concentrations of calcium and with variations in pH levels (North, 2002), both of which are altered in many situations including spinal cord injury (Moriya et al., 1994; Wang et al., 2004). Therefore understanding the ways that alteration of these factors affects the responses of CSFcCs to ATP could shed light on the impact of this spatial variation of P2X receptors in CSFcCs.

$P2X_3$ subunit-containing receptors, are highly expressed within a subset of primary nociceptive afferents (Chen et al., 1995; Adriaensen et al., 2015) and are involved in peripheral pain mechanisms (Burnstock, 2001; North, 2004). Understanding the roles of $P2X_3$-containing receptors in pain signalling can provide insight into how characteristics such as desensitisation and response to calcium levels provide reliable signalling without excessive or inappropriate signalling. Homomeric $P2X_3$ receptors show a number of characteristics that are different from other P2X receptors, particularly from $P2X_2$ receptors, with the heteromeric $P2X_{2/3}$ receptors resembling $P2X_2$ receptors in some aspects, $P2X_3$ in other aspects and showing intermediate characteristics in the others (see section 3.3.4 for further information on these differences and features). These features of $P2X_3$-containing receptors are significant in the functions they perform. A key feature of $P2X_3$ receptors is that they show both fast and slow desensitisation and a desensitisation that can be mediated

even by concentrations of ATP which are unable to elicit a detectable change in membrane potential (Sokolova et al., 2004; Pratt et al., 2005). This is a process called high affinity desensitisation. It may be that the desensitisation and high affinity desensitisation shown by $P2X_3$ play a role in reducing inappropriate excitation of nociceptive fibres against physiological releases of ATP such as those seen after exercise (Mortensen et al., 2011), preventing nociception due to ATP released from causes related to non-noxious stimuli. The desensitisation of $P2X_3$ receptors means that only large bursts of ATP release can overcome the desensitisation with alterations to the desensitisation properties playing a role in the development of chronic pain (Burnstock, 2001). Modulation of the desensitisation could contribute to the development of chronic pain and particularly inflammatory pain (North, 2004; Giniatullin and Nistri, 2013). One way in which this desensitisation could be reduced is by reconfiguration of the receptors from the homomeric $P2X_3$ receptors which show the high affinity desensitisation to the heteromeric $P2X_{2/3}$ receptors which do not.

It could be that some of these characteristics that enable successful functioning of $P2X_3$-containing receptors in nociceptive afferent neurons also play a role in the functioning of $P2X_3$-containing receptors in CSFcCs. The impact of the response to ATP being mediated by $P2X_3$-containing receptors in a subset of CSFcCs compared to the $P2X_2$-mediated response in the majority of CSFcCs could be of value to help elucidate the importance of the two receptor types. To investigate how modulation of the external environment differentially affects these two CSFcC populations further electrophysiological experiments could be done under different conditions. Modulation of the pH of the extracellular solution and of the concentration of extracellular calcium could be examined as both of these characteristics have been shown to differentially affect $P2X_2$ and $P2X_3$-containing receptors. This could provide insight as to how the two groups of cells function in the changes of conditions seen in spinal cord injury.

Another aspect of the $P2X_3$-containing receptor involvement in CSFcC functioning that deserves further investigation is the location of the $P2X_3$ subunit expression. Immunohistochemistry did not show strong expression within the CSFcCs, instead the strong expression appeared to be within a bundle of fibres just ventral to the central canal. Clarification of whether the depolarisation to ATP was mediated directly by $P2X_3$ located post synaptically, could be achieved by blocking synaptic signalling using a combination of Na^+ channel blockers, excitatory and inhibitory amino acid

receptor antagonists for example TTX, DNQX, AP-5, bicuculline and strychnine (as has been used previously in Deuchars et al. (2001)). A lack of response under these conditions would indicate that the $P2X_3$-containing receptors were located pre-synaptically while continued presence of the response would suggest a post-synaptic expression on the CSFcCs.

Another important part to investigate would be the role of the $P2X_3$-mediated responses in CSFcCs in the responses to spinal cord injury. This is particularly pertinent due to the fact that the Ca^{2+} levels rise after spinal cord injury (Moriya et al., 1994) which affect $P2X_2$ receptors and $P2X_3$-containing receptors in very different ways. Increased levels of extracellular Ca^{2+} inhibit the responses of $P2X_2$ to ATP (Evans et al., 1996), while the raised levels of Ca^{2+} have the effect of attenuating the desensitisation of $P2X_3$-containing receptors (Cook et al., 1998). This could be investigated using the organotypic slice culture model using the selective $P2X_3$/$P2X_{2/3}$ antagonist ARL 67156. In addition to examining the behaviour of cells around the central canal in this experiment, it would also be worth determining if there were any differences along the ventral midline and on the ventromedial edge of the slice as $P2X_2$ is expressed in the axons of the CSFcCs (Stoeckel et al., 2003), with the expression of $P2X_3$ not having been previously investigated.

6.4 Conclusions

This study has provided an in-depth characterisation of the role of purinergic signalling around the central canal of the adult spinal cord. The responses of both ependymal cells and CSFcCs to application of nucleotide triphosphates indicate the importance of these as intercellular signalling molecules and the results from the organotypic slice cultures show the role of this signalling in “injury” situations. Within this situation purinergic signalling, via suramin-sensitive channels, facilitates the migration of the ependymal cells away from the central canal. This could occur via enhancement of chemokine chemotaxis as is seen in other migratory cell types. The value of proliferation and differentiation in ependymal cells in spinal cord injury has been demonstrated so the effect of purinergic signalling on the migration of ependymal cells warrants further study. Better understanding of the factors that control the proliferation, migration and differentiation of ependymal cells in spinal cord injury and spinal cord pathologies such as multiple sclerosis and ependymomas will

allow optimisation of response of ependymal cells to produce the best functional outcome. These findings provide greater understanding of the complexity of electrophysiological responses of cells around the central canal to purinergic signalling and demonstrate a novel role for this signalling in an experimental model with features of spinal cord injury.

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