Pharmacological characterisation

of GABA receptors

in potential spinal cord neural stem cells

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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 works of others.

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ABSTRACT

Modulation of GABA mediated inhibition is one of the most important approaches for the treatment of central nervous system (CNS) diseases. Precise targeting of such treatments depends on identification and characterisation of the different subunit complexes that exist. In the CNS, there are pools of neural stem cells (NSC), which can differentiate to become neurones, astrocytes or oligodendrocytes while progenitor cells have limited lineage. In addition to brain regions, where NSCs are now known to exist, the spinal cord area also has a neurogenic potential in the form of ependymal cells.

Ependymal cells surrounding the central canal displayed typical properties of glial cells; lack of voltage-gated channels, and showed coupling indicative of the presence of gap junction. Ependymal cells showed responses to GABA as previously observed (Corns et al., 2013). The effects of GABA on ependymal cells were concentration dependant.

Ependymal cells in our study showed atypical GABA receptors since it was not fully blocked by $GABA_A$ receptor antagonist; bicuculline. Hyperpolarising responses to low GABA were antagonised by the GABAρ receptor antagonist, (1, 2, 5, 6-Tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA). On further confirmation of this, Trans-4-aminocrotonic acid (TACA), a selective agonist of $GABAρ_1$ elicited robust and reproducible hyperpolarising responses, similar to those observed with low concentrations of GABA. This provides evidence of a combination or coassembly of $GABA_A$ and ρ receptors in the ependymal cells or the presence of two different receptors.

One potential site of modulation of GABA receptors is through diazepam binding inhibitor (DBI). DBI with its breakdown product, octadecaneurapeptide (ODN) acts as

positive and negative modulator at central benzodiazepine receptor (CBR). The responses to high concentrations of GABA were modulated by midazolam suggesting a potential involvement of CBR and another receptor, Translocator Protein (TSPO). On testing TSPO, N, N-Dihexyl-2-(4-fluorophenyl) indole-3-acetamide (FGIN-1-27); selective agonist at TSPO, increased the responses in ependymal cells to the higher concentration of GABA. When combining FGIN-1-27 with 1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide (PK-11195); a selective TSPO antagonist, the augmented GABA responses were not observed, suggesting that this effect was due to activation of TSPO. These results indicate that modulation of GABA receptors by midazolam occurred at both CBR and TSPO. However, the responses of GABA in the presence of ODN (20 μM) did not show significant changes regardless of the GABA concentration used. Nanomolar ODN application alone causes fast hyperpolarisations similar to low GABA and TACA effects. Responses to nanomolar ODN were significantly but not fully blocked by cyclo1-8[dleu5] OP (cDLOP), an ODN metabotropic receptor antagonist. ODN is somehow directly activating the GABA receptors that contain rho subunits since the hyperpolarising effects of ODN were significantly blocked by TPMPA. So there is an evidence there are atypical GABA receptors responsible for this action with ODN.

Baclofen (a selective $GABA_B$ receptor agonist) also hyperpolarised ependymal cells, an effect antagonised by CGP 55845 ($GABA_B$ antagonist), indicating a further role for $GABA_B$ receptors on ependymal cells. The numbers of proliferative cells in the central canal region were significantly lower in baclofen treated slices compared with control.

Activation of the 5-HT receptor caused both depolarisation and hyperpolarisation contribute to the proliferative capabilities of ependymal cells. Blockade of 5-HT receptor mediated depolarisations with cinanserin showed the presence of $5\text{-}HT_2$ receptors in the spinal cord. Furthermore, hyperpolarisations with 8-OH-DPAT and

cisapride indicate that 5-HT $_{1A}$ and 5-HT$_4$ receptors are also present in the central canal area of spinal cord.

Overall, our study has shown a complex and varied response profile of ependymal cells to GABA and its modulators. This could have important implications in the proliferative capabilities of spinal cord neural stem cells and thus functional consequences. The modulation of proliferation and differentiation by GABA may be an interesting future therapeutic target for conditions where there is over proliferation such as in spinal cord ependymomas or in conditions such as in multiple sclerosis where replacement cells may be useful to aid recovery.

List of Abbreviations

ACh	acetylcholine
AKH	akhirin
$AKH^{-/-}$	akhirin negative mutant mice
$AKH^{+/+}$	akhirin positive wild type mice
APC+	mature oligodendrocyte marker
BLBP+	brain lipid binding protein progenitor cells
BMP	bone morphogenetic proteins
BrdU	Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
Ca^{2+}	calcium ions
Cl^-	chloride ion
CBR	central benzodiazepine receptor
CSF	cerebrospinal fluid
CSFcCs	cerebrospinal fluid contacting cells
Cx43	connexin 43
C57BL/6	Wild type mouse
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DBI	diazepam binding inhibitor
DBI (KD)	DBI knockdown
DBI (OE)	DBI overexpression
DCX+	doublecortin
DIC	differential interference contrast
dKO	double knockout
DNA	deoxyribonucleic acid

DMSO	dimethyl sulfoxide
DG	dentate gyrus
EAP	extraavidin peroxidase
EC_{50}	effective concentration for half-maximal response
EdU	5-ethynyl-2'-deoxyuridine
EPSP	excitatory depolarizing postsynaptic
FGIN-1-27	*N*,*N*-Dihexyl-2-(4-fluorophenyl)indole-3-acetamide
Fox J1	Forkhead box protein J1
GABA	Gamma aminobutyric acid
[GABA]	GABA concentration
$GABA_A$	GABA subunit A
$GABA_{B1}^{-/-}$	GABA subunit B
GABAρ	GABA subunit rho
GAT3	GABA transporter 3
GAD	glutamic acid decarboxylase
GAD67-GFP	glutamate decarboxylase green fluorescent protein
GFAP	glial fibrillary acidic protein
GCaMP	genetically encoded calcium indicator, a fusion of green fluorescent protein, calmodulin, and M13
GIRK	G protein-coupled inwardly-rectifying potassium channels
Gi/o	heteromeric G protein subunit that inhibits cAMP-dependant pathway
Gs	heteromeric G protein subunit that activates cAMP-dependant pathway

Gq	heteromeric G protein subunit that activates phospholipase C
GPCR	G-protein coupled
GΩ	gigaOhm
Gβγ	G beta-gamma complex composed of one G_{β} and one G_{γ} subunit
HPLC	high-performance liquid chromatography
H_2O_2	hydrogen peroxide
i.e	that is
I.P	intraperitoneal
IPSP	inhibitory postsynaptic
IR	input resistance
IML	intermediolateral nucleus
KCC2	K^+-coupled Cl^- cotransporter 2
K^+	Potassium ion
kDa	kiloDalton
Ki67	cellular marker for proliferation, MKI67
kdyn	kilodynes
Lamina X	lamina ten
MCM2+	proliferating marker
ml	millilitre
mM	milimolar
mOsm	milliosmole
mV	millivolt
MΩ	megaohm
NAN-190	as an antagonist to 5-HT_{1A}
nAChR	nicotinic acetylcholine receptor
NeuN	marker

NKCC1	Na^+-K^+-$2Cl^-$ cotransporter 1
nm	nanomolar
*nm*1054	a deletion of 400 kb on chromosome 1 that includes DBI gene
Olig2	oligodendrocyte transcription factor
ODN	octadecaneuropeptide
OE	DBI overexpression
OPCs	oligodendrocyte progenitor cells
pA	picoAmp
PBS	phosphate buffer saline
PC	computer
PCNA	proliferating cell nuclear antigen
PCA	parachloroamphetamine
PFA	paraformaldehyde
PLC	phospholipase c
PK-11195	1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide
P15-P21	postnatal fifteen to postnatal twenty one
RER	rough endoplasmic reticulum
RMS	rostral migratory stream
SERT	serotonin transporter
SGZ	subgranular zone
SVZ	subventricular zone
TACA	trans-4-aminocrotonic acid
TPMPA	tetrahydropyridin-4-yl)methylphosphinic acid
TSPO	translocator protein

TTN	triakontatetraneuropeptide
T-type Ca^{2+}	*T-type calcium* channels are low-voltage activated calcium channels
THP	tryptophan hydroxylase
α_3	alpha subunit 3
α7*nAChRs	alpha7 nicotinic acetylcholine receptors
β-gal	labels the ependymal cells
µl	microlitre
µm	micrometre
µM	micromolar
%	percentage
3CB2	radial glial marker
40x	fourty times (magnification)
400 kb	four hundred kilobyte
5-HT	5-hydroxytryptamine
($^3H^-$)-baclofen	radiolabelled form of baclofen

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Chapter 1

General Introduction

1.1 The spinal cord

The spinal cord is the most important structure for relaying sensory and descending information between the body and the brain. It extends from the foramen magnum, continuous with the medulla to the level of the first or second lumbar vertebrae.

The main structure of the spinal cord comprises the cellular grey matter, surrounded by neuronal tracts of white matter (De Leener et al., 2016). At the centre of grey matter, there is a central canal in which the cerebrospinal fluid flows.

The spinal cord is contained within 3 protective layers of membrane, known as meninges. The innermost delicate layer is the pia mater which closely wraps the spinal cord and roots. The next layer is the arachnoid mater, with the space between the pia and the arachnoid mater called the subarachnoid space. The most outer side of the meninges is the tough protective layer called the dura mater.

The grey matter of the spinal cord is organised into 10 layers called the Rexed laminae (Rexed, 1952). Laminae I-VI are responsible for sensory processing while laminae VIII and IX are considered as ventral horn, where the somatic motor neurones and their associated interneurons reside. The intermediate zone is found between the dorsal and ventral horn and incorporates the lateral horn, which contains the intermediolateral column (IML) and is associated with autonomic output. It can be

divided into laminae VII and X. Lamina X is the area of grey matter at the centre of spinal cord surrounding the spinal cord.

1.2 Lamina X

Lamina X is the area surrounding the central canal containing the grey commissures and the substantia gelatinosa centralis. It is also termed as area X or the central grey of the spinal cord. It consists of small to medium-sized multipolar, triangular and spindle shaped neurones. The density of nerve fibres in the lamina is relatively low (Rexed, 1954). The neurones found in lamina X of mice are triangular, multipolar and spindle shaped (Sengul and Watson, 2012).

Studies have shown that Lamina X may be the site of spinal neural stem cells that are generated from the ependymal layer (Rusanescu and Mao, 2015) and this potential neurogenic niche is the main focus of my thesis.

1.3 What is neurogenesis?

Neurogenesis is often defined as a process of generating functional neurones from adult neural precursors which occurs during embryonic and perinatal stages (Ming and Song, 2005). However many researchers use this term to describe generation of new oligodendrocytes and astrocytes as well (Yang, H. et al., 2006). Thus, neurogenesis encompasses the production of not only new neurones but also oligodendrocytes and astrocytes from neural stem cells. There are two main steps of neurogenesis; i.e., proliferation and differentiation of neural stem cells. Neural stem cells include neuroepithelial stem cells, radial glial cells, basal progenitors, and

astrocytes. Neurogenesis is present not only during embryonic development but also occurs throughout adult life in specific regions or niches.

The adult central nervous system was thought to have a static condition because it showed little cell turnover. Altman and Das (1965) then proved that there is evidence for the presence of newly generated dentate granule cells in animal models i.e. in the area of postnatal rat hippocampus. New neurones are continuously created and functionally integrated into existing neuronal networks in the adult brain. Active adult neurogenesis is mainly confined to two distinct locations: the subventricular zone (SVZ) of the lateral ventricles in the forebrain and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (Ming and Song, 2011). The peak time of neurogenesis in the dentate gyrus of a rat is seen postnatally during the first week of life with a progressive decline after postnatal day 9 (Liu, H. et al., 2003). Neurogenesis appear to be comparable between the rodent (Kuhn et al., 1996) and the human dentate gyrus (Eriksson et al., 1998). As in rodents, neurogenesis continues throughout life in the human dentate gyrus.

Numerous studies have reported that neurogenesis does not normally occur in postnatal mouse cortex (Bauer and Patterson, 2005; Zhao and Brinton, 2005), which reflects an intrinsic limitation of the endogenous neural precursor potential, or a lack of appropriate micro-environmental signals necessary for neuronal differentiation or survival. Additional sites of neurogenesis which were previously known as non-neurogenic areas occur in hypothalamus in the mouse (Kokoeva et al., 2005) and striatum in the non-human primates (Bedard et al., 2006) and in human (Ernst, A. et al., 2014). However there is evidence that new neurones are generated in the human brain comparable to those observed in rodents especially in the dentate gyrus of hippocampus (Eriksson et al., 1998).

1.3.1 Regulation of neurogenesis

The cells that are critical in neurogenesis are the neural stem cells which are found throughout the central nervous system and are defined by common cellular properties: they proliferate, self-renew, and give rise to differentiated progeny. Cells defined by these proliferative and differentiative properties are present during the development of the central nervous system and persist into adulthood in certain locations. It is well confirmed that the multipotent neural stem cells are found in many regions of the adult central nervous system. The exact origin of these multipotent neural stem cells, whether from subventricular, ependymal or parenchymal areas, together with the induction signals which are essential for proliferation, migration, or differentiation remains controversial. Many experimental studies provide contradictory data regarding cell origin, type and the factors that induce neurogenesis (Rice et al., 2003; Horner, P. et al., 2000).

Regulation of neurogenesis can be categorized as either intrinsic or extrinsic. Many intrinsic (cell autonomous such as via transcription factors) and extrinsic (cell non-autonomous such as via secreted factors) factors that modulate neurogenesis have been identified (Aimone et al., 2014). Extrinsic and intrinsic mechanisms regulate different aspects of neurogenesis (Figure 1-1). Molecular factors and signalling pathways including niche factors or receptors, cytoplasmic factors, transcriptional factors and genetic regulators may cause neurogenesis in both adult and embryogenic situations. Between embryonic and adult neurogenesis, there is significant similarity in signalling pathways (Mash1, Notch etc.) although the origin and nature of extrinsic signals (growth factors, neurotrophins, and hormones) could be different. Neurotransmitters regulate brain development, changes in their levels have also been shown to influence neurogenesis in adulthood. These neurotransmitter systems are subject to regulation by pharmacological manipulations (Ming and Song, 2011).

FACTORS CONTROLLING NEUROGENESIS SIGNALLING PATHWAY	
EXTRINSIC FACTOR	INTRINSIC FACTOR
Growth factors: EGF,HB-EGF, VEGF, CTNF, TGFα, BDNF	Neurogenic Factors: PAX6/WNT/MASH1 Gliogenic factors: OLIG2/Notch Stem Cell renewal : SHH/Notch
Neurotransmitters: GABA, 5-HT, DA, Ach, NO	cytokines
prolactin, pregnanolone	
Stress, environment	

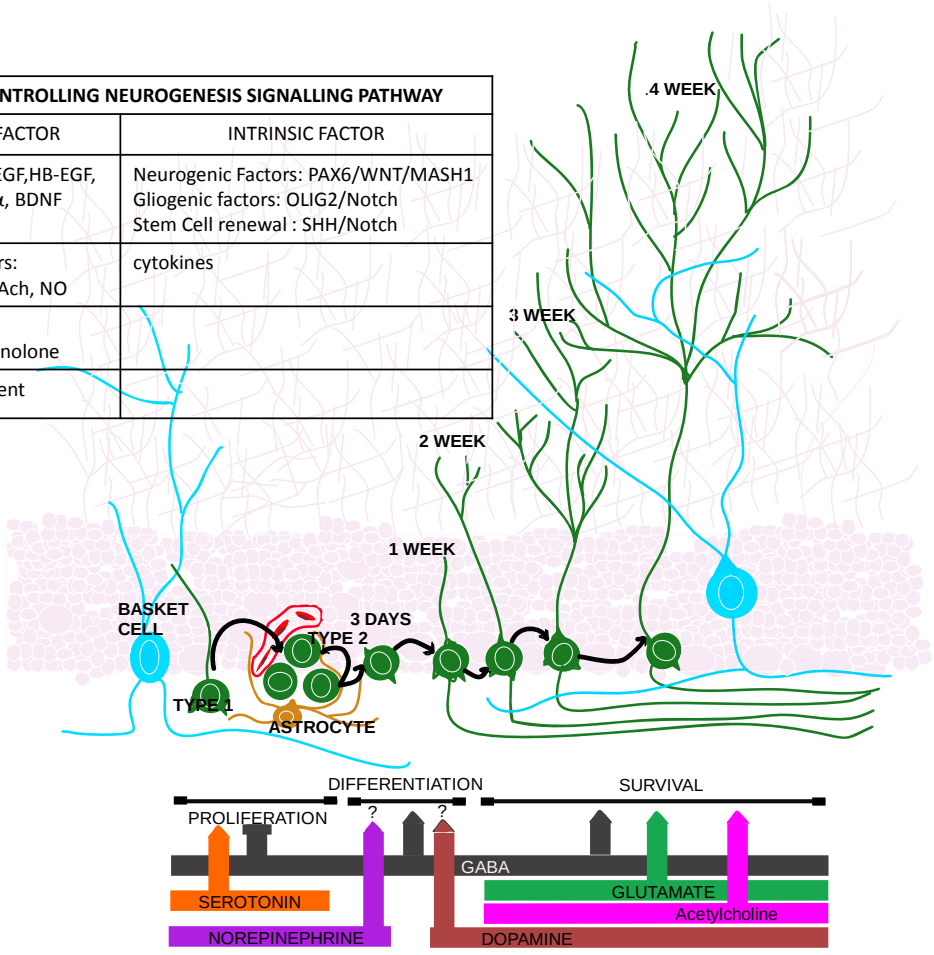


Figure 1-1 Intrinsic and extrinsic factors in the neurogenesis signalling pathway

Diagram demonstrating the intrinsic and extrinsic factors in the neurogenesis signalling pathway in the dentate gyrus of the hippocampus (modified from Aimone et al. (2014); Lledo et al. (2006)).

Among these neurotransmitters, gamma-aminobutyric acid or ɣ-aminobutyric acid (GABA), often considered an inhibitory neurotransmitter, regulates the neural stem cell niche at the level of both stem cells and young neurones. GABA released by nearby interneurones activates neural stem cells in a quiescent state (Song, J. et al., 2012). Then GABA acts on neuroblasts and increases their survival and maturation (Song, I. et al., 2013).

1.4 Neurogenesis in spinal cord

1.4.1 The identity of the neurogenic niche

The cells surrounding cells in the central canal of the spinal cord (see Figure 1-2) have similar features to the stem cell niches of the brain (Hamilton et al., 2009) since they express the same markers (Meletis et al., 2008) as neural stem cells and have the ability to proliferate (Horner et al., 2000).

Due to growing evidence that neural stem cells may originate from the periventricular region along the entire axis of ventricular area, including the forebrain SVZ, the ependymal layer of the central canal has been considered as the neurogenic niche in the spinal cord by many researchers (Sabelström et al., 2013; Cusimano et al., 2012; Barnabé-Heider et al., 2010; Danilov et al., 2006).

The ependymal cells of the spinal cord has been shown in several studies to be capable of undergoing division (Barnabé-Heider et al., 2010; Cizkova et al., 2009; Danilov et al., 2006; Johansson et al., 1999). Endothelial cells, astrocytes, ependymal cells, cerebral spinal fluid contacting cells, microglia, mature neurones, and progeny of adult neural precursors are among the major cellular components of the adult neurogenic niche (Marichal et al., 2012; Barnabé-Heider et al., 2010; Meletis et al.,

2008; Adrian Jr and Walker, 1962). More recently, Alfaro Cervello (2012) suggested that biciliated ependymal cells are the primary neural stem cells in the spinal cord. Proliferation of ependymal cells is common during embryonic and early postnatal periods of development in most species; however, ependymal cell turnover declines significantly postnatally (Bruni, 1998).

The neurogenic potential of astrocytes is regionally specified for neurogenesis. The astrocytes from adult hippocampus retain the potential to promote neurogenesis, but astrocytes from adult spinal cord do not (Barnabé-Heider et al., 2010). Barnabe-Heider (2010) showed that there were less BrdU incorporation in astrocytes compared to 10 times higher BrdU incorporation in ependymal cells and astrocytes in the spinal cord only give rise to new cells of the same fate whereas ependymal cells differentiates to different cells. Most of the postnatal neurogenesis involves ependymal cells which then rely on the extrinsic factors such as neurotransmitters.

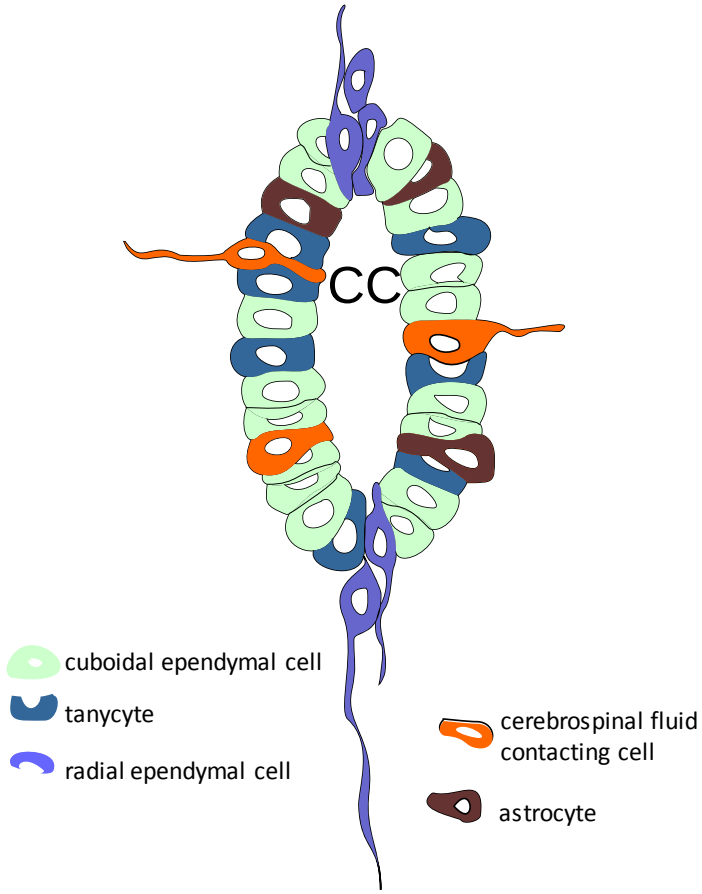


Figure 1-2 Ependymal cell layer and the various types of cells

Diagram depicting the ependymal cell layer and the various types of cells in the central canal region (Adapted and modified from Marichal et al. (2012)).

1.4.2 Cell types that may be involved in neurogenesis-

1.4.2.1 Ependymal cells

Ependymal cells are epithelium cells lining the ventricular surface from the lateral ventricles to throughout the length of the spinal central canal. Ependymal cells are remnants of primitive neuroepithelium from which neural progenitor cells originate during pre- and perinatal development (Chenn and McConnell, 1995). In this developmental stage, from neuroepithelial cells, the neural stem cells differentiate into radial glial cells and then progress into the ependymal cells lining the ventricles and central canal.

The cell types observed and their general organization are similar at cervical, thoracic, and lumbar levels of the spinal cord (Alfaro-Cervello et al., 2012). The layer of ependymal cells in the majority of the central canal area is only a single cell thick however in some places it is thickened such that it contains multiple layers of ependymal cells.

The ependymal cells in the cervical and thoracic regions are mainly round, but at the lumbar level, they are more likely to be oval-shaped. Ependymal cells around the lateral regions of the central canal are predominantly pseudostratified cuboidal ependymal cells forming the lining of the channel through which the cerebrospinal fluids flows (Bruni and Reddy, 1987).

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. Ependymal cells do not rest on basement membranes but the bases of the cells taper and then break up into fine branches which ramify into the underlying layer of processes derived from astrocytes. The cells are tightly bound together at their luminal surfaces by the usual epithelial

junctional complexes; with their surface covered by microvilli and they contain central clusters of long cilia (Bruni and Reddy, 1987; Sturrock, 1981). At luminal surfaces there are a variable number of cilia and microvilli, which probably have absorptive and secretory functions. Beating of the cilia of ependymal cells during development appears to set up concentration gradients of guidance molecules to direct migration of neuroblasts.

Ependymal cells in spinal cord identified by Meletis et al. (2008) were not the classical multiciliated ependymal cell classified as E1 in brain by Mirzadeh et al. (2008) but three subpopulations of cells with one to three cilia. The most common cell type in the spinal cord epithelium had two cilia with some similarities to the E2 (characterized by only 2 cilia and complex basal bodies) cells described in the lateral ventricular wall by Alfaro-Cervello et al. (2012) and Mirzadeh et al. (2008). Alfaro-Cervello et al. (2012) also observed smaller subpopulations of cells with one, three and four cilia with highly polarized and had lipid droplets. Those spinal cord ependymal cells with two cilia had two unique large electron-dense basal bodies. The cilia of biciliated cells were long (7–9 μm) and had a 9+2 microtubule axoneme structure (Alfaro-Cervello et al., 2012). The cell types observed in spinal cord were similar at cervical, thoracic and lumbar level. Regardless of numbers of cilia, all ependymal cell cilia are highly polarized since they have lipid droplets towards the apical membrane which may be important for membrane synthesis (Spassky et al., 2005).

The nuclei of ependymal cells are large and round, located at the basal part, whereas the cytoplasm is at the apical region (Bruni and Reddy, 1987). Under electron microscopy ependymal cells have large nuclei and electron dense cytoplasm rich in intermediate filaments (Alfaro-Cervello et al., 2012; Bruni and Reddy, 1987). They also have numerous mitochondria and Golgi apparatus in a horse-shoe shape (Alfaro-Cervello et al., 2012).

Ependymal cells are connected to neighbouring ependymal cells via a number of connections. Long zonulae adherens junctions running along the lateral sides of the ependymal cells (Alfaro-Cervello et al., 2012) are formed of cadherins and are linked to the actin cytoskeleton. Cadherins are calcium-dependant transmembrane adhesion molecules that form the adherens junctions.

The other type of junctional connection through which ependymal cells are connected is gap junctions. Gap junctions provide a reciprocal direct cytoplasmic linkage between adjacent cells for electrotonic and metabolic cell-to-cell communication. Gap junctions are formed by different connexin types which have different functional properties. There are 3 main types of connexin found in the ependymal cells of spinal cord. These are connexin 43 (Cx43) (Belliveau and Naus, 1995), connexin 45 (Chapman et al., 2013) and connexin 50 (Rodriguez-Jimenez et al., 2015). Expression of these connexins has been shown to produce functionally active gap junctions between the cells as demonstrated during electrophysiological experiments. Dye-coupling of cells, which suggests the presence of gap junctions, was observed in many of the ependymal cells recorded in mouse spinal cord slices but the extent of dye coupling was lower in the dorsal and ventral poles and in these regions immunohistochemistry for Cx43 revealed less dense labelling in these regions (Marichal et al., 2012).There are increases in input resistance and voltage changes with application of gap junctions blockers (Corns et al., 2013) indicating that gap junction could be one way how ependymal cells modulate their responses to signalling.

Ependymal cells in the mammalian spinal cord express many of the immunohistochemical markers which are associated with neural stem cells in other regions (Sabelström et al., 2014; Marichal et al., 2012; Hamilton et al., 2009), although not all markers were found in all regions. The markers are nestin, S100β, SOX2 and some expressed the radial glial marker 3CB2 or vimentin (Corns et al.,

2015; Marichal et al., 2012). These ependymal cells have specific electrophysiological characteristics. Marichal et al. (2012) made patch clamp recordings of cells in the lateral domains of the ependymal layer of mice and found that these cells had linear voltage current relationships, low input resistance and hyperpolarized resting membrane potentials. From their morphological study, the cells on the whole belonged to ependymocytes lining substantial portions of the central canal. They were also able to record from the midline domains and these cells exhibited most of the markers of neural stem cells and were considered the radial ependymal cells. Their electrophysiological properties included a hyperpolarized resting membrane potential, high input resistance and sometimes potassium and calcium voltage gated currents. To determine whether the properties of these cells persisted into adulthood, Marichal et al. (2012) explored the results in P15-P21 and even some P40 rats where they found that the properties of the two cell types were similar in these older animals to the neonatal recordings. Furthermore, nestin and 3CB2 (a radial glial marker) were still expressed in cells contacting the poles (midline domain) and a substantial number of cells on the lateral aspects were positive for proliferating cell nuclear antigen (PCNA). They concluded that progenitors in the ependymal layer of mature rat spinal cord maintain the basic properties of neonatal rodents.

SOX2, a transcription factor essential for maintenance of pluripotency of undifferentiated stem cells and related proteins is widely expressed in ependymal cells around the central canal (Corns et al., 2015; Barnabé-Heider et al., 2010; Hamilton et al., 2009). Nestin is expressed in a subset of ependymal cells, predominantly found dorsal to the central canal (Alfaro-Cervello et al., 2012; Hamilton et al., 2009). Nestin is an intermediate filament found in developing cells and cells after injury, suggesting that ependymal cells around the central canal may act as stem cells. Ependymal cells also expressed CD24, a glycoprotein expressed by mature

ependymal cell (Alfaro-Cervello et al., 2012; Mirzadeh et al., 2008), CD133 (Alfaro-Cervello et al., 2012) which is observed in ependymal cell and astrocytes, and also expressed transcription factor FoxJ1 which acts as a marker for ciliated epithelia and cells of ependymal lineage (Alfaro-Cervello et al., 2012; Meletis et al., 2008).

Ependymal cells also express Akhirin (AKH), a soluble molecule that might enhance the stem cell proliferation and differentiation during development. Using Akhirin negative mutant mice ($AKH^{-/-}$), Abdulhaleem M et al. (2015) showed that the size of neural tube was smaller in $AKH^{-/-}$ compared with positive mutant mice ($AKH^{+/+}$). The smaller size could be due to a significant reduction of the proliferative activity in the spinal cord of $AKH^{-/-}$ during development.

Vimentin is another neural stem marker found in central canal studies by Hamilton et al. (2009) and Meletis et al. (2008). Using postnatal mice at day 0, they performed immunohistochemistry on secondary spheres which were generated from primary spheres after 7 days. The central canal in the spinal cord is lined with cells that express Vimentin around the central canal in circular manner (Hamilton et al., 2009). Other neural stem/progenitor cell markers found in the central canal includes CD15, brain lipid binding protein (BLBP) and GFAP. They are expressed by subpopulations of cells lining the central canal (Hamilton et al., 2009; Meletis et al., 2008). The GFAP expressing astrocytes and Nestin expressing cells were found by Hamilton et al. (2009) at the dorsal area of central canal. In line with this finding, GFAP radial glial like cells located in the dorsal pole ependymal region have been proposed to have the ability to generate neurospheres producing astrocytes, oligodendrocytes and neurones (Sabourin et al., 2009).

Looking specifically at biciliated cells of spinal cord identified by Alfaro-Cervello et al. (2012), they showed that these expressed vimentin, CD24, FoxJ1, SOX2 and CD133. They were negative for Nestin and GFAP. This was in contrast with Sabourin et al. (2009) who found abundant GFAP positive cells around the central canal. Differences

in these studies highlight the issues regarding consistent classification of these different cells.

1.4.2.2 Sub classification of ependymal cells

There is some controversy about the subtypes of cells in the spinal cord ependymal niche, but cuboidal, tanycytic and radial classes of lumen-contacting, ciliated ependymal cells have been identified (Alfaro-Cervello et al., 2012; Hamilton et al., 2009; Meletis et al., 2008; Bruni and Reddy, 1987). Some authors differentiate ependymal cells and tanycytes (Mothe and Tator, 2005), others define three subtypes; cuboidal ependymal, radial ependymal (in dorsal and ventral poles) and tanycytes (radial) (Meletis et al., 2008).

The ependymal layer in the spinal cord consists of roughly equal numbers of cuboidal ependymal cells and tanycytes, based on electron microscopy analysis (Meletis et al., 2008) but this depends on what cells are counted as tanycytes and other studies suggest that the cuboidal ependymal cells are the most numerous (Bruni and Reddy, 1987).

<u>Cuboidal ependymal cells</u>

Alfaro-Cervello et al. (2012) described a subpopulation of cells in lateral walls of central canal of spinal cord that did not have radial processes. These cells may be grouped as cuboidal ependymal cells because they reside mostly at lateral region of spinal cord. They had pseudostratified organization, electron-dense rich cytoplasm with intermediate filaments, horse-shoe shaped Golgi apparatus polarized towards lumen, numerous dark mitochondria throughout basal and apical cytoplasm, and apically located lipid droplets. The rough endoplasmic reticulum (RER) was small, with few free ribosomes. Nuclei were mostly located in the apical row of the

pseudostratified epithelium. The chromatin was condensed in small clumps, with three to four nucleoli associated with the nuclear envelope. Long zonulae adherens junctions with a beaded appearance comprised of electron-dense clumps alternating with thin, tightly apposed electron-dense membranes were observed between adjoining cells. Intercellular spaces and deep interdigitations were also observed on the apical surface of ependymal cell.

The full function of cuboidal ependymal cells is not fully understood but beside potential proliferative capabilities, they might have other important functions such as propulsion of CSF flow (Sabelström et al., 2013). Using whole mounts of the central canal and placing fluorescent microbeads on the central canal surface, the cilia of these cells have been shown to move back and forth, which would allow movement of fluid (Alfaro-Cervello et al., 2012). The cells may also be important in providing a barrier to prevent harmful substances from entering the spinal cord from the CSF. This is a role known to occur in other regions (Del Bigio, 2010), along with potential metabolic regulation since the ependymal cells express glucose transporters that may allow them to take up glucose from the CSF to regulate the levels. Ependymal cells can be infected by various viruses such as measles, mumps and Herpes simplex type 1 (Del Bigio, 2010) and may almost act as a first line of defence in the response to infection, although it is not clear how this may occur.

Tanycytes and radial ependymal cells

Tanycytes have a basal process, which can contact blood vessels or neurones suggesting interactions between ependymal cells and the surrounding niche (Meletis et al., 2008). Researchers also used immunoelectron microscopic analysis of CreER-immunoreactive cells in FoxJ1-CreER mice and reported that these cells have a single cilium. Tanycytes have been observed along the length of the spinal cord at an average frequency of 1-3 cells per 0.5 μm section, and the numbers were higher in

lumbar and sacral regions than in cervical and thoracic levels (Bruni and Reddy, 1987).

The least numerous ependymal cell subtype is the radial ependymal cell, which resides in the dorsal and ventral poles of the central canal and extends long processes aligned with the dorsoventral axis (Lacroix et al., 2014; Meletis et al., 2008). Hamilton et al. (2009) described a subpopulation of tanycyte and radial ependymal cells that are the same based on their long, basally projecting fibres and expression of Nestin. In the mouse spinal cord, ependymal cells are derived from Nkx6.1 expressing ventral neuroepithelial cells (Fu et al., 2003) while the mouse forebrain ependymal cells are derived from radial glia and appear to be post mitotic (Spassky et al., 2005). From the ependymoglial cells, the cells persist at the dorsal and ventral midlines, maintaining long filament-rich fibres which persist from embryo time day. Ependymal cells do not fully differentiate until late in postnatal development in rodents or during the third trimester in humans, when they possess multiple motile cilia and significant adherens junctional proteins at their apical surface (Spassky et al., 2005). The dorsal fibres can still be traced along the dorsal grey matter by which at postnatal day 5, their trajectory within the dorsal column is obscured by white matter (Sturrock, 1981). The cells in most dorsal and ventral tips of spinal cord central canal showed a very long radial expansion containing intermediate filaments running towards pial surface of spinal cord and there are groups of cells which also had dorsally located radial processes but did not reach pial surface identified by Alfaro-Cervello et al. (2012). The processes also identified by others in both rat (Rafols and Goshgarian, 1985) and mouse (Seitz et al., 1981).

Dorsal GFAP positive cells identified by (Sabourin et al., 2009) have a radial morphology and some expressed BLBP (radial glial marker). These cells have been classified as tanycytes by others (Bruni and Reddy, 1987; Rafols and Goshgarian, 1985; Seitz et al., 1981). A subpopulation of tanycyte ependymal cells express the

neural precursor marker Nestin and extend long process from the dorsal and ventral poles of the central canal (Hamilton et al., 2009).

In human study by Cawsey et al. (2015), Nestin-positive cells with tanycyte morphology were identified in the ventral and dorsal regions of the central canal of spinal cord and they suggested that these cells act as neural progenitor cells.

1.4.2.3 Cerebrospinal fluid contacting cells

Cerebrospinal fluid contacting cells (CSFcC) are also commonly found in the central canal region and distributed around the area among neurones. CSFcCs are found in the subependymal layer surrounding the central canal. The cell size is smaller than nearby interneurones (Barber et al., 1982). The prominent feature of CSFcCs is the process that extends from the cell body and through the ependymal layer to contact the cerebrospinal fluid in the central canal (Vigh et al., 1977). It has been proposed that CSFcCs could connect the internal cerebral spinal fluid (CSF) with the external CSF surrounding the entire spinal cord (Vígh et al., 2004) and they could become excited through changes in CSF flow either through pH changes or neurotransmitter release. The primary function of these cells may therefore be to regulate the local environment of central canal.

1.4.3 Evidence that the ependymal cells are the neural stem cells of the spinal cord

Ependymal cells are considered unique according to the following properties, which I will describe in detail in the following sections:

1. Self-renewal capacity

2. Ability to increase in number in injured tissue

3. They are multilineage progenitors

1.4.3.1 Self-renewal capacity

An important characteristic of ependymal cells in spinal cord area is the self-renewal capability in normal tissues. Some of the first evidence looking at this capacity was carried out in neurospheres grown from ependymal cells in culture which showed that ependymal cells in the spinal cord are capable of neurogenesis (Shihabuddin et al., 1997). They reported that the majority of cells expressed vimentin, which is a marker of immature proliferating cells but some did differentiate into neurones, astrocytes and oligodendrocytes. Then in 2000, Horner et al. (2000) studied this in vivo, using 5′-Bromo-2-deoxyuridine (BrdU) as a marker of dividing cells since it is incorporated into cells during the S-phase, and showed that, not only did cells proliferate but that they colocalised with markers of immature glial cells or to a lesser extent, mature astrocyte or oligodendrocytes. In a study using transgenic FoxJ1-CreER mice where all cells with motile cilia or flagella are labelled with β-gal (which labels the ependymal cells) or nestin-CreER mice which labels progenitor cells and BrdU labelling of dividing cells, it was shown that 19% of the ependymal cells were labelled in the adult spinal cord (Meletis et al., 2008). They showed that in the intact spinal cord, ependymal cell proliferation occurs at a slow rate and just enables self-renewal of

these cells. In comparison, in the lateral ventricles, the ependymal cells were unlabelled with tritiated thymidine and BrdU without injury suggestive that in normal conditions, ependymal cells of lateral ventricle are not dividing cells (Spassky et al., 2005).

Bone morphogenetic proteins (BMP) are multifunctional growth factors (Navarro Quiroz et al., 2018) that are present in neural stem cells in brain neurogenic niches and have been shown to play important roles in generation of astrocytes over oligodendrocytes during development (Bonaguidi et al., 2005). In adult brain, BMP2 and 4 may inhibit neurogenesis and Lim et al. (2000) found out that ependymal cells in the SVZ expressed Noggin; an antagonist to BMP, which may block BMP signalling in neural stem cells. In spinal cord, the expression of various BMPs and noggin in ependymal cells indicates that these cells are involved in neurogenesis and that BMP modulates this ability (Miyagi et al., 2012).

1.4.3.2 Ability to increase in cell number in injured tissue

Much evidence shows that ependymal cells in the spinal cord are capable of increasing their number in response to injury (Lacroix et al., 2014; Barnabé-Heider et al., 2010; Cizkova et al., 2009; Meletis et al., 2008; Danilov et al., 2006; Johansson et al., 1999). Cell fate mapping studies using the FoxJ1 promoter to label the ependymal cell in the spinal cords revealed that ependymal cells migrate to the lesion site following injury and give rise to cells other than spinal cord ependymal cells such as astrocytes and oligodendrocytes (Li, X. et al., 2016; Barnabé-Heider et al., 2010; Meletis et al., 2008).

Using transgenic FoxJ1-CreER mice where all cells with motile cilia or flagella are labelled with β-gal (which labels the ependymal cells) or nestin-CreER mice which labels progenitor cells, Meletis et al. (2008) showed that in the intact spinal cord,

ependymal cell proliferation occurs at a slow rate and just enables self-renewal of these cells. They then introduced transverse spinal injury at the dorsal funiculus of the spinal cord in mid thoracic level. Their findings showed that there were higher SOX9 immunoreactive cells compared to non-injured segments after 4 weeks. FoxJ1 +ve ependymal cells migrated to the injury site with evidence of increased numbers of recombined cells and there were patches of new and old ependymal cells. Even in dissociated spinal cord cell cultures, the transgenic mice showed a high proportion of recombined neurospheres in both types of transgenic mice, showing that these are derived from ependymal cells. Barnabé-Heider et al. (2010) took this work a little further, when they looked at proliferation and differentiation of all cell types in the ependymal cell layer. They showed that ependymal cells are able to generate progeny of both astrocytes (identified through Sox9 labelling) and oligodendrocytes (identified as either Olig2 or APC positive). In the uninjured cord, oligodendrocytes are produced in greater numbers than the ependymal cells or the astrocytes. However all cells only self-renew and there is little differentiation. After injury was introduced (transverse cut without reaching grey matter or central canal at thoracic level), astrocytes, ependymal cells, and oligodendrocytes progenitors showed higher progeny production. After injury, glial cells and ependymal cells were produced in higher amounts than oligodendrocyte progenitors, but only ependymal cells were able to give rise to cells of different fates, producing astrocytes and oligodendrocyte. Other studies also support this finding - ependymal cells migrate from the region of the central canal after 3 days post injury and differentiate into astrocytes showed by nestin expression on a spinal cord injury rat model (Mothe and Tator, 2005). Furthermore, after spinal cord compression injury, there was an increase in cells expressing proliferating cell nuclear antigen (PCNA) (showing increased proliferation) and some of these cells were double labelled with GFAP (a marker of astrocytes) or with nestin and GFAP; indicating a differentiation of ependymal cells into reactive astrocytes. Nestin or PCNA immunoreactivity showed increases in levels by day 1

post injury and showed its peak expression by 7 days post injury. Most of the ependymal cells were able to divide and proliferate according to the severity of injury without experiencing apoptosis.

A few studies in the past also showed that spinal cord ependymal cells contribute to tissue repair to promote functional recovery after spinal cord injury (Moreno-Manzano et al., 2009; Takahashi, T. et al., 1998). In a recent study by Sabelström et al. (2013) the contribution of ependymal cell proliferation to the glial scar formation was highlighted by using a mouse with all Ras genes (required for mitosis) deleted in ependymal cells (FoxJ1-rasless mouse). This meant that ependymal cells were not able to proliferate. These FoxJ1-rasless mice had abnormal scar formation and increased neuronal loss and spinal cord atrophy after spinal cord injury, compared to control injured mice.

In contrast, a recent study looked at the contribution of ependymal cells to scar formation after spinal cord injuries of varying magnitude and suggested that there was no contribution of these cells to the recovery process following small injuries, such as stab injuries. Even large crush injuries involving damage to the central canal only led to proliferation of minimal numbers of ependymal cell-derived contributing to the scar (Ren et al., 2017).

The ability of ependymal cells to proliferate after injury may also be preserved in humans, which is very pertinent for potential therapies for spinal cord injury. In a study of post-mortem spinal cords of patients that had experienced a spinal cord injury (Cawsey et al., 2015), there was a significant increase in nestin immunoreactive cells around the central canal compared to control tissue. Furthermore, there was a positive correlation between survival time and numbers of nestin positive cells. Only one group has been able to perform in vitro assays with human ependymal cells. This ependymal neurospheres may give rise to cells expressing glial and neuronal markers but they cannot be passaged. When cultured, even though these cells can be

passaged, they produced cells compatible with mesodermal cell types (Garcia-Ovejero et al., 2015).

The most recent evidence also questions the role of ependymal cells in contributing to a proliferative responses after spinal cord injury in humans. Even with a strong stimulus, the ependymal remnant in adult humans does not proliferate at any distance from the lesion of injury either at early times and months after injury. The area covered by the nuclei of cells in the ependymal region is not significantly different between control and spinal cord injury individuals. There were only 6 cells labelled with Ki67 or MCM2+; out of 7607 cell nuclei counted in the ependymal region including all the slices from all the individual tested which they labelled the overall labelling index as <0.08%. This result is really small compared to the strong induction of ependymal proliferation after spinal cord contusion in rats. They found that in rodent, with moderate/severe spinal cord contusion (200 kdyn), the peak of ependymal proliferation was 20% in the spinal cord and this occurred at 3 days post-injury. This result challenges the view of the spinal cord ependymal cell layer as a neurogenic niche and target cell for neural repair in human (Paniagua-Torija et al., 2018).

1.4.3.3 Ability to be the multilineage progenitors

In normal spinal cord, Barnabe-Heider et al (2010) suggested ependymal cells, astrocytes and oligodendrocyte progenitors were able to self-renew based on their constant quantity of cell numbers over the 4 months of observation. They then went on to test with an incision at the dorsal funiculus in vivo using transgenic mice left for two weeks and four months and the results showed an increase in cell proliferation at the site of injury. The FoxJ1-CreEr mice (labelling ependymal cells) showed 4 to 5 fold increment while Cx30-CreEr (astrocytes) and Olig2-CreER (oligodendrocytes) mice showed double increment. Ependymal cells of FoxJ1-CreEr mice were found in

the centre of the glial scar caused by the injury compared to astrocytes of Cx30-CreEr mice and oligodendrocytes of Olig2-CreER mice which both found at the injured and non-injured area. Ependymal cells were suggested to act as multilineage progenitors as FoxJ1-CreEr ependymal cells gave rise to neurospheres which then differentiated into neurones, astrocytes and oligodendrocytes compared to cells from the other two progeny (Cx30-CreEr/Olig2-CreER) which did not produce new neurospheres.

Most of the studies have showed that ependymal cell proliferation was induced with spinal cord injury; which explains that in intact spinal cord, the turnover and proliferation of ependymal cells is limited (Barnabé-Heider et al., 2010; Cizkova et al., 2009; Meletis et al., 2008). However the ependymal cells were considered as neurosphere forming cells that can self-renew after several passages indicating that ependymal cell have stem cell potential in adult spinal cord and are able to differentiate into all neural cell types (Sabelström et al., 2013).

Therefore there is dispute about whether spinal cord ependymal cells can be regarded as neural stem cells or neural progenitor cells in the adult spinal cord. However many studies have focussed on how these cells respond to an injury rather than determining whether they can be made to contribute to recovery by specific modulation of the intrinsic or extrinsic factors that are known to modulate neurogenesis in other brain regions.

1.4.4 What may affect the proliferation and differentiation of uninjured ependymal cells?

Both cell-intrinsic and -extrinsic factors contribute to changes in cell production and affect the growth of central nervous system cells. Similar extrinsic and intrinsic factors to those that are important in other neurogenic regions may also modulate the ependymal cells which are the spinal cord neural stem cells. For example, adult neural stem cells have been reported to proliferate and migrate either laterally or dorsally towards the lesion site following experimental spinal cord injury (Cizkova et al., 2009). Cizkova group also compared the effects of spinal cord injury with the effects of exercise on ependymal cell proliferation. They found that the injured group of rats had ependymal cell proliferation mainly dorsally or laterally towards the injured site whereas running rats exhibited a different pattern of proliferation, with the BrdU nuclei restricted to medial regions and only in a minor population.

It is critical to understand how these potential neural stem cells in the spinal cord can be manipulated. As mentioned, one method is by activation of specific neurotransmitter receptors. There is little information regarding the neurotransmitters that may regulate proliferation in the spinal cord but some recent research has started to address this and we now know that neurotransmitters can influence proliferation and differentiation of spinal neural stem cells. Progenitors in stem cell niches in the brain have been shown to be regulated by GABA, glutamate, acetylcholine, dopamine and serotonin (Jang et al 2008). Ependymal cells of the brain (Alfonso et al., 2012) and the spinal cord (Corns et al., 2013) have been shown to respond to a neurotransmitter i.e. GABA. Moreover in the dentate gyrus, GABA can modulate both proliferation, differentiation and migration of neural progenitor cells (Tozuka et al., 2005). Application of GABA receptor agonists increased the number of calbindin

positive BrdU neurones, suggesting that $GABA_A$ receptor activation promotes their differentiation.

In SVZ, GABA released from newborn neurones inhibits the proliferation of neighbouring progenitors to control the neurogenesis (Liu, X. et al., 2005; Haydar et al., 2000; LoTurco et al., 1995). Although less is known about the regulation of spinal stem cell niches by neurotransmitters, some recent studies have shown neurotransmitter mediated influences on stem cell niches especially the central canal contacting progenitors (Corns et al., 2015; 2013; Marichal et al., 2012; Reali et al., 2011). One example, acetylcholine was shown to induce cell proliferation in the spinal cord neurogenic niche which was mediated by α7*nAChRs (alpha-7 nicotinic receptor) and could be enhanced with a selective α7*nAChR positive allosteric modulator (PNU 120596) (Corns et al., 2015). A neuropeptide; substance P (SP) can influence neural stem/progenitor cell proliferation and neurogenesis in a compression type of spinal cord injury (Kim et al., 2015) in adult Sprague Dawley rats. Through immunoblot analysis, they were able to show that SP also increases the phosphorylated ERK and phosphorylated p38 kinases in neural stem/progenitor cell proliferation.

In my work, I will be examining the role of GABA and 5-HT on ependymal cell proliferation and so the next sections will now focus on these neurotransmitters.

1.5 Neurotransmitters and their receptors

The cellular response is determined by which specific receptors the transmitter binds to, which can be either excitatory or inhibitory. The magnitude of the response is determined by receptor number, the state of the receptors and the amount of transmitter released.

There are two major classes of neurotransmitter receptors; ionotropic and G-protein coupled (GPCR) or metabotropic (Owens and Kriegstein, 2002). Ionotropic receptors are ligand-gated ion channels where binding of a ligand to these receptors results in a change that opens a pore in the receptor and permits ion transfer across the cell membranes. Metabotropic receptors are associated with G proteins which transduce the extracellular signal into an intracellular response.

The ionotropic receptor is a relatively large, multi-subunit complex composed of four or five individual proteins that combine to form an ion channel through the membrane. According to the amino acid sequence, there are 2 distinct families. One family includes the nicotinic acetylcholine (ACh) receptor (nAChR), the GABA receptor, the glycine receptor and one of 5-HT receptor, the 5-HT_3 receptor. The other family is a group of ionotropic glutamate receptors (Traynelis et al., 2010).

1.6 GABA

GABA has been known to exist in brain since 1950 (Awapara et al., 1950; Roberts and Frankel, 1950). GABA is a four-carbon, non-protein amino acid found in all prokaryotic and eukaryotic organisms as a significant component of the free amino acid pool.

One function of GABA is to act as a neurotransmitter; the most used inhibitory neurotransmitter in the mammalian central nervous system. It acts at inhibitory synapses in the brain, and plays a key role in modulating neuronal activity. GABA acts by binding to specific transmembrane receptors in the plasma membrane of both pre and postsynaptic neuronal processes. There are three different receptor subtypes which mediate the inhibitory actions of GABA; the ionotropic $GABA_A$ and $GABA_c$ receptors and metabotropic $GABA_B$.

GABA is synthesized from glutamate, using the enzyme glutamic acid decarboxylase (GAD) and pyridoxal phosphate (active form of vitamin B6) as a cofactor to convert into inhibitory neurotransmitter (Bradford, 1995).

$$L\text{-}Glu + H^{+} \rightarrow GABA + CO_2$$

The enzyme GAD appear in two isoforms; GAD65 and GAD67. Most neurones express both GAD isoforms. GAD67 is involved in the synthesis of GABA for general metabolic activity whilst GAD65 is involved in the synthesis of GABA for vesicular release (Erlander et al., 1991). After being synthesized in the cytoplasm, GABA is transported to the nerve terminal and packaged into vesicles by a chain of vesicular transporters (Erlander et al., 1991). GABA molecules are released from the presynaptic terminals to act on postsynaptic receptors and/or as presynaptic auto receptors. Action potentials reaching the axon terminals induce Ca^{2+} influx which triggers the release of GABA into the synaptic cleft (Südhof and Rizo, 2011). The effect of GABA depends on the type of receptor it binds to and, in the case of the ionotropic receptors, on the intracellular Cl^- concentration (Bormann, 1988). GABA has a chloride equilibrium potential of approximately -70 mV, close to the membrane resting potential in neurones (Olsen and Sieghart, 2009).

The Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) and K^+-Cl^- cotransporter 2 (KCC2) determine the intracellular Cl^- concentration. Intracellular Cl^- concentration compared to extracellular Cl^- concentration dictates whether GABA is inhibitory or excitatory. Low intracellular Cl^- leads to Cl^- influx when $GABA_A$ receptors are activated as in mature neurones while high intracellular Cl^- concentrations leads to Cl^- efflux when $GABA_A$ receptors are activated as in neural stem cells and oligodendrocyte progenitor cells. GABA can promote or suppress proliferation depending on the developmental stage, brain area and the fate of distinct progenitor populations (Duveau et al., 2011). GABAergic signalling has already been shown in the neurogenic niche of the

postnatal turtle (Dervan and Roberts, 2003) and the mammalian spinal cord (Corns et al., 2013; Shechter et al., 2007).

1.7 GABA receptors

The most abundant GABA receptor is the $GABA_A$ receptor ($GABA_ARs$). $GABA_ARs$ found in the CNS can be grouped into two types: synaptic and extrasynaptic $GABA_ARs$. The first mediate synaptic communication and are located in the underlying postsynaptic density. These receptors can be activated by GABA release from presynaptic vesicles increasing the membrane permeability to chloride and bicarbonate ions for a brief period of time producing inhibitory postsynaptic (IPSC) or excitatory/depolarizing postsynaptic (EPSC) currents in mature and immature neurones, respectively. In contrast, the extra synaptic $GABA_ARs$ usually cause slow tonic inhibition. The presence of $GABA_A$ receptors was also demonstrated in stem-like cells and neuroblasts (Alfonso et al., 2012). Ambient GABA was shown to derive from neuroblasts and it was proposed that it provides a feedback mechanism for neuronal proliferation (Wang, D. et al., 2003; Nguyen et al., 2003).

There are 3 main types of GABA receptor that may be important in neurogenesis and they each show different properties and sensitivity to modulators. These receptors are $GABA_A$, $GABA_B$ and GABAρ (Table 1-1) and I will discuss each in turn and their potential role in neurogenesis.

Table 1-1 Composition of GABA receptors and their actions and antagonists

RECEPTOR	STRUCTURE	ACTION	MODULATORS	ANTAGONISTS	REFERENCE
$GABA_A$	Heterooligomeric - The subunits are α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-3}, δ, ε, Θ and π -Ligand gated chloride ion channel	-entering of chloride ion caused hyperpolarisation, exit of chloride causes depolarisation -net uptake of chloride ion generates a source of inward depolarising current - intracellular Ca^{2+}, if the spike preceded with Ca^{2+} the GABA receptor mediated response inhibited	Benzodiazepine able to give enhancement and reduction of $GABA_A$ function -Steroids -barbiturates	Alkaloid bicuculline	Corns et al. (2013); Duveau et al. (2011); Liu, X. et al. (2005); Nguyen et al. (2003); Wang, D. et al. (2003)

$GABA_B$	-G protein-coupled receptor -7 transmembrane receptors -3 subunits have been cloned i.e. $GABA_BR1_a$ $GABA_BR1_b$ $GABA_BR_2$	-activate 2nd messenger systems phospholipase C & adenylate cyclase -activate potassium & calcium ion channels via G-coupled proteins -slow prolonged inhibitory signals & function	Methylphosphinic acid analogue of GABA (3 amino-propyl)-potent agonist at $GABA_B$ receptor- with no activity of $GABA_A$Rs	CGP 55845 CGP 52342	Liu, X. et al. (2005); Wang, D. et al. (2003)
GABAρ or $GABA_C$	-Homooligomeric -selective chloride channel	-conducted less current compared to $GABA_A$ - had a longer channel opening time & desensitization.	Activated selectively by CACA (cis-4-aminocrotonic acid)	blocked competitively by TPMPA (1,2,5,6-Tetra hydropyridin-4-yl) methylphosphinic acid) and noncompetitively by picrotoxinin	Sigel and Steinmann (2012)

1.7.1 $GABA_A$ receptor

1.7.1.1 Structure, composition and function of $GABA_A$ receptors

The $GABA_A$ receptor is an ionotropic receptor which is a GABA-gated ion channel. The endogenous ligand is GABA. The receptors are made up of five subunits, each of which contains four transmembrane domains. There are 19 different $GABA_A$ receptor subunits that have been identified (D'Hulst et al., 2009). The subunits are α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-3}, δ, Ɛ, Θ and π. The most common receptor is comprised of two α_1- and two β_2-subunits with one γ_2-subunit (Olsen and Sieghart, 2009; Olsen and Sieghart, 2008) see Figure 1-3.

$GABA_A$ receptors are distributed widely throughout the central nervous system and they are permeable to chloride and bicarbonate. $GABA_A$ receptors that contain an α_{1-3}, π and γ_2 subunit are mainly synaptic, whereas α_{4-6} and *δ*–containing receptors are mainly peri- or extrasynaptically located (Belelli et al., 1997). Thus there are two types of transmission mediated by $GABA_A$ receptors, these are synaptic, also be known as phasic transmission and extrasynaptic which is also known as tonic/paracrine transmission. GABA is known to exert phasic transmission which results in rapid and direct transmission of signals from one neurone to another with GABA containing vesicles released from presynaptic terminal to bind to GABA receptors at the post synaptic density of the postsynaptic terminal while tonic transmission involves the extrasynaptic terminals. Many processes are known to modulate $GABA_A$ receptor number and function and these are likely to be relevant to both phasic and tonic inhibition involving their different subtypes (Farrant and Nusser, 2005).

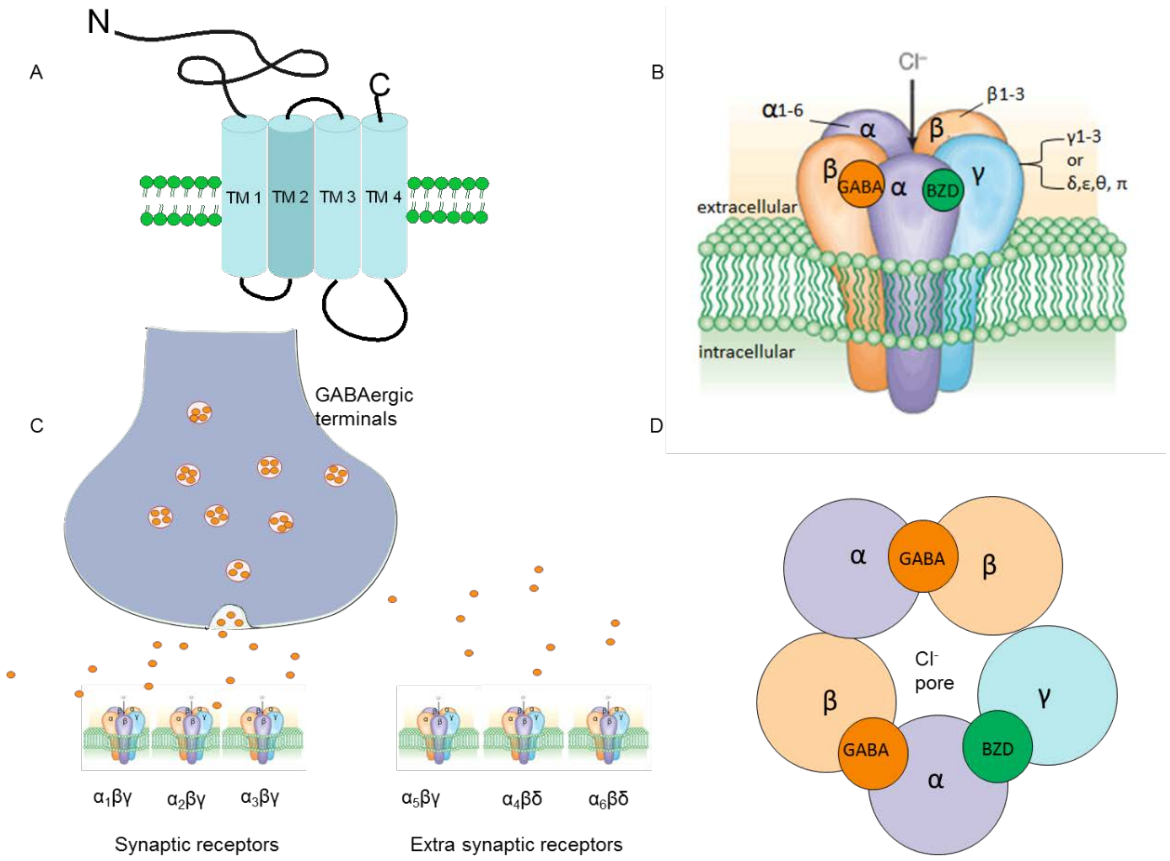


Figure 1-3 $GABA_A$ subunits

Diagram showing the $GABA_A$ subunits. (A).$GABA_A$ receptor subunit consist of four hydrophobic transmembrane domains (TM1-4), with TM2 believed to be the line pore of the channel. (B & D).The subunits are α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-3}, δ, Ɛ, Θ and π , most $GABA_A$ receptors expressed in the central nervous system consist of two α subunits, two β subunits and one γ subunit; the subunit γ can be replaced by δ, Ɛ, Θ and π . Binding of neurotransmitter GABA occurs at the interface between the α and β subunits and triggers the opening of the channel, allowing the rapid influx of Cl^- into the cell. BZ binding occurs at the interface between the α and γ subunits, (C) $GABA_A$ receptors composed of $\alpha_{(1–3)}$ subunits together with β and γ subunits are thought to be primarily synaptically localized, whereas $\alpha_5\beta\gamma$ receptors are located largely at extrasynaptic sites. Both these types of $GABA_AR$ are benzodiazepine sensitive. By contrast, receptors composed of $\alpha_{(4\ or\ 6)}$ βδ are benzodiazepine insensitive and localized at extrasynaptic sites (Adapted and modified from Jacob et al. (2008)).

1.7.1.2 Maturation switch in GABA-mediated effects

Synaptic GABAergic inputs were detected at postnatal day 7; i.e, when the granule cells were integrated to become innervated by synaptic inputs in the pre-existing functional circuit (Ge et al., 2006). Mature neurones express the K^+-coupled Cl^- transporter 2 (KCC2), which exports Cl^- ions from the cell, leading to a lower intracellular Cl^- concentration. Binding of GABA to the receptor leads to influx of Cl^- into the cell, which causing a hyperpolarization of the cell membrane. Thus, in mature neurones GABA acts as an inhibitory neurotransmitter. Neural progenitors and young neurones express the Na^+-K^+-$2Cl^-$ transporter NKCC1, and have a higher Cl^- intracellular concentration compared to the extracellular space (Ge et al., 2006). GABA is depolarizing when binding to the GABA receptor in immature neurones since Cl^- exits the cell down its concentration gradient. During neuronal maturation, the expression of NKCC1 decreases while KCC2 starts being expressed which leads to a reversal of chloride gradient and as a consequence to a switch from depolarising to a hyperpolarising action (Ge et al., 2006; Rivera et al., 1999).

1.7.1.3 Modulation of $GABA_A$ receptor

GABA binds to the $GABA_A$ receptor at the GABA binding site situated extracellularly at the interface between α and β (Ramerstorfer et al., 2011). Most of $GABA_A$ receptors comprise two α and two β subunits and have two GABA binding site (Sigel and Steinmann, 2012). Binding of GABA to one of the site can induce opening of the channel and occupation of both sites can enhance the opening probability (Baumann et al., 2003). Besides the binding site of GABA between α and β subunits, there is evidence of presence of another GABA binding site at the interface of δ subunit in the receptors containing it (Stell et al., 2003).

$GABA_A$ receptors can be modulated by: 1) post-translational modification by different protein kinases, 2) receptor associated protein, 3) endogenous molecules and 4) exogenous molecules.

$GABA_A$ receptors can be modulated by phosphorylation through several protein kinases such as protein kinase A (PKA) (Bohnsack et al., 2016), protein kinase C (O'Neill and Sylantyev, 2018; Bohnsack et al., 2016) and other tyrosine kinases such as glycogen synthase 3β, which phosphorylates the GABA receptor scaffolding protein, gephyrin and subsequently affects $GABA_A$ receptor diffusion (Battaglia et al., 2018). Different receptor associated proteins such as GABARAP also play a role in modulating the $GABA_A$ receptor function (Wang, H. et al., 1999).

Examples of modulators are benzodiazepines and other drugs which can act at sites on the receptor; barbiturates, etomidate, the competitive antagonist bicuculline and the channel blocker picrotoxin (Sigel and Steinmann, 2012). The activity of $GABA_A$ receptors can also be altered by intrinsic modulators including neurosteroids, endozepines and zinc (Bormann, 2000).

The primary neural action of benzodiazepines and related compounds is augmentation of inhibitory transmission which occurs through allosteric modulation of the GABA-induced current at the $GABA_A$ receptor (Majewska, 1992).

Based on $GABA_A$ receptor structure, the benzodiazepine binding site has been mapped to the extracellular part of the receptor at the interface between α and γ subunit (Ernst, M. et al., 2003). The discovery of the benzodiazepine binding site has uncovered a variety of endogenous ligands such as diazepam binding inhibitor (DBI) and its fragment. The next section will discuss more on DBI and its fragment octadecaneuropeptide (ODN) and sites of action i.e.; central benzodiazepine receptor (CBR) and translocator protein (TSPO).

Diazepam binding inhibitor and central benzodiazepine receptor

DBI also known as endozepine or acyl-CoA binding protein is an endogenous 10 kDa peptide (Knudsen et al., 1993). DBI was first characterised and isolated from rat brain by Guidotti et al. (1983). DBI is able to bind and displace radiolabelled diazepam from $GABA_A$ receptors (Guidotti et al., 1983). In the nervous system, DBI is expressed in astrocytes, in Bergmann glia, in ependymal cells and also in thalamus (Christian et al., 2013). Highest expression levels of DBI in the brain are in dentate gyrus, rostral migratory stream, SVZ, in the ependymal layer lining of third ventricles, in area of postrema, in cerebellar cortex, in hypothalamus, in amygdala and certain area of thalamus and cerebral cortex (Alfonso et al., 2012). There are high concentrations of DBI mRNA in ependymocytes bordering the third ventricle and tanycytes in the median eminence (Burgi et al., 2000).

DBI binds to an extracellular domain between α and γ_2 subunits of the $GABA_A$ receptor i.e., central benzodiazepine receptors (D'Hulst et al., 2009) and also can bind to peripheral benzodiazepine receptor (PBR) also known as translocator protein (TSPO) (Papadopoulos et al., 2006). The CBR is in fact a part of $GABA_A$ receptor while TSPO is localized mainly in the mitochondrial membrane in the cells of the central nervous system and peripheral organs and there is also some evidence that it may be in plasma membranes (Gandolfo et al., 1997). In the postnatal central nervous system, DBI is present in glial and ependymal cells but not in neurones (Papadopoulos, Vassilios et al., 2006; Bormann, 2000; Bormann, 1988). DBI undergoes tryptic digestion within cell to forms its two main processing products; ODN and triakontatetraneuropeptide (TTN) (Alfonso et al., 2012; Slobodyansky et al., 1989). There are no in depth studies addressing the question whether DBI is cleaved intra- or extracellularly. Also the identity of the enzymes responsible for DBI cleavage has remained elusive. DBI might be capable of self-cleavage and self-activation as it comprises several putative endoproteases (Farzampour et al., 2015). DBI and its

cleavage products have the ability to displace benzodiazepines from the binding site and therefore considered as endozepines. However only DBI and ODN preferentially bind to CBR site of $GABA_A$ receptor while TTN had a preferential affinity for the PBR (Costa and Guidotti, 1991). DBI and its cleavage products ODN, and to a lesser extent, TTN bind to CBR site of $GABA_A$ receptor and act to reduce $GABA_A$ receptor mediated Cl^- currents.

DBI has multiple roles within CNS. One important action of DBI, pertinent to this thesis, is that it affects proliferation of cells within the neurogenic niche of the SVZ (Alfonso et al., 2012). Using primary SVZ cultures which are allowed to differentiate and form neurospheres treatment with specific CBR and TSPO ligands; flumazenil and PK-11195 induced cell proliferation, (Alfonso et al., 2012). This DBI induced proliferation was blocked by flumazenil, not PK-11195 suggesting that the effects of DBI on proliferation are likely to be mediated via CBR within the $GABA_A$ receptor. The actions of DBI at CBR is to reduce the GABA activity and counterbalance the effect on neurogenesis, therefore promoting proliferation.

Alfonso et al. (2012) characterised the expression of DBI in SVZ and rostral migratory stream (RMS) using immunohistochemistry and found that DBI was detected in astrocyte like GFAP/Nestin+ stem cells and also in Mash1+ fast dividing progenitors; however it was absent from doublecortin (DCX+ cells, marker of neuronal precursor and immature neurones) migrating neuroblasts. This finding suggests that DBI is expressed in the early stages of neurogenesis and subsides when cells progress to the neuroblast stage. While DBI is shown to expressed strongly in neural stem cells and transit amplifying cells of SVZ (Alfonso et al., 2012), Dumitru et al. (2017) investigated the presence of DBI in SGZ, both in juvenile (3 weeks old) and adult (2 months) mice. The site of expression localized in the radial glial-like stem cells and amplifying neural progenitors. DBI expression reduced in level along with the developmental stage of SGZ neural progenitors, shown as no co-localization with

DCX+ and NeuN marker, resembling its expression pattern in the SVZ as done by Alfonso et al. (2012). In addition to SGZ, Dumitru and group also found that DBI is strongly expressed in all Nestin+, SOX2+ in tanycytes lining the walls of 3rd ventricle. These tanycytes were previously shown to act as neural stem cells (Dumitru et al., 2017).

Dumitru and colleagues (2017) also showed the presence of DBI is important for regulating the balance between preserving the progenitor pool and generating neurones. They manipulated DBI expression in SGZ neural stem cells using 3 mechanisms : they induced a genetic mouse line in which DBI can be deleted specifically in the nestin expressing stem cells and their progeny using tamoxifen administration, a combination of Nestin-cre mouse with lentiviral injection in dentate gyrus which caused DBI knockdown (KD) and strong DBI overexpression (OE) in stem cells and the progeny and injections of lentiviruses that knockdown or overexpressed DBI in dentate gyrus of wild type mice. All three manipulations produced changes in the population of SGZ neural stem cells. The viral DBI KD group showed a decrease in number of transit amplifying cells, astrocytes and neuroblasts and an increase in number of adult born neurones. However the DBI OE group demonstrated on increase in transit amplifying cells, neuroblasts and astrocytes and a decrease in number of adult born neurones. In a long-term group of mice (3 months post injection), the number of neuroblasts and adult-born neurones produced by the cells overexpressing DBI were comparable to controls. After a long period of DBI OE the progenitors overexpressing DBI would have enough time to exit the cell cycle and become adult-born neurones. Thus, DBI knockdown increases neuronal differentiation while DBI OE expands the pool of early progenitors.

DBI was shown before to act as a negative modulator of the $GABA_A$ receptor through the CBR receptor in cultured spinal cord neurones (Costa and Guidotti, 1991; Bormann, 1991). The DBI cleavage product ODN, when overexpressed, reproduced

the phenotype of DBI OE. ODN is known to bind with high affinity to the CBR receptor, but not to the peripheral one (Alfonso et al., 2012). Furthermore, electrophysiological recordings showed that ODN inhibits GABA receptor-mediated currents in transit amplifying cells in SVZ area. Similar effects of ODN were seen on SGZ stem cells (Dumitru et al., 2017). To investigate whether DBI exerts its effects on dentate gyrus neurogenesis by binding externally to $GABA_A$ receptor, they injected in the dentate gyrus of wild type and gamma2 F77I mice at postnatal day 7 with DBI OE and control lentiviruses and these animals were sacrificed after 2 weeks. The proportion of DBI-overexpressing SOX2+ precursor cells was significantly increased compared to that of control infected SOX2+ cells in wild type mice. If DBI is regulating SVZ and SGZ neurogenesis by modulating $GABA_A$ currents, DBI and ODN should have a much smaller effect in the mutant mice. Indeed overexpression of DBI in mutant mice prevented the phenotype found in wild type mice; namely an expansion of the pool of SOX2+ cells. Their results (Dumitru et al., 2017; Alfonso et al., 2012) provide evidences that DBI/ODN affects SVZ and SGZ niche by modulating the $GABA_A$ receptor in stem cells. Thus, DBI and ODN enhance postnatal and adult SVZ neurogenesis by negatively modulating the GABA induced currents (Alfonso et al., 2012).

ODN-immunoreactive material was only detected in glial and ependymal cells (Tonon et al., 1990), contradicting findings from (Alho, H et al., 1991) who reported that DBI immunoreactivity is located in non-GABAergic neurones of cerebral cortex and GABAergic neurones of cerebellum and hippocampus. There is therefore some controversy about expression patterns of ODN. To add to the complexity of the story, ODN was initially reported to act just as an agonist at CBR (Ferrero et al., 1986), however more recently it has been shown that ODN can also act as agonist at a metabotropic receptor coupled either to adenyl cyclase (AC) or to phospholipase C (PLC) (Leprince et al., 2001). Astrocytes synthesize and released endozepines

including DBI and ODN. More recently, an endogenous molecular form of ODN; bisphosphorylated ODN has been found to have stronger affinity for CBR than ODN (Gach et al., 2015). As well as being shown to stimulate neurogenesis in the adult mouse brain (Alfonso et al., 2012), ODN endogenously also contributes to the protection of astrocytes and neurones (Ghouili et al., 2018).

These data suggest that ODN might play a role in function of glial and ependymal cells perhaps through interaction at both CBR and metabotropic receptors as a modulator regulating proliferation and/or survival of neuronal cells.

Numerous reports show that DBI acts as a negative allosteric modulator of the GABA receptor both in cultured spinal cord neurones as well as in neural progenitors (Dumitru et al., 2017; Alfonso et al., 2012; Costa and Guidotti, 1991; Bormann, 1991). However, a study found out that DBI can also enhance GABA currents (Christian et al., 2013). DBI has been shown to act as a positive allosteric modulator in reticular thalamic nucleus. Mice lacking DBI gene showed an absence of endogenous potentiation of GABAergic synaptic transmission within the reticular thalamic nucleus. The potentiation was absent in the DBI knockout mice (*nm*1054- a deletion of 400 kb on chromosome 1 that includes DBI gene) and in the mice with a mutation that abolishes the binding of benzodiazepines at the benzodiazepine binding site in the α3 subunit. These data suggested that DBI in the thalamic reticular nucleus acts as a positive modulator of $GABA_A$ receptors by binding to the benzodiazepine site. Christian and colleagues also found that DBI reduces thalamo-cortical oscillations which might be the mechanism to prevent seizures. Therefore they proposed low DBI concentrations enhance GABA induced currents, while high DBI concentrations would negatively modulate the activity of the receptor.

In summary, GABA negatively regulates cell division by inhibiting proliferation and promotion of progenitor differentiation (Tozuka et al., 2005) and DBI opposes this inhibition. If DBI is indeed found in the central canal of spinal cord, it is possible that

it is also reside within the niche to counter balance the negative effects of GABA on proliferation.

Translocator protein (TSPO)

In addition to CBR, DBI binds to a second benzodiazepine receptor named as peripheral benzodiazepine receptor (PBR).This receptor was discovered in 1977; primarily within the outer mitochondrial membrane of peripheral tissues (Braestrup and Squires, 1977). PBR, also known as translocator protein (TSPO) is a 18 kDa protein. It consists of five transmembrane α helices (Papadopoulos, Vassilios et al., 2006). It is the minimal functional unit that acts as a binding site for all PBR ligands (Papadopoulos et al., 2006). In the CNS, TSPO is mostly found in glia, particularly within microglia and astrocytes. It was discovered due to its ability to bind to diazepam with an affinity closest to that of CBR. But this receptor shows no other structural or pharmacological similarities to $GABA_A$ receptors (Papadopoulos et al., 2006). TSPO roles include a role in steroid biosynthesis (Papadopoulos, V et al., 1997), acting as a component of the mitochondrial permeability transition pore complex in the inner and outer mitochondrial membranes which contain the anion channel and proteins necessary for benzodiazepine and endozepine binding (McEnery et al., 1992).

The DBI cleavage product TTN binds with low affinity to the $GABA_A$ receptor but binds with high affinity to TSPO (Mukhin et al., 1989). There are a number of drugs that have been established as typical TSPO-binding compounds, the benzodiazepine Ro5-4864, which has no affinity for $GABA_A$ receptors, instead acting as a ligand at TSPO, the anxiolytic drug FGIN-1-27 and a non-benzodiazepine antagonist, PK11195 [1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3 isoquinolinecarboxamide] which bind to TSPO but not to $GABA_A$ receptor. An indirect role of DBI in steroidogenesis is through the handling of mobilisation of cholesterol which involves the function of TSPO.

Allopregnanolone is a neurosteroid formed in response to ligand binding at TSPO and this neurosteroid is known to have potent positive allosteric modulator effects on $GABA_A$ receptor (Belelli and Lambert, 2005; Belelli et al., 1997). Thus TSPO activation may have downstream positive modulatory effects on the GABA response due to a neurosteroidogenic pathway. This is supported by the observation that in isolated mitochondria from a glioma cell line, endozepines activate the formation of the steroid hormone precursor pregnenolone (Papadopoulos, V et al., 1997) suggesting that endozepines also act as intracrine factors stimulating the synthesis of neurosteroids in astrocytes.

Because several neuroactive steroids are potent allosteric modulators of the $GABA_A$ receptor, it seems that endozepines can indirectly regulate the activity of the $GABA_A$-benzodiazepine receptor complex through their ability to control the production of neurosteroids through TSPO (Tokuda et al., 2010). TSPO can also be located at the plasma membrane, leading to an influx of Ca^{2+} through voltage-dependent calcium channels in glial cells (Papadopoulos, V et al., 1997).

Regulation and manipulation of neurotransmitter levels and GABA receptors within the area of the spinal cord may play a major role in determining how resident neural stem cells in this area behave. Studies into the effects of exogenous compounds which can affect the environment in which neural stem cell exist may explain the importance of particular neurotransmitters in the maintenance of the niche.

1.7.1.4 Role of $GABA_A$ receptor activation in neurogenesis

GABA plays a different role in regulating neurogenesis in the early postnatal period compared to the adult brain (Stefovska et al., 2008). The activity of GABA receptors can be altered by modulators including neurosteroids. Precise targeting of such

treatments depends on identification and characterization of the different subunit complexes that exist.

It has been proposed that the effects of GABA in embryonic CNS is to stimulate proliferation. GABA is important for development as injection of pentobarbital (a $GABA_A$ receptor agonist) stimulated dendritic growth of dentate gyrus cells (Ge et al., 2006). In adults, the most common reported action of GABA on proliferation is inhibitory.

GABA also promotes migration of spinal cord cells during development. Embryonic spinal cord cells of rat were dissected and dissociated into single-cell suspension for a microchemotaxis assay experiment. This chemotaxis assay revealed that these cells migrated toward GABA and muscimol ($GABA_A$ agonist). In contrast, bicuculline and picrotoxin ($GABA_A$ antagonist) inhibited this muscimol-induced migration (Behar et al., 1994).

In the spinal cord, the electrophysiological effect of GABA on GABA receptors in CSFcCs has been shown in rat (Marichal et al., 2009). In whole cell patch clamp recordings in spinal cord slices, 100 μM GABA produced currents that reversed at the Cl^- equilibrium potential. 10 μM Gabazine (a $GABA_A$ receptor antagonist) blocked this GABA-induced current, suggesting that it was mediated through $GABA_A$ receptors.

Most of the research on ependymal cells has focussed on the role of ependymal cells as neural stem/progenitor cells and their importance in neurogenesis following spinal cord injury. Thus to date, little is known about GABA-mediated changes in proliferation but it is known that ependymal cells can respond to GABA. The effects of GABA on ependymal cells in turtle spinal cord showed that GABA depolarised the brain lipid binding protein progenitor cells (BLBP+) (Reali et al., 2011). A GABA transporter 3 (GAT3) selective blocker (SNAP 5114) and non-selective GABA transporter blocker (nipecotic acid) were able to reduce, but not abolish the GABA-

induced currents in these BLBP+ progenitor cells. BLBP+ progenitor cells showed positive staining for anti GAT3, S100β (marker for ependymal cells) and anti-NKCC1. When gabazine or bicuculline and nipecotic acid were applied onto to the cells, the GABA-induced current was abolished, which suggests that both GABA transporters and receptors were involved in the response (Reali et al., 2011).

Furthermore in rat, ependymal cells were depolarised by GABA, mediated through $GABA_A$ receptors (Corns et al., 2013) suggesting that neural stem cells can respond to the pressure application of GABA, although whether this effect was direct was not investigated. Bicuculline or gabazine decreased the amplitude of the depolarisation but nipecotic acid had no effect, suggesting that GABA transporters were not involved in these responses, unlike the turtle. The subunit composition of this response was also not investigated. This is important since in other regions, involvement of more unusual GABA subunits is reported. For example, the influence of GABA on ependymal cells was shown by work on cerebellar slices from P13-P15 mice (Reyes-Haro et al., 2013). GABA applied to ependymal cells was blocked by the GABAρ subunit antagonist TPMPA as well as the $GABA_A$ antagonist bicuculline.

Both in the SVZ and SGZ, GABA regulates the activation and proliferation of stem cells (Song et al., 2016; Song et al., 2014; Liu et al., 2005). At the level of the SGZ, GABA regulates also the survival and development of transit amplifying cells (TACs) and neuroblasts (Song et al., 2013).

The table (Table1-2) below summarizes the effects of a few drugs and neurosteroids on the receptors and any known effect on neurogenesis.

Table 1-2 The effects of a few drugs and neurosteroids on the receptors and any known effect on neurogenesis:

DRUG/MODULATOR	RECEPTOR INVOLVED	EFFECTS INCLUDING ANY KNOWN EFFECT ON NEUROGENESIS	REFERENCE
Neurosteroids: 3α, 21-dihydroxy-5α-pregnan-20-one (THDOC), pregnanolone	$GABA_A$ receptors (specifically on delta subunit) and γ_2 subunit	Acting with 2-arachidonoyl glycerol (2-AG) (lipid signalling network) potentiates phasic inhibition in synaptic receptors, and tonic inhibition in extrasynaptic receptors during neurogenesis	Sigel et al. (2011), Ranna et al. (2006)
Etomidate	$GABA_A$ receptors (β_2- or β_3 subunit)	Gave a large direct inward current in cerebellar granule cells mediated through the $GABA_A$ receptors	Ranna et al. (2006), Belelli et al. (1997)
Pentobarbitone	$GABA_A$ receptors α_3, α_2, β_1 and β_3 subunits	Significantly increased the number of new neurones in the adult dentate gyrus	Tozuka et al. (2005)
Phenobarbital	$GABA_A$ receptor	Significantly increased the number of new neurones in the adult dentate gyrus	Tozuka et al. (2005)

DRUG/MODULATOR	RECEPTOR INVOLVED	EFFECTS INCLUDING ANY KNOWN EFFECT ON NEUROGENESIS	REFERENCE
Diazepam	$GABA_A$ receptor	Acts on α and γ_2 subunit to enhance response. Does not induce any current by themselves but increase the current amplitude	Sigel and Steinmann (2012)
Bicuculline	$GABA_A$ receptor	Widespread effect on tonically active GABA receptors on sympathetic neurones of intermediolateral region of rat spinal cord	Wang, L. et al. (2008)
Gabazine	$GABA_A$ receptor	Gabazine will modulate tonic inhibition and influences firing properties	Wang, L. et al. (2008)
Endocannabinoid	$GABA_A$ receptor	The endocannabinoid 2-arachidonyl glycerol (2-AG) potentiates $GABA_A$ receptors at low concentrations of GABA. Tonic inhibition in extrasynaptic receptors	Sigel et al. (2011)
Dehydroepiandrosterone (DHEA)	-	Neural stem cell renewal, differentiation i.e. induces neurogenesis in human cortical-derived neural stem cells and enhances proliferation of newly formed rat neurones, blocking corticosterone effects	Charalampopoulos et al. (2008)

1.7.2 $GABA_B$ receptors

$GABA_B$ receptors were identified by Bowery et al. (1980). $GABA_B$ receptors are defined by their selective activation by baclofen (Kerr and Ong, 1995; Bowery et al., 1980) and lack of sensitivity to bicuculline. $GABA_B$ receptors belong to the family III G protein-coupled receptors (GPCRs) that are activated by GABA. These receptors use second messenger systems through binding and activation of guanine nucleotide-binding proteins (G proteins) (Bettler et al., 2004). The receptor has a central core domain, consisting of 7 transmembrane helices, that is responsible for G-protein coupling. It is an obligatory heterodimer and is formed by 2 subunits; i.e., $GABA_{B1}$ and the $GABA_{B2}$ (Kaupmann et al., 1998). The $GABA_{B1}$ subunit contains a large extracellular domain that binds GABA or other ligands such as baclofen, and $GABA_{B2}$ subunit couples the receptor with the effector G protein (see Figure 1-4).

G protein activation causes interactions with ion channels and other proteins to open or close ion channels through intracellular messengers (Bettler et al., 2004). They produce slow postsynaptic responses and can be activated in conjunction with ionotropic receptors to induce both fast and slow postsynaptic potentials. The delayed action of $GABA_B$ is due to the relay action of the second messenger, G protein to mediate its response. These metabotropic GABA receptors use potassium channels instead of chloride and opening of these channels causes hyperpolarisation of postsynaptic cells through efflux of potassium. They can also close calcium ion channels presynaptically to reduce neurotransmitter release (Kerr and Ong, 1995).

1.7.2.1 Mechanism of action

$GABA_B$ receptors are present both pre- and postsynaptically. Pre- and postsynaptic-$GABA_B$ receptors have different properties. The pre-synaptic receptors are composed of $GABA_{BR1a}$ and $GABA_{BR2}$ subunits. At the presynaptic site, $GABA_B$ receptors mediate inhibition of neurotransmitter release through an autoreceptor-like mechanism by activation of K^+ conductances and inhibition of Ca^{2+} conductances (Dunlap and Fischbach, 1981). The post- synaptic receptors are composed of $GABA_{BR1b}$ and $GABA_{BR2}$ subunits (Koulen et al., 1998).

$GABA_B$ receptor activation leads to three major effector cascades: adenyl cyclases, voltage-sensitive Ca^{2+} channels and inwardly rectifying K^+ channels (Benke et al., 2012) (Figure 1.4).

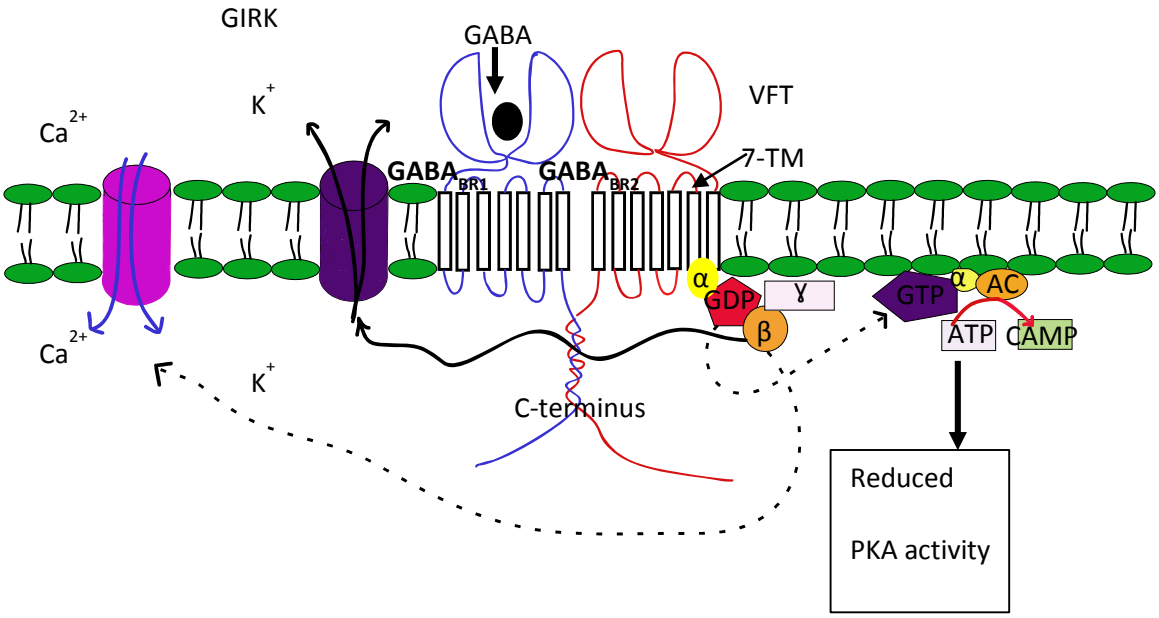


Figure 1-4 Structure and effector mechanism of $GABA_B$ receptors.

When the GPCR is activated by its extracellular ligand, a conformational change is induced in the receptor is transmitted to an attached intracellular heterometric G protein complex. The Gi/o alpha subunit of the stimulated G protein complex exchanges GDP for GTP and is released from the complex. The activated Gi/o alpha subunit binds to and inhibits adenylyl cyclase which in turn catalyzes the conversion of ATP into cyclic adenosine monosphosphate (cAMP). GABA = γ-aminobutyric acid; AC = adenylyl cyclase; ATP = adenosine triphosphate; cAMP = cyclic adenosine monophosphate (Edited from Kumar et al. (2013) and Xu et al. (2014)).

One target of excitability control mechanism at post-synaptic membranes are G-protein-gated inwardly rectifying K^+ (GIRK or Kir3) channels, which generate slow inhibitory post-synaptic potentials following activation of Gi/o-protein-coupled receptors and leads to decreasing neuronal excitability. The GIRK also can play a role at presynaptic sites (Fernández-Alacid et al., 2009).

Postsynaptic $GABA_B$ receptors inhibit adenylate cyclase via the Gi/o subunits of the G-protein, resulting in a reduced level of intracellular cyclic adenosine monophosphate (cAMP) (Knight and Bowery, 1996). The βγ complex released from the G protein is coupled to inwardly rectifying potassium channels (GIRK) which mediate the membrane potential response (Nicoll, 2004). The receptor agonist baclofen hyperpolarizes neurones expressing postsynaptic $GABA_B$ receptors in vitro, associated with decreased input resistance (Howe et al 1987).The hyperpolarisation has a reversal potential close to the potassium equilibrium potential. Postsynaptic $GABA_B$ receptors mediate slow GABAergic currents which can be blocked by $GABA_B$ antagonists. The current induced by $GABA_B$ receptors hyperpolarizes the membrane and shunts the current by coupling to GIRK3 via G proteins (Lüscher et al., 1997).

Baclofen remains the only available $GABA_B$ medication. Baclofen, a lipophilic derivative of GABA, was synthesized in 1962 in an attempt to enhance blood brain barrier penetrability of the endogenous neurotransmitter. Baclofen is used to treat spasticity and skeletal muscle rigidity in patients with spinal cord injury and multiple sclerosis (Fox et al., 1978).

1.7.2.2 G protein-coupled inwardly-rectifying potassium channels

G protein-coupled inwardly-rectifying potassium channels (GIRK) channels are members of a large family of inwardly rectifying K^+ channels. GIRK channels are mainly concentrated at postsynaptic sites. Studies in cerebellum showed that GIRK

channels contain three subunits, GIRK 1, GIRK 2 and GIRK 3 where they associate with $GABA_B$ receptors (Lüscher and Slesinger, 2010). The three primary GIRK subunits exist in the brain and form heterotetramers (GIRK1–GIRK2, GIRK2–GIRK3 and GIRK1–GIRK3; (Krapivinsky et al., 1995)) and homotetramers (GIRK2–GIRK2; (Lesage et al., 1995)). Electrophysiological studies have shown that the binding of GABA to $GABA_B$ receptors activates GIRK channels at postsynaptic sites (Jones et al., 1998). GIRKs associated with $GABA_B$ receptors are also present at presynaptic sites - the mechanism of GIRK-dependent effects here is not fully clear, but it might involve the inhibition of neurotransmitter release following activation of $GABA_B$ receptors (Luján et al., 2014; Fernández-Alacid et al., 2009; Ladera et al., 2008). A study has shown that excess accumulation of GABA which is released in the synaptic cleft will diffuse to neighbouring $GABA_B$-GIRK complexes (Nicoll, 2004).

1.7.2.3 Interaction of $GABA_B$ with other receptors

Interaction of $GABA_A$ and $GABA_B$

$GABA_B$ receptors may be located at different regions on neurones in relation to the other GABA receptors. $GABA_A$ receptors are Cl^- ion channels which produce fast electrical signals, whereas $GABA_B$ receptor activation induced long term modulation through G protein regulated gene transcription and protein synthesis (Lüscher et al., 1997). There is some evidence showing that in rat hippocampal pyramidal cells, $GABA_B$ receptors are not located on the cell soma but rather on the dendrites while $GABA_A$ receptors are located in both regions. This means that $GABA_A$ and $GABA_B$ receptors on the dendrites may both be activated by synaptic release of GABA and the $GABA_B$ receptor activation may modulate the effectiveness of fast $GABA_A$ receptor-mediated inhibitory postsynaptic potentials (Newberry and Nicoll, 1985). In

some situations, GABA released spontaneously or driven by a single action potential produces a fast inhibitory post-synaptic potential mediated by $GABA_A$ channels. In contrast to this, a stronger stimulation is needed to elicit the slow inhibitory potential, suggesting that GABA released into synaptic cleft diffuses and activates perisynaptic $GABA_B$ receptors coupled to GIRK channels (Nicoll, 2004).

It appears that activation of $GABA_A$ receptors containing γ_2 subunits can potentially cause internalization of the $GABA_B$ receptors comprised of $GABA_{BR1/R2}$ subunits through an interaction of this γ_2 subunit with the $GABA_B$ receptor (Balasubramanian et al., 2004). Although this work was only done in oocytes, it may be relevant to ependymal cells. Furthermore, $GABA_B$ receptors may also be able to influence the cell surface expression of $GABA_A$ receptors through $GABA_B$ receptor mediated secretion of brain-derived neurotrophic factor (BDNF). This in turn increases expression of $GABA_A$ receptors that contain $\beta_{2/3}$ subunits (Kuczewski et al., 2011). These experiments provide a mechanism for quite complex co-ordination of the neuronal responses to GABA, depending on the subunit composition of neighbouring receptors.

Interaction of $GABA_B$ and 5-HT

Interaction of 5-HT and baclofen works by activation of separate receptors which use a common conductance mechanism (Andrade et al., 1986).

In hippocampal neurones, it is possible that some serotonin receptors (5-HT_1 and 5-HT_5) and $GABA_B$ receptors are coupled via the G proteins to the same GIRK channels. Andrade and colleagues (1986) showed that simultaneous activation of both receptors elicited slow currents in these neurones that were smaller than what would be expected. If there was a simple summation of the currents due to activation of the two receptors separately. They concluded from their various manipulations of

the second messenger pathway that the G protein enabled this direct coupling of the receptors to the same potassium channels.

Looking at the multiple interactions of $GABA_B$ receptors, we can say these differences may be due to variation in the composition of tetramers of $GABA_B$ receptors, variants of subunits and isoforms, cell surface expression and also crosstalk with interacting proteins.

1.7.2.4 Role of $GABA_B$ receptors in neurogenesis

Adult hippocampal neural stem cells are quiescent but reversible in response to stimuli such as neurotransmitter modulation (Bonaguidi et al., 2012). Excitation of the cell with extrinsic and intrinsic factors such as growth hormones, neurogenic factors, cytokines and as well as specific neurotransmitters can activate the quiescent stem cell pool and there is evidence showing that $GABA_B$ receptors can contribute to the process (Giachino et al., 2014; Felice et al., 2012). Most mouse hippocampal neural stem cells and their progeny express metabotropic $GABA_B$ receptors (Schuler et al., 2001). A study done by Giachino et al. (2014) showed newly generated adult granule neurones express $GABA_B$ receptors. Next they used $GABA_B$ genetic deleted mice $GABA_{B1}^{-/-}$ or $GABA_{B2}^{-/-}$ and compared them with the wild type for numbers of proliferating cells. Thirty days after injection of BrdU, newborn neurones were identified by BrdU and in the $GABA_{B1}^{-/-}$ or $GABA_{B2}^{-/-}$ mice, the number of newly generated cells was significantly higher in these animals and these showed enhanced neuronal differentiation. The increased progenitor proliferation in $GABA_{B1}^{-/-}$ mice suggested that activation of $GABA_B$ receptors is needed to maintain neural stem cells in a quiescent state.

Felice et al. (2012) studied how the $GABA_B$ receptor antagonist CGP 52432 can increase hippocampal neurogenesis in the ventral hippocampus. The increase in cell

proliferation in the adult hippocampus in response to CGP 52432 was observed after chronic (21days) administration of CGP 52342 but not the subacute (7 days) or acute (1 injection). Cell proliferation was upregulated following chronic administration of CGP 52342; consistent with the lags for actions of drugs. They proposed the molecular mechanism of cell proliferation may due to brain-derived neurotrophic factor. BDNF is important in the regulation of antidepressant-induced increases in hippocampal neurogenesis as in previous study (Enna and McCarson, 2006). In another study, acute administration of baclofen induced a decrease of BDNF mRNA expression in the dentate gyrus (Khundakar and Zetterström, 2011). The decreased of BDNF was attenuated by pre-treatment with $GABA_B$ antagonists CGP 46381 and CGP 55845.

Little is known about the role of $GABA_B$ receptors in spinal cord proliferation but they are widely expressed in the spinal cord (Wang, L. et al., 2008) and may have effects on the neurogenic niche of the spinal cord.

Since GABA has significant effects on ependymal cells in the spinal cord and modulators of GABA receptors are expressed in these cells, I sought to determine how responses of ependymal cells to GABA were affected by application of specific drugs acting on or modulating different GABA receptors.

1.7.3 GABAρ receptor

The third type of GABA receptor, termed $GABA_C$ has been proposed to have a distinct molecular subunit composition and can be distinguished pharmacologically from $GABA_A$ receptors even though it also gates the Cl^- currents (Johnston, G., 1996). $GABA_C$ receptors, which were insensitive to bicuculline, were found in various parts of vertebrate brain and were first described in interneurones of the mammalian spinal

cord (Johnston, G.A., 1997), although the name of $GABA_C$ was not used by this group.

It was in a study on rat cerebellum by Drew et al. (1984) where this new receptor was designated the $GABA_C$ receptor. CACA; the folded conformationally restricted analogue of GABA did not inhibit the binding of ($^3H^-$)-baclofen to rat cerebellar membranes. Since this compound was also known to act at GABA receptors that were bicuculline insensitive and therefore was not a $GABA_A$ receptor ligand the authors suggested that there was a third type of receptor, $GABA_C$ receptors. In further experiments in retina, $GABA_C$ receptors were shown to be insensitive to $GABA_A$ receptor antagonists particularly bicuculline and not activated by the $GABA_B$ receptor agonist baclofen (Bormann and Feigenspan, 1995), providing further support for a new receptor.

Most of the studies on $GABA_C$ were done in the retina (Feigenspan et al., 1993; Qian and Dowling, 1993; Cutting et al., 1992; Woodward et al., 1992) to establish the native $GABA_C$ receptor. Later, $GABA_C$ receptors were observed by Polenzani et al. (1991), who showed that GABA activated Cl^- currents were made up of two different components, one mediated by $GABA_A$ receptors and the other mediated by $GABA_C$ receptors. This gave evidence of different receptors with distinct protein subunits which associate with each other.

Identification of ρ subunits in the $GABA_C$ receptor was based on Cutting et al. (1991) where they cloned the ρ_1 subunit from human retina complementary DNA (cDNA) library. When expressed in Xenopus oocytes, the ρ subunits formed a channel that was sensitive to GABA and these responses were not blocked by bicuculline. Two distinct clones were isolated differently through DNA sequencing. The clone contained a single exon from the rho_1 gene (GABBR1) while the second clone encompassed an exon with 96% similarity to rho_1 gene. The second gene had an amino acid sequence of cDNA with 30-38% similarity to α,β,γ and δ GABA receptor

subunits and 74% similarity to GABA rho$_1$ subunit and this suggested that this was a newly member of rho subunits. The ρ_2 subunits were cloned from human retina in 1992 (Cutting et al., 1992) and were also found in hippocampus, cerebellum and pituitary gland (López-Chávez et al., 2005a). The subunit ρ_3 has been detected in retina and also in higher brain regions (Boue-Grabot et al., 1998). Currently the name $GABA_C$ is in disuse and the three GABAρ genes are included in the $GABA_A$ receptor family. However, for the sake of this thesis, I will consider these receptors as potentially mediating different effects.

1.7.3.1 Structure and properties of GABAρ

GABA ρ-subunits have been found in retina (Enz et al., 1995), cerebellum (Pétriz et al., 2014) , hippocampus (Rozzo, Aldo et al., 2002; Enz et al., 1995), pituitary gland (Boue-Grabot et al., 1998), spinal cord (Rozzo, Aldo et al., 2002; Rozzo, A et al., 1999), gastrointestinal (Jansen et al., 2000) and sperm cells (Li, S. et al., 2008).

The GABAρ receptors were originally thought to be homopentameric ligand gated ion channels composed of ρ subunits (Figure 1-5). It required five subunits to assemble a single ion channel. Subunits in the ligand gated ion channels exhibit 10-20% sequence homology and have a similar distribution of hydrophobic and membrane-spanning segments and are assumed to have similar structure. Hence, $GABA_A$ and $GABA_{C/\rho}$ receptors are believed to be comprised of five subunits which assemble to form an ion channel. The general structure of every subunit consists of an extracellular amino terminal domain, four transmembrane domains, and an extracellular carboxy terminus. Each subunit is thought to have four transmembrane domains: TM1-TM4 (Chebib and Johnston, 2000). TM2 is a significant transmembrane domain. It crosses the membrane as an α–helix, forms the wall of the channel pore and is involved in the gating process (Chebib and Johnston, 2000). The

binding site of GABA is located in the extracellular amino-terminal domain which when activated, leads to the opening of ion channel with subsequent flux of Cl^- through it (Figure 1-5).

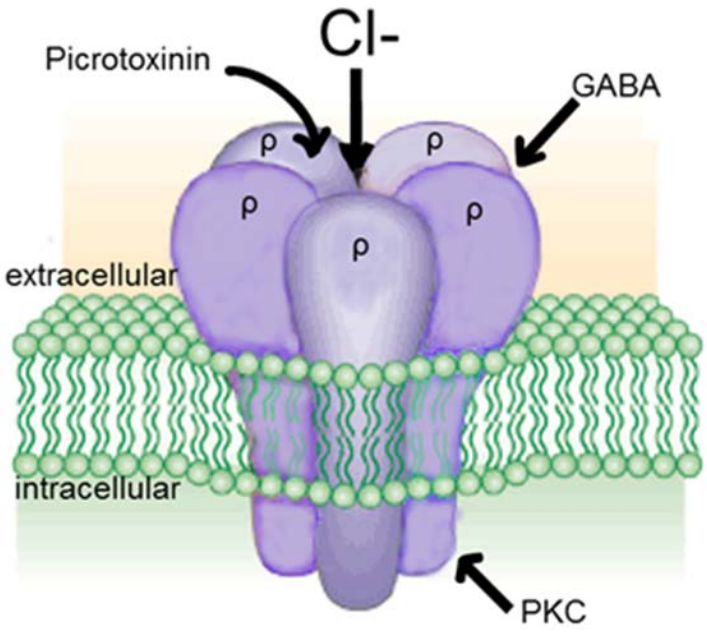


Figure 1-5 GABAρ receptor structure.

Five ρ subunits assemble to form a pentameric protein complex with the Cl^- channel as a central pore. The binding sites for agonists (e.g. GABA) as well as antagonists and modulators are located in the extracellular part of the protein complex. Picrotoxinin binds in the channel pore but shows also an use-dependent effect, indicating an allosteric mechanism. The intracellular loop between transmembrane regions 3 and 4 may contain sequences for protein kinases such as PKC (Image modified from Enz and Cutting (1998)).

A study regarding GABAρ subunits also suggests pseudoheteromeric channels consisting of different ρ subunits (Connolly et al., 1996). Some studies also support co-assembly of ρ subunits with $GABA_A$ subunits i.e. with α_1 and γ_2 (Milligan et al., 2004; Pétriz et al., 2014) and also with α_1 and α_2 subunits (Duke et al., 2000). There are three specific types of ρ–subunits, ρ_1-ρ_3 and these form different complexes depending on their distribution. In adult rats, the distribution of heteromeric complexes of ρ subunits is scattered and the presence of the different complexes is reported by researchers who found only receptors composed of $\rho_{1,2}$ (Cutting et al., 1991) while others found only $\rho_{2,3}$ heteromers (Ogurusu et al., 1995). In 2005, Alakuijala demonstrated all three ρ subunits are expressed in several regions in rats' postnatal developing brain.

To group the GABAρ receptors based on pharmacological interaction, basically this receptor has greater sensitivity to GABA and shows bicuculline and baclofen insensitivity. Their currents are smaller than other receptor currents and do not desensitize. In general, GABA is between 10-100 fold more potent at GABAρ receptors than $GABA_A$ receptors. Furthermore, GABA acting at GABAρ receptors produces responses with slow activation and deactivation (Feigenspan and Bormann, 1994; Feigenspan et al., 1993).

EC_{50} values for GABAρ_1 and GABAρ_2 are in the range of 0.8 to 2.2 μM (Polenzani et al., 1991; López-Chávez et al., 2005b) and around 7.5 μM for GABAρ_3 receptors (Zhang et al., 2001). However a study by (Schmidt et al., 2001) on rat superior colliculus showed that at lower agonist concentrations, which most likely activate GABAρ, the effects of GABA on evoked excitatory and inhibitory responses were not uniform. Evoked responses were suppressed by GABA (applied at concentrations of between 10 and 50 μM) in 48% of neurones, the remaining cells exhibited enhanced responses. But all effects of GABA at low concentrations were blocked by TPMPA but not bicuculline.

There are a number of compounds available that seem to selectively or preferentially act on ρ subunit-containing receptors. An early study by Drew et al., (1984) showed that the

GABA analogue cis-4-aminocrotonic acid (CACA) had activated selectively GABAρ receptors in cerebellar neurones. The study showed CACA had a bicuculline insensitive depressant action on firing neurones, but had no effect on the binding of (^{3}H)-baclofen to the rat cerebellar membranes and thus it is unlikely that this drug acts as an agonist on $GABA_A$ or $GABA_B$ receptors.

Other than GABA and CACA, trans-3-aminocrotonic acid (TACA) has greater potency than GABA at ρ receptors but is non-selective, also acting as an agonist at heteromeric $GABA_A$ receptors (Kerr and Ong, 1995; Woodward et al., 1992).

GABAρ receptors are not sensitive to other modulators of $GABA_A$ receptors such as benzodiazepines, and barbiturates. There are no benzodiazepine (diazepam 10^{-4} M, β-CCE 10^{-4} M and bicuculline 10^{-6} M) and barbiturate (pentobarbital 10^{-4} M) sites on this receptor (Shimada et al., 1992).

Neuroactive steroids can modulate the ρ_1 subunit in xenopus laevis oocytes (Morris et al., 1999). The modulation of ρ_1 receptors by neurosteroids compounds were dependant on GABA concentration. These effects were prominent in the presence of low concentrations of GABA (0.2-0.4 μM). The differential modulation by neurosteroids depends on the type of derivatives. The 5α derivatives (allopregnanolone, alphaloxone and 5α-tetrahydro- deoxycorticosterone (5α-THDOC)) were potentiators of GABAρ while the 5β-tetrahydrodeoxy- corticosterone (5β-THDOC) were the inhibitors to GABAρ. Finally the effects of the neuroactive steroids on ρ_1 receptor were shown to be long lasting because the application of GABA did not return to the control level for few minutes after neuroactive steroid treatments. Differently, these effects of neurosteroid modulation did not occur in an earlier study by (Woodward et al., 1992) using GABA (1-2 μM) on retina RNA of Xenopus oocytes.

A study by Shimada et al., (1992) expressed the GABAρ subunits in oocytes and reported no effects of benzodiazepine on responses to GABA 1 μM but these responses were blocked by both the $GABA_A$ receptor Cl^- channel blocker (picrotoxin 10^{-4} μM) and

the GABA receptor antagonist (tert butylbicyclo- phosphoro- thionate 10^{-6} μM). This suggests that some antagonists have effects at both types of receptor.

Picrotoxinin is an active isomer from picrotoxin and a pore blocker at the GABAρ receptor ligand site (Goutman and Calvo, 2004). It binds to TM2 to inhibit chloride flux through the anionic ligand-gated channels (Olsen, 1982). Picrotoxinin has different effects at ρ_1 and ρ_2 homomeric receptors and from different species. Picrotoxinin is able to block current induced in GABAρ_1 mRNA-injected Xenopus oocytes superfused with GABA (1 μM) (Shimada et al., 1992) but rat GABAρ_2 receptors are relatively insensitive to picrotoxinin (Feigenspan et al., 1993).

There are relatively few antagonists that selectively distinguish GABAρ receptors from $GABA_A$ receptors; the most highly used being (1, 2, 5, 6-Tetrahydropyridine-4-yl) methylphosphinic acid (TPMPA). The distinct antagonist profiles of $GABA_A$ and GABAρ showed that the agonist and antagonist binding area/site is not the same for these receptor classes. TPMPA is a selective GABAρ antagonist that is 8 times more potent at ρ_1 over ρ_2 subunit containing receptors (Chebib et al., 2007) and is 100 times weaker as an inhibitor of $GABA_A$ receptors (Murata et al., 1996). In astrocyte primary culture, only 10 μM TPMPA was needed to antagonise the effect of GABA by 80% (Pétriz et al., 2014). In contrast, 100 μM was needed to block 68% of GABA responses in ependymal cells from mouse cerebellar slices ages postnatal days13 to 35 (Reyes-Haro et al., 2013). Similar concentrations were needed to block 52% of GABA responses in striatal astrocytes (Reyes-Haro et al., 2017). However in a study by Schlicker et al. (2009), in the presence of TPMPA, a TPMPA sensitive current persisted in GABAρ_1 knockout stratum griseum superficial cells. The differences of results in the studies may due to the drug potency using TPMPA or it may involve a particular subunit arrangement.

GABAρ may even coassemble with subunits of $GABA_A$ receptor (Harvey et al., 2006; Pan et al., 2005; Hartmann et al., 2004; Qian and Ripps, 1999). Qian and Ripps (1999) and Pan et al. (2005) examined the interactions between GABAρ and γ_2 subunits. In

heteromeric $\rho_1\gamma_2$ receptors they found that responses to GABA were not affected by bicuculline and diazepam but were antagonised by picrotoxin (Qian and Ripps, 1999). The γ_2 subunit co-immunoprecipitated with ρ_{1A}, ρ_{1B} and ρ_{2B} but not with the ρ_{2A} when co-expressed (Pan et al., 2005). Using GABA induced currents in CA1 pyramidal cells in rat hippocampal slices, the currents were partially suppressed by TPMPA while responses to CACA were more fully antagonised. Both GABA and CACA currents were sensitive towards bicuculline and this showed a differences between the features of this receptor and features of GABAρ (Hartmann et al., 2004). In brainstem neurones, application of CACA requires the ρ_1 subunit to elicit responses but these are blocked by bicuculline and TPMPA. The responses were also modulated by the $GABA_A$ modulator (pentobarbitone) (Milligan et al., 2004). In addition to these, zolpidem (500 nM) which acts on α_1 and γ_2 enhanced the action of CACA. From immunohistochemistry analysis, it was indicated that ρ_1 and α_1 subunits were colocalized at both at both light and electron microscopic level. Furthermore they showed that co-immuno- precipitation indicated that ρ_1 subunit formed complexes with α_1 and γ_2 subunits. Harvey et al. (2006) also reported that an ionotropic GABA receptor in mouse cerebellar Purkinje cells showed atypical mixed $GABA_A$ and GABAρ when the currents were not affected by TPMPA but were blocked by bicuculline. Immunohistochemical analysis showed the presence of ρ subunit and it was co-immunoprecipitated with α_1 subunit. This might mean that responses in ependymal cells may be mediated through $GABA_A$, $GABA_C$ or mixed $GABA_{A/C}$ receptors.

1.7.3.2 Role of GABAρ in neuronal function and neurogenesis

To date, the precise function of GABAρ is unknown. GABAρ has been suggested to have physiological roles in the apoptosis process in hippocampal neurones (Yang, L. et al., 2003), hormone release in pituitary (Boue-Grabot et al., 1998), and may be involved in paired-pulse depression of inhibitory postsynaptic currents (Jacquet et al., 2009). Receptors expressing ρ_1 subunits are involved in learning and memory of chicks and

rats (Chebib et al., 2009), in the inhibitory modulation of olfactory bulb (Chen et al., 2007), and in inhibitory pain transmission in spinal cord (Zheng et al., 2003).

The role of GABAρ in neurogenesis is unknown. It is proposed that GABAρ_1 and ρ_2 subunits may contribute to the regulation of glial development in cerebellum. GABA ρ_1 and ρ_2 subunits, either in homomeric form or combination with other $GABA_A$ subunits (α_1 and γ_2) have been identified in cultured astrocytes. Coimmunoprecipitation demonstrated that there is interaction between GABAρ_1 and GABAα_1 and double immunofluorescence showed that these subunits were co-localised in in the plasma membrane of astrocytes (Pétriz et al., 2014).

Initial studies indicate that the spinal cord contained only ρ_2 subunits (Enz et al., 1995), later more studies indicated that spinal cord expressed ρ_1 and ρ_2 subunits (Rozzo, Aldo et al., 2002). Detailed investigations of GABAρ at spinal cord level are still lacking and its functional role in spinal cord remains to be established.

1.8 5-hydroxytryptamine

Serotonin, also referred as 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter and has numerous roles in CNS. It is a neurotransmitter that appears early in development and extends widely in the CNS. Serotonin is synthesized in neurones of the brain stem raphe nuclei (Gaspar et al., 2003). It is also synthesized extensively in the enterochromaffin cells of the gastrointestinal tract. It is synthesized from the amino acid tryptophan by a short metabolic pathway, by hydroxylation and decarboxylation of the amino acid L-Tryptophan using the neurone-specific enzyme tryptophan hydroxylase (THP) 2 (Walther et al., 2003). Once synthesized, serotonin is packed in synaptic vesicles by the vesicular monoamine transporter. Serotonin is released from serotonergic vesicles (mediated through increased intracellular Ca^{2+}-level) and activates 5-HT receptors located on the dendrites, cell bodies and presynaptic terminals of adjacent neurones. 5-HT receptors are located on membrane of neurones and other cells. Extracellular levels of serotonin are regulated by its re-uptake into the presynaptic cell through the serotonin transporter (SERT) (Gaspar et al., 2003).

Serotonin plays two key roles: 1) in early developmental periods, it acts as a growth factor to regulate the development of its own (Bonnin et al., 2007) and related neural systems; and 2) as a neurotransmitter (Lauder, 1990) regulating cognition, attention, emotion, pain, sleep and arousal. In its developmental role as a trophic factor, 5-HT regulates processes such as cell division, differentiation, migration, myelination, synaptogenesis and dendritic pruning (Gaspar et al., 2003). The concept of 5-HT as a neurotransmitter which also works as hormone/growth factor and differentiation factor in foetal brain emerged decades ago (Lauder and Krebs, 1976).

Studies in animal models have shown that 5-HT modulates neuronal progenitor cell proliferation, neuronal migration and axonal wiring during foetal and early postnatal periods which did not occur in adult brain (Banasr et al., 2004).

1.9 5-HT receptors

5-hydroxytryptamine receptors or 5-HT receptors or serotonin receptors are a group of G protein-coupled receptor and ligand gated ion channels found in the central and peripheral nervous system (McCorvy and Roth, 2015). They mediate both excitatory and inhibitory neurotransmission. The serotonin receptors are activated by the serotonin neurotransmitter which acts as their natural ligand (McCorvy and Roth, 2015).

Fifteen different transmembrane receptors have been classified into seven groups ($5HT_1$ to $5HT_7$) largely based on their structural and functional characteristics. The receptors have been further classified as 5-HT_{1A-F}, 5-HT_{2A-C}, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7. Among these, 5-HT_{3A-3B} are ionotropic (Mattson et al., 2004) while the remaining receptors are coupled to different G proteins (Albert and Tiberi, 2001). The receptors which are coupled to G proteins are further categorized into four groups according to their second messenger coupling pathways (Raymond et al., 2001). The 5-HT_1 (5-HT_{1A}, 5-HT_{1B-C} and 5-HT_{1D-F}) receptors are coupled to $G_{i/o}$ proteins, 5-HT_2 (5-HT_{2A-C}) receptors are coupled to G_q proteins, 5-H_{T4}, 5-H_{T6} and 5-H_{T7} receptors, which are coupled to G_s proteins, and 5-HT_5 (5-HT_{5A} and 5-HT_{5B}) receptors whose transduction cascade is not clear (Raymond et al., 2001) (see Figure 1.6).

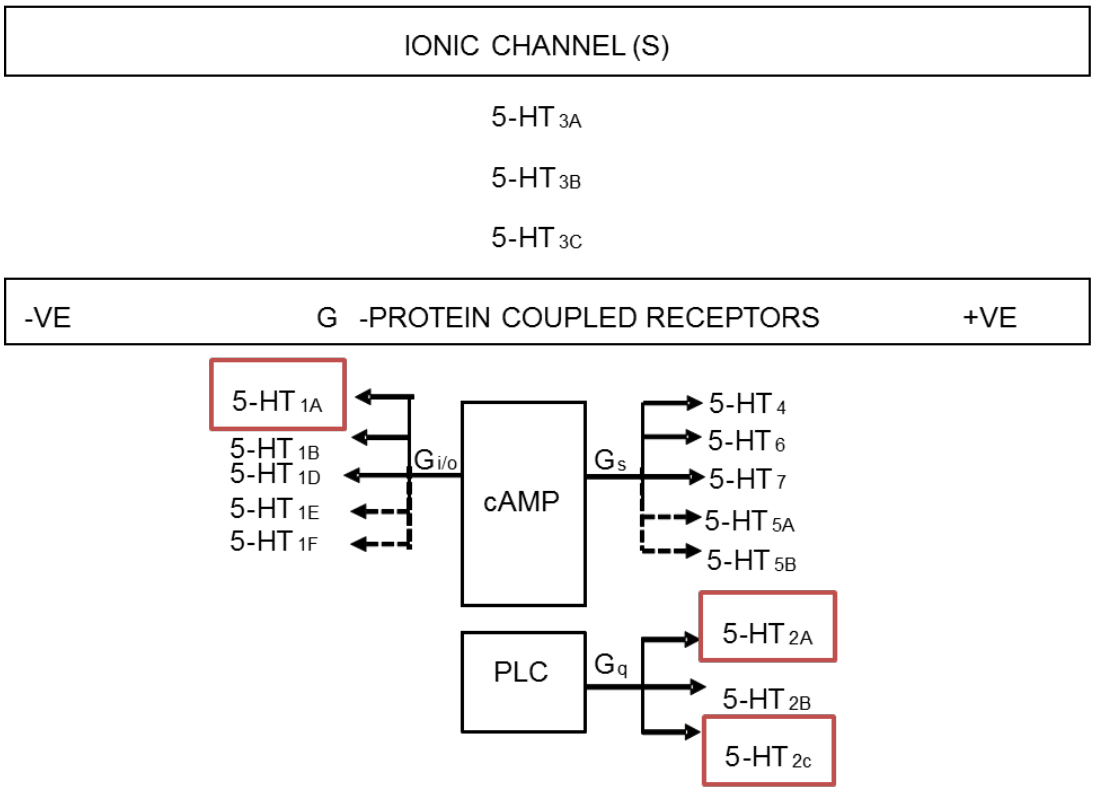


Figure 1-6 Classification of serotonin (5-HT) receptors.

They belong to a G-protein coupled family with one exception; the 5-HT_3 receptors are ligand-gated ion channels. Regulation of adult hippocampal neurogenesis is mediated by 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2B} (red squares). (–ve, negative; +ve, positive; cAMP, cyclic adenosine monophosphate; PLC, phospholipase C; G, guanine nucleotide binding proteins: Gi/o, heteromeric G protein subunit that inhibits cAMP-dependant pathway; Gs, heteromeric G protein subunit that activates cAMP-dependant pathway and Gq, heteromeric G protein subunit that activates phospholipase C) (Image edited from Hoyer et al. (1994)).

Because of the diversity of 5-HT receptors, it is quite difficult to define their actions on brain development. Most immunohistochemical studies showed that these receptors are expressed early during embryonic development and regulated postnatally (Gaspar et al., 2003). In human brain, the serotonergic neurones are first evident as early as 5 weeks of gestation (Sundström et al., 1993). By 15 weeks of gestation, the raphe nuclei already contain a typical arrangement of serotonin neurones (Takahashi, H. et al., 1986).

Developmental studies however have paid little attention to spinal cord even though it constitutes one of the major targets of 5-HT neurones. One day after serotonergic neuronal generation, raphe neurones can synthesize 5-HT and begin to extend to axon tracts, the caudal group project into spinal cord and descend in the lateral and anterior funiculi to terminate in gray matter (Carlsson et al., 1964). Most of 5-HT containing fibres in the spinal cord descend ipsilaterally and some fibres may cross the midline in thoracic and lumbosacral cord (Ni et al., 2014).

1.9.1 Distribution of 5-HT receptors

Very high concentrations of 5-HT_1 receptors are localized in the choroid plexus, lateroseptal nucleus, globus pallidus and ventral pallidum, dentate gyrus, dorsal subiculum, olivary pretectal nucleus, substantia nigra, reticular and external layer of the entorhinal cortex, hippocampus, amygdaloid complex, hypothalamic nuclei and also dorsal raphe (Pazos and Palacios, 1985). In claustrum, olfactory tubercle, accumbens, central grey and lateral cerebellar nucleus there were intermediate densities, while in other brain areas; pons, medulla and spinal cord, only low or very low concentrations of 5-HT_1 receptors were found. From the areas strongly enriched in 5-HT_1, dentate gyrus and septal nucleus contained 5-HT_{1A} while globus pallidus, dorsal subiculum, substantia nigra and olivary pretectal nucleus were enriched in 5-

HT_{1B} (Pazos and Palacios, 1985). For 5-HT_2 mapping, very high concentrations were localized in claustrum, olfactory tubercle, anterior olfactory nucleus, piriform cortex, and layer 1 and IV of neocortex while in thalamus, hippocampus, brainstem, medulla, cerebellum and spinal cord showed lower densities (Pazos and Palacios, 1985).

Cross sections of brain ventricular walls of mice and rats revealed the presence of axons containing serotonin; 5-HT (Aghajanian, G. and Gallager, 1975; Mathew, 1999). The importance of this is that the axons are not within the SVZ itself but are found inside the ventricles on the ependymal surface (Mathew, 1999). An in depth study of these cells showed that serotonergic axons originating from the raphe nucleus form intimate contacts with the B1 cells (morphological properties and expression profiles of astrocytes) and ependymal cells of SVZ. B1 cells expressed the 5-HT receptors 2C and 5A (Tong et al., 2014).

Although multiple 5-HT receptor subtypes have been defined in the central nervous systems, four types (5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_4) of 5-HT receptors have been identified in the spinal cord (Fonseca et al., 2001; Hoyer et al., 1994; Marlier et al., 1991).

A study by Nagatsu et al. (1988) showed there was no serotonin immunopositive staining in central canal area; evidenced in CSF-contacting neurones as well as the non-CSF-contacting neurones of rats and mice. However unpublished observations from our laboratory report dense innervation of 5-HT immunopositive fibres around the central canal.

The abundance of several types of 5-HT receptors in nervous system may explain why and how each receptor, alone or combination with others may influence brain development. With different cellular localisation of the receptors which is not well understood, finding the exact receptor which may be responsible in the cellular proliferation and differentiation is extremely complex. However a few studies showed that each of main 5-HT receptors are expressed early in embryonic life and regulated

during pre and postnatal development and a few studies of effects of 5-HT on neural stem cells are considered here.

1.9.2 Role of 5-HT on regulation of neurogenesis

5-HT influence and modulation of neurogenesis can be divided according to the effects of pharmacological 5-HT depletion (Brezun, J.M. and Daszuta, A., 2000; Malberg et al., 2000; Yan et al., 1997), pharmacological receptor targeting studies (Banasr et al., 2004; Malberg et al., 2000; Yan et al., 1997), genetically modified 5-HT system using mouse models specifically on the receptor which can cause neurogenesis (Santarelli et al., 2003), the effects of antidepressant action on neurogenesis and functional role of serotonin in neurogenesis (Klempin et al., 2010; Abbas et al., 2007) .

Since 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} have been shown to have established effects on neurogenesis, this section will elaborate on these receptor effects on neurogenesis.

5-HT is one of the most important neurotransmitters in the brain. A depletion or low supply of serotonin can contribute to symptoms of neurogenic decline, changes in appetite and mood disorders (depression) due to impaired serotonin synthesis, release, reuptake or serotonin receptor abnormalities. In the case of 5-HT depletion, the effect of the depletion can decrease adult hippocampal neurogenesis (Brezun, J. and Daszuta, A., 2000; Malberg et al., 2000). Thus 5-HT stimulates granule cell proliferation in adult hippocampus. A lesion induced by injection of 5, 7-dihydroxytryptamine (5,7-DHT) into the medial and dorsal raphe nuclei caused complete serotonergic denervation of hippocampus (Brezun, J.M. and Daszuta, A., 2000). In these animals, there was a 50% reduction in BrdU labelled proliferating cells in the hippocampus. In another group of animals, one month after 5, 7-DHT injections, a unilateral graft of mesencephalic raphe (for n=10 rats) or spinal tissue (for n=5 rats,

serving as control) from embryonic day-14 rat foetuses was placed into the right hippocampus. The rats were sacrificed one month after the graft. Results showed by 1 month after transplantation, the hippocampus exhibited intense 5-HT re-innervation from raphe (but not spinal cord) graft and in these raphe grafted animals, the levels of proliferation were restored.

Cell proliferation and the numbers of newborn neurones derived from the neurogenic niche in adult hippocampal dentate gyrus are increased after prolonged treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Malberg et al., 2000; Jacobs and Fornal, 1999). Malberg et al. (2000) used a selective serotonin reuptake inhibitor (SSRI); fluoxetine (5mg/kg) which was administered for 14 days intraperitoneally to adult male Sprague Dawley rats (250-300gm) followed by BrdU labelling 4 d after the last drug treatment. In this chronic treatment, the numbers of proliferating cells were increased. However, acute administration of fluoxetine for 1 or 5 days did not significantly affect the number of BrdU-positive cells compared with control. Therefore there is no acute effect of fluoxetine but a delayed increase in the number of proliferating precursors cells in dentate gyrus after 14 days. The finding is quite similar with the preliminary study by (Jacobs and Fornal, 1999).

The dentate gyrus is enriched with 5-HT_{1A} receptors (Pazos and Palacios, 1985; Azmitia et al., 1996) and may have an effect on developing dentate granule cells (Yan et al., 1997). Yan et al. (1997) used buspirone (1.0mg/kg) as an agonist and NAN-190 (1.0 and 3.5mg/kg) as an antagonist to 5-HT_{1A} in vivo and used parachloroamphetamine (PCA) to deplete 5-HT in postnatal rats (given on postnatal day 3 and postnatal day 4). PCA reduced total dendritic length, an effect that was reversed with treatment of buspirone. If NAN-190 (3.5mg/kg) was injected from postnatal day 3 to 14 these animals exhibited a significant reduction in total dendritic length that was comparable to the reduction seen with PCA alone. This drug study showed that specific 5-HT receptors are responsible for the structural changes in

neuronal cell. Loss of dendritic spines is a function of decreased 5-HT_{1A} receptor stimulation.

Several studies report that 5-HT_{1A} receptors contribute to the modulation of mood as well as to induce hippocampal neurogenesis (Abbas et al., 2007; Banasr et al., 2004; Santarelli et al., 2003). Santarelli et al. (2003) compared the effect of increased 5-HT levels for antidepressants (fluoxetine) delivered to wild type mice and 5-HT_{1A} receptor knockout mice. The results indicated that fluoxetine had no effect in knockout mice. This suggests that 5-HT_{1A} receptors are required for fluoxetine-induced neurogenesis. Receptor subtypes other than 5-HT_{1A} may be involved in the stimulation of cell proliferation and neurogenesis in brain induced by activation of 5-HT transmission (Banasr et al., 2004). Using 8-OH-DPAT (1 mg/kg) for 5-HT_{1A} receptor activation, significant increases in numbers of BrdU labelled cells were found in dentate gyrus, SVZ and olfactory bulb after 15 days of treatment. Banasr and group also compared with 5-HT_{2A} receptor blockade using (ketanserin, 1mg/kg), a 5-HT_2 antagonist and observed decreased cell proliferation in the dentate gyrus area while RO 600175 a 5-HT_{2C} agonist was able to increase cell proliferation in SVZ of adult mice. In contrast to Banasr et al. (2004), Abbas et al. (2007) found out that the antagonist to 5-HT_{1A}; WAY 100635 induced an increase in 5-HT_{1A} receptor expression. However, the antagonistic action may naturally lead to more receptor expression as a compensating effect. More recent work showed that activation of serotonin receptors 5-HT_{1A}, 5-HT_2 and 5-HT_{2C} affected different stages and aspects of the adult neurogenesis and cell survival process (Klempin et al., 2010). They suggested that effects mediated by these different receptors may be responsible for the fact that 21 days treatment with fluoxetine increased the number of BrdU-positive cells in the dentate gyrus, while a single injection of the drug followed by 20 days of saline had no effect. They found that in the acute stages, 5-HT exerts opposing effects on proliferation and differentiation through effects of different receptors. *In vivo*, acute

treatment with 8-OH-DPAT (2 h pre BrdU treatment) significantly increased precursor cell proliferation whereas 1 week of treatment had no significant effect. However, *in vivo* treatment with the 5-HT_2 receptor antagonist cinanserin resembled the effects of the 5-HT_{1A} receptor agonist. There was an acute increase in cell proliferation but no effect after 7 days of treatment of cinanserin. Furthermore the 5-HT_2 receptor agonist α- methyl-5HT decreased cell proliferation after both 1 and 7 days of treatment. To define the receptors further, the 5-HT_{2C} agonist WAY 161503 elicited a shift in the numbers of cells at different developmental stages such that there was a decrease in type 1 cells but an increase in type 3 precursor cells, which indicated that this receptor was more critical for the differentiation of proliferating cells. They suggested an indirect effect of activation of 5-HT_{2C} receptors on surrounding GABAergic cells rather than neural progenitor cells themselves (Klempin et al., 2010).

This suggestion that there are mechanisms that involve 5-HT_{2C} receptor mediated activation of GABAergic neurones is supported by other studies (Boothman et al., 2006; Cumming-Hood et al., 1993). Given the synaptic contacts of 5-HT fibres onto GABAergic neurones, GABA release may lead to an inhibitory action on cell proliferation but may affect differentiation. Thus, it is possible that activation of 5-HT_2 receptors via serotonin may affect neurogenesis in the spinal cord.

All interactions of GABA receptors, and 5-HT receptors raise the possibility that GABA and 5-HT induced effects on spinal cord neurogenesis are worth consideration but firstly it is critical to establish what effects if any are observed by activation of specific receptors on ependymal cells in the spinal cord.

1.10 Aims and Hypothesis

There is currently little understanding of how cells at the central canal of spinal cord especially ependymal cells are affected by GABA and serotonin neurotransmitters. There are therefore a number of hypotheses and aims designed to examine this:

1. To characterise further the ependymal cells, which are the proposed neural stem cells of spinal cord central canal. Ependymal cells recorded from postnatal mouse spinal cord will be characterised using whole cell patch clamp recordings, dye fills and gap junction blockers.

2. To identify the $GABA_A$ receptor subunits expressed by postnatal spinal cord ependymal cells. It is hypothesised that ependymal cells express $GABA_A$, $GABA_B$, GABAρ. Using whole cell patch clamp and immunohistochemistry, ependymal cells isolated from postnatal mouse spinal cord will be examined for the presence of $GABA_A$ receptor subunits, $GABA_B$ and GABAρ receptors.

3. To characterise the responses of ependymal cells to GABA after application of specific modulators. It is hypothesised that these modulators will change the GABA actions on ependymal cells.

4. To test the outcome of manipulation of $GABA_B$ receptor on cell proliferation at the central canal of postnatal spinal cord. Using Edu labelling to reveal the effects of receptor manipulation on newly divided cells in spinal cord acute slice cultures, it is hypothesised that $GABA_B$ will contribute to the proliferation process.

5. To characterise the effects of 5-HT receptor activation on ependymal cells. It is hypothesised that these cells will respond to 5-HT application potentially through a number of different receptors.

Chapter 2

General Methods

2.1 Animals and ethical approval

Animals were obtained internally from the Central Biological Services with approval of animal ethics under ethical review committee at the University of Leeds. All experiments were conducted under UK Home Office License and in accordance with the regulations of the UK Animals (Scientific Procedures) Act, 1986. Postnatal 8 to 15 day wild type C57BL/6 mice of either sex were used in electrophysiology and acute slices for study of proliferation. Every effort was made to minimize the number of animals used and their suffering.

2.2 Spinal cord slice preparation

Animals were anaesthetized with Euthatal (sodium pentobarbitone, 60 mg/kg Merial Animal Health Ltd, UK) intraperitoneally. Using scissors, an incision was made through the skin from the caudal aspect of the sternum to the distal aspect of the clavicle on each side of the animal to allow access to the rib cage. The rib cage was removed to expose the heart. The mice were perfused transcardially through the left ventricle with ice cold sucrose artificial CSF (aCSF) which was previously gassed (95% O_2: 5% CO_2) (for composition of aCSF see Table 2-1).

Table 2-1 Composition of extracellular solutions for electrophysiological experiments

Composition	Sucrose aCSF Concentration (mM)	aCSF Concentration(mM)
Sucrose	217	
Sodium chloride		124
Sodium bicarbonate		26
Potassium chloride	3	3
Magnesium sulphate heptahydrate	2	2
Sodium phosphate monohydrate	2.5	2.5
Glucose	10	10
Calcium chloride	1	2

Animals were considered sufficiently perfused when blanching of liver, tail, and paws became apparent. Animals were then killed by decapitation using scissors. A dorsal laminectomy was performed using blunted scissors, starting from the rostral end. First the skin and muscle were removed followed by cutting of vertebrae surrounding the spinal cord using Vannas Scissors (8 cm, angled, standard; World Precision Instruments UK). The nerve rootlets were severed to allow the whole spinal cord to be removed and then it was transferred into a petri dish containing ice cold, gassed (95% O2: 5% CO2) sucrose aCSF. Under a dissecting microscope (SM2 2B, Nikon, Japan) for visualisation, fine forceps (Dumont # 55, Dumostar, 11cm straight) were used to remove the dura and pia mater. The spinal cord then was embedded in a 3% agar (Sigma Aldrich, UK made at 3% in aCSF) which was allowed to cool before

cutting. The block of agar containing spinal cord was then attached to a larger supporting block of agar (4% in 0.9% saline) before gluing onto a cutting platform and then was cut into 300 μm thick transverse spinal cord slices using an Integraslice 7550 PSDS, Campden Instruments, UK with ceramic blades. The settings of the integraslice were 108 Hz, 0.8 mm amplitude and 0.5 mm/s advance speed. Slices were mostly originated from thoracic and lumbar spinal segments. Slices were transported to a holding chamber containing aCSF at room temperature (gassed with 95% O2: 5% CO2). They were left for 60 minutes at room temperature to equilibrate.

2.3 Electrophysiology

Spinal cord slices were immersed in a recording chamber and the border of 3% agar was securely pinned under tightly stretched crossed threads to immobilise the slice. From a storage flask, the oxygenated aCSF was gravity fed to flow across the recording chamber at a rate of 2-5 ml/minute and was subsequently removed by suction and discarded. A silver chloride coated wire was attached to the electrode holder, this allowed application of positive and negative current to the electrode tip. A reference electrode which is a silver chloride pellet (1 mm X 2.5 mm, 70 mm exposed wire, Science Products, GmbH, Germany) placed in the bath was connected to complete the circuit. A micromanipulator (Burleigh, Thorlabs) which was attached to a head stage (Axon Instruments, USA) allowed coarse and fine control of the microelectrode. Signals from the recording electrode were preamplified by the head stage, filtered by a Bessel low pass filter set at 2-5kHz and fed into an Axopatch-1D amplifier (Axon Instruments, USA before being filtered for mains noise by a Humbug (Quest Scientific via Digitimer, UK). The signals were then converted from analogue to digital using an analogue to digital converter unit (Digidata, DD1440) before being

captured by Signal version 1.903 and Spike2 version 7 (CED) or Clampex 10.5 (Axon Instruments) software on a PC (Figure 2-1).

A Master-8 stimulator (A.M.P.I., Israel) was used to control the timing and duration of current and voltage pulses. Channel 1 of the Master-8 triggered the start of a sweep in the pClamp or Signal software via the Digidata DD1440, whilst also triggering channel 2 of the Master-8. Channel 2 of the Master-8 then initiated a current or voltage pulse from the Axopatch 1D amplifier down to the electrode.

2.3.1 Positioning of electrodes and obtaining whole cell patch recording

The first electrode lowered down into the recording chamber was the puffing electrode which was filled with either GABA or ODN. The electrode was fully filled with the drug and was ejected at desired controlled puff after positioned at the central of the slices. Firstly, the electrode was viewed by x10 objective, the tip of the electrode was positioned above the central canal using the coarse controls of the micromanipulator (Burleigh®). The microscope was changed to x60 magnification and the tip of electrode was then lowered towards a cell of interest using the fine controls of the micromanipulator (Burleigh®). Then the recording electrode was filled about a 1/3 of the way with intracellular solution (see table 2-2 for composition of intracellular), 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).

Table 2-2 Composition of intracellular solution

Composition	Working concentration (mM)
For 25 ml intracellular solution:	
Potassium gluconate	110 mM
Ethylene glycol tetraacetic acid	11 mM
20 ml distilled water were added to the above mixture and the solution was adjusted to pH 9 using potassium hydroxide 1M solution, the solution then was stirred for 2 hours before adding the following:	
Magnesium chloride	2 mM
Calcium chloride	0.1 mM
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	10 mM
Disodium salt hydrate (Na_2ATP)	2 mM
Guanosine 5′-triphosphate sodium salt hydrate (NaGTP)	0.03 mM
The solution was adjusted to pH 7.2 using potassium hydroxide 1M solution (usually 1or 2 drops). To 20 ml of solution, 100 mg of Neurobiotin tracer (Sp-1120, Vector Laboratories, Peterborough, UK) were added. Remnant of solution was discarded. *The final solution was adjusted to osmolarity of 285 mOsm using 1M Potassium gluconate or with distilled water; 0.5 ml aliquots of solution were stored in -40°C freezer for no longer than 1 month. After this time, unused aliquots were discarded.	

Positive pressure was applied to the electrode tip before lowering it into the solution. Viewed by x10 objective of Nikon Eclipse E600FN microscope, the tip of the electrode was positioned above the central canal using the coarse controls of the micromanipulator. Once in the solution, and in the small distance just above the slice, in current clamp mode, the off-set 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 microscope was changed to x60 magnification The visualisation was 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 Pro7 software to enable the individual cells to be seen and guide the recordings. This also helped with the identification of the cell and the tip of electrode was then lowered towards a cell of interest until the positive pressure caused a dimple to appear in the cell membrane. The positive pressure was released and the seal formation was observed by monitoring the current response to a voltage pulse of 25 mV. The holding voltage of -60 mV was removed. The amplifier was switched back to current clamp; the current pulse was reduced to -50 pA and a continual current was also applied to maintain the cell at -60 mV to -80 mV. A seal was considered enough when the current response equated to a 3GΩ resistance. A sharp suction was applied to achieve whole cell patch clamp configuration and seal formation. A staggered current step was applied to the patched cell and voltage responses monitored. The effects of drugs applied (Table 2.3 and Table 2.4) were monitored for each cell type.

The recordings were set up to identify the cell type and responses to drugs. The bath application of drug solutions was achieved by switching a three way tap to allow the drug to be fed into the recording chamber. A micropipette was filled with intracellular

solutions (Table 2.2). The micropipette was pulled using a Sutter P97 micropipette puller (Sutter Instruments, USA), from borosilicate glass capillary tubes (inside diameter 0.94mm, and outside diameter 1.2mm; Warner Instruments, USA). The pulled electrodes had resistance of 7-14 MΩ and a tip diameter of approximately 1-3 μM. Neurobiotin (0.5%; Vector Laboratories, Peterborough, UK, https: // www. Vector labs.com/ UK) and tetramethylrodamine (0.02%; Life Technologies, Paisley, UK), were added to the intracellular solution to visualize the cells post-recording.

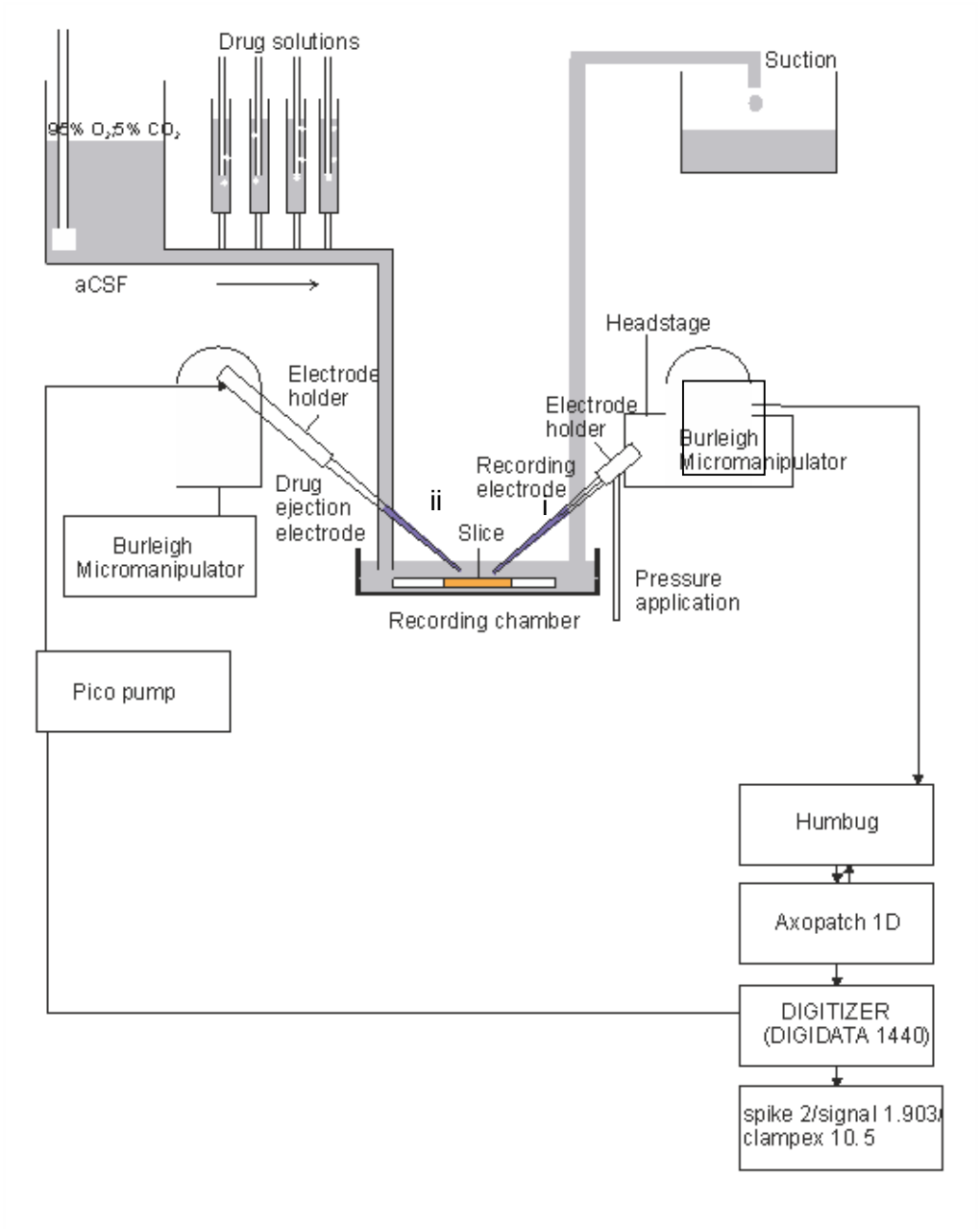


Figure 2-1 Experimental set up for whole cell patch clamp

Illustration of the electrode placement during the electrophysiology recordings. Recording electrode (filled with intracellular solution; numbered as i) was attached to and recording from either ependymal cell or CSFcC. The pressure ejection electrode containing drug to be pressure ejected (numbered as ii) was positioned near the cell being recorded from but not directly pointing towards it to minimise movement of the cell.

2.3.2 Application of drugs

The main drugs (GABA, ODN and TACA) were used in a pressure ejected manner through a borosilicate glass electrode (drug ejection electrode) (Borosilicate with filament, internal diameter 0.94, Model G 120 TF-4; Warner Instruments,USA) (resistance 3-5 MΩ, tip diameter 3-4 μm). It was 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 a micromanipulator (Scientifica Patchstar, UK). The PV800 was connected to the pClamp9/Clampex program via the molecular devices Digidata1440A, and thus allowed the program to record the timing of the pressure ejection of the drug.

The cells were hold at least at -40 mV if it is a CSFcC and at more negative potentials ≥-70 mV for ependymal cells. When recording started, an even baseline recording was maintain before any drugs were given. If the cell is not stable, the recording were either repolarised or tried to stabilise the baseline recording or the recording were get rid of. Only one successful recording was done per slices.

The two drug application methods were used to apply GABA or ODN or TACA. At my initial trials, GABA was introduced via bath method, 2 consecutive bath applications with 5 minutes bathing and 5 minutes gap each of bath applications. Due to unstable bathing controlled set and stable recording results of GABA via puff application, GABA, ODN and TACA then were introduced via puff ejection before, during and after bath application of modulators. GABA or ODN or TACA were set for ejection at 2 puffs before bath application of modulators, each ejection lasted for 1-2 sec, with a pressure of 20 mmHg with a gap of 3-5 minutes each ejection and the application of modulators will commence 5 minutes after the second ejection of GABA or ODN or TACA. At each step, the recordings were repolarised to the baseline. Modulators will be applied for 10-15 minutes before GABA or ODN or TACA were puffed again within bath of modulators. Before starting any drugs or modulators, and at the end of modulators bathing, the

recordings were repolarised accordingly. Washout were done for 10-15 minutes after stopping any modulators and were proceeded with post recording puffer of GABA or ODN or TACA.

A list of drugs applied during the electrophysiological experiments are listed in Table 2-3 and Table 2-4. For bath application of drugs, a concentrated solution was dissolved in water or DMSO and stored frozen in aliquots and then diluted at 1:1000 in the aCSF for applying to the bath. This ensured that the DMSO was at very low concentration (1:1000) in the bath. For puff experiments, all drugs were dissolved in aCSF solutions. Pressure ejection electrode was kept opposite but a bit lower position to the recording electrode with a gap of 30 nm (as in Figure 2-2).

Table 2-3 Drugs used in puffing electrode

DRUG NAME	KNOWN FUNCTION	SUPPLIER	CONCENTRATION USED	REFERENCE
GABA (γ-Aminobutyric acid),	GABA receptor agonist	Sigma Aldrich, UK	10-500 μM for high concentration doses and 1.25-2.5 μM for low concentration doses	Corns et al. (2013) Schmidt et al. (2001)
TACA (trans-4-aminocrotonic acid)	GABAρ agonist	Sigma Aldrich, UK	0.1 -15 μM	Chebib et al. (1997)
ODN	Diazepam binding inhibitor (DBI)-related peptide	**	15 nM-20 nM	Gandolfo et al. (1997)

**ODN peptide; ODN (H-Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lysup-OH), 65mg > 95% purity was prepared using the high-performance liquid chromatography (HPLC) method specifically by Prof Andy Wilson from School of Chemistry, University of Leeds and also from Assoc. Prof Julien Chuquet and group from INSERM Lab, University of Rouen, France.

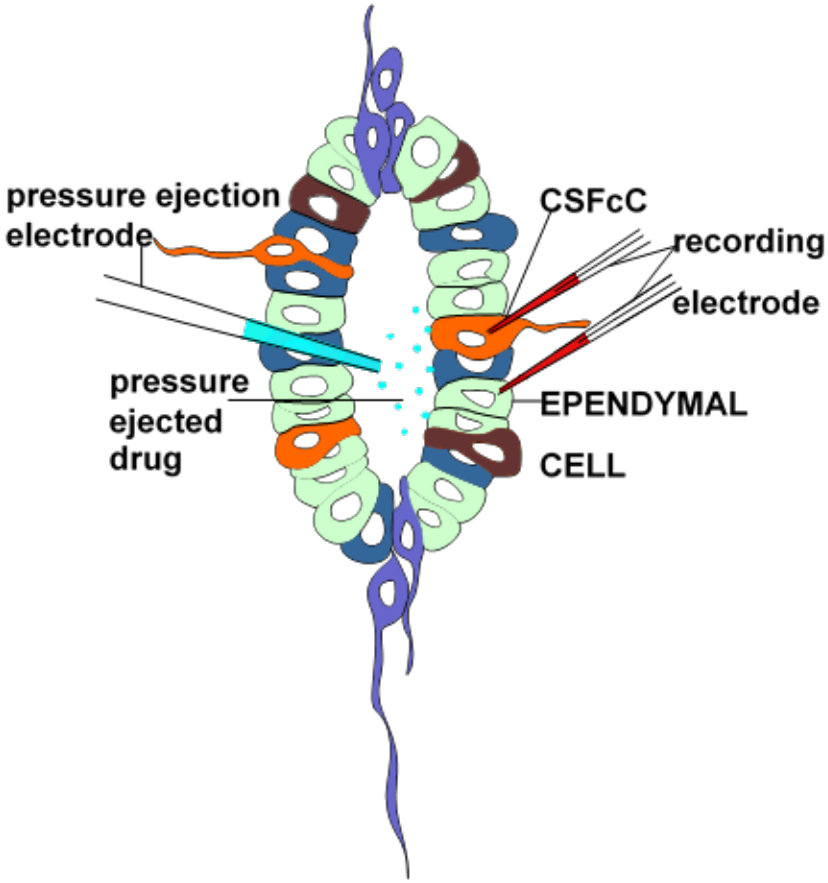


Figure 2-2 Configuration of electrodes for whole cell patch clamp electrophysiology on central canal area

Image depicting of recording electrodes and pressure ejection electrode position during recording and puffing drugs in whole cell patch clamp electrophysiology in central canal area.

Table 2-4 Drugs applied in the bath solution

DRUG NAME	KNOWN FUNCTION	SUPPLIER	CONCENTRATION USED	REFERENCE
GABA γ-Aminobutyric acid	GABA receptor agonist	Sigma Aldrich, UK	400 μM	Corns et al. (2013)
Bicuculline	$GABA_A$ antagonist	Sigma Aldrich, UK	100 μM	Park-Chung et al. (1999)
Picrotoxin*	$GABA_A$ receptor antagonist	Tocris Bioscience, UK	1 μM	Park-Chung et al. (1999)
Midazolam	Central benzodiazepine receptor & TSPO (agonist)	Sigma Aldrich, UK	0.1 μM	Tokuda et al. (2010)
N,N-Dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN-1-27)*	TSPO (agonist)	Bio-Techne, UK	1 μM	Tokuda et al. (2010)
Diazepam	mixed CBR/TSPO agonist	Sigma Aldrich, UK	1 μM	Alfonso et al. (2012)

DRUG NAME	KNOWN FUNCTION	SUPPLIER	CONCENTRATION USED	REFERENCE
8-OH-DPAT	5-HT_{1A} agonist	Tocris Bioscience	40 μM	Middlemiss and Hutson (1990)
Serotonin Hydrochloride	Non-selective 5-HT agonist	Sigma-Aldrich, UK	10 μM	Wang, M. and Dun (1990)
Cinanserin	5-HT_2 antagonist	Tocris Bioscience	20-30 μM	Klempin et al., (2010)
Cisapride	5-HT_4 receptor agonist	Tocris Bioscience	10 μM	Bockaert et al., (2008)
Niflumic acid	Chloride channel antagonist	Sigma-Aldrich, UK	250 μM	Woodward et al., (1993)
Nipecotic acid	glial and neuronal GABA uptake	Tocris Bioscience	300 μM	Barrett-Jolley (2001)

DRUG NAME	KNOWN FUNCTION	SUPPLIER	CONCENTRATION USED	REFERENCE
Baclofen	$GABA_B$ receptor (agonist)	Tocris Cookson, Bristol, UK	1 μM and 5 μM	Wang, L. et al. (2010)
(2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl)phosphinic acid hydrochloride (CGP 55845)	$GABA_B$ receptor (antagonist)	Tocris Cookson, Bristol, UK	200 nM	Wang et al. (2011)
1,2,5,6-Tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA)	$GABA_A$ρ antagonist	Sigma-Aldrich, UK	80 μM	Milligan et al. (2004)
18β-glycyrrhetinic acid	Gap junction inhibitor	Sigma-Aldrich, UK	100 M dissolved in 50% ethanol	Momose-Sato et al. (2012)

*drugs were diluted using DMSO and kept stored frozen in aliquots and then diluted at 1:1000 in the aCSF for applying to the bath

**ODN peptide; ODN (H-Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lysup-OH), 65mg > 95% purity was prepared using the high-performance liquid chromatography (HPLC) method specifically by Prof Andy Wilson from School of Chemistry, University of Leeds and also from Assoc. Prof Julien Chuquet and group from INSERM Lab, University of Rouen, France.

2.4 Cell identification

Differential interference contrast imaging was used to identify healthy cells by their even grey tone and visible but not overly distinct outline. 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 tend to be larger and more likely to have a sub ependymal location. After recording, further morphological characteristics could be viewed due to the injection of 0.02% dextran tetramethyl rhodomine in the intracellular solution. Visualisation of recorded cell was used in conjunction with 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 was a CSFcC. Ependymal cells were identified by passive, no 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 Q Capture Pro 7 (Q Imaging Canada) as in Figure 3-1 (A).

2.4.1 Electrophysiological cell characterisation

For the characterisation of the electrophysiological behaviour of the recorded cell, 1 second current pulses were applied to the cell with the resulting voltage responses measured and analysed. Positive current pulses (+10 to +80 pA) were used to determine if there were any spiking or action potentials produced in response to the depolarisation created by the current pulses. Negative current pulses (-10 to -50 pA) produced hyperpolarisations. These were then used to calculate the cell input

resistance (IR) by using Ohm's Law (Input resistance=voltage response/current injected).

2.4.2 Post fixative identification of cells using rhodamine and/or neurobiotin

The recorded cells were identified using rhodamine and/or neurobiotin. Following recording, the recovered slices were fixed in 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (PFA) and 0.25% glutaraldehyde and were stored at 4°C. For visualisation of the rhodamine, the slices were washed x3 using the 0.1 M phosphate buffer saline (PBS) for 2 times with 5 minutes gap. Then the slices were incubated in a fresh solution of 1% hydrogen peroxide (H_2O_2) using 10 ml methanol and 330 µl H_2O_2 stock solution. The slices were incubated in the solution for 30 minutes to prevent endogenous peroxidase staining of blood vessels and blood cells. Then the slices were wash well in PBS to remove all traces, 3 times, 5 minutes apart. The slices then were incubated in extraavidin peroxidase (EAP; 1:250) with PBSt (PBS+ 0.2% triton). The slices were left overnight or longer at 4°C on a shaker. The slices were washed 2 times, 5 minutes apart with PBS. Vector lab Peroxidases DAB kit (Vector Laboratories, UK; 5 ml distilled water, 2 drops buffer+ 4 drops DAB solution+ 2 drops H_2O_2 solution) were used to incubate slices for 2 minutes and the staining done with dark brown staining of filled cell. CUBIC 1 (25% urea, 25% NNN tetrakis 2 hydroxypropyl ethylenediamine and 15% tritonx100) were used to clear the slices. The slices mounted on a glass coverslips. The slices then were visualised using microscope Eclipse E600 (Nikon, UK) and captured with Micropublishing 5.0 Camera (Q-Imaging, UK). Images were taken at 20x, 40x and 60x magnification. An Acquis imaging software (Synoptic, Cambridge, UK) was used to visualize the pictures taken from the microscope.

2.4.3 Post fixative identification of cells using streptavidin

The recorded cells were identified using streptavidin 555 (1:500 in PBS, Life Technologies, UK). The recovered slices were fixed in 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (PFA) and were stored in 4°C. For visualisation, the slices were washed x3 using the 0.1 M PBS for 3 times with 5 minutes gap. The slices were incubated in PBSt 0.3% (PBS+ 0.3% triton) for 1 hour. The slices then were washed 2 times, 5 minutes apart with PBS. And lastly, the slices were mounted onto slides with vectashield with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride DAPI (Corns et al., 2015) and viewed using Zeiss confocal microscope.

2.5 Immunohistochemistry

Animals used in this study were C57BL/6 mice as for the electrophysiology experiments, and also GAD67-GFP mice that express green fluorescent protein (GFP). The GAD67-GFP mice originally sourced from Yuchio Yanagawa (Tamamaki et al., 2003). The GAD-67 GFP mice were bred in-house in the University of Leeds. These animals have been used previously to identify CSFcCs since these cells will be visualised by the GFP (Gotts et al., 2016b; Corns et al., 2015) and have been validated by Tamamaki et al. (2003).

The GAD67-GFP mice are a transgenic strain of mice that express GFP under the control of the endogenous GAD67 promotor due to the insertion of GFP cDNA into the GAD67 locus. The GFP is obtained from a bioluminescent jellyfish. The jellyfish produced a fluorescent product light when expressed in cells. This fluorescence does not require any exogenous substrates and cofactors, hence GFP expression can be

used to monitor gene expression and protein localisation (Chalfie et al., 1994). A cDNA-enhanced GFP was targeted to the locus encoding GAD67 using embryonic stem cells homologous recombination to generate chimeric male mice by 8 cell stage injection. GAD67-GFP knock in mice were obtained by breeding the male mice with C57BL/6 female mice. The expression of GFP in the GAD67-GFP can be evaluated by visualisation under an ultra violet light at 2-3 days of age and at this age, the fluorescent in the brain could be identified through the mice thin skull. For this experiment, only a few slices (1-3 slices) from a GAD67-GFP mouse aged 4-6 weeks old used to show the result.

Nestin-GFP mice were also used to identify the ependymal cells since it is expressed by neural stem and progenitor cells. These transgenic mice are generated to express GFP under the control of regulatory elements of the nestin gene and have been previously validated (Mignone, J. et al., 2016).

Nestin, an intermediate filament protein is expressed in majority of mitotically active CNS and peripheral nervous system progenitors that give rise to both neurons and glia. Nestin was found in the dorsal and ventral regions of the central canal but this was not found in all ependymal cells but a subset of ependymal cells with large basal bodies and typically two long cilia, referred to as central canal ependymal cells (Alfaro-Cervello et al, 2012). Presence of this proteins identified the neural stem and progenitor cells and the regulatory elements of the nestin gene were used to create a reporter line, thereby allowing the identification and isolation such cells in transgenic mouse (Michalczyk et al., 2005; Lendahl et al., 1990).

Transgenic mouse lines in which the neural stem cells of the embryonic and adult central nervous system expressed GFP have been proven to be a useful study tool to show neurogenesis (Mignone, J. et al., 2016; Encinas et al., 2011).

Nestin is downregulated in all cells upon differentiation (Zimmerman et al., 1994) but reappears transiently after injury to CNS where it has been found in reactive astroglia

of the brain and in ependymal cells of the rat after spinal cord injury (Namiki and Tator, 1999; Holmin et al., 1997).

The expression of GFP in the nestin-GFP can be evaluated by visualisation under an ultra violet light since postnatal day-1 of age and the fluorescent in the brain could be identified through the mice thin skull. Deuchars Lab had generated a nestin-GFP mouse transgenic line according to Mignone, J.L. et al. (2004). The male nestin-GFP were bred with 2 C57BL/6 female in the Central Biomedical Services Unit, University of Leeds and for this experiment, I only used 2-3 spinal cord slices from a 4 weeks old nestin-GFP mouse.

2.5.1 Fixed spinal cord section preparation

Animals were anaesthetized with Euthatal (sodium pentobarbitone, 60 mg/kg Merial Animal Health Ltd, UK) intraperitoneally. Using scissors, an incision was made through the skin from the caudal aspect of the sternum to the distal aspect of the clavicle on each side of the animal to allow access to the rib cage. The rib cage was removed to expose the heart. The animals were then perfused via the left ventricle with 20 ml of 0.1M phosphate buffer (PB) containing 4% paraformaldehyde (PFA). The whole vertebral column was dissected out and the spinal cord was carefully removed then the dura and pia mater. The spinal cord was post fixed in the same PFA solution overnight then a block of thoracolumbar spinal cord was sectioned at 50 μM on a Leica VT100 S vibrotome (UK) and collected into 0.1 M PBS. After 3 x 10 minute washes in PBS, slices were incubated in appropriate primary antibodies overnight in 0.1M PBS or 0.1% PBST (0.1M PBS containing 0.3% Triton X100, 3% donkey serum, 0.8% bovine serum albumin) at the concentrations given in Table 2.5. Triton X100 is a detergent that permeabilises the cell membranes allowing access to

intracellular antigens. Donkey serum and bovine serum albumin are used to reduce the amount of unspecific binding of the antibodies used to the tissue.as listed below (Table 2.5). CD24 biotin (Miltenyi Biotec) with concentration of 1:100 and diluted in Tween, were added to each of slices of C57BL/6 mice slices and were incubated overnight together with $GABA_{B1A}$ antibody and $GABA\rho_1$ antibody.

The next day, the slices then were washed 3 times, 5 minutes apart with PBS. After repeated washing, the slices were incubated in secondary antibodies (refer Table 2-5) conjugated to either Alexa Flour 488 or 555 (1:500; Invitrogen) for 1-2 hour and washed 3 times, 5 minutes apart. For $GABA_{B1A}$ and GABAρ C57BL/6 mice slices, streptavidin pacific blue (Invitrogen, S11222) was added to the slices to visualise the CD24 biotin and incubated for another 1-2 hour. Antibodies used in this thesis were for established markers with well-known patterns of expression.

Lastly, the slices were mounted onto slides with vectashield with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride DAPI (Corns et al., 2015) and viewed using Zeiss confocal microscope, Zeiss LSM 700. The images were analysed using software Zen 2.3, Carl Zeiss Microscopy, GmbH, 2011. The pictures from the Zen 2.3 were exported in jpeg/tiff format into CorelDRAW® software for additional input such as labelling.

2.6 Acute slices for study of $GABA_B$ proliferation

Spinal cord slices were prepared similar to previous method (in 2.2) except that the thickness of the transverse slices was increased to 500 μm, a thickness that has been used previously to look at spinal cord oscillations (Pierce et al., 2010). The spinal cord slices were divided into 3 groups which were the control group, the 1 μM baclofen group and the 5 μM baclofen group. The slices were incubated in vials filled with aCSF

(4 ml for each vial) for 30 minutes then 5-ethynyl-2'-deoxyuridine (EdU) (4 μl of 1 mM) was added to each vial to give a final concentration of 1 μM (Figure 2.3). Baclofen at the appropriate concentrations was added to their respective vials to achieve the final working concentrations of 1 and 5 μM. EdU is a nucleoside analogue of thymidine that is incorporated into deoxyribonucleic acid (DNA) during DNA synthesis and it labels proliferating cells (Chehrehasa et al., 2009). All vials were oxygenated with 95% oxygen and 5% carbon dioxide and placed in a water bath at 37°. The agonist and EdU were changed every 2 hours and after 4 hours' treatment with the drug, the spinal cord slices were fixed in 4% PFA 0.1 M PB for 1 hour (Stoppini et al., 1991). The 4% PFA was changed to 0.1 M PB solution. Then the 500 μm slices were embedded in 10% gelatine, which was further fixed for 4 hours and slices were resectioned at 50 μm before proceeding with EdU localization.

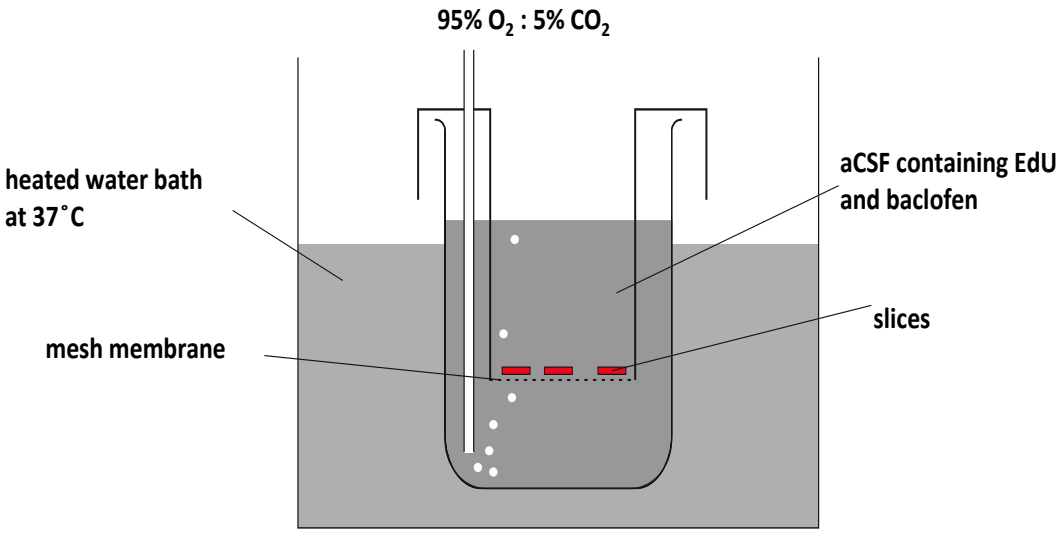


Figure 2-3 Experimental set up for acute slices with baclofen incubation

2.6.1 EdU localization

For EdU detection, sections were incubated in 320 μl of distilled water, 25 μl of 2 M Tris Buffer, 50 μl of 10 mM of copper sulphate, 5 μl of biotinylated azide 1 mM and 100 μl of ascorbic acid for 30 minutes followed by washing with Tris (0.1 M). Then the sections were incubated in streptavidin 555 (1:1000 in PBS, Life Technologies, UK) before mounting onto slides with vectashield with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Corns et al., 2015). DAPI binds to the DNA of cells and emits blue fluorescent colour so this is used to label the nucleus of cells. The proliferating cells were labelled using the streptavidin 555 and appeared red in colour and scattered throughout the whole area of spinal cord. The main area of interest was the central canal and the surrounding lamina X.

2.6.2 EdU-positive cell counts

EdU-positive cells were counted in central canal region. The central canal region and part of lamina X was identified as the area within 10 μm of the abluminal edge of the ependymal cells and could be visualized directly through the NIKON 600 microscope under 40x magnification (Corns et al. 2015). Cells were mapped and counted manually.

2.6.3 $GABA_B$ immunohistochemistry on ependymal cells and CSFcCs

2.6.3.1 Fixed spinal cord section preparation

As previously stated, all experiments were carried out in line with the animal ethics and regulations. Wild type mouse (C57BL/6) and GAD67-GFP mice (21 days old or adult) were anaesthetised by administration of pentabarbitone (60 mg/kg). The spinal cord was post fixed in the same PFA solution overnight then a block of thoracolumbar spinal cord was sectioned at 50 μm on a Leica VT100 S vibrotome (UK) and collected into 0.1 M PBS. After 3x 10 minute washes in PBS and prepared for incubation with primary antibody. The primary antibody $GABA_{B1A}$ was diluted 1 in 100 in PBST 0.1% and added with CD24-biotin REA743 (Miltenyi Biotec GmbH) diluted in 1 in 100 in Tween solution. Tween was used as the detergent here in preference to Triton since the antibody worked better. The slices were incubated with these antibodies overnight. For GAD-GFP mouse, the slices were incubated overnight with primary antibody $GABA_{B1A}$, diluted in 1 in 100 in PBS.

The next day, the C57BL/6 and GAD-GFP mouse slices then were washed 3 times, 5 minutes apart with PBS. After repeated washing, C57BL/6 spinal cord slices were incubated in secondary antibodies. The C57BL/6 mouse slices were added with secondary antibody donkey anti-sheep conjugated with Alexa flour 555 (Invitrogen) for 1-2 hour and washed 3 times, 5 minutes apart. Then streptavidin (blue) was added to the plate and incubated for another 1-2 hour. CD24 is used as an ependymal stem cell marker. The GAD67-GFP mouse was used to show any co localisation of $GABA_{B1A}$ on CSFcC cells.

2.6.4 5-HTergic fibres and nestin positive cells in spinal cord of nestin-GFP mouse immunohistochemistry

The immunofluorescence method was used to determine the location and apposition of 5-HTergic fibres with nestin positive cells in spinal cord. The animal was anaesthetised with sodium pentobarbitone (60 mg/kg) and then was intracardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). After the perfusion was completed, the spinal cord was removed and was kept in 0.1% PFA for 24 hours. After that, the solution was replaced with 0.1 M PB. Immunohistochemistry was performed. In order to detect 5-HTergic fibres, an antibody against 5-HT (1:1000; RA 2000; Neuromics) was used. Full details of the methods and antibodies used are in the table below:

Table 2-5 Primary and secondary antibodies used for pre-fixed spinal cord immunohistochemical

1º ANTIBODY & SOURCES	DILUTION	2º ANTIBODY	DILUTION	REFERENCE
$GABA_{B1A}$ antibody	1:100 in PBST 0.1%	Donkey anti-sheep	1:1000 in PBS	Charles et al. (2003)
$GABA\rho_1$ antibody, Santa Cruz	1:400 in PBST 0.1%	Donkey anti-goat	1:1000 in PBS	Milligan et al. (2004)
ODN antibody, from (Tonon et al., 1990)	1:500 in PBST 0.1%	Donkey anti-rabbit 488	1:1000 in PBS	Tonon et al. (1990)
TSPO antibody, Abcam (ab 118913)	1:100 in PBS	Biotin donkey anti-goat 1:1000 for 2h and followed with SA 555	1:250 in PBS and followed with SA 555 1: 1000	New et al. (Deuchars Lab)
DBI antibody, Frontier Institute	1:2000 in PBST 0.1%	Donkey anti-rabbit 1:1000	1:1000 in PBS	Christian et al. (2013)
5-HT, Neuromics	1:500 in PBST 0.1%	Donkey anti-rabbit 488	1:1000 in PBS	Ghani et al. unpublished (Deuchars Lab)

2.7 Analysis of data

2.7.1 Data collection

Electrophysiological data were collected on the Spikes2 version 7.0 files data (Cambridge Electronic Design) and Clampex program (Molecular Devices, USA). Amplitudes for 2 bath or puff recordings were taken and mean for these 2 were used for amplitude for each results. Averages for according to pharmacological group were calculated and expressed as the mean ± standard error mean (S.E). For drugs in bath and puff solution, comparisons were made before during and after application of antagonist and modulators. The measurements of recordings were: amplitude of voltage response and input resistance (IR; as calculated from the current steps using Ohm's Law). Other data of cell localisation, immunostaining were collected and analyse and recorded in Excel table sheet.

2.7.2 Statistical analysis and statistical tests

All data were entered and analysed using the IBM SPSS statistic 21 and Excel software. All the data were checked for normal distribution and tests chosen accordingly. To compare responses to drugs before and after application in the same cells, a paired t-test (parametric for normally distributed) or Wilcoxon ranked sum test (non-parametric) was used. To analyse and compare means between two groups and two-sample assuming unequal variances, the independent T-test was used. A Spearman's Rank order of correlation for used to test correlation and One-Way ANOVA was used to compare means between groups for the $GABA_B$ experiments. Data were expressed as mean ± s.e.m (standard error mean). P values less than 0.05 (*p<0.05) were indicated as the level of significance. Data that

produced significant results (*p<0.05) were further tested using post-hoc tests. The data are presented in the graphs and table forms.

Chapter 3

Effects of GABAergic modulation on ependymal cells

3.1 Introduction

There is little information regarding the neurotransmitters that may regulate proliferation in the spinal cord but some recent research has started to address this and we now know that neurotransmitters can influence proliferation and differentiation of spinal neural stem cells. Progenitors in stem cell niches in the brain have been shown to be regulated by GABA, glutamate, acetylcholine, dopamine and serotonin (Berg et al., 2013). In subventricular zone, GABA released from newborn neurons inhibits the proliferation of neighbouring progenitors to control the neurogenesis (Liu, X. et al., 2005; Haydar et al., 2000; LoTurco et al., 1995). Although less is known about the regulation of spinal stem cell niches by neurotransmitters, recent findings show influences of neurotransmitters on the stem cell niches especially the central canal contacting progenitors (Corns et al., 2015; Corns et al., 2013; Marichal et al., 2012; Reali et al., 2011). Acetylcholine can induce cell proliferation in the spinal cord neurogenic niche which is mediated by α7*nAChRs (alpha-7 nicotinic receptor) and can be enhanced with a selective α7*nAChR positive allosteric modulator (PNU 120596) (Corns et al., 2013). A neuropeptide; substance P (SP) can influence neural stem/progenitor cell proliferation and neurogenesis in spinal cord injury (Kim et al., 2015). The researchers used a compression type spinal injury in adult Sprague Dawley rats. Through immunoblot analysis, they were able to show that SP also

increases the phosphorylated ERK and phosphorylated p38 kinases in neural stem/progenitor cell proliferation.

Ependymal cells have been characterised as having a low input resistance (124 ± 24 MΩ), a resting membrane potential of -84 ± 2 mV and passive response properties to current pulses in rat (Marichal et al., 2012). There is a presence of gap junction and dye coupling in the area of ependymal cells. However the significance of these have yet to be considered.

In my work, I will be examining the characteristics of ependymal cells in the central canal of mice, determining how the cells communicate in the central canal of spinal cord and how they respond to GABA.

3.2 Hypothesis and aims

Here I first aimed to establish whether ependymal cells in spinal cords from mice aged P8-15 have similar properties to those observed in other species. It is hypothesised that ependymal cells from the spinal cords have passive electrophysiological properties with a high degree of gap junction coupling, thus differing from CSFcCs. The main hypothesis of this chapter proposes that ependymal cells are capable of responding to GABA and that these responses are mediated by specific receptor subtypes. The secondary hypothesis proposes that modulators at specific sites on the GABA receptors or ependymal cells can affect the responses of these cells to GABA.

3.3 Results

The results in this chapter are obtained from spinal cord slices prepared from postnatal 8 to 15 day mice of either sex as described in general method.

Basic electrophysiological characteristics of 118 ependymal cells in total, were recorded and used for different parts of this chapter. The ependymal cells recorded in this study had a mean resting membrane potential of -74.0 ± 0.57 mV, similar to the recorded resting membrane potential (-76 mV) observed by Corns et al. (2013).

Whole cell patch clamp methods were used, which sets the reversal potential for chloride according to the chloride concentration in the aCSF (127 mM) and the intracellular solution (2.1 mM). The reversal potential is -103 mV.

The majority of cells recorded in the central canal region were ependymal cells and were distinguished from the other major cell group in this region (the cerebrospinal fluid contacting cells (CSFcC)) by their electrophysiological properties. Using Q-capture software images (Figure 3-1 A), the cells were identified around the central canal with a faint outline of cuboidal shape cells, and were tightly bound together at the luminal surfaces (Figure 3-1 A, C, D). Electrophysiological characterisation showed that ependymal cells in this study had negative membrane potentials, and show passive electrophysiological properties, where the cells do not show any spiking activity (Figure 3-1 B). There was a complete absence of spikes or action potentials in response to depolarising or hyperpolarising current pulses.

There is a linear relationship between the size of the current pulse and the change in voltage (Figure 3-2 A). Input resistances for ependymal cells were small and ranged from 10 MΩ to 200 MΩ (Figure 3-2 B) compared to cells previously recorded by Corns et al. (2013). The ependymal cells in this study had an average input

resistance of 72.6 ± 3.9 MΩ (n=118). The highest numbers of cells have input resistances falling in the range of 30-40 MΩ (n=18).

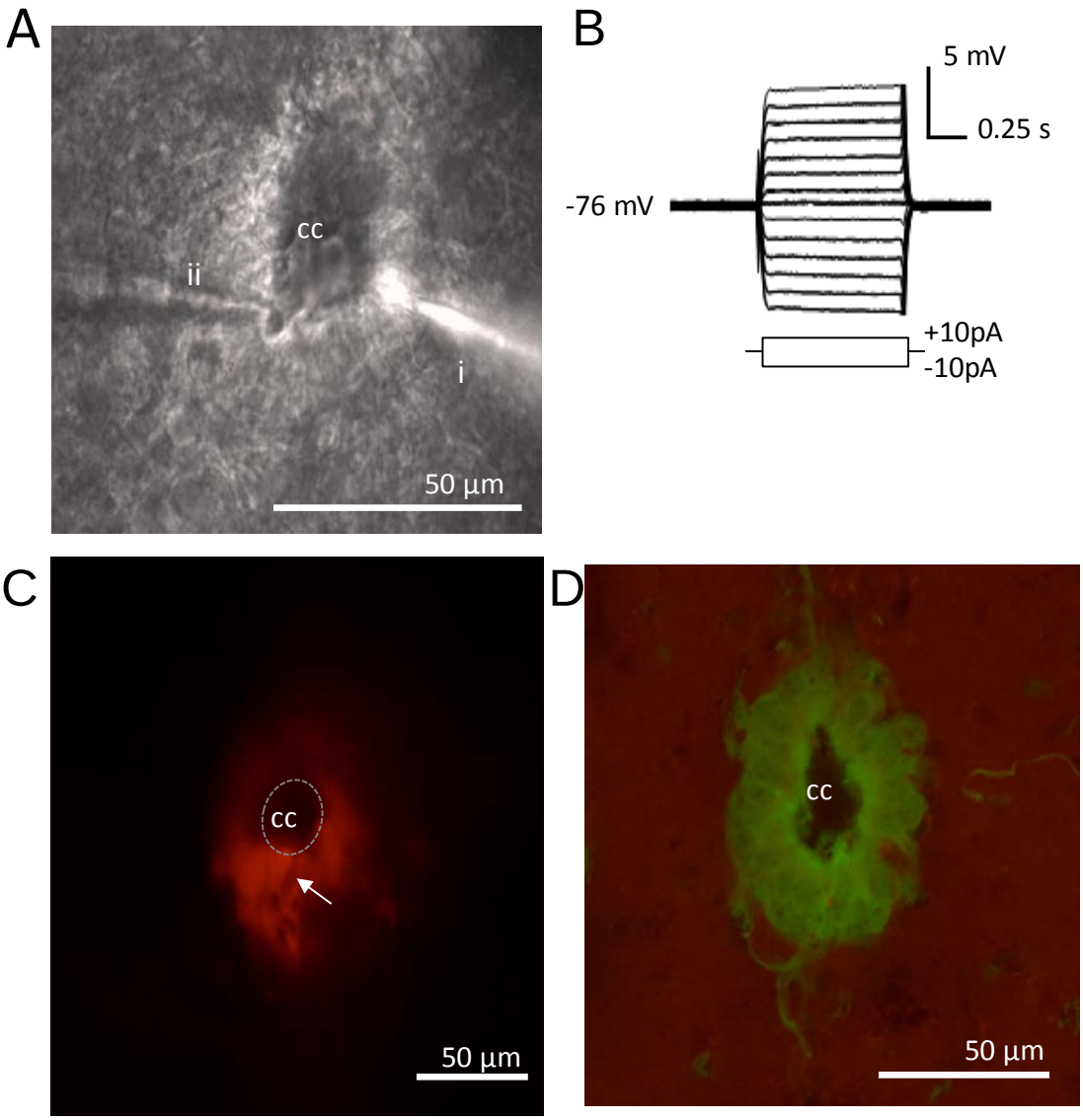
Figure 3-1 Basic electrophysiological characteristics of ependymal cells

An example of DIC image of the spinal cord slice, showing the ependymal cell layer around the central canal and the pipettes used to obtain single cell patch clamp recordings or to enable puff application of drugs from ependymal cell with the i) recording electrode and ii) drug application pipette. Note the cell was filled with fluorescent dye and was seen post recording, imaged using Q-capture software (A).

Example of whole cell patch clamp trace recorded in current clamp of an ependymal cell responding to the injection of positive and negative current pulses (B).

During recording, the cell was filled with neurobiotin and for posthoc confirmation the cell was an ependymal cell. The arrow points to the patched and filled cell and there is clear dye spread to other ependymal cells around the central canal (C).

A spinal cord slice section from a nestin-GFP mouse showing ependymal cells around the central canal (cc) (D).


A
cc
ii
i
50 µm
B
5 mV
0.25 s
-76 mV
+10pA
-10pA
C
cc
50 µm
D
cc
50 µm

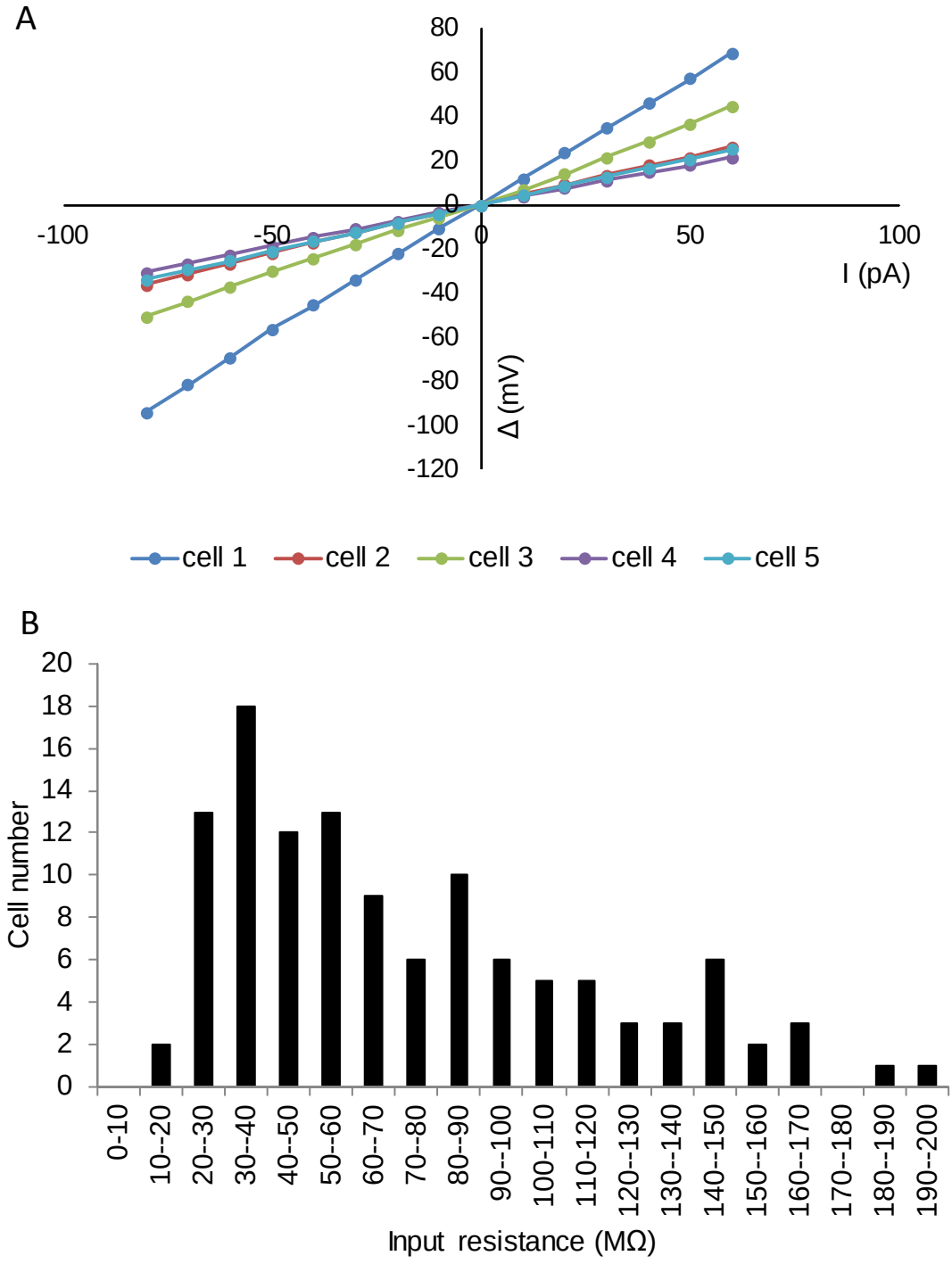


Figure 3-2 Current voltage relationship and histogram of input resistances

Pooled data for 5 ependymal cells showing similar changes in voltage in response to positive and negative current pulses injected for ependymal cells (A). Frequency histogram for mean input resistances for ependymal cells (n = 118 cells) (B).

3.3.1 Dye coupling reveals that ependymal cells are coupled to each other.

Tracers were included in the intracellular solution such as neurobiotin, which is a gap junction permeable tracer (Kita and Armstrong, 1991; Vaney, 1991) and when the recorded ependymal cell is coupled to other ependymal cells through the gap junctions, the neurobiotin could diffuse through the gap junctions to fill some of the connected ependymal cells. In this study, the visualisation of neurobiotin with DAB revealed that the ependymal cells also showed gap junction coupling since there was dye in neighbouring cells to the neurobiotin-filled ependymal cell detected by post-fixation DAB images. In those cells that were recovered (n = 27), the majority (n = 26) showed dye coupling.

The extent of neurobiotin labelling demonstrated different levels of coupling between ependymal cells; sometimes this involved the whole ependymal layer surrounding the cc that was dye filled (Figure 3-3 A), while in others, not all of the ependymal cell layer (Figure 3-3B) was filled. In other recordings, a smaller group of ependymal cells within one region of central canal (Figure 3-3 C and D) was observed. Visualisation of neurobiotin and/or rhodamine revealed that some of the ependymal cells had processes as seen in Figure 3-3 (C) and (D). Processes were found originating from the dorsal (D) and ventral (A) pole of central canal, from which they extended dorsally and ventrally along the midline. In the same group of cells, there was also a number of processes observed extending from the lateral aspects of central canal (C and D).

Imaging of cells with rhodamine, which is not gap junction permeable, showed no coupling of cells indicating that there was no spillage of tracers. This is observed using the Q-capture software, when a single rhodamine-filled cell is observed (Figure 3-1 A).

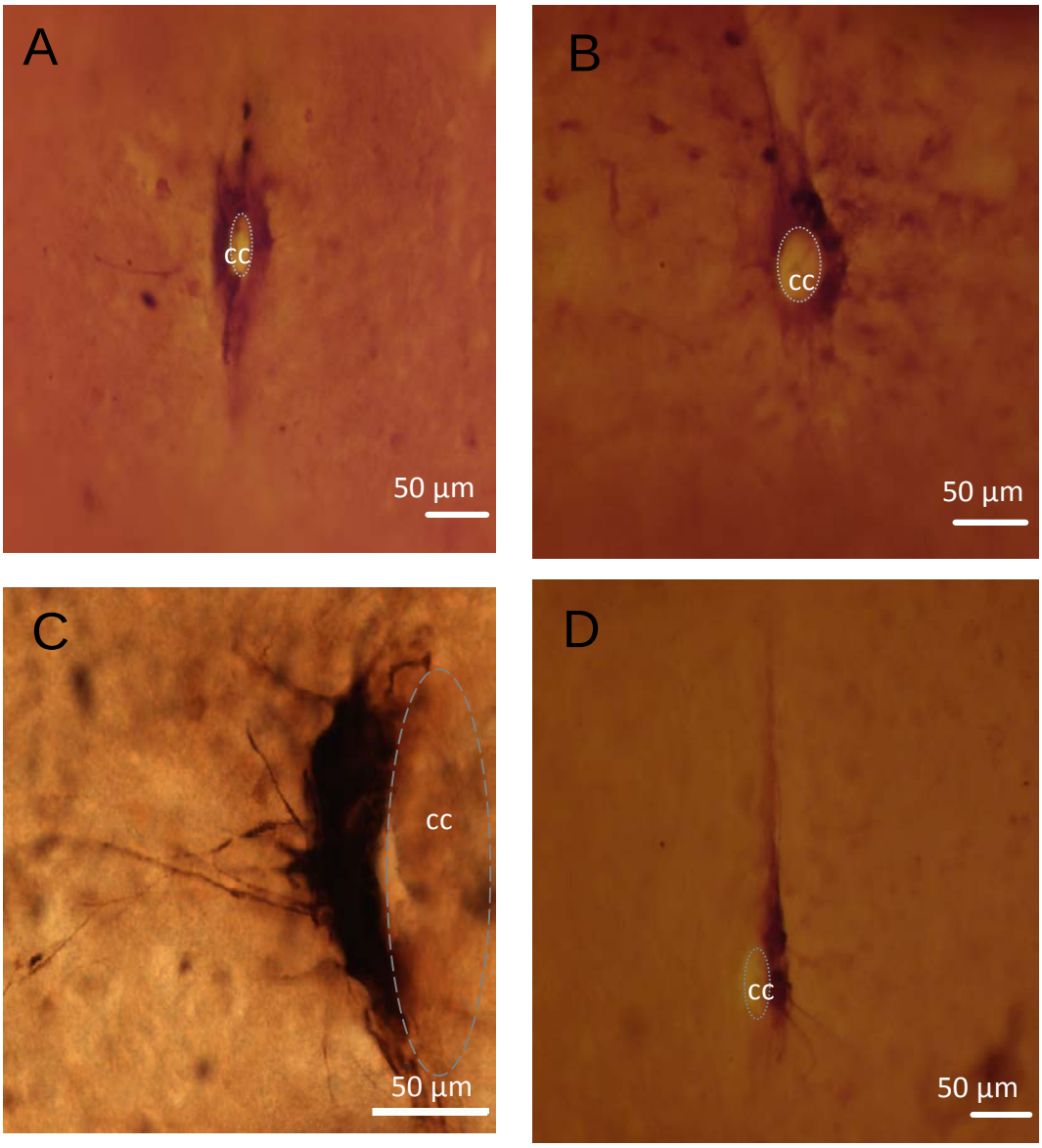


Figure 3-3 Intracellular dye-loading with neurobiotin revealed coupling between ependymal cells

Visualisation of neurobiotin by DAB revealed ependymal cells that are dye coupled around the whole central canal of the (A and B), a multiple layer of ependymal cells that were dye-coupled around a region of the central canal (C), and dorsal processes extending from some of the cells within a group of dye-coupled ependymal cells located at the dorsal pole of the central canal (D). Images are all orientated with dorsal at the top, A, B and D using x20 magnification and C using x60 magnification, cc=central canal.

3.3.2 Effects of 18β-Glycyrrhetinic acid on dye coupling of ependymal cell

To determine the effect that gap junction coupling was having on the electrophysiological properties of ependymal cells, the non-selective gap junction blocker 18β-glycyrrhetinic acid (100 μM) (Davidson and Baumgarten, 1988) was bath applied. The input resistance was increased in five of the six cells tested following bath application of 18β-glycyrrhetinic acid (Figure 3-4 A). Four of the six cells showed changes of membrane potential even though overall, the change in these 4 cells did not reach significance (Figure 3-4 C). With 18β-glycyrrhetinic acid, the input resistance of ependymal cells tested significantly increased (n=6, Figure 3-4, A, D) from 42.45 ± 9.52. MΩ to 127.9 ± 32.70 MΩ. The wide range of changes in input resistance suggests that the ependymal cells are coupled to different degrees.

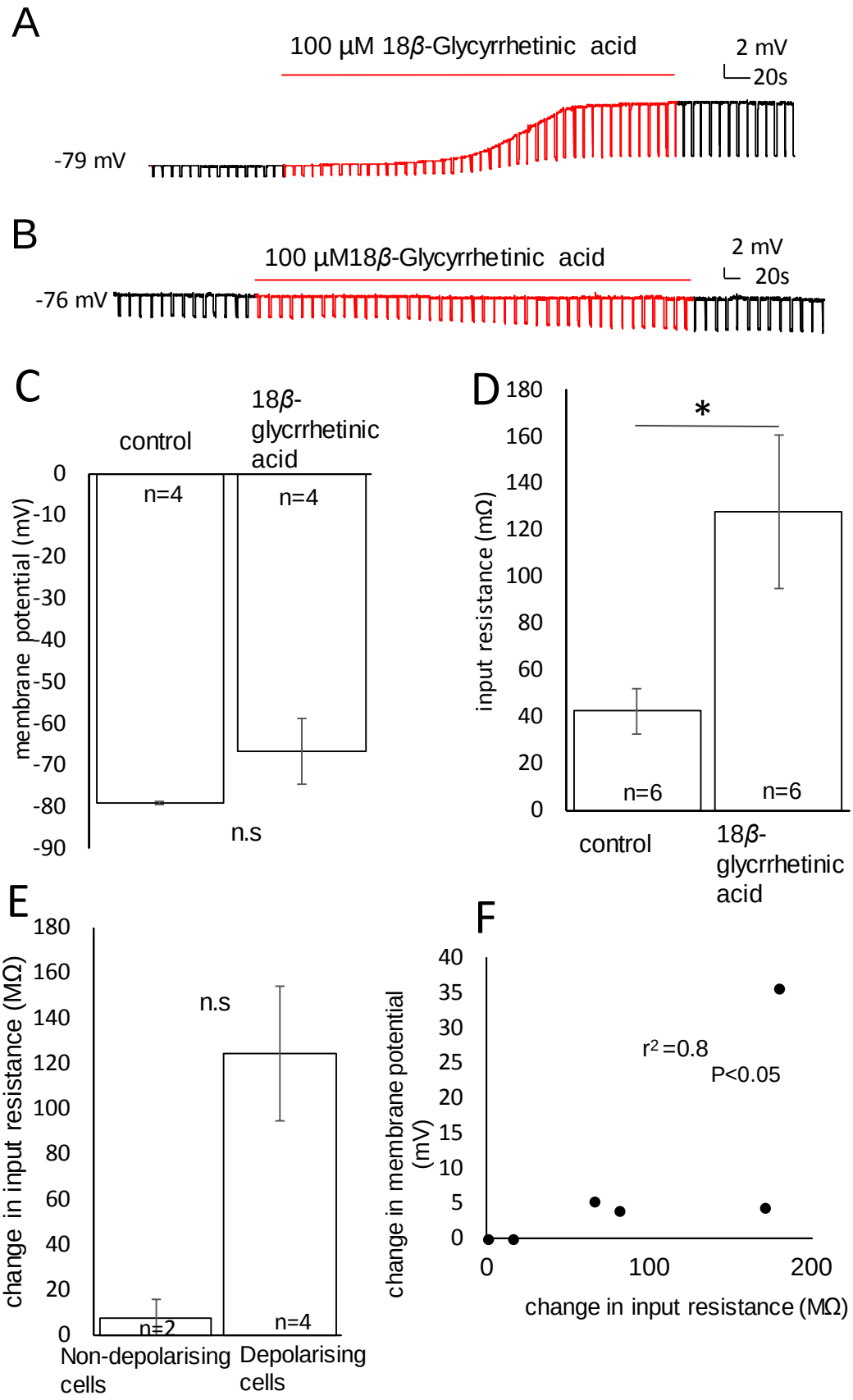
18*β*-glycyrrhetinic acid also had an effect on membrane potential in 4 out of 6 ependymal cells, causing depolarisation in these 4 cells (Figure 3-4 E). Interestingly, the change of input resistances was 124.27 ± 29.51 MΩ for the depolarising cells. The average change in input resistance for the cells that did not depolarise in response to 18β-glycyrrhetinic acid was much smaller (7.8 ± 7.8 MΩ). The changes of IR between depolarising and non-depolarising cells were not significantly different but only 2 cells were considered in the non-depolarising group.

The correlation between the change in input resistance and change in membrane potential was examined. A Spearman's Rank order of correlation showed a significant correlation between the change in input resistance and the change in membrane potential (r^2=0.8, P<0.05, Figure 3-4 F). This suggests that the depolarisation that occurs in response to application of 18β-glycyrrhetinic acid is related to blockade of gap junctions. As the slices were not pre-incubated with 18*β*-glycyrrhetinic acid prior

to recording, the effect of 18β-glycyrrhetinic acid on dye coupling could not be investigated.

Figure 3-4 A gap junction blocker, 18β-glycyrrhetinic acid, increases input resistance of ependymal cells

Example whole cell patch clamp traces showing effects of bath application of 18β-glycyrrhetinic acid (A and B). The top trace shows an increase in input resistance is accompanied by a large depolarisation (A) while on the bottom trace, an increase in input resistance is observed with little change in membrane potential (B). Pooled data for 4 cells of the changes in membrane potential caused by 18β-glycyrrhetinic acid (C). Pooled data for 6 cells of the input resistance changes elicited by 18β-glycyrrhetinic acid (D). Grouped data (mean ± SE) of the change in input resistance for the cells classed as non-depolarising cells compared to those classed as depolarising cells in response to bath application of 18β-glycyrrhetinic acid (E). Correlation between the change in input resistance and the change in membrane potential in response to bath application of 18β-glycyrrhetinic acid. Plots of changes in input resistance against changes in membrane potential showed significant correlation value *p<0.05 (F).


A
100 µM 18β-Glycyrrhetinic acid
2 mV
20s
-79 mV
B
100 µM18β-Glycyrrhetinic acid
2 mV
20s
-76 mV
C
control
18β-glycrrhetinic acid
n=4
n=4
membrane potential (mV)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
n.s
D
*
input resistance (mΩ)
180
160
140
120
100
80
60
40
20
0
n=6
n=6
control
18β-glycrrhetinic acid
E
change in input resistance (MΩ)
180
160
140
120
100
80
60
40
20
0
n.s
n=2
n=4
Non-depolarising cells
Depolarising cells
F
change in membrane potential (mV)
40
35
30
25
20
15
10
5
0
r2 =0.8
P<0.05
0
100
200
change in input resistance (MΩ)

3.3.3 The properties of cerebral spinal fluid contacting cells differed from those of ependymal cells

To aid identification of CSFcCs, these cells were rounded and larger than the remaining ependymal cells and were usually seen as brighter cells located around the central canal using a contrast image camera software. A process with an end bulb often was observed extending into the central canal.

Electrophysiology revealed that CSFcCs were different from ependymal cells and consisted of a varied population of cells. Their phenotype ranged from the one resembling ependymal cells to a phenotype resembling a repetitively firing neurone. Due to this varied phenotype, the criteria used to define as a CSFcC were the shape of the voltage responses to current pulses, visualisation of a CSF-contacting process post-recording using either rhodamine/neurobiotin or using a combination of rhodamine/neurobiotin with streptavidin alexafluor 555 to show that most cells were not coupled.

Recordings were made from 13 CSFcCs that were identified microscopically and using Q capture software. The cells were found interspersed among the cuboidal cells around the CC. In contrast to ependymal cells, the majority of CSFcCs had spikelets or full action potentials, The mean IR of CSFcCs was significantly higher (703.6 ± 86.1 MΩ, n=13) than that of ependymal cells while their resting membrane potential was more depolarised (-56.9 ± 4.4 mV, n=13). (Figure 3-5).

Only 1 Subtype 1 CSFcC was identified that had a phenotype almost indistinguishable from ependymal cells (Figure 3-5 A, Ai). The cell had a resting membrane potential, in the range of the recorded resting membrane potential of ependymal cells; -73.5 mV, a low input resistance (18.5 MΩ) and a lack of spontaneous or evoked activity. Within subtype 1 CSFcCs, previous research has further categorised the cells into subtype 1A and 1B by visualisation of neurobiotin post recording (Corns et al., 2015).

Subtype 1A were defined as cells which were coupled to neighbouring cells resembling ependymal cells. Neurobiotin can pass through gap junctions, therefore if cells are coupled to neighbouring cells, these cells may be visualised (Figure 3-5 Ai). Subtype 1B were defined as cells which were visualised alone and not coupled to neighbouring cells. In this study, no recordings were made of Subtype 1B CSFcCs.

Subtype 2 CSFcCs were defined by the production of a single fast depolarisation in response to the injection of positive current (Figure 3-5 B). Subtype 2 had membrane potential oscillations that followed the single spike. These cells also had synaptic activity with negative current (Figure 3-5 B, black arrow). Rebound depolarisations were often observed following a negative current pulse (Figure 3-5. B; black arrowhead). Rebound depolarisations suggested the presence of T-type Ca^{2+} channels (Leresche and Lambert, 2018; Surges et al., 2006). Visualisation of subtype 2 CSFcCs post-recording revealed a single cell with a single process (Figure 3-5 Bi). A single cell dye fill indicated that these types of cells were not coupled to neighbouring cells.

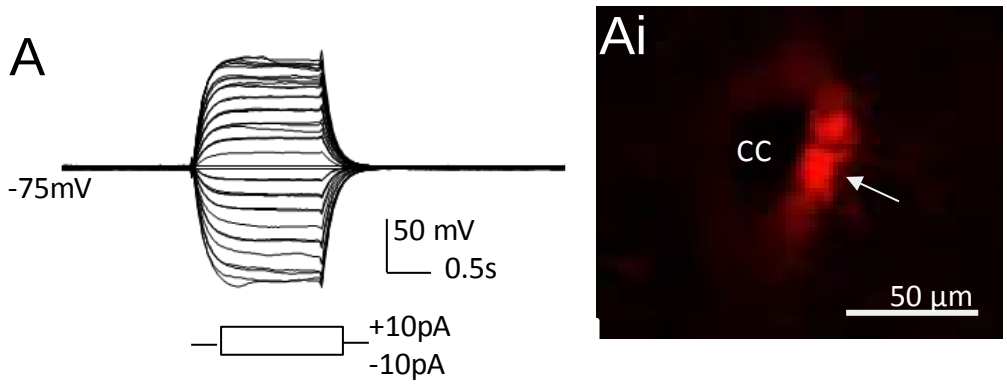
Subtype 3 CSFcCs are defined by their ability to produce multiple spikes or action potentials in response to the injection of positive currents but only one of this subtype was recorded (Figure 3-5 C). This subtype 3 cell displayed spontaneous activity in form of EPSPs and sometimes spikes or action potentials and synaptic activity (Figure 3-5 C, black arrow), followed by rebound depolarisations with negative current injections (Figure 3-5 C, arrowhead).

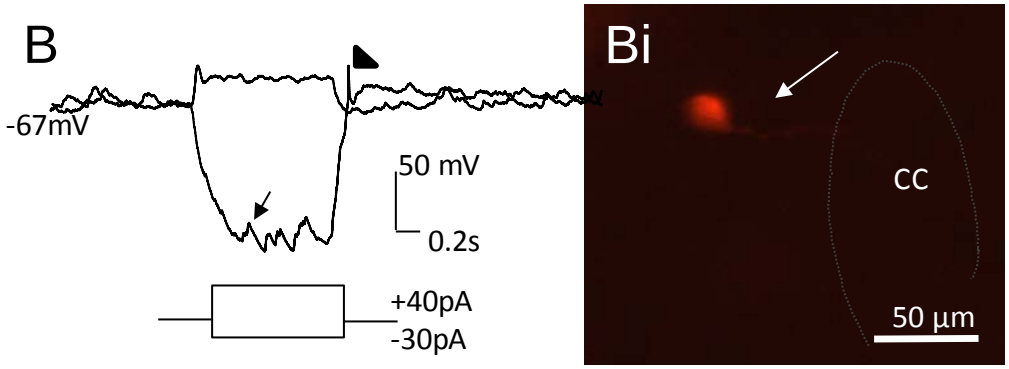
The subtype recorded most frequently in this study was subtype 2 (n=11). A previous study of CSFcCs also showed a majority of Subtype 2 CSFcCs recorded in spinal cord of rodents (Corns et al., 2015). The visualisation of rhodamine post-recording allowed the observation of CSF-contacting processes. These processes were better identified following the visualisation of neurobiotin. Not all cells were successfully visualised post recording and sometimes the processes were lost in the

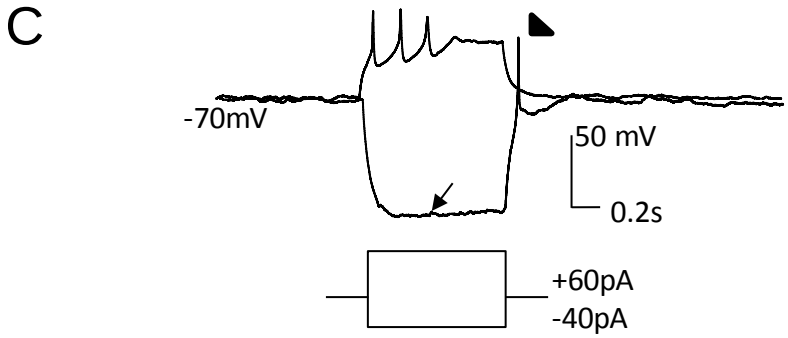
resectioning procedure. Generally, the processes projected from the cells ventrally towards ventral area (Figure 3-5 Bi).

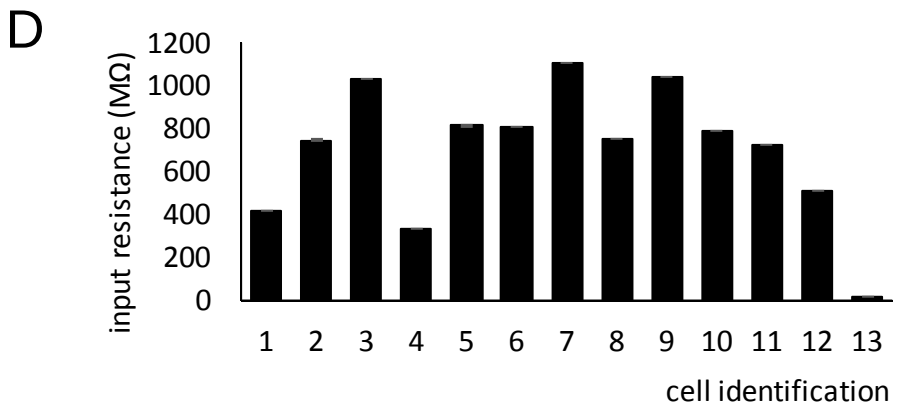
Figure 3-5 CSFcCs subtypes and their input resistance

Example trace of whole cell patch clamp characterisation of Subtype 1 CSFcC (A). Subtype 1 had a phenotype that was almost indistinguishable from ependymal cells, a similar resting membrane potential, low input resistance, and a lack of spontaneous activity. This cell can be categorised further into 1A subtype by visualisation of neurobiotin, coupled to neighbouring cells, resembling ependymal cells (A). Example of whole cell patch clamp of subtype 2 CSFcC, producing a single spike and rebound depolarisation following injection of negative current was observed (B). The visualisation of neurobiotin by fluorescent microscopy image confirmed the cell as CSFcC (Bi). Example trace of a subtype 3 CSFcC producing multiple action potentials in response to the injection of positive current (C). All images are orientated, so dorsal at the top. Black arrows indicate spontaneous synaptic activity and black arrowheads indicate rebound depolarisations. Histogram showing CSFcCs with large input resistances, varying over a wide range (D).


A
-75mV
50 mV
0.5s
+10pA
-10pA
Ai
CC
50 µm


B
-67mV
50 mV
0.2s
+40pA
-30pA
Bi
CC
50 µm


C
-70mV
50 mV
0.2s
+60pA
-40pA


D
input resistance (MΩ)
1200
1000
800
600
400
200
0
1 2 3 4 5 6 7 8 9 10 11 12 13
cell identification

3.3.4 Ependymal cells respond to GABA

Given the response to GABA in ependymal cells in rats as reported in previous work of Deuchars Lab (Corns et al., 2013), on CSFcCs (Corns et al., 2013; Marichal et al., 2009) and on radial glia surrounding the central canal in the turtle (Reali et al., 2011), responses of GABA were further investigated on ependymal cells in mouse.

GABA caused both depolarising and hyperpolarising responses and the type of response was dependent on the concentration of GABA applied. Of 26 ependymal cells tested, the higher concentration; 400 μM GABA bath, elicited a depolarisation with amplitude of 3.85 ± 0.29 mV compared to an amplitude of only 1.54 ± 0.13 mV with lower concentration 200 μM GABA bath (Figure 3-6 A, B). With high concentrations of bath applied GABA, depolarising responses were always observed. GABA at a concentration of 400 μM was therefore chosen to elicit the desirable amplitude response in ependymal cells.

Second applications of GABA usually elicited a reduced amplitude response with bath (Figure 3-6 C) and puff application (not shown).

Puff application of GABA with two different concentrations was also tested; high concentration 400 μM (Figure 3-7 A) and low concentration 1.25 μM (Figure 3-7 B) on ependymal cells, which elicited both depolarisations and hyperpolarisations. With puff application, as the concentration was increased, the responses were more likely to be depolarising (Figure 3-7A and C) and as the concentration was decreased, the responses were hyperpolarising (Figure 3-7 B and C).

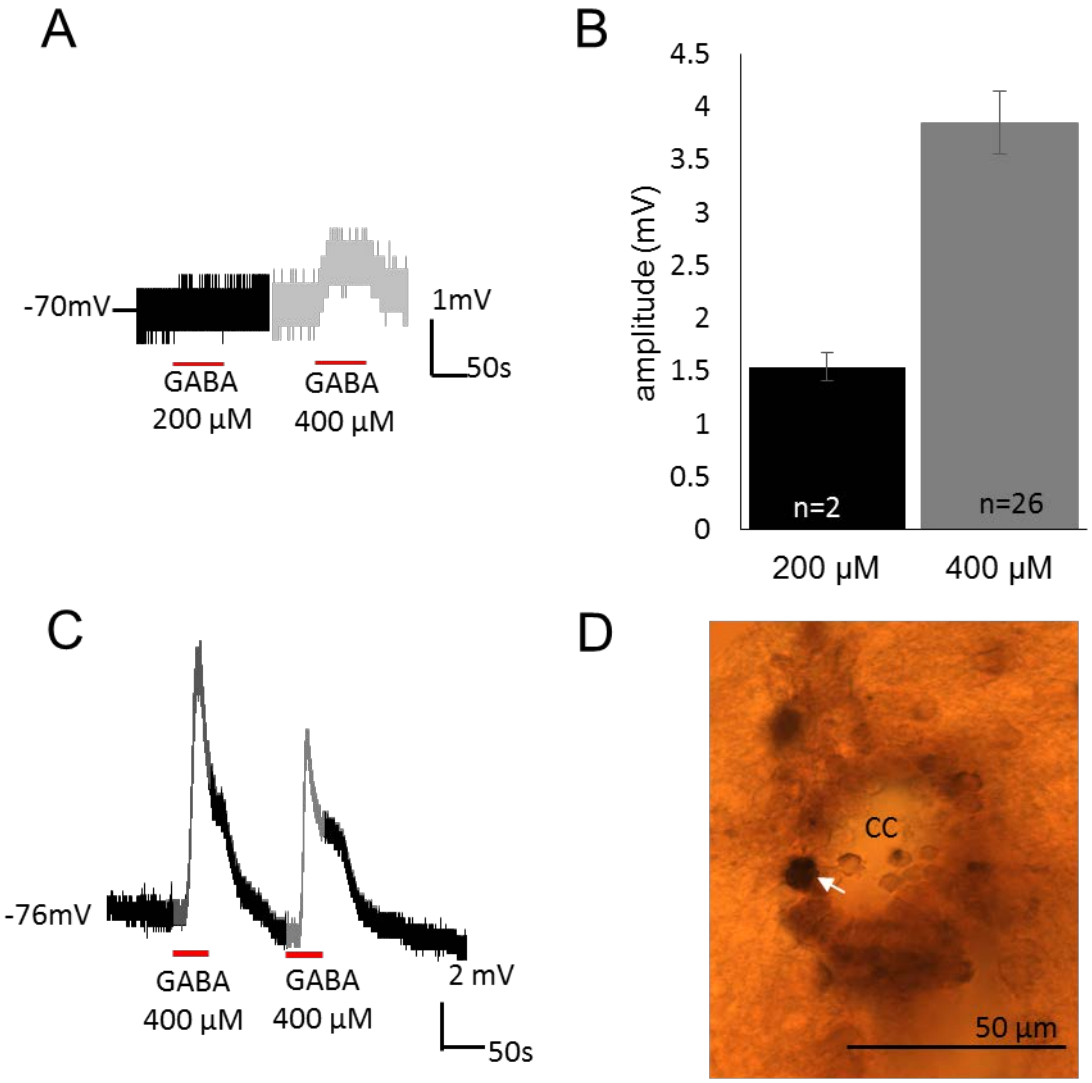


Figure 3-6 Effects of GABA on ependymal cells were dependant on concentration.

Bath application of GABA with two different concentration; 200 µM and 400 µM on same ependymal cell (A). There is an increase in amplitude for the dose of 400 µM compared to 200 µM (A) and (B). With high concentrations of bath applied GABA, depolarising responses were always observed. Second applications of GABA usually elicited a reduced amplitude response with bath application (C). Post-hoc confirmation with DAB; it was an ependymal cell (D) (cell rotated with dorsal at top, white arrow point to the patched cell, cc=central canal).

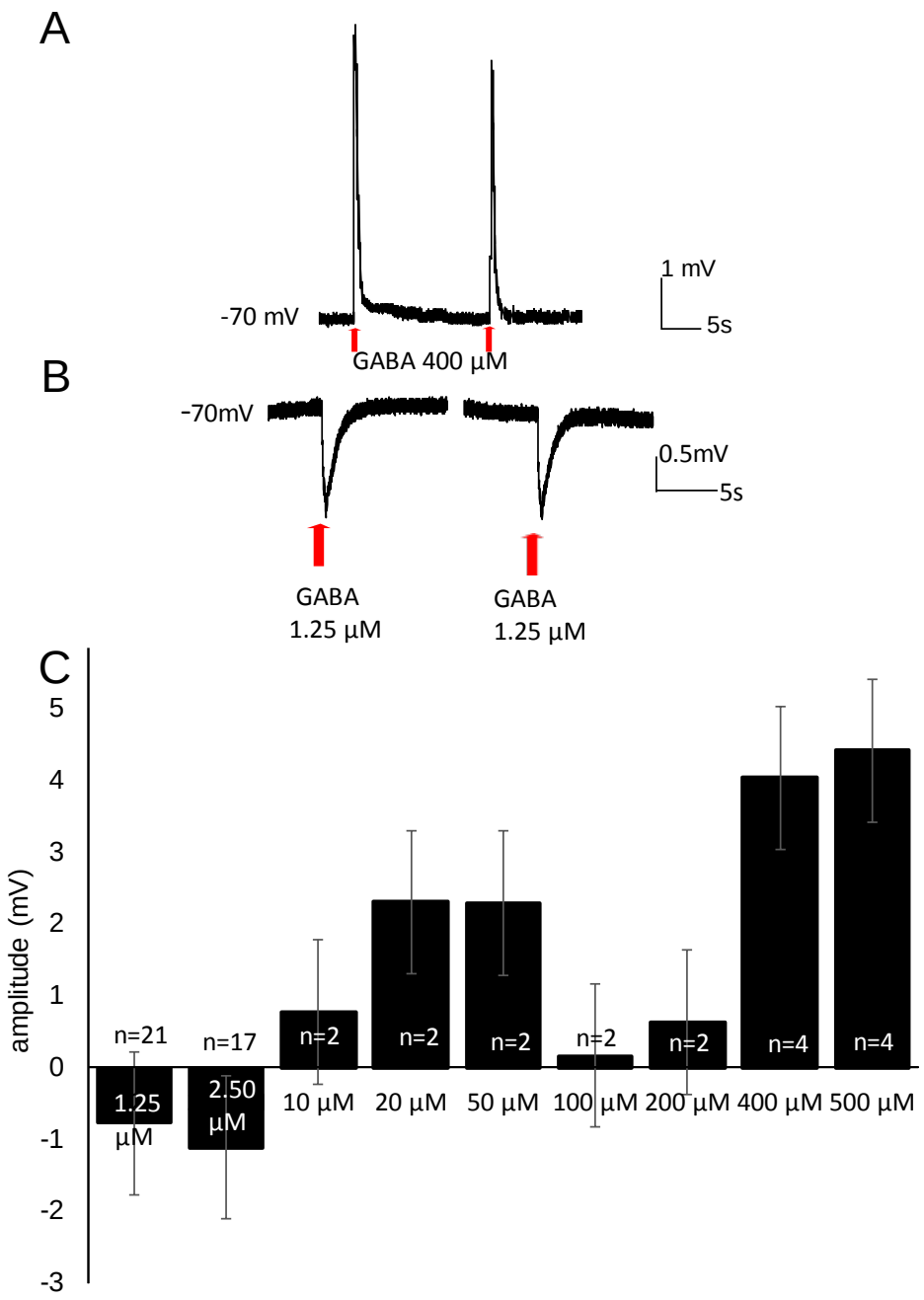


Figure 3-7 Ependymal cells respond to puff application of GABA both with depolarisation and hyperpolarisation.

Puff application of GABA with two different concentration; high concentration 400 µM (A) and low concentration 1.25 µM (B) on ependymal cells. Ependymal cells respond to GABA both with depolarisation and hyperpolarisation. With puff application, as the concentration was increased, the responses were more likely to be depolarising (A and C) and as the concentration was decreased, the responses were hyperpolarising (B and C).

To establish whether the location of the cell affected the response observed, ependymal cells were recorded from around the central canal and the location and response were noted. It was clear that there was no location-specific response profile (Figure 3-8).

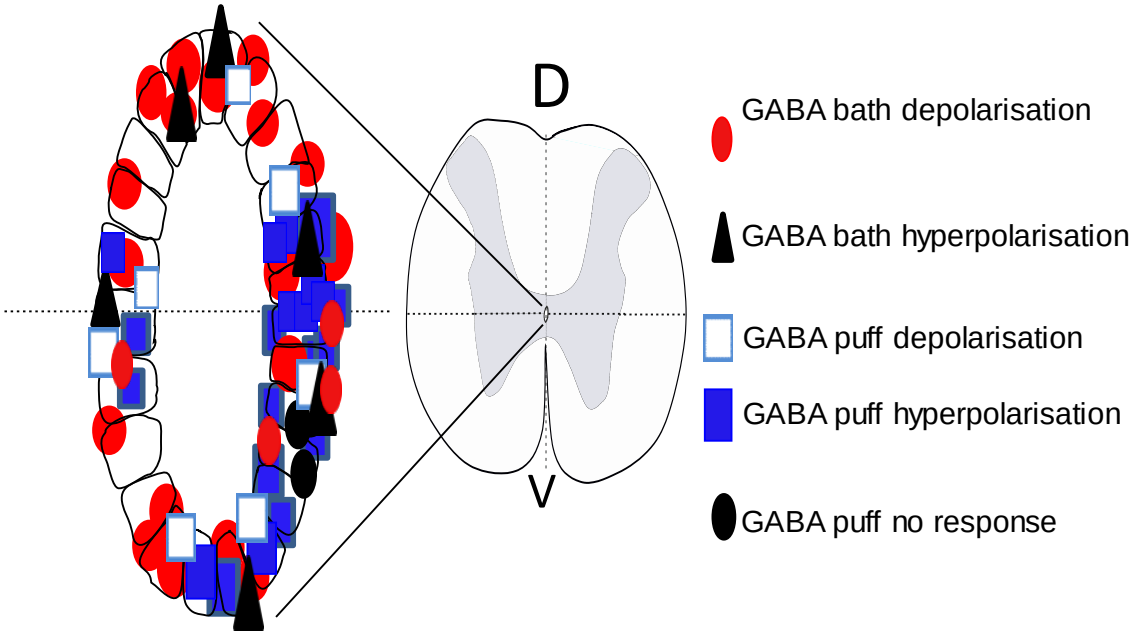


Figure 3-8 Location of ependymal cells exhibiting the different responses to GABA.

Whole cell patch clamp recordings were taken from regions throughout the area of central canal. There is no location specificity for the type of response observed. GABA was applied locally using a puff electrode or applied in the bath solution.

3.3.4.1 The GABAergic response in ependymal cells is mediated in part by $GABA_A$ receptors.

To determine which receptors are mediating the GABA response in ependymal cells, GABA was applied in the presence of the $GABA_A$ antagonists; bicuculline 50-100 μM (n=3). The depolarising GABA responses could not be antagonised by bicuculline (Figure 3-9 A). The amplitudes of control responses 3.02 ± 0.44 mV were slightly reduced to 2.59 ± 0.36 mV in the presence of bicuculline (n=3, n.s). The hyperpolarising responses to GABA using lower concentration (1.25 μM) responses could not be antagonised by bicuculline, amplitudes showed no significant change from -0.73 ± 0.30 to -0.80 ± 0.28 mV (n = 4, Figure 3-9 B).

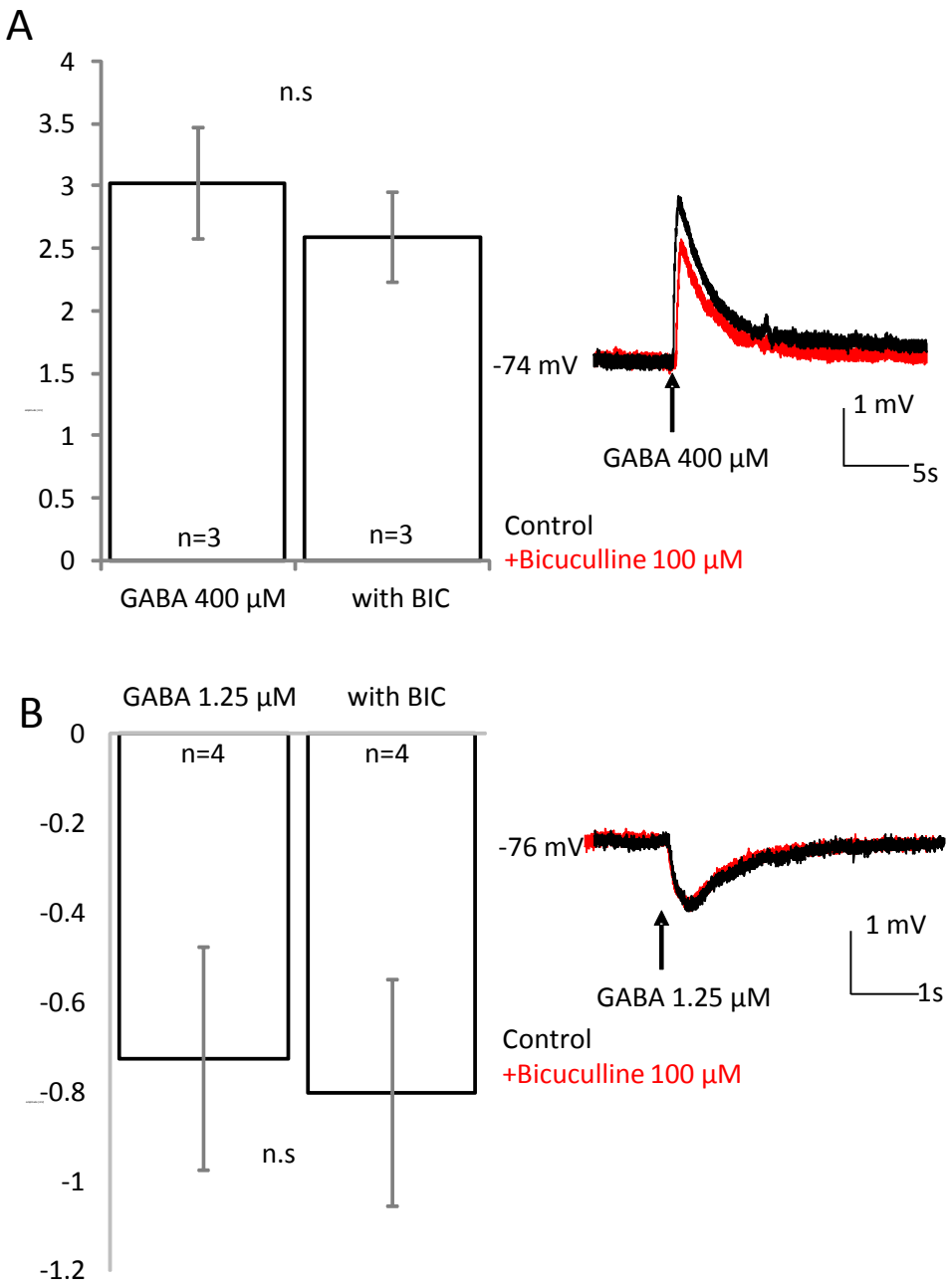


Figure 3-9 Antagonism with $GABA_A$ antagonists was dependant on GABA concentration.

Bicuculline (100 µM) slightly reduced the responses to high concentrations of GABA (400 µM). Examples of responses to high concentrations of GABA in the absence (black) and presence (red) of bicuculline are observed. Bicuculline (100 µM) could not antagonise the responses to low concentrations of GABA (1.25 µM). Examples of responses to low concentrations of GABA in the absence (black) and presence (red) of bicuculline are observed.

3.3.4.2 Effects of TPMPA on responses of ependymal cells to low concentrations of GABA

Since low concentrations of GABA preferentially activate GABA receptors containing ρ subunits (Schmidt et al 2001), the effects of the GABAρ receptor antagonist TPMPA on responses to low concentrations of GABA were tested. TPMPA at a concentration of 80 μM significantly reduced the amplitude of the responses to low GABA from -2.17 ± 0.83 mV to -1.21 ± 0.69 mV (*$p \leq 0.05$, n =5, Figure 3-10).

Immunohistochemistry using an antibody against GABAρ_1 on spinal cord sections revealed GABAρ_1 immunopositive staining on CD24 immunopositive cells (Figure 3-11 A) but levels of immunopositive structures were less numerous in the vicinity of GAD-GFP cells (Figure 3-11 B) which labelled the CSFcCs.

To further characterise whether there was a contribution of ρ subunits, TACA as a GABAρ agonist was used. Puffing TACA at 0.1-0.8 μM always produced hyperpolarising responses (Figure 3.12 A) and these responses reduced in amplitude as the cell was hyperpolarised. Responses to TACA were reduced by TPMPA 80 μM, but this reduction did not quite reach significance for the five ependymal cells tested. The amplitude before TPMPA was -2.73 ± 0.71 mV and after TPMPA was -1.70 ± 0.50 mV (*$p = 0.07$, n=5, Figure 3-12 B).

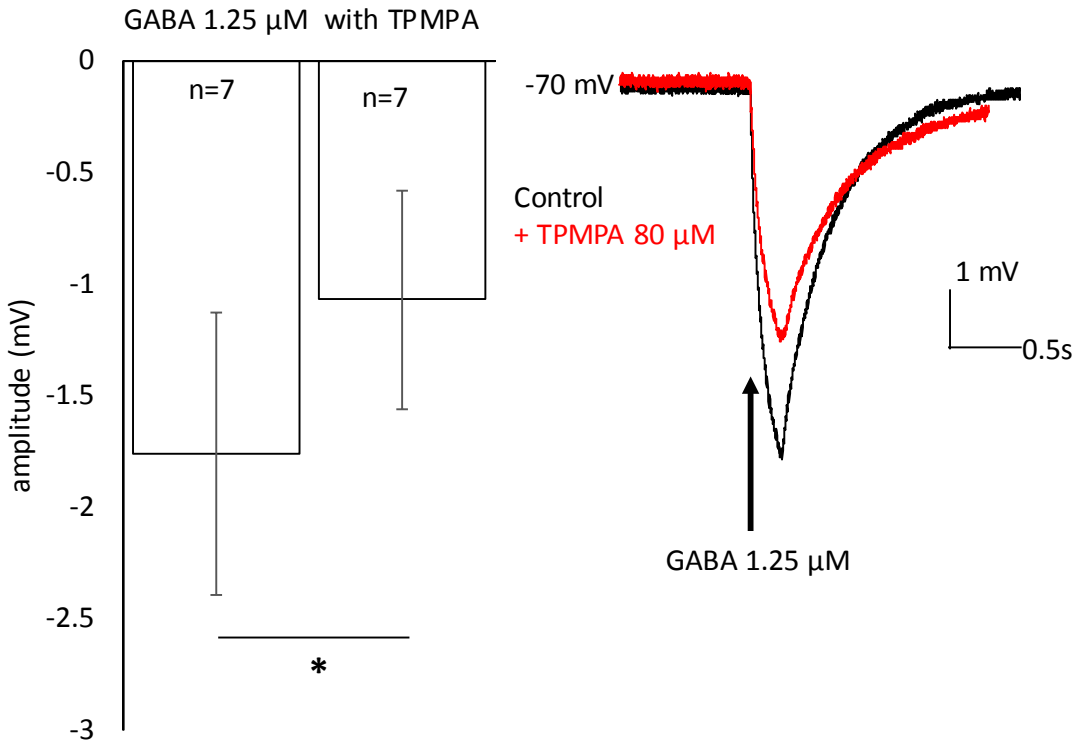


Figure 3-10 Ependymal cell responses to low GABA 1.25 µM

Responses to low GABA concentrations were antagonised by the rho antagonist TPMPA. TPMPA antagonised the responses of low concentrations of GABA. An example of the response of a single ependymal cell to GABA in the absence (black) and presence (red) of TPMPA (*$p \leq 0.05$, n=7).

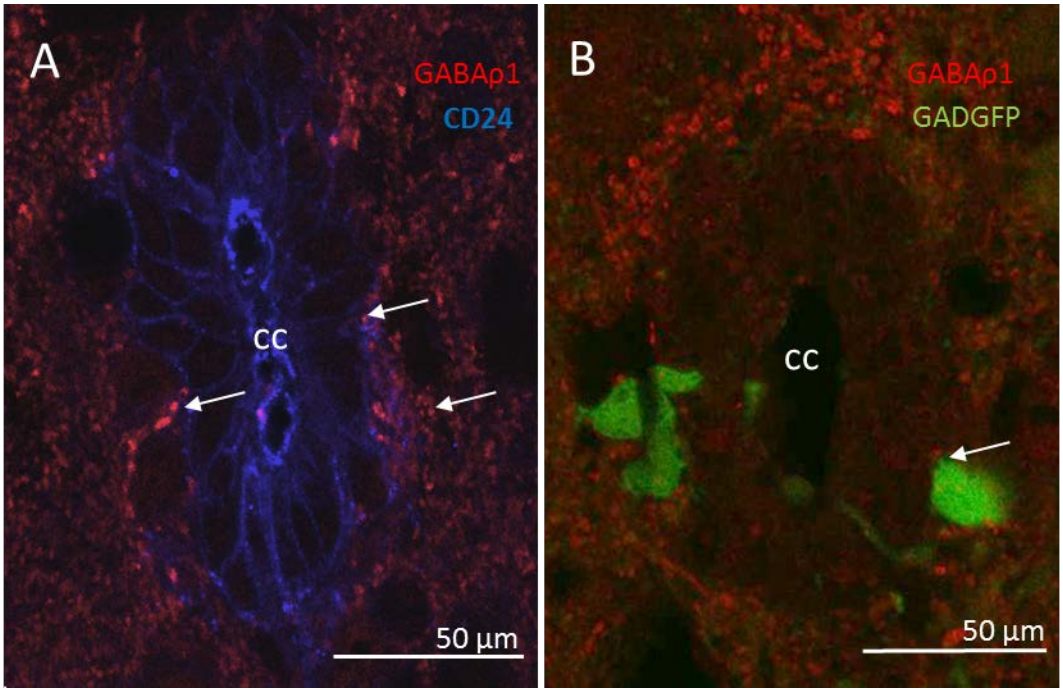
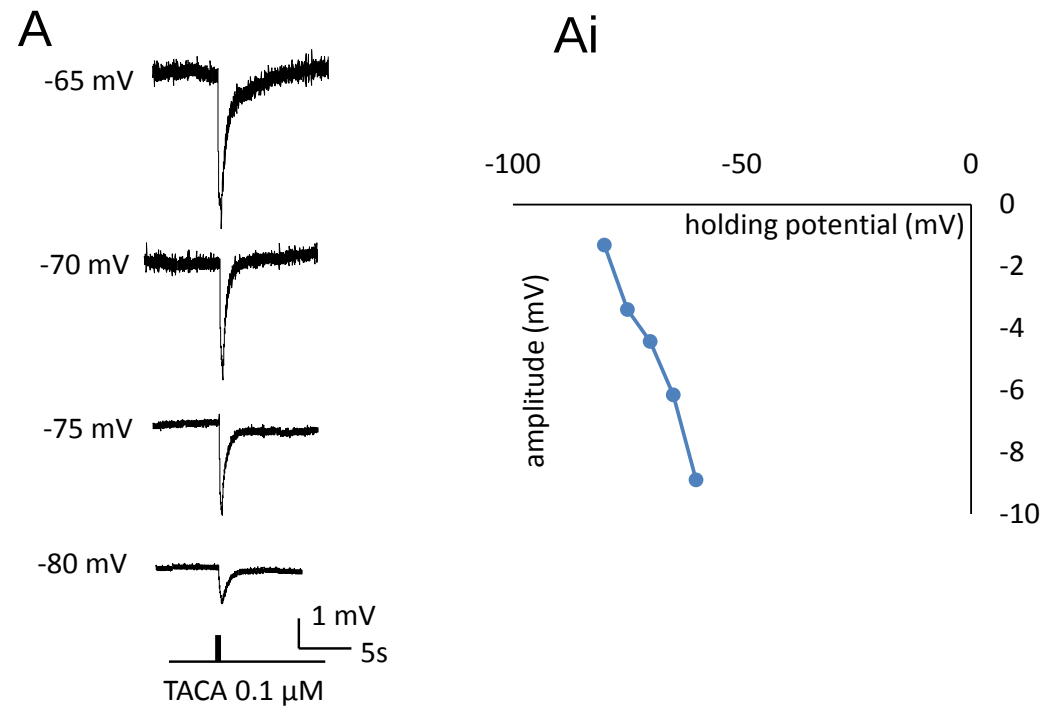


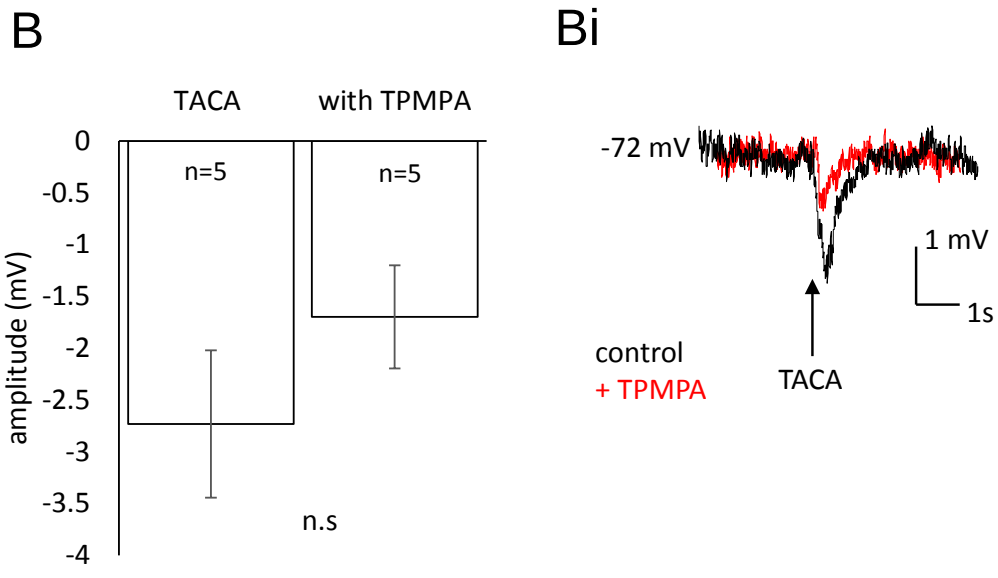
Figure 3-11 GABAρ_1 immunofluorescence was observed around the central canal area of spinal cord

Ependymal cells were immunopositive for CD24 (blue) (A) while GAD-GFP (green) tissue was used to label CSFcCs (B). Arrows point to GABAρ_1 immunopositive punctate staining observed in close apposition to cells, cc=central canal.

Figure 3-12 Ependymal cells were hyperpolarised by TACA

This ependymal cell responded to TACA with hyperpolarisations (A). Responses to TACA were measured at 4 different holding potentials (-80 mV, -75 mV, -70 mV and -65 mV). The amplitude of hyperpolarisations decreased as the membrane holding potential was made more negative (Ai). Responses to TACA were reduced slightly by the rho antagonist, TPMPA but this did not reach significance ($p = 0.07$, $n=5$) (B). An example of the response of a single ependymal cell to TACA in the absence (black) and presence (red) of TPMPA (Bi).


A
-65 mV
-70 mV
-75 mV
-80 mV
1 mV
5s
TACA 0.1 µM
Ai
-100
-50
0
holding potential (mV)
amplitude (mV)
0
-2
-4
-6
-8
-10


B
TACA
with TPMPA
n=5
n=5
amplitude (mV)
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
n.s
Bi
-72 mV
1 mV
1s
control
+ TPMPA
TACA

3.3.5 GABA actions may be modulated at different sites

The GABA receptor has a number of modulatory sites located on specific subunits that form the overall receptor (Sigel et al., 2011). The presence of these sites enables selective and fine control of responses of cells to GABA. We focussed on one such site, the benzodiazepine site since it can be modulated by the endogenous peptide, diazepam binding inhibitor. DBI is broken down into two peptides, ODN that selectively activates the central benzodiazepine site in the GABA receptor, CBR and TTN which acts at another site that is located on the mitochondrial surface (Costa and Guidotti, 1991). DBI is strongly expressed in brain neurogenic niches (Alfonso et al., 2012). The function of DBI in neurogenesis of brain neurogenic niche raised the question whether the protein can have an effect on the ependymal cells in spinal cord neurogenic niche.

Firstly, immunofluorescence labelling was carried out in tissue taken from nestin-GFP mice using antibodies specific for DBI and TSPO. In both cases, there was strong DBI and TSPO immunofluorescence located around the central canal area of spinal cord (Figure 3-13 A), extending into the fibres of these cells in particular for the case of the DBI-immunofluorescence.

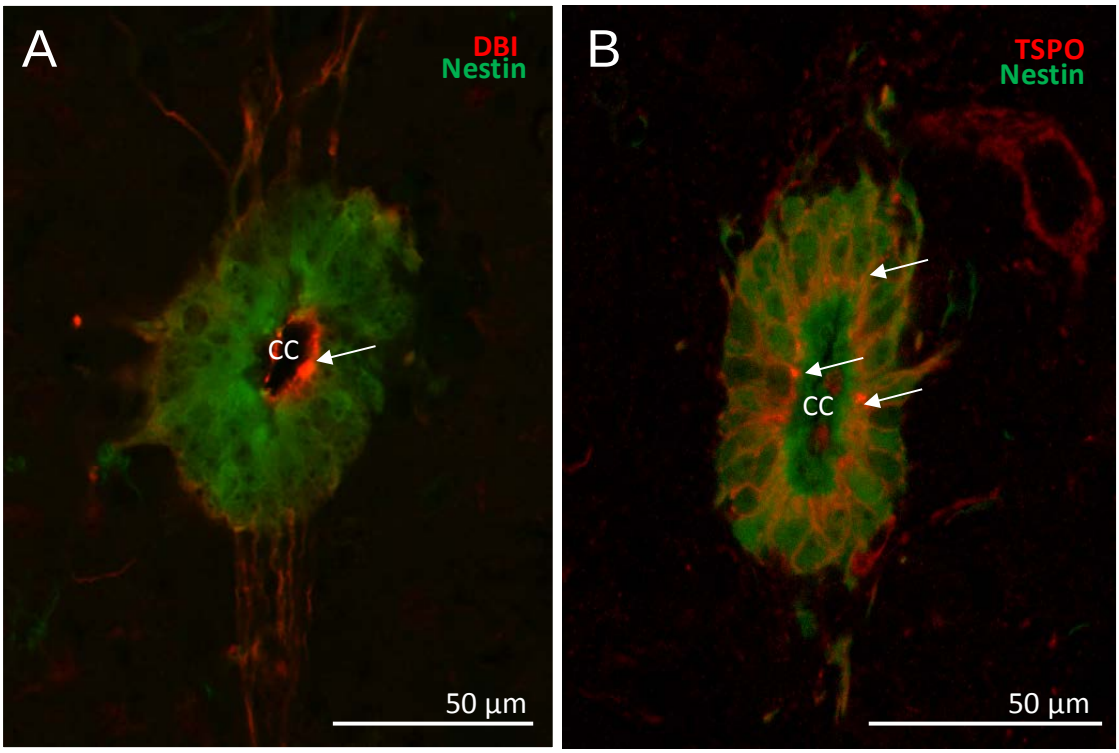


Figure 3-13 DBI immunopositive structures around nestin-GFP ependymal cells in central canal area

DBI immunopositive structures (A) in the central canal area of spinal cord and TSPO immunostaining (B) in the central canal area of spinal cord. White arrows indicate DBI and TSPO structures, cc=central canal.

3.3.5.1 Is there a role for both central benzodiazepine receptor and translocator protein in modulating responses to GABA?

To establish whether activation of these sites can affect the responses of ependymal cells to high concentrations of GABA, I first applied midazolam, which acts as non-selective modulator at both CBR and TSPO. Application of midazolam (0.1 μM) significantly reduced the amplitude of responses to 400 μM GABA from 12.06 ± 2.26 mV to 9.50 ± 2.25 mV (*$p \leq 0.05$, n = 14, Figure 3-14 A).

Since midazolam has effects at both CBR and TSPO, I next tested the effects of activating TSPO alone since DBI peptides can modulate GABA through an alternative pathway distinct from binding to the DBI site on $GABA_A$ receptor. FGIN-1-27 is an anxiolytic drug which acts as a selective agonist at TSPO. FGIN-1-27 (1 μM) was able to increase the responses to GABA from baseline 5.53 ± 1.43 mV to 6.29 ± 1.57 mV (*$p \leq 0.05$, n = 12, Figure 3-14 B). The effects of FGIN-1-27 on GABA responses showed a latent plateau only in this single cell.

PK-11195 is a selective antagonist for TSPO (Gandolfo et al., 1997). It is the most commonly used TSPO ligand due to its high affinity to TSPO determined in glial cells of ependymal walls, choroid plexus and olfactory bulb. Combining the application of FGIN-1-27 and PK-11195 before puffing GABA resulted in a blockade of the effects of FGIN-1-27 alone, such that GABA responses were no longer enhanced in ependymal cells (Figure 3-15, n=6). This indicates that the effects of FGIN-1-27 are mediated through the TSPO site.

FGIN-1-27 is thought to have effects at TSPO by stimulating steroidogenesis of neuroactive steroids such as allopregnanolone, also known as 5-alpha-pregnane-3alpha-ol-20-one. Allopregnanolone binds to an allosteric site on $GABA_A$ receptor distinct from the DBI site and potentiates GABA function. Allopregnanolone applied

at two different concentrations (1 μM, n=5 and 10 nM, n=4) slightly increased responses of ependymal cells to GABA (from 3.21 ± 1.23 mV to 5.9 ± 1.18 mV and from 1.62 ± 0.58 mV to 3.17 ± 1.14 mV respectively) but these did not reach significance (*p*= 0.10, Figure 3-16 A and *p*=0.09, Figure 3-16 B).

The effects of these modulators were also tested on responses of ependymal cells to low concentrations (1.25 μM) of GABA. For FGIN-1-27 and allopregnanolone, neither modulator caused significant changes to the amplitude of the hyperpolarisations elicited by low GABA (Table 3.1). One further cell was tested with midazolam and this modulator also did not change the amplitude of the response to low GABA but this could not be tested for significance due to low numbers.

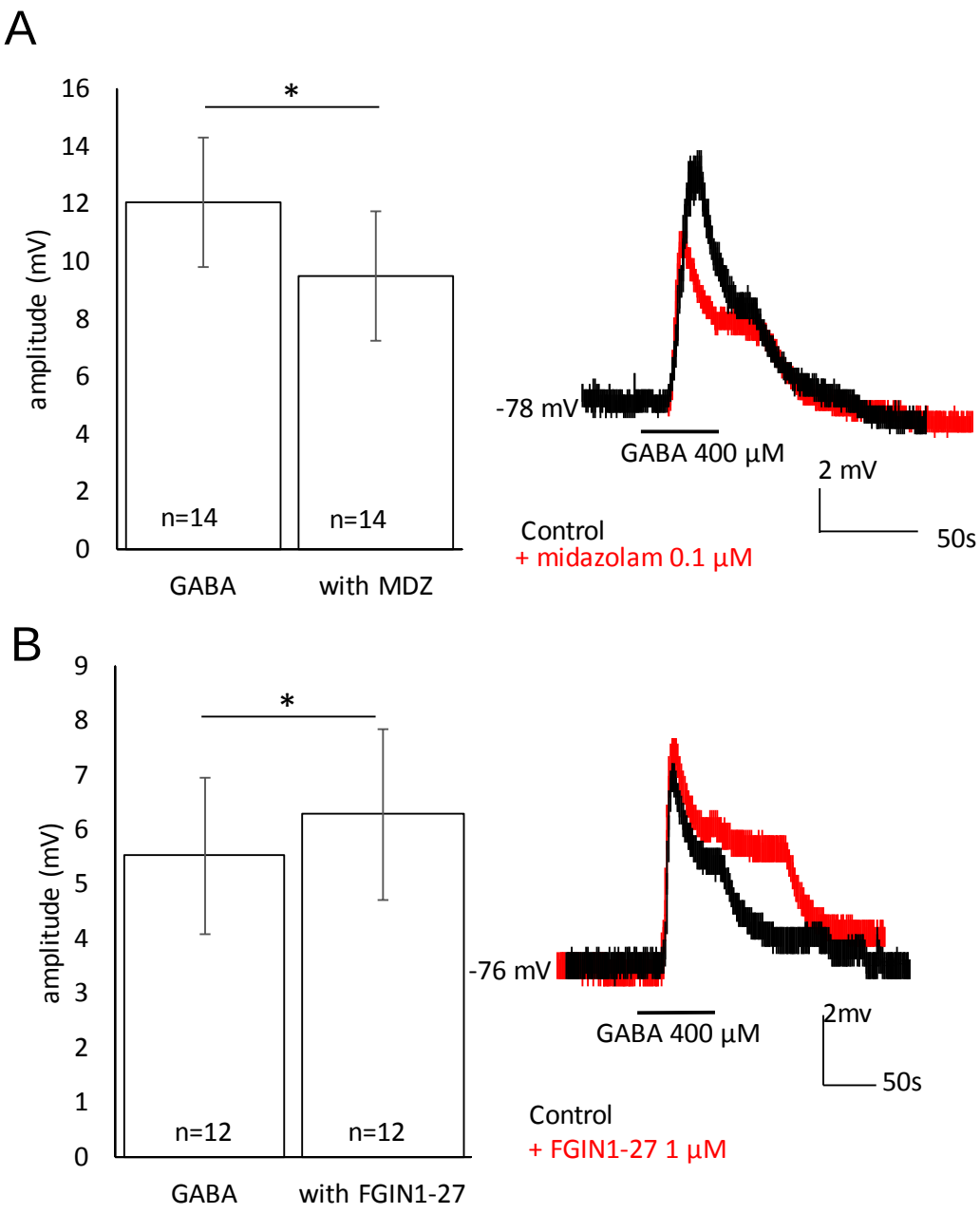


Figure 3-14 Receptor modulators have differing effects on the responses of ependymal Cells to high [GABA]

Midazolam (0.1 µM) decreases responses of ependymal cells to GABA (400 µM). An example of the response of a single ependymal cell to GABA in the absence (black) and presence (red) of midazolam (*$p \leq 0.05$, n=14) (A). Effects of FGIN1-27 (1 µM) on high concentration of GABA (400 µM). An example of the response of a single ependymal cell to GABA in the absence (black) and presence (red) of FGIN-1-27 (*$p \leq 0.05$, n=12) (B).

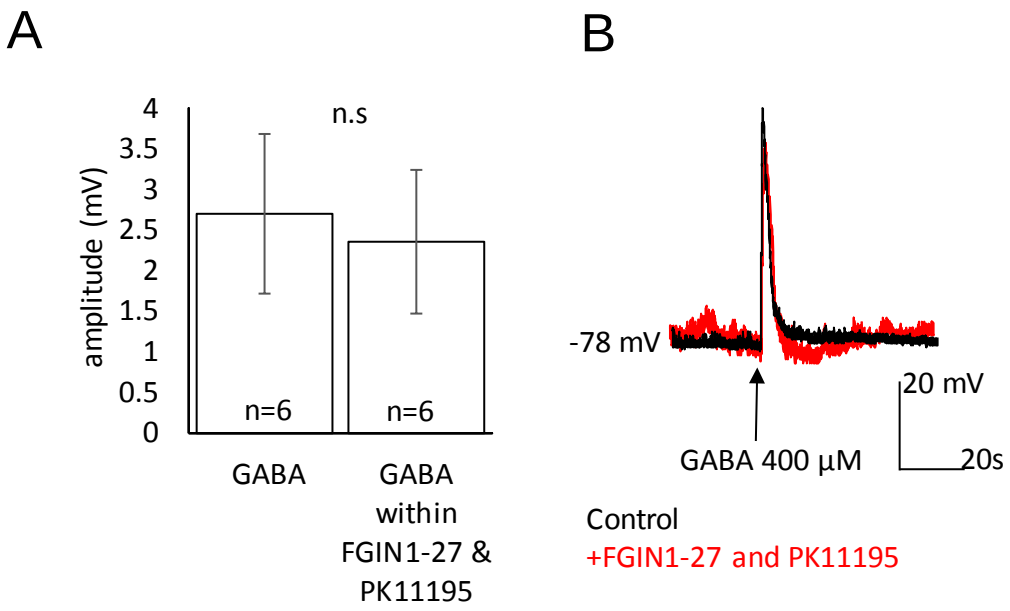


Figure 3-15 Effects of combination of FGIN-1-27 and PK-11195 on the response to high concentration of GABA (400 µM).

Pooled data showing the effects of a combination of FGIN-1-27 and PK-11195 on the response to high concentration of GABA (400 µM) (n=6) (A) and an example of the response of a single ependymal cell to GABA in the absence (black) and presence (red) of FGIN-1-27 and PK-11195 (B).

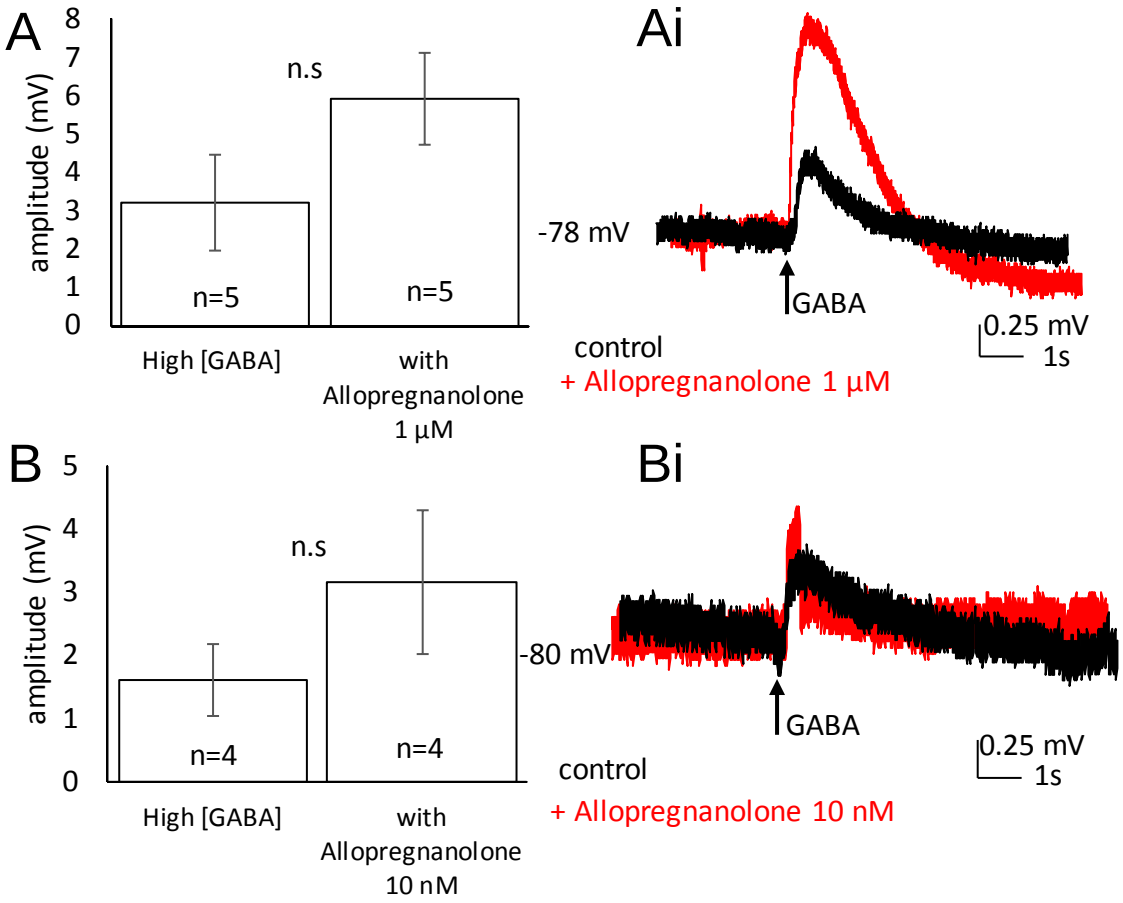


Figure 3-16 Allopregnanolone does not significantly change responses to high GABA concentration

Allopregnanolone at both concentrations 1 µM (A) and 10 nM (B) does not significantly increase GABA responses on ependymal cell. Example of the response of a single ependymal cell to GABA in the absence (black) and presence (red) of allopregnanolone in 1 µM (n=5) (Ai) and 10 nM (n=4) (Bi).

Table 3-1 Modulators have no effects on the responses of ependymal cells to low [GABA]

Modulator	Concentration	Baseline depolarisation/ hyperpolarisation amplitude (mV)	With modulator depolarisation/hyperpolarisation amplitude (mV)	n	N	P value
FGIN-1-27	1 μM	-0.63 ± 0.22	-0.57 ± 0.20	5	4	0.29
Bicuculline	100 μM	-0.97 ± 0.25	-1.02 ± 0.25	4	4	0.16
Allopregnanolone	1 μM	-0.57 ± 0.13	-0.43 ± 0.08	6	5	0.38
Allopregnanolone	10 nM	-0.68 ± 0.25	-0.61 ± 0.23	3	3	0.10
ODN	20 μM	-0.87 ± 0.15	-0.74 ± 0.16	6	5	0.42
Cyclo1-8[dleu5]OP	50-300 nM	-0.59 ± 0.06	-0.63 ± 0.06	4	5	0.54
Picrotoxin	1 μM	-0.53 ± 0.15	-0.51 ± 0.14	3	2	0.65

Modulators such as FGIN-1-27, ODN, allopregnanolone and picrotoxin had no effect on the responses to low [GABA], suggesting that the rho containing receptors are not modulated in the same way as the $GABA_A$ receptors. n values denote numbers of neurones, while N denotes numbers of mice.

3.3.6 Can octadecaneuropeptide modulate responses of ependymal cells to GABA?

Similar to DBI, ODN binds with high affinity to the CBR on the $GABA_A$ receptor; however, it binds only with low affinity to the TSPO.

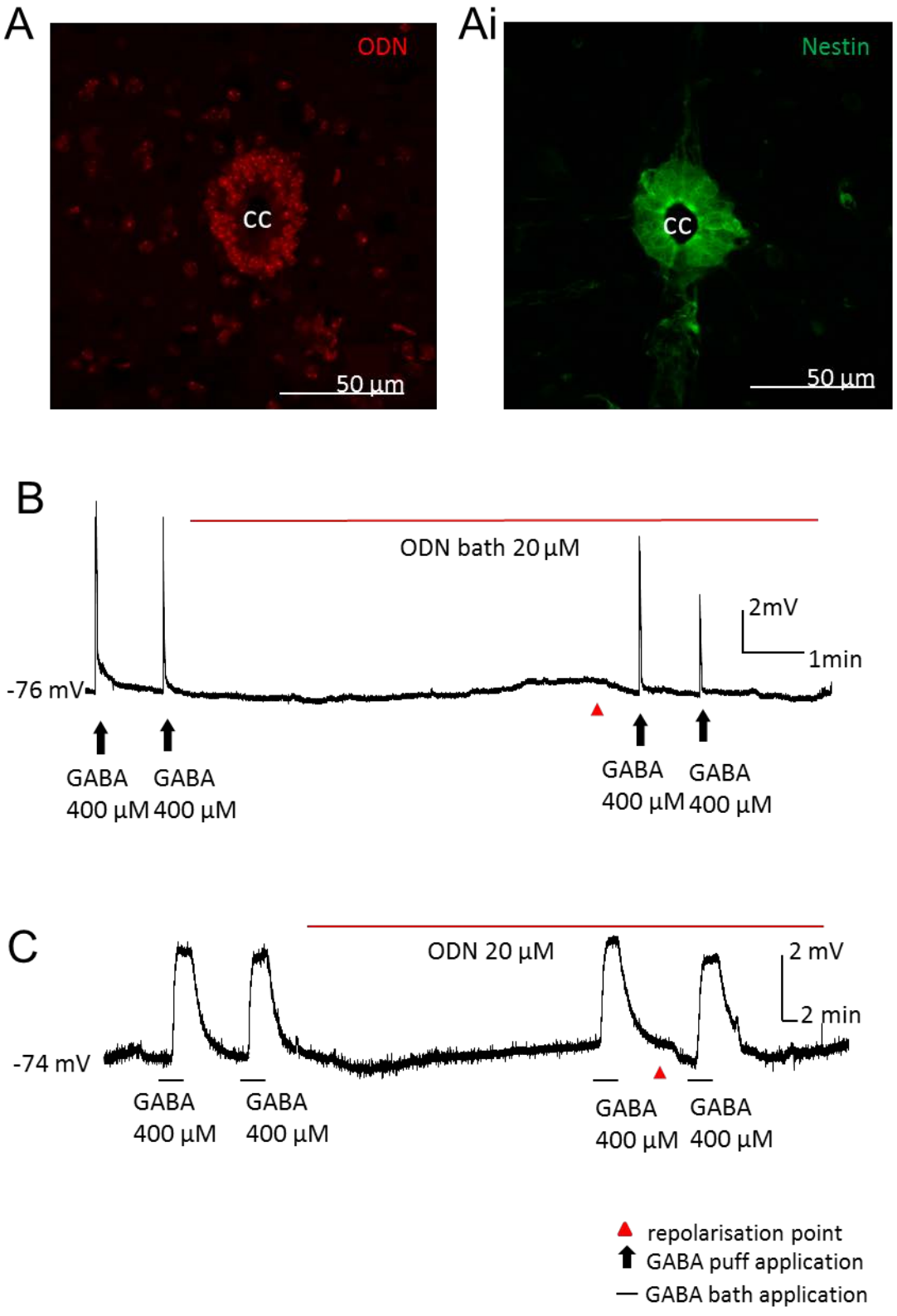
Firstly, the presence of ODN in the vicinity of ependymal cells was deduced using specific antibodies to ODN in spinal cord tissue taken from nestin-GFP mice. There was intense and localised ODN-immunofluorescence in the vicinity of nestin-GFP positive ependymal cells in the central canal region (Figure 3-17A), suggesting that ODN may be able to influence the activity of these ependymal cells.

Indeed, when bath applied alone, ODN, the endogenous ligand of the central benzodiazepine receptor caused both depolarising (Figure 3-17 B) and hyperpolarising effects on ependymal cells (Figure 3-17 C). Regardless of whether responses to ODN were either depolarising or hyperpolarising, there were no significant differences in responses to GABA in the presence or absence of ODN.

For each cell, the membrane potential was repolarised to test the effects of GABA. However, ODN did not significantly modulate the effects of either bath or puff applied GABA, regardless of the initial response to ODN, the direction of response to GABA and the concentration of GABA used (Figure 3-18 A and B).

Figure 3-17 ODN expression and effects on ependymal cells in central canal area of spinal cord

ODN immunopositive staining using ODN antibodies showed ODN is widely expressed around the central canal area of spinal cord of nestin-GFP mouse (A, Ai). ODN at a concentration of 20 μM (bath application) caused both depolarising (B) and hyperpolarising (C) effects on ependymal cells. The responses to GABA on (B) are using puff application and responses to GABA on (C) are using bath application. Red arrow head showed the point at which the membrane potential was repolarised to retest GABA at the same membrane potential as prior to the ODN.


A
ODN
cc
50 µm
Ai
Nestin
cc
50 µm
B
ODN bath 20 µM
2mV
1min
-76 mV
GABA
400 µM
GABA
400 µM
GABA
400 µM
GABA
400 µM
C
ODN 20 µM
2 mV
2 min
-74 mV
GABA
400 µM
GABA
400 µM
GABA
400 µM
GABA
400 µM
repolarisation point
GABA puff application
GABA bath application

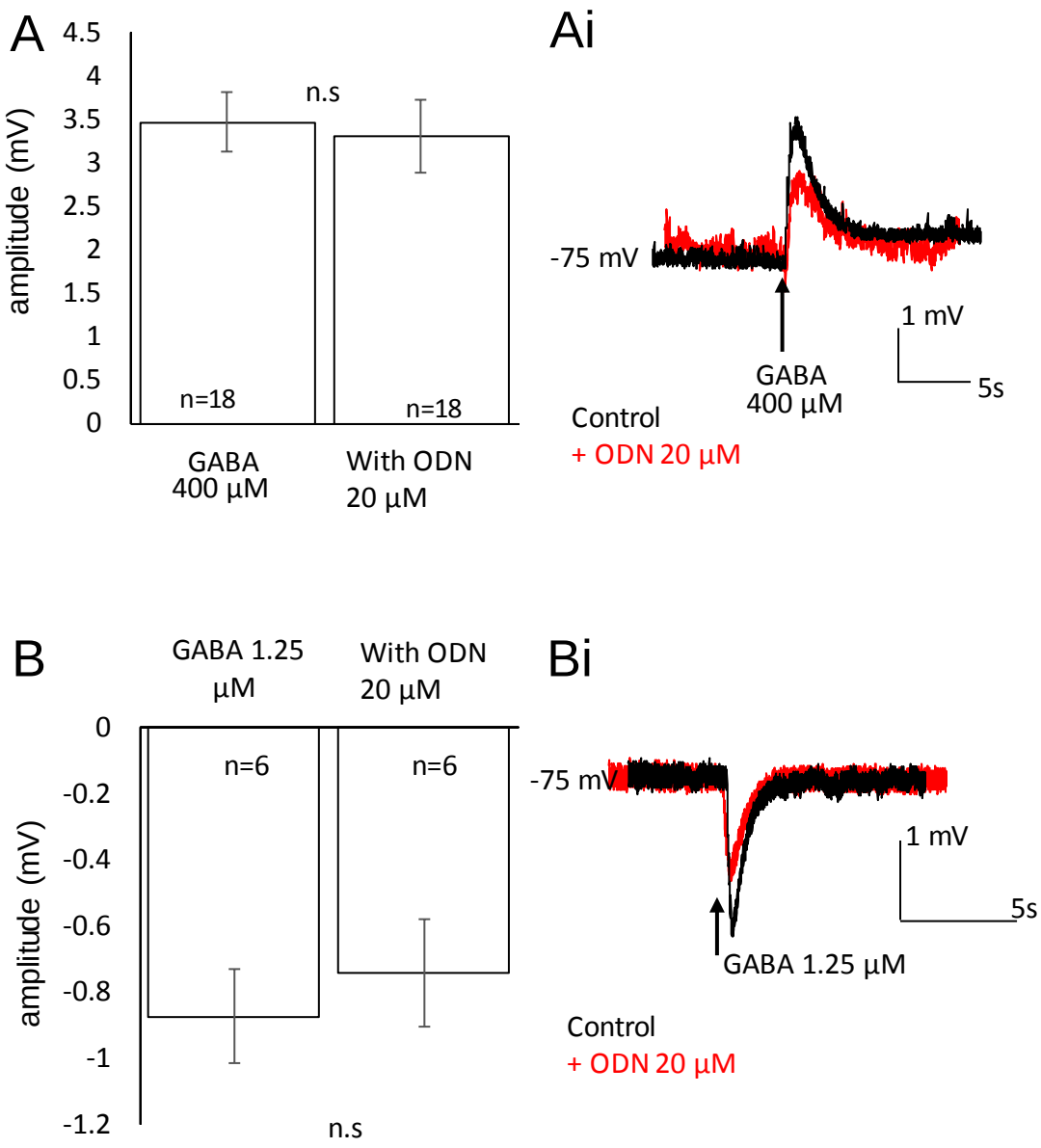


Figure 3-18 ODN did not significantly change responses of ependymal cells to different concentrations of GABA

ODN at a concentration of 20 µM did not significantly changed the GABA effects on ependymal cells; in higher concentration (A) and low concentration (B) of GABA. Example of the response of a single ependymal cell to GABA at high concentration (400 µM) and low concentration (1.25 µM) in the absence (black) and presence (red) of ODN 20 µM. Pooled data and an example of the response of a single ependymal cell to GABA in high concentration (400 µM, n=18, A) and low concentration (1.25 µM, n=6, B) in the absence (black) and presence (red) of ODN 20 µM. Red arrow head indicate point of repolarisation.

3.3.7 Octadecaneuropeptide alone hyperpolarised ependymal cells

Since ODN had effects on the membrane potential of ependymal cells when bath applied alone, I tested responses of ependymal cells to puff applications of low concentrations of ODN (15-20 nM). ODN caused robust and repeatable hyperpolarising responses of 1.26 ± 0.25 mV (n = 45) in ependymal cells (Figure 3-19 B). These hyperpolarising responses were observed in ependymal cells at all locations of the central canal (Figure 3-19 A).

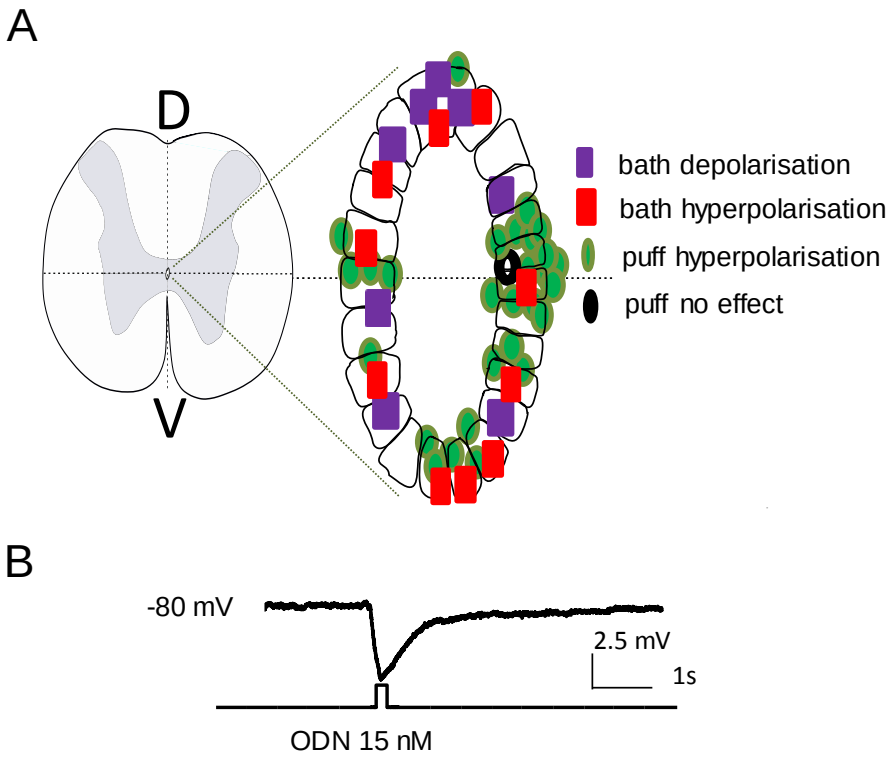


Figure 3-19 Ependymal cells responses to ODN.

Location of ependymal cells exhibiting the different responses to ODN. Whole cell patch clamp recordings were taken from throughout the area of central canal. There is no location specificity for the type of response observed. ODN was applied locally using a puff electrode and also in the bath solution (A). ODN applied alone elicited hyperpolarising responses in ependymal cells. Clear effects of puffing ODN on ependymal cells (B).

3.3.8 Effects of TPMPA on responses of ependymal cells to octadecaneuropeptide

Since TPMPA blocked similar hyperpolarising responses to low concentrations of GABA, I applied this antagonist and observed significant reductions in the amplitude of responses to ODN, from -1.31 ± 0.26 mV to -0.91 ± 0.2 mV. (*$p \leq 0.05$, n=8, Figure 3-20 A). However, there was no significant change in responses to ODN in the presence of the $GABA_A$ antagonist bicuculline (-1.95 ± 0.95 mV to -1.81 ± 0.84 mV, n=6). There were also no significant changes of responses to ODN with other antagonists, modulators or channel blockers as shown in Table 3.2.

Interestingly however, the responses to ODN were antagonised by selective antagonist of metabotropic endozepine receptors, using cyclo (1-8) [dLeu (5)] OP. The responses were reduced from 0.59 ± 0.05 mV to 0.47 ± 0.05 mV (*$p \leq 0.05$, n=9, Figure 3-20 B).

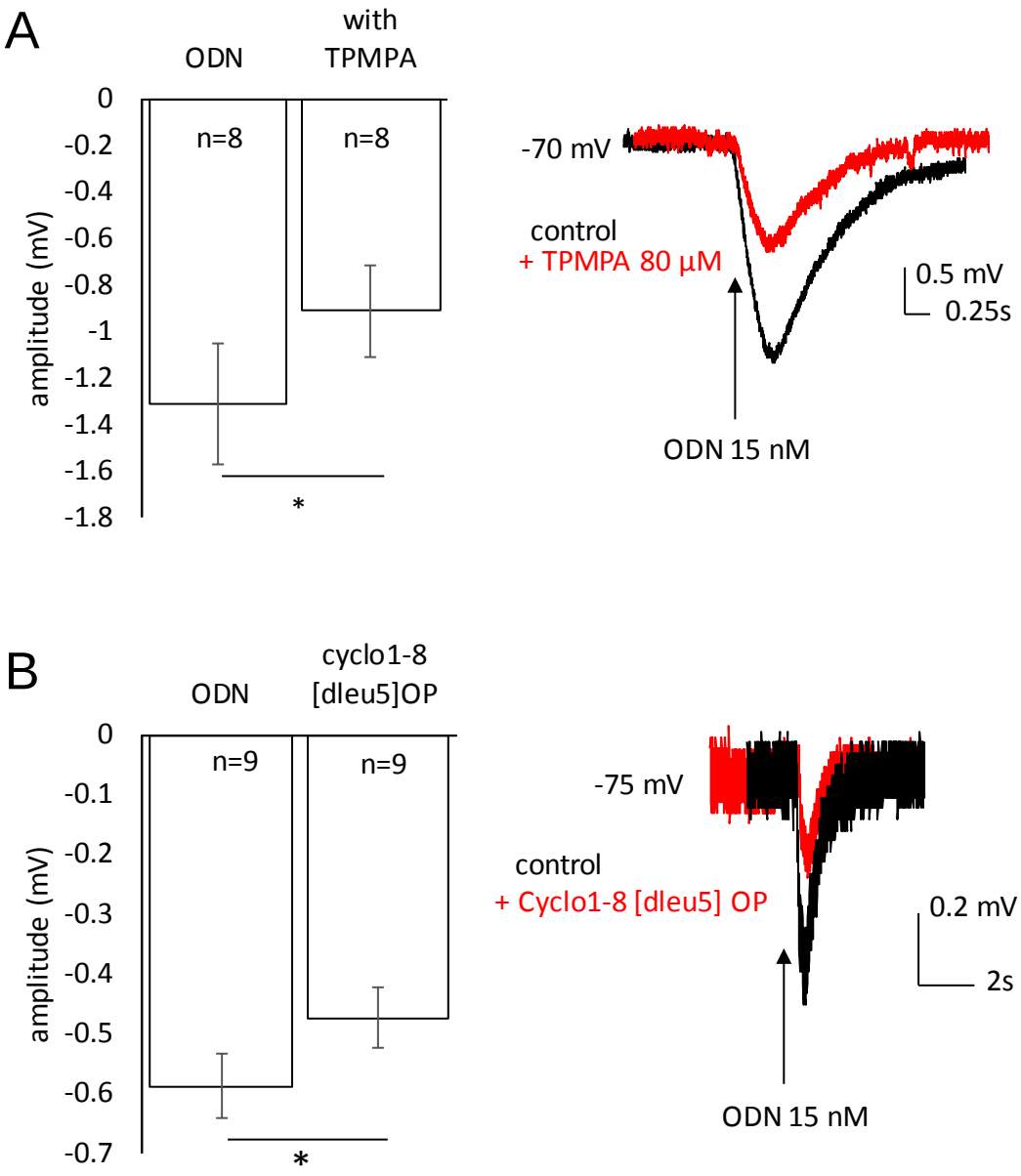


Figure 3-20 ODN effects on ependymal cells.

Antagonism of ODN (15 nM) responses on ependymal cells. ODN effects are antagonised by both TPMPA (A) and cyclo1-8[dleu5] OP (B) (**p*≤0.05). On the right are examples of the response of a single ependymal cell to ODN (1.25 µM) in the absence (black) and presence (red) of TPMPA (**p*≤0.05) and cyclo1-8[dleu5] OP.

Table 3-2 ODN responses were not significantly reduced by other antagonists, modulators or channel blockers

Antagonists	Concentration	Baseline hyperpolarisation amplitude (mV)	Antagonists hyperpolarisation amplitude (mV)	n	N	P value
Nipecotic acid	300 μM	-0.49 ± 0.11	-0.45 ± 0.12	5	4	0.070
Niflumic acid	250 μM	-0.47 ± 0.18	-0.81 ± 0.02	2	2	0.135
Bicuculline	100 μM	-1.95 ± 0.95	-1.81 ± 0.84	6	5	0.292
FGIN-1-27	1 μM	-0.58 ± 0.02	-0.71 ± 0.11	2	2	0.500
Flumazenil	20 μM	-2.41 ± 1.33	-2.22 ± 1.42	5	5	0.444
PK-11195	10 μM	-3.32 ± 1.76	-3.56 ± 1.75	3	3	0.398
Midazolam	0.1 μM	-0.58 ± 0.07	-0.63 ± 0.09	5	4	0.415

Modulators had no effect on the responses to ODN, suggesting that ODN is not modulated in the same way as GABA. n values denote numbers of neurones, while N denotes numbers of mice.

3.4 Discussion

This chapter provides a characterisation of the pharmacological and electrophysiological characterisation of $GABA_A$ receptors in central canal area of spinal cord.

Electrophysiological characteristics of ependymal cells surrounding the central canal displayed typical findings of glial cells: with negative resting membrane potentials of -74 mV, and no spontaneous or evoked activity, indicating a lack of voltage-gated channels. These properties differ from CSFcCs, which had more depolarised membrane potentials (-56.9 ± 4.4 mV), larger input resistance, varied phenotype of electrical activity and some also had passive responses. CsFcCs with active response properties have been previously reported under the name of CSF-contacting neurones (CSFcNs) (Marichal et al., 2009) and as CSFcC (Corns et al., 2015; 2013).

The visualisation of neurobiotin following intracellular loading revealed not only that ependymal cells were coupled but that different degrees of coupling were present whereas in CSFcCs, only subtype 1 showed coupling indicative of the presence of some degree of gap junction. The coupling in ependymal cells can be reduced in the presence of gap junction blocker 18β-glycyrrhetinic acid as evidenced by the large increases in input resistance. Dye coupling with neurobiotin showed gap junctions present between these cells. Since these characteristics were similar to those observed previously in rats (Corns et al 2015), we are confident that the cells are the ependymal cells of the neurogenic niche.

My study provides data on ependymal cells within the postnatal mammalian spinal cord which respond to GABA with 2 different characteristics. The higher concentration of GABA will produce mostly depolarising activity while the lower concentration will produce mostly hyperpolarising activity. There is no location specificity for the type of

response observed and the type of GABA application (puff or bath) did not contribute to the response.

Our study further shows a complex role of GABA receptors in modulating the activity of ependymal cells since it is likely that $GABA_A$ receptors are present on ependymal cells and further modulation of these receptors through CBR and TSPO elicits a variety of effects. On further characterising the GABA receptors, it was observed that the GABAρ antagonist TPMPA reduced responses to low concentrations of GABA while TACA, a GABAρ agonist mimicked the responses to low GABA. This indicates the presence of atypical GABA receptors and/or mixed pharmacological properties of $GABA_A$ and GABAρ receptors. ODN effects on ependymal cells that may be mediated through sites other than at the CBR site suggest yet another mechanism by which the activity of ependymal cells may be modulated, through activation of a metabotropic ODN receptor.

3.4.1 Basic electrophysiological characteristics are consistent with ependymal cells as previously observed

The resting membrane potential of ependymal cells determined in this study in rodents, -74 mV is relatively similar to those determined previously , -84 mV for ependymal cells in rat spinal cord (Marichal et al., 2012), -80 mV for ependymal cells in the rat third ventricle (Jarvis and Andrew, 1988) and -76 mV (Corns et al., 2013). In other species, ependymal cells in turtle spinal cord had a resting membrane potential of -87 mV (Reali et al., 2011). The variations of membrane potential among studies may be due to variations in extracellular K^+ concentration within aCSF or due to intracellular K^+ concentration within intracellular patch pipette solution, differences in the region of the ventricle or central canal studied and differences of age of animals used.

The input resistance of ependymal cell determined in this study was 72.6 ± 3.9 MΩ. The input resistance is lower compared to that previously determined by Marichal et al. (2012); 124 MΩ, 167 MΩ in the turtle spinal cord (Reali et al., 2011) and 96 ± 13 MΩ in ependymal cells of spinal cord (Corns et al., 2013). The lower input resistance suggests that the cells recorded in this study could be highly coupled since this would result in increased “leakiness” of the cells (Davidson and Baumgarten, 1988). The study from Marichal et al. (2012) recorded from cervical spinal cord, whereas in this study, most cells were recorded from thoracolumbar spinal slices. The other factor which can contribute to the differences are the age of animals, Marichal et al.(2012) used a younger animal i.e. P0-P5, while in this study, recorded ependymal cells were from taken from mice aged P8-P15. The expression of ion channels has been shown to change during postnatal development (Chvátal et al., 1995) and expression of connexins can vary during CNS development. Connexin 43 mRNA expression and protein expression increased from P0 to adulthood (Leung et al., 2002). If the same pattern of expression occurs in the ependymal cell, the more that cells are coupled towards P21, the greater the likelihood of lower input resistances, supporting the lower input resistance in this study compared to previous study.

The lack of spontaneous activity and the linear voltage-current relationship showed similar findings with previous studies (Corns et al., 2013; Marichal et al., 2012; Reali et al., 2011). Ependymal cells showed lack of spontaneous activity because they do not express voltage-gated ion channels. Marichal et al. (2012) did not observe any outward current in their leak subtraction protocol in ependymal cells. This suggests that ependymal cells of postnatal mammalian central canal do not express voltage-gated channels. For CSFcCs, the input resistance was 703.6 ± 86 MΩ, relatively low compared to the observation of Subtype 2 by Marichal et al. (2009) : 3950 ± 290 MΩ and also observation by Corns et al. (2015) : 2668 ± 130 MΩ; The difference may be due to different age and type of animals used.

Dye coupling occurs between ependymal cells. The different degree and variation of coupling has been shown to be similar to other studies (Corns et al., 2013; Russo et al., 2008). A degree of variation of coupling has been found in ependymal cell progenitor population in turtle where they referred to different sized clusters of cells; from quadrants of dye-coupled cells to small clusters containing a few cells only (Russo et al., 2008). The degree of coupling and the increase in input resistance observed in the presence of gap junction blocker, 18β-glycyrrhetinic acid is consistent with dye-coupling and the gap junctions present between ependymal cells. Connexin 43 (Marichal et al., 2012) and connexin 45 (Chapman et al., 2013) were identified in previous studies of ependymal cells in rat and the connexin 43 subunit was revealed with immunohistochemistry in the ependymal cells of turtle (Russo et al., 2008). The correlation between the change in input resistance and the change in membrane potential in response to 18β-glycyrrhetinic acid indicates that the depolarisation is a direct effect of gap junction blockade.

The ependymal region consists of cells arranged in lateral and dorsal-ventral domains (Marichal et al., 2012; Trujillo-Cenóz et al., 2007). The majority of cells have two motile cilia but some have 1, 3 or 4 cilia (Alfaro-Cervello et al., 2012). At least three different morphologically different cell types were described by these researchers in the central canal area: 1. ciliated ependymal cells with short basal processes, 2. ciliated ependymal cells with long processes reaching the pial surfaces (Radial glial like cells and 3. Typical CSFcC which is a single cell with a single long processes (Marichal et al., 2012; Meletis et al., 2008; Trujillo-Cenóz et al., 2007). Most researchers seems to agree that on the lateral regions of the central canal, cells are either cuboidal ependymal cells or tanycytes and these can have short or long radial processes. However, many researchers fail to agree on how cells should be grouped or what characteristics are important to focus on when identifying these cells. Most researchers refer to the majority of lateral cells as cuboidal ependymal cells (Marichal

et al., 2012; Alfaro-Cervello et al., 2012). However, according to Meletis et al. (2008), tanycytes and cuboidal types are the most abundant cell types among the ependymal cells and they are usually found at the lateral up to each pole while radial cells are usually situated at the dorsal and ventral poles. Only Meletis reports that tanycytes can be differentiated from cuboidal cells using electron microscopy because they have darker cytoplasm and can have two cilia or multiciliated (Meletis et al., 2008). We did not split the lateral cells into tanycytes or cuboidal ependymal cells in this study due to the problems in definitive identification. In our study, the first criterion to choose cells was based on the shape of faint but distinct outline of the cells – these were cuboidal in shape and were tightly bound together at the luminal surfaces. Our observation using DAB immunostaining with rhodamine showed ependymal cells had a multiple radial processes extending laterally from a single cell compared to CSFcCs which had a single long process arising from the main cell body which extended into the central canal. This suggests that like Alfaro-Cervello. (2012), we see longer processes in the lateral cells that may make them tanycytes. Factors to differentiate the ependymal cell from the subtype 1 CSFcC were the shape of the voltage responses to current pulses and visualisation of processes post-recording using either rhodamine or neurobiotin or using a combination of rhodamine/neurobiotin.

Our acute slices were prepared from mice similar to Meletis et al. (2008). Tanycytes were only reported by Meletis et al. (2008), while other types of ependymal cell were described in rat (Marichal et al., 2012) and turtle (Trujillo-Cenóz et al., 2007).

In one of a study of ependymal cells in SVZ, ependymal cell possess a fractone bulbs that appeared at the apical membrane of ependymal cells at the end of first week after birth (Nascimento et al., 2018). If we consider that the ependymal cells of the spinal cord also have the same function in neurogenesis as the ependymal cell in SVZ area, their structures would be similar. In future plans, a study using electron microscopy could be done on neurobiotin filled cells recovered from patched acute

slices to observe the morphological characteristics of individual ependymal cells from the spinal cord. Future studies to consider the complex morphology of the different cell types may also be aided by using fate-labelling of cells (see general discussion).

Since these characteristics were similar to those observed previously (Corns et al., 2015; Marichal et al., 2012; Marichal et al., 2009), we are confident that the cells are the ependymal cells and CSFcCs of the neurogenic niche. In our study, tanycytes, cuboidal and radial ependymal cells were included in the observations as ependymal cells but the locations of the cells were taken into account.

3.4.2 Ependymal cell responses to GABA

Ependymal cells showed responses to GABA as in previous study (Corns et al., 2013). The effects of GABA on ependymal cells were dependant on concentration. With bath application of high concentration of GABA, the effects were mainly depolarisations. From my observation, only 1 cell showed hyperpolarisation using the higher concentration of GABA (400 μM). The cell was not included in the analysis of modulation of GABA since it alone exhibited hyperpolarisation. A few possibilities could explain the depolarisations seen in ependymal cells. Firstly this may arise because of intense activation of GABA receptors (Milligan et al., 2004; Staley and Proctor, 1999) which can lead to $HCO3^-$ efflux and Cl^- influx. The imbalance can shift the excitatory value. The opposing $HCO3^-$ and Cl^- fluxes through the $GABA_A$ ionophore diminishes the electrochemical gradient driving the hyperpolarising Cl^- flux so that the depolarising $HCO3^-$ flux dominates.

The depolarisations could also be due to contribution of GABA uptake transporters which transport GABA back into the cells with two Na^+ ions and one Cl^- ion, which caused a net positive charge into the cell. A study of GAT1, a family member of GABA transporter (Fattorini et al., 2017), revealed that GABA is transported back into the

cells with two Na^+ ions and one Cl^- ion. Thus, application of GABA can induce depolarisation in cells expressing GABA transporter. GAT3 has been observed to contribute to the mediation of GABA responses in progenitor cells surrounding the turtle central canal (Reali et al., 2011). The presence of GABA transporters in ependymal cells was not investigated in this study, however it is likely that expression of glial GABA transporter; GAT3 could contribute to the depolarisation in response to GABA. Finally, GAT1 was observed to be expressed in oligodendrocytes (Fattorini et al., 2017). To verify which GABA transporter is responsible in ependymal cells, a future experiment to apply GABA with GABA transporter antagonists could be done.

The depolarisation induced by GABA using the perforated patch method is thought to be due to the presence of Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) (Marichal et al., 2012). Immature neurones express high levels of NKCC1 (Clayton et al., 1998) which drive Cl^- into the cell in exchange for the extrusion of Na^+ and K^+, maintaining the high intracellular Cl^- concentration. On activation of GABA receptors, the Cl^- ions exit the cell due to the concentration gradient, causing a depolarisation. This is less likely to be the case in our study though since the chloride equilibrium is set through the whole cell patch clamp method that fills the cell with a preset chloride concentration but it may be possible that at a local level, near the membrane and the chloride transporters, the concentration of chloride may differ from the rest of the cell. Another point to make is that since these cells are coupled, it may be that in our recorded cell, where the equilibrium potential is set, a hyperpolarising response is observed. However in the gap junction coupled cells, where the equilibrium potential is set by chloride transporters, NKCC1 may be high thus causing depolarising responses to the bath application of GABA in these cells and this depolarisation is transmitted through the gap junctions to be recorded as an overall depolarisation in our cell.

It is most likely that the depolarisation of ependymal cells to higher concentration of GABA is not a result of one of the above causes but a combination of some of or all of the causes.

On bath application, there is an increase in amplitude for the dose of 400 μM compared to 200 μM. Because of the low amplitude induced by 200 μM GABA, the dose of 400 μM was chosen to induce higher amplitude effects. Corns et al. (2013) used GABA dose of 200 μM to show amplitude of 3.85 ± 0.37 mV in ependymal cells similar to changes of amplitude in this study 3.85 ± 0.29 mV using 400 μM. The EC_{50} GABA observed by Wang, D. et al. (2003) was 196 μM. The observation was done on postnatal neuronal progenitor cells of SVZ. Hence our GABA dose (200-400 μM) is optimal to induce $GABA_A$ receptor responses. The second bath and puff application usually showed a reduced amplitude of response because of receptor desensitisation. Receptors may change their sensitivity including becoming desensitized. Low doses may reduce desensitization.

The whole cell patch clamp method was used which sets the reversal potential according to the chloride concentration in the aCSF (131 mM) and the intracellular solution (2.2 mM). Since the Cl^- reversal potential is -103 mV and the cells are sitting at around -74 mV, it would be expected that the responses to GABA would be hyperpolarising. Puff application of GABA with concentration of 1.25 μM causes hyperpolarisations. With puff application, as the concentration was increased, the responses were more likely to be depolarising and as the concentration was decreased, the responses were hyperpolarising. A concentration of 50 μM was used during recording on ependymal glial cells (EGCs) of cerebellar slices from P13-P35 mice and hyperpolarisations were observed (Reyes-Haro et al., 2013). The differences of responses of GABA may involve factors such as different types of

animals, different location of the cells which could contribute to the receptor expression.

The membrane potential of ependymal cells was repolarised to the resting membrane potential during the application of GABA to ensure that any opening and closing of voltage-gated channels in response to changes in the membrane potential did not affect the input resistance of ependymal cells. A change in input resistance would normally be expected to accompany a response to GABA, as opening of channels associated with $GABA_A$ receptors allows the movement of ions and thus input resistance would be decreased, but the input resistance for ependymal cells were already low due to extensive coupling, so the relative effect of ion channels opening on the size of the input resistance would quite low. If GABA had been given in the presence of gap junction blockers, which would increase input resistance, the responses observed would be expected to be bigger but this was not carried out in this study since the gap junction blockers often destabilised the cells, making it difficult to get consistent responses to drug applications.

Since ependymal cells are found throughout the neural stem cell niche, but there is heterogeneity in the properties of these cells (which leads to the sub-classifications described above), ependymal cells were investigated based on location to identify any differences. No location specificity for the type of responses of GABA was observed in the central canal area of spinal cord, based on the widespread hyperpolarisations and depolarisations using whole cell patch clamp. GABA signalling reflects the different degrees of maturation, so no localisation of specificity should be observed in any of the region even though there are differences in origin of the cells. Reali et al. (2011) also had similar findings of varied responses of depolarisations and hyperpolarisations of the ependymal cells using GABA with concentration of 100-1000 μM around the lateral aspects of central canal. These cells were electrically coupled as revealed by dye coupling of Alexa 488 and were in close proximity with

GAD+ terminals. The dorsal and ventral cells originate from different embryonic origins (Yu et al., 2013). Even though our observation did not characterise the neural stem cell marker individually, other studies showed all these type of ependymal cells expressed SOX2 (Alfaro-Cervello et al., 2012; Barnabé-Heider et al., 2010; Sabourin et al., 2009). Using different neural stem markers, they also showed only dorsally situated ependymal cells express nestin/GFAP. This implies that ependymal cells are most likely heterogenous in their NSC potential and that all ependymal cell may possess different properties regardless of location.

3.4.3 Atypical GABA receptor properties?

GABA-gated ion channels may be comprised of different subunits and the exact composition defines the properties of the receptor. Subunits from the $GABA_A$ and $GABA_C$ receptor have multiple configurations and thus have different responses to agonists and antagonists.

The depolarising GABA responses could be slightly reduced by $GABA_A$ receptor antagonists, bicuculline, quite similar to that observed by Corns et al. (2013). As GABAergic responses could be slightly reduced by the $GABA_A$ antagonist, bicuculline, it indicates that the response to GABA is in part being mediated by $GABA_A$ receptors. Bicuculline binds at the orthosteric site to stabilize the receptor in a closed state. Bicuculline acts as a competitive antagonist in which it competitively inhibits GABA binding to $GABA_A$ receptors, and GABA competitively inhibits bicuculline binding. Our ependymal cells may not show a maximum blockade with bicuculline for a number of reasons: 1. The concentrations of GABA used are too high for the bicuculline to overcome, 2. The residual GABA response could be a result of GABA transporter activity 3. There may be a presence of bicuculline resistant types of receptor subunit.

The presence of different $GABA_A$ receptor subunits may contribute to the different properties of the receptors which make them respond differently to lower or higher concentrations of GABA and also affects the degree of antagonism that is observed. Even though $GABA_A$ receptor expression subunit was not investigated for our ependymal cells of the spinal cord, a few immunohistochemical studies revealed high expression of α_1, α_3 , β_2/β_3 and γ_2 subunits with lower expression of α_2, α_5, α_6 and δ subunits in lamina X of spinal cord (Bohlhalter et al., 1996; Wisden et al., 1991). Due to these different subunits being present in the spinal cord, the degree of antagonism would be different even in similar animals. Another factor may be due to residual GABA action on the investigated cells. The residual GABA response could be a result of GABA transporter activity. To investigate this possibility, the GABA transporter blocker such as SNAP 5114 (GAT3 antagonist) could be co applied with bicuculline to see if they can antagonise the GABA response. We also need to consider the presence of $GABA_B$ receptors, which may also be present on the ependymal cells (see chapter 4) but this receptor solely causes hyperpolarisations so would not be expected to contribute to depolarising GABA responses.

The hyperpolarising responses to GABA were not blocked by the $GABA_A$ receptor antagonist bicuculline at a very high dose of 100 μM. What was most interesting was that responses to low GABA concentrations were antagonised by the GABAρ receptor antagonist; TPMPA. Bicuculline is known to have little effect on GABAρ. The effects of GABA 50 μM were not fully antagonised (90.5 ± 3.7%) by 100 μM bicuculline in cerebellar astrocyte culture and were confirmed, by electron microscopy, the presence of GABAρ in the plasma membrane of GFAP+ cells (Pétriz et al., 2014). When TPMPA 10 μM was added to bicuculline, GABA responses were then 100% blocked in an observation by Pétriz et al. (2014). Therefore in cerebellar astrocytes, the GABAρ subunit underlies a bicuculline-resistant component of responses to GABA. In wild type mouse stratum griseum superficiale cells, muscimol at a low

concentration of 0.5 μM which selectively activates GABAρ receptors, also elicited responses that could not be blocked by a lower concentration of bicuculline, 10 μM (Schlicker et al., 2009). With both low and high concentrations of bicuculline, the receptors were insensitive, so regardless of concentration, the distinct ρ subunit confers a bicuculline-insensitive component.

In all cases of effects of high doses GABA 400 μM (n=3) with bicuculline, bicuculline (100 μM) had no effect on the membrane potential (same as baseline reading; data not shown) of ependymal cells when applied alone similar with Corns et al. (2013) observation. Post washout GABA after bicuculline had a mean amplitude of 2.86 ± 0.37 mV (n=3, data not shown), with 95 ± 1.8% changes towards the initial GABA responses (3.02 ± 0.44 mV, n=3). The possibility of bicuculline is not working can be excluded as post washout GABA had reached to initial baseline changes from the co application of GABA and bicuculline responses. The stock and the drug safety checking were done during bicuculline stock preparation. The drug certificate of analysis had been reviewed before using the bicuculline.

In a study using knockout rho1 ($\rho^{-/-}$) mice, the effects of GABA on evoked potentials in spinal cord lumbar segment (L2-L6) recordings were no longer antagonised with TPMPA, but in the wild-type animals, the effects of GABA on these potentials had a significant TPMPA-sensitive component (Zheng et al., 2003). Without using knockout mice, previous studies in central nervous system observed a mixed response involving $GABA_A$ and GABAρ components (Reyes-Haro et al., 2017; Milligan et al., 2004). Reyes-Haro et al. (2017) observed hyperpolarised evoked GABA currents induced by GABA 100 μM in GFAP-GFP astrocytes that were partially antagonised by bicuculline 100 μM but the remaining current persisted then the responses were fully abolished when TPMPA 100 μM was added to the bicuculline. An observation by Milligan et al. (2004) reported that the responses induced by CACA were reduced by preincubation with TPMPA 160 μM, but were then fully antagonised with further

addition of bicuculline 10 μM and had partial recovery from bicuculline. Application of picrotoxin, which is thought to act as a non-competitive inhibitor of chloride channels (Akaike et al., 1985), also abolished the responses to CACA. This showed evidence of a mixture of $GABA_A$ and GABAρ effects. Our responses of low concentration of GABA were not antagonised with picrotoxin, which was somewhat surprising but this was only tested on 3 cells so may need further investigation.

Interestingly our data showed a significant reduction of amplitude of low GABA concentration responses with TPMPA 80 μM. On further confirmation to this, TACA, selective agonist of $GABA\rho_1$ elicited robust and reproducible hyperpolarising responses, similar to those observed with low concentrations of GABA. On application of TPMPA, the responses of TACA were reduced (n=5) but did not quite reach significance. Therefore, it may be worthwhile repeating in more cells to see if this reaches significance. TACA has greater potency than GABA at ρ receptors but also acts as an agonist at heteromeric $GABA_A$ receptors (Woodward et al., 1993), so the response effects may be divided in between $GABA_A$ and GABAρ receptor. Hence, there were a slight combination of action at both $GABA_A$ and $GABA_C$ in this study.

GABAρ receptors are formed by functional homo- or heteropentamers composed of ρ_1, ρ_2 and ρ_3 subunits (Enz and Cutting, 1999). The distribution of the three GABAρ subunits during postnatal development in the brain was described by Alakuijala et al. (2005). To date, there are still no characterisation or distribution studies of GABAρ subunits observed in the rodent postnatal development in spinal cord. Immunopositive staining on central canal area using $GABA\rho_1$ antibodies (Santa Cruz, sc-16879) was observed in the close vicinity of CD24 cells and also on GAD-GFP tissue. The immunopositive punctate staining observed in closed apposition to CD24 and GAD-GFP cells. The appositions were seen in quite a number in CD24 cells while it was sparse at GAD-GFP cells, so this supports the observation of hyperpolarisations seen mainly from the ependymal cells. Rho1 subunits are

expressed abundantly at brainstem, while ρ_2 and ρ_3 are found at much lower level (Milligan et al., 2004). Rho1 subunits also found in the synaptic junctions in brainstem neurones in their observation. Milligan et al. (2004) used a pre embedding technique to detect ρ_1 subunit immunoreactivity in synaptic junctions. Furthermore, from their observations, ρ_1 and α_1 subunits were co-localised at light and electron microscopy levels in brainstem neurons. In a study of rat cerebellum development, all subunits were expressed in the qRT-PCR of RNA isolated from postnatal developing cerebellum with relative comparative expression ratio $\rho_3>\rho_1>\rho_2$ (Mejía et al., 2008). So there is a variant of expression of rho subunit in the central nervous system. We choose to focus on the ρ_1 subunit because of the above immunopositive findings and previous results of immunoreactivity by Milligan et al. (2004). To confirm the expression of ρ_1 subunits in the spinal cord, we could perform reverse transcription (RT)-PCR with primers specific cDNA to ρ_1. If we used the same techniques to our spinal cord slices, so the expression of rho subunit/s can be identified.

Our ependymal cells did not respond well with CACA and therefore this was not included in the results section. CACA is inactive at heteromeric $GABA_A$ receptors (Naffaa et al., 2017), but is a partial agonist (70% efficacy) at $GABA\rho_1$ recombinant homomeric receptors. So if ependymal cells showed a mixture of different receptor subtype, CACA would not be a suitable agonist to show responses. The restricted cyclic analogue, (+)-cis-2-(aminomethyl) cyclopropane carboxylic acid (CAMP) and trans-2-(aminomethyl) cyclopropane carboxylic acid (TAMP) have been shown to differentiate between subtypes of GABAρ receptor (Duke et al., 2000). The (+) CAMP has greater potency at GABAρ compared to CACA and TACA. The (+) CAMP is also a weak antagonist at $GABA_A$ and is able to inhibit GABA reuptake transporters, while TAMP is an antagonist for ρ_3 subtypes (Vien et al., 2002). If we were able to proceed with further agonists and antagonists, CAMP and TAMP would be the choice to differentiate the effects on $GABA_A$ and among GABAρ subtypes. By using specific

agonists and antagonists, this would give us understanding of what type of channel was mediating the GABAergic effects with respect to rho subtypes and may also shed light on the structural organization of the pore of rho receptor, the permeability and ion selectivity of the channel.

Indeed responses to lower concentrations of both GABA (1.25-2.5 μM) and ODN (15-20 ηM) application were antagonised by TPMPA (80 μM) and not fully antagonised by $GABA_A$ receptor antagonist; bicuculline. So there is evidence of combination or coassembly of both receptors in the ependymal cells.

3.4.4 Responses to GABA can be further modulated by endogenous peptides

One potential site of modulation of GABA receptors is through the polypeptide discussed in the introduction, diazepam binding inhibitor (DBI). Two main breakdown products of DBI are triakontetraneuropeptide (TTN) and the octadecaneuropeptide (ODN). ODN is reported to have an action on central type benzodiazepine receptors (CBR) (Slobodyansky et al., 1989; Ferrero et al., 1986), while TTN activates peripheral type benzodiazepine receptors also known as translocator protein (TSPO).

There was strong DBI and TSPO immunofluorescence located around the central canal area of spinal cord. DBI immunopositive staining extended into the fibres of these cells in particular for the case of the DBI-immunofluorescence, whereas TSPO immunofluorescence was markedly positive at most of the neural stem cells in nestin-GFP mice. DBI immunopositive structures were found in embryonic slices of rodents and extended until postnatal age at the ventral and lateral white matter (Dickinson et al., 1996). Very sparse immunostaining was seen in the central canal area, compared to our study here. Extensive work by Dumitru et al. (2017) showed positive immunostaining in neural stem cells of SGZ and tanycytes in the third ventricle. Tonon

et al. (1990) used a different antibody complex that is rabbit antiserum to ODN, goat anti-rabbit gamma-globulins and the peroxidase-antiperoxidase (PAP) complex. ODN-immunoreactive structures were detected in glial and ependymal cells of the brain. Our finding showed extensive expression of DBI and TSPO in the region of the central canal neural stem cells possibly at the ependymal cell membrane.

An important initial finding has been made to demonstrate the reduction of GABA induced Cl^- currents by DBI in a study by Bormann et al. (1985), using cultured mammalian unidentified spinal cord neurons. Bormann et al. (1985) reported that reduction of GABA responses by DBI did not occur in the presence of DBI antagonist, flumazenil. DBI is indeed mediated by benzodiazepine receptors, rather than due to direct blockade of the Cl^- channel by the DBI peptide itself (Bormann, 1991).

Using the non-selective modulator, midazolam which acts on both CBR and TSPO (Tokuda et al., 2010), the responses to GABA were reduced. In characterising which subunits in the GABA receptor formed the binding site for midazolam using expression studies comprised of different subunits, it was found that in cells expressing the γ_2 subunit with α_1 and β_1 subunits, midazolam produced high-affinity binding for CBR ligands (Pritchett et al., 1989). The fact that the responses to high concentration of GABA were modulated by midazolam suggests a potential involvement of central benzodiazepine receptors as shown by decreases in GABA responses with midazolam activity but does not rule out a role for TSPO as well. To test whether some of the response was due to activation of TSPO, FGIN-1-27, a selective TSPO agonist was applied. In our experiments, FGIN-1-27 (1 μM) also increased the GABA mediated response in ependymal cells to the higher concentration (400 μM) of GABA.

The fact that the responses to GABA are inhibited by midazolam while the response to GABA is augmented by FGIN-1-27 suggests that the effect at the CBR predominates with midazolam, causing an overall inhibition. However, the responses

of GABA in the presence of ODN did not show significant changes regardless of the GABA concentration used. Even though our results with benzodiazepine receptor ligand ODN did not show any significant results, it was interesting that in cells responding to GABA by depolarising, the effects were reduced with ODN without quite reaching significance. This lack of effect of ODN is in sharp contrast to GABA responses in the sub ventricular zone where GABA responses in neural stem cells were significantly reduced by the same concentration of ODN that we applied (Alfonso et al., 2012). The reduction in GABA currents induced by ODN was strongly diminished in gamma2 F77I mice which represent a mutation in the gamma2 subunit of $GABA_A$ receptor that decreases binding to the benzodiazepine binding site. Their mouse line constitutes an optimal system to test whether DBI modulates neurogenesis via the $GABA_A$ receptor. In our observations, using C57BL/6 mice, ODN with the dose of 20 μM causes both depolarisation and slow hyperpolarisation effects on ependymal cells. The spill over of ODN into both synaptic and peri-synaptic effects on the cell may affect CBR and TSPO simultaneously. It may be therefore that the $GABA_A$ receptors are not in the appropriate configuration to enable binding of ODN to the CBR alone. One cannot exclude the possibility of ODN acting via TSPO or via its function as acyl-CoA esters reservoir even though ODN only binds with low affinity to TSPO. Another option to directly test the effects of activation of the CBR on responses to GABA, rather than inhibiting binding at this site, which is how ODN exerts its effects would be to use diazepam, which produces a biphasic potentiation of GABA receptor especially at receptors containing $\alpha_1\beta_2\gamma_2$ subunits (Walters et al., 2000). The experiments by Walters et al. (2000) were done on Xenopus Laevis oocytes and parameters were obtained from fitting the incorporation of different subunits. The nanomolar component of diazepam action depends on the presence of the γ subunit and their ρ_1 receptor channels are insensitive to diazepam. This

experiment would at least elucidate whether it is possible to enhance GABA responses through an action at the CBR.

To test the effects of activating TSPO alone, FGIN-1-27 was used. With FGIN-1-27, GABA responses increased significantly. So there is a positive modulation likely mediated through TSPO. We also tested using a combination of FGIN-1-27 and PK-11195; and this combination of TSPO agonist and antagonist, had no overall effect on amplitude of GABA responses, thus positive modulation of GABA was reduced as TSPO were blocked by PK-11195. An action of GABA on TSPO can promote the synthesis of GABA-enhancing neurosteroids. In a study by Tokuda et al. (2010) using hippocampal slices, activation of TSPO by the selective agonist FGIN-1-27 caused neurosteroidogenesis, observed as an increase in the intensity of allopregnanolone immunofluorescence, within just 30 minutes of application. Next, Tokuda et al. (2010) used finasteride to function as a neurosteroidogenesis inhibitor and showed that this blocked the increased levels of neurosteroidogenesis in response to midazolam so they suggested that a component of the effect of midazolam is to stimulate neurosteroidogenesis, presumably via the TSPO. Therefore a further experiment here would be to test this directly, using FGIN-1-27 in the presence of finasteride to investigate whether in the spinal cord, activation of TSPO may stimulate neurosteroidogenesis. I also studied the effects of application of a neurosteroid, allopregnanolone on responses of cells to GABA. The potency of allopregnanolone is dependent on the subunit composition of the GABA receptors (Belelli et al., 2002) and so two concentrations were tested here. The lower concentration is more likely to act at receptors that include α_1 and α_3 subunits plus β_1 and γ_2 while the higher concentration is likely to also target those receptors containing α_2, α_4 and α_5. We did show, that the responses of GABA were increased in ependymal cells in the presence of both high and low concentration of allopregnanolone (1 μM and 10 nM) but this did not reach significance for either situation. This was surprising since many studies

have showed that allopregnanolone has a permissive effect on modulating responses of GABA receptor activation (Puia et al., 2003; Pinna et al., 2000). The lack of response may be due to a high degree of variation in the amplitude of the responses of ependymal cells to GABA, both before and after allopregnanolone, especially since the n numbers are low (n = 5 and 4 for the high and low concentrations respectively). The allopregnanolone mechanism of action on $GABA_A$ is concentration dependant. In lower nanomolar concentrations it acts allosterically while at higher concentration (micromolar range) it directly gates the $GABA_A$ receptor complex (Puia et al., 2003; Callachan et al., 1987). Furthermore neurosteroids may directly gate $GABA_A$ receptor also at lower concentration at around 100 nM (Shu, H.-J. et al., 2004). My doses of allopregnanolone: 1 μM and 10 nM may act both as allosteric modulators and directly at the $GABA_A$ receptor but neither of these mechanisms would explain our lack of significance.

The positive modulation of allopregnanolone on GABA receptor is thought to be due to allosteric modulation and also in hybrid system, increasing both frequency and duration of the chloride channel opening (Lambert et al., 2009). However it is possible that allopregnanolone may become sulphated since the enzyme 3α-hydroxysteroid sulfotransferase is present in tissue (Driscoll et al., 1993) and thus some of the effects of the this drug are due to its sulphated metabolite. This is relevant since sulphated neurosteroids have been shown to antagonize $GABA_A$ receptors in a non-competitive manner where allopregnanolone inhibits channel activity in either a closed state prior to GABA agonist activation or in an open state, such as blocking activity in instances where GABA is bound (Akk et al., 2001). With high concentration of GABA, the effects of sulphated neurosteroids is thought to be due to the different binding site and mechanism (synaptic) and due to this (which depends on the subunit type of receptor), this group of neurosteroids can serve as a homeostatic balance to the positive modulator (Reddy and Kulkarni, 1998). More recently, the sulphated

neurosteroids may display biselectivity of modulation on $GABA_A$ as well due to the presence of δ and γ subunit (Shu, H.J. et al., 2012). So the effects of neurosteroids are primarily affected by GABA subunit rather than the binding site of GABA receptor.

Our observations on low GABA concentrations showed no significant effect with modulators such as FGIN-1-27, ODN and allopregnanolone (1 μM and 10 nM). Most of GABAρ receptor studies did not use FGIN-1-27 and ODN as modulators, while a study with low GABA concentration action with neurosteroids found out that there is differential modulations of ρ_1 receptor channel (Morris et al., 1999). However, neuroactive steroids can modulate the ρ_1 subunit in xenopus laevis oocytes (Morris et al., 1999). The modulation of ρ_1 receptors by neurosteroid compounds was dependant on GABA concentration. These effects were prominent in the presence of low concentrations of GABA (0.2-0.4 μM). The differential modulation by neurosteroids depends on the type of derivatives. The 5α derivatives (allopregnanolone, alphaloxone and 5α-tetrahydro- deoxycorticosterone (5α-THDOC)) were potentiators of GABAρ while the 5β compounds (5β-tetrahydrodeoxy-corticosterone (5β-THDOC), 5β-Dihydroprogesterone; 5β-DHP) were the inhibitors to GABAρ. Morris et al. (1999) showed the 5α derivatives potentiate and prolong decay time whereas 5β compounds were inhibitors of GABA currents. Morris et al. (1999) hypothesised that this was because of the difference in the structure component of hydrogen atom in the neurosteroid itself. The structural differences of the neurosteroids can influence the relative position within their effector site which can influence ρ_1 ion channel in an opposing way (Morris et al., 1999). Finally the effects of the neuroactive steroids on ρ_1 receptor were shown to be long lasting because the application of GABA did not return to the control level for few minutes after neuroactive steroid treatments. In contrast, these effects of neurosteroid modulation did not occur in an earlier study by Woodward et al. (1992) using GABA (1-2 μM) on retina RNA of Xenopus oocytes. Our allopregnanolone doses may have similar

structure components but maybe due to different doses used, the degree of modulation may be different.

These suggests that the rho containing receptors are not modulated in the same way as the $GABA_A$ receptor.

3.4.5 How can ODN be exerting direct effects on ependymal cells?

ODN immunopositive staining using ODN antibodies supplied by Tonon et al. (1990), (1:500) on Nestin-GFP mouse showed ODN is expressed in the central canal area of spinal cord. No previous immunofluorescence study using ODN antibodies has been done in spinal cord. ODN can be detected in non-neuronal cells throughout the rat brain and it was restricted to glial ependymal cells (Tonon et al., 1990). Different results have been obtained by Alho, H et al. (1989) who, using antibodies against DBI, found that immunostaining was associated with neurons in several brain regions that are hypothalamus, amygdala, hippocampus and cerebellum. Furthermore in later studies, cultured astrocytes are strongly labelled with specific ODN antibodies (Lamacz et al., 1996).

On further characterising the modulation of GABA responses through an effect on CBR, direct responses of the cell to ODN were observed. This was of interest since ODN, which exert anxiogenic activity, has been previously shown to increase intracellular calcium concentration in cultured rat astrocytes through activation of a metabotropic receptor, not the CBR (Leprince et al., 1998; Gandolfo et al., 1997). The nanomolar concentration was found to induce marked increases in Ca^{2+} in cultured rat astrocytes (Lamacz et al., 1996). Cultured astrocytes were also found in their experiments to release substantial amounts of ODN, which showed evidence of an ability of astrocytes to synthesize DBI in vitro. They were able to block the effect of

ODN on Ca^{2+} with pertussis toxin. However, the surprising finding using ODN from our study was that, when puffed alone at nanomolar concentration, ODN caused fast hyperpolarising responses in ependymal cells that were strikingly similar to those elicited by the lower concentrations of GABA and TACA responses.

In our study, the effects of ODN puff showed no location specificity in the central canal area. Our responses to nanomolar ODN were significantly but not fully blocked by cyclo1-8[dleu5]OP. Cyclo1-8[dleu5]OP is an ODN metabotropic receptor antagonist, so a component of the responses may be due to an action at this receptor, which in turn may elicit calcium influx but it is still surprising that the effect is hyperpolarising, rather than depolarising, as would be expected with calcium influx. One possible explanation may be that ODN is somehow directly activating the GABA receptors that contain rho subunits since the hyperpolarising effects of ODN were significantly blocked by TPMPA. Our ODN did not show any significant blockade with other antagonists, modulators or channel blockers. So there is an evidence there are atypical GABA receptors responsible for this action with ODN.

One study of interest to us, showed that ODN protects astrocytes against hydrogen peroxide induced apoptosis (Hamdi et al., 2012). Their ODN seemed to reside on the cell membrane of astrocytes and keep the cell membrane integrity. It would be interesting to see if application of ODN on spinal cord slices could act to protect ependymal cells against hydrogen peroxide induced apoptosis.

3.4.6 How does GABA affect proliferation in the spinal cord?

A study by Reali et al. (2011) showed the effects of GABA on BLBP immunoreactive progenitor cells that are found in central canal of turtle spinal cord. These cells appear to be similar to the ependymal cells observed surrounding the mammalian central canal (Corns et al., 2013; Marichal et al., 2012; Meletis et al., 2008). Many studies

carried out in brain showed that GABA was able to control proliferation of cells in subventricular ventricular zones (SVZ) and dentate gyrus (DG) (Wang, D. et al., 2003; Haydar et al., 2000). It is reported that GABA reduced the number of BrdU labelled cells in the SVZ. Similar outcomes were reported by Nguyen et al. (2003) where GABA reduced the number of BrdU- incorporated neural progenitor cells compared to control and by (Tozuka et al., 2005); $GABA_A$ receptor agonists decreased while the antagonist increased the BrdU labelled progenitor cells. Even though those studies conclude that GABAergic inputs had blocked the cell cycle progression and further neurogenesis, GABA is indeed an important regulator of neurogenesis in subventricular zone and dentate gyrus. Moreover, with the findings in Corns et al. (2013), GABA acts significantly on spinal ependymal cells and GABA in this study showed significant increase in amplitude with modulation of FGIN-1-27; the selective agonist at TSPO, which is thought to induce neurosteroidogenesis (Tokuda et al., 2010). In SVZ, DBI negatively modulates the activity of the $GABA_A$ receptor in transit amplifying cells and enhances their proliferation through this mechanism and not through its binding to TSPO. Similar action is seen in SGZ NSCs and transit amplifying cells through binding to the $GABA_A$ receptor and enhances the proliferation of NSCs (Dumitru et al., 2017). In other unpublished experiments in our research group (New, Deuchars and Deuchars), we have shown that midazolam can modulate the levels of cell proliferation in the spinal cord. So GABA through $GABA_A$ receptor can enhance proliferation of NSCs.

3.5 Conclusion

This study concludes that the ependymal cells surrounding the central canal of postnatal mammalian spinal cord showed the characteristics of neural stem cells as previously described with extensive gap junctions. The presence of gap junctions showed that this cell functioning unit was through a group of cells and not as a single unit. The ependymal cells responded to the neurotransmitter GABA possibly through expression of multiple combination of subunits and multiple receptors. Ependymal cells in our study showed atypical GABA receptors since it was not blocked by $GABA_A$ receptor antagonist; bicuculline while hyperpolarising responses to low GABA were antagonised by the GABAρ receptor antagonist, TPMPA. With TACA, a selective agonist of $GABA\rho_1$, GABA responses may partially being blocked and this provides evidence of a combination or coassembly of $GABA_A$ and ρ receptors in the ependymal cells or the presence of two different receptors.

The modulation of GABA receptors is through multiple sites on the subunit and membrane of the cell including both CBR and TSPO. DBI, TSPO and GABA regulate spinal cord neurogenesis in a close partnership. The balance between DBI polypeptide action, and actions through other GABA receptors then may be able to balance the modulation of the GABA in neurogenesis.

Chapter 4

Activation of $GABA_B$ receptors in ependymal cells

4.1 $GABA_B$ receptors

$GABA_B$ receptors belong to the G-protein coupled receptors (GPCR) family that act slowly and maintain the inhibitory tone (Bettler et al., 2004). The $GABA_B$ complex were composed of $GABA_{B1}$ and $GABA_{B2}$ subunit with a 1:1 stoichiometry and both are required in forming functional receptors (McCarson and Enna, 1999). Results from recombinant studies suggest that the $GABA_{B1}$ subunit provides the G-protein coupling site whilst the $GABA_{B2}$ subunit is necessary for both cell surface expression and agonist activation (Margeta-Mitrovic et al., 1999; Jones et al., 1998).

The $GABA_{B1}$ subunit exists in two isoforms ($GABA_{B1A}$ and $GABA_{B1B}$) which are generated from $GABA_{B1}$ gene by differential promoter usage. $GABA_B$ receptors can modulate activity of voltage gated calcium channels thereby regulating neuronal excitability (Ulrich and Bettler, 2007).

Within SGZ of adult mouse hippocampus, $GABA_B$ receptors are expressed by multiple cell types including neural stem cells, neuroblasts and newborn neurons and $GABA_B$ receptor activation is able to inhibit dentate gyrus neural stem cell proliferation (Giachino et al., 2014), while chronic antagonism of this receptor increased cell proliferation in the ventral but not dorsal hippocampus (Felice et al., 2012). Expression of $GABA_B$ in the SVZ area has not been well addressed but $GABA_B$ receptor blockade has been

observed in SVZ to influence which the migration of tangential interneurons (López-Bendito et al., 2003).

Little is known about the role of $GABA_B$ receptors in spinal cord proliferation but they are widely expressed in the spinal cord (Wang, L. et al., 2008) and may have effects on the neurogenic niche of the spinal cord. Several studies provide evidence for the influence of $GABA_B$ receptors on neurogenic niches in the brain (Jurčić et al., 2018; Giachino et al., 2014; Felice et al., 2012) and a study on brainstem CSFcCs showed $GABA_B$ receptor mediated effects on these cells (Jurčić et al., 2018). However no-one has looked at the contribution of these receptors in the neurogenic niche of the spinal cord.

4.2 Hypothesis and aims

As ependymal cells have been shown to respond to GABA in the previous chapter, the aim of this chapter is to establish whether ependymal cells in spinal cords from mice aged P8-P15 also express $GABA_B$ receptors. This was determined using both electrophysiology and immunofluorescent methods. The main hypothesis of this chapter is that ependymal cells are capable of responding to baclofen, an agonist of $GABA_B$ receptors and that antagonism of this receptor can affect the responses of these cells to baclofen. Furthermore, since previous reports have shown that activation of $GABA_B$ receptors can inhibit neurogenesis in other neurogenic niches, I hypothesise that application of baclofen will reduce ependymal cell proliferation in spinal cord slices

4.3 Results

4.3.1 $GABA_{B1A}$ subunits were expressed in central canal of spinal cord

$GABA_{B1}$ immunopositive punctate staining was observed throughout the region of the central canal and especially observed in close apposition to cells expressing the ependymal cell marker (CD24) (Figure 4-2 A), or near GFP positive cells in GAD-GFP mouse (Figure 4-2 B). At high magnification (x40), it is not known whether the immunofluorescent structures close to the cells are either in the membrane or in close appositions onto the membrane, which would signify post and pre-synaptic labelling respectively.

The $GABA_{B1}$ staining was found throughout the central canal region and did not show any regional localisation. This is relevant when considering the locations of the cells that were tested for responses to baclofen.

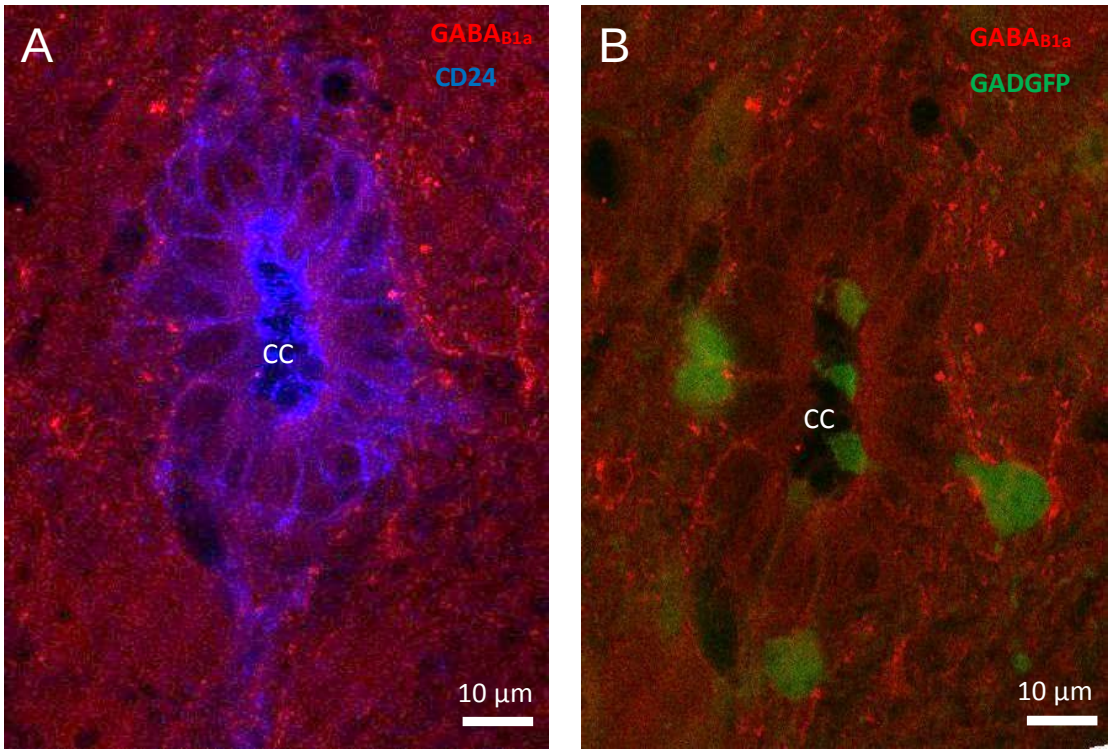


Figure 4-1 $GABA_{B1A}$ immunopositive staining at the central canal of spinal cord

Fluorescence immunohistochemistry using $GABA_{B1A}$ antibody together with antibodies for ependymal cell marker (CD24) (A), or on GAD-GFP mouse (B) in the central canal area. There are close appositions of the $GABA_{B1A}$ immunopositive punctate structures with both ependymal cells and CSFcCs in central canal (cc).

4.3.2 Hyperpolarising effects of baclofen on ependymal cells in the spinal cord

To determine the effects of activation of $GABA_B$ receptors, baclofen was applied in the bath solution. The effects of bath application of baclofen were tested on 15 ependymal cells located at different sites around the central canal (Figure 4-3). Regardless of their position, 15 cells responded to baclofen with a hyperpolarisation. No ependymal cells were depolarised by baclofen. In response to 1 μM baclofen, the hyperpolarisations were on average -0.91 ± 0.11 mV in amplitude (Figure 4-3 A) while the higher dose 5 μM were on average of -0.73 ± 0.15 mV in amplitude (Figure 4-3 B). No significant differences were observed between the amplitudes in response to the 2 concentrations (p=0.37).

The hyperpolarising effect could be antagonised by the selective $GABA_B$ antagonist CGP 55845 (200 nM) since the hyperpolarising response to baclofen was reduced from -0.91 ± 0.11 mV to -0.27 ± 0.07 mV (*$p \leq 0.05$, n=11, Figure 4-3 A & Ai) for baclofen (1 μM) and from -0.73 mV ± 0.15 to -0.11 ± 0.07 mV (*$p \leq 0.05$, n=4, Figure 4-3 B & Bi) for baclofen (5 μM).

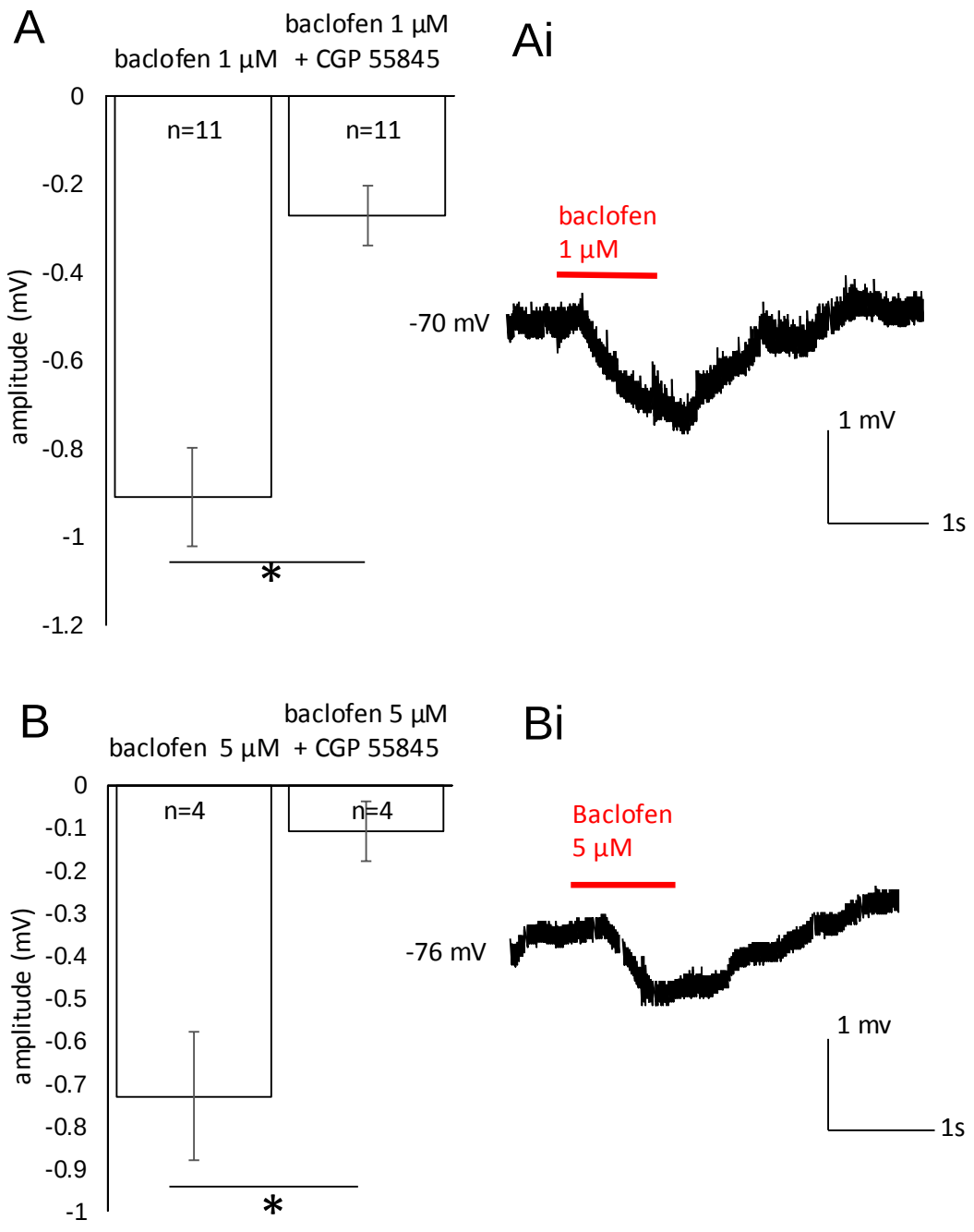


Figure 4-2 Effects of baclofen on ependymal cells

Effects of baclofen (1 µM) (A) and (5 µM) (B) with CGP 55845 (200 nM) on ependymal cells. Both baclofen (1 µM) and (5 µM) caused hyperpolarising responses in ependymal cells and these were antagonised with the selective $GABA_B$ antagonist CGP 55845 (200 nM) (Ai & Bi). Two concentrations are used to confirm if there is any difference. (*$p \leq 0.05$, n = 11 for baclofen (1 µM) and *$p \leq 0.05$, n=4 for baclofen (5 µM)).

To establish whether the location of the cell affected the response observed, ependymal cells were recorded from around the central canal and the location and response were noted. It was clear that there was no location-specific response profile (Figure 4-4). This fits well with the immunofluorescent data showing that $GABA_{B1A}$ immunopositive staining was located around the whole of the central canal.

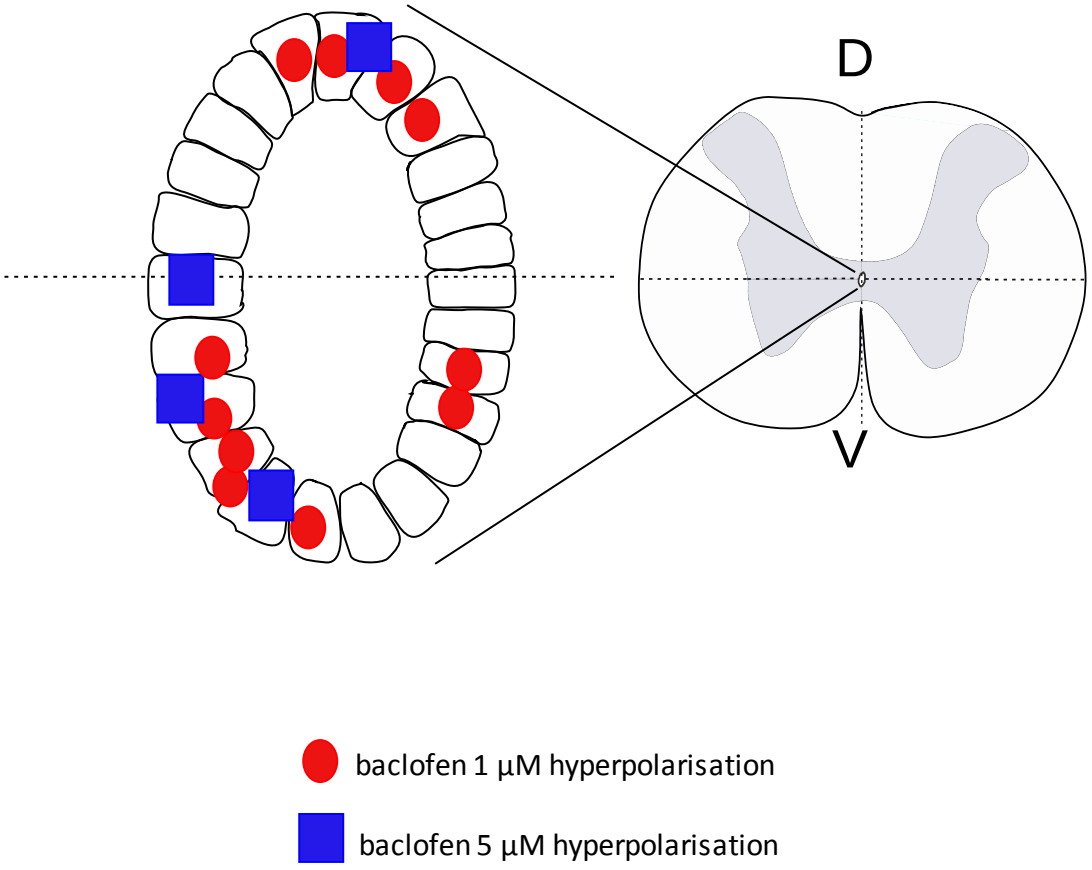


Figure 4-3 Location of ependymal cells exhibiting the responses to baclofen.

Whole cell patch clamp recordings were taken from throughout the area of the central canal. There is no location specificity for the responses observed. Baclofen 5 µM and 1 µM were applied in the bath solution.

4.3.3 Activation of $GABA_B$ receptors decrease levels of proliferating cells in the central canal area

Since the responses to baclofen were always hyperpolarising, we were interested to know whether activation of the $GABA_B$ receptor with baclofen could affect the level of proliferating cells in the neurogenic niche. Using EdU as a marker of cell proliferation and applying baclofen at two different concentrations (1 and 5 μM) to spinal cord slices incubated for 4 hours, there was a significant reduction in the numbers of newly proliferating cells in those sections treated with baclofen compared to control slices ((control=2.81 ± 0.21 cells/50 μm section, 5 μM=1.91 ± 0.15 cells/50 μm section and 1 μM=1.79 ± 0.17 cells/50 μm section), N=4 mice, n = 237 sections) (Figure 4.5). The slices were viewed using a fluorescent microscope and images were noted to show reduced numbers of fluorescently labelled cells in the area of central canal for both concentrations of baclofen (1 μM) and (5 μM) (Figure 4.6).

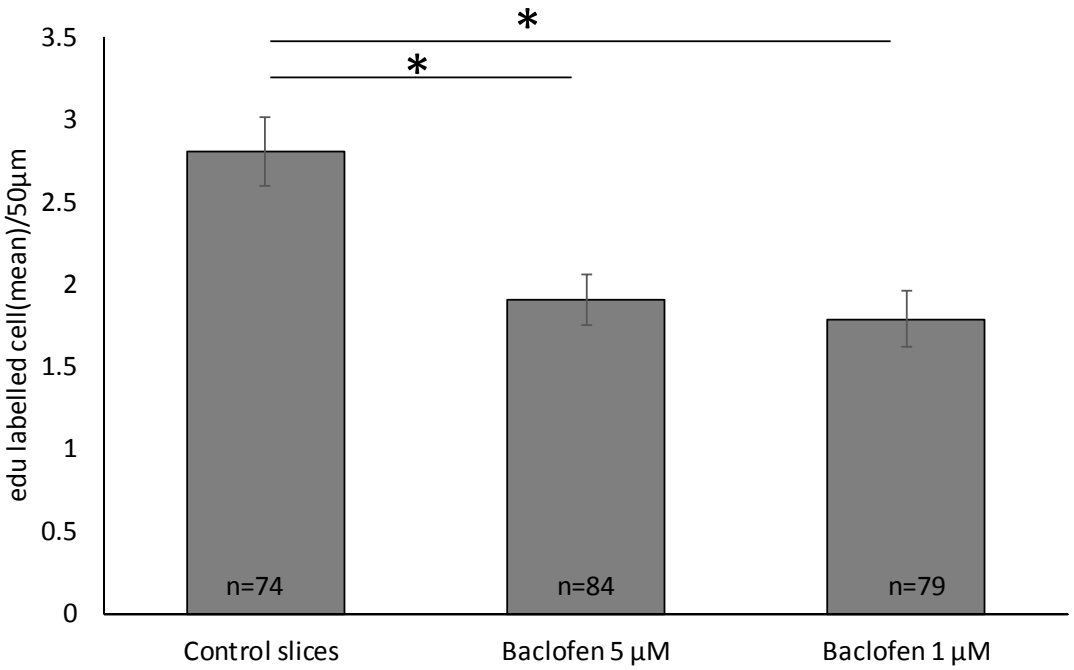


Figure 4-4 Effects of baclofen on EdU-labelled cells in the central canal area

Bar chart showing the numbers of EdU-labelled cells in the central canal area for each group; control, baclofen (5 µM) and baclofen (1 µM). The numbers of EdU-labelled cells in baclofen (1 µM) and baclofen (5 µM) treated slices were reduced significantly when compared to control. There is no difference between baclofen (1 µM) and baclofen (5 µM). (*$p \leq 0.05$, N=4 mice, control; n=74 slices, baclofen (1 µM); n= 84 slices and baclofen (5 µM); n= 79 slices.)

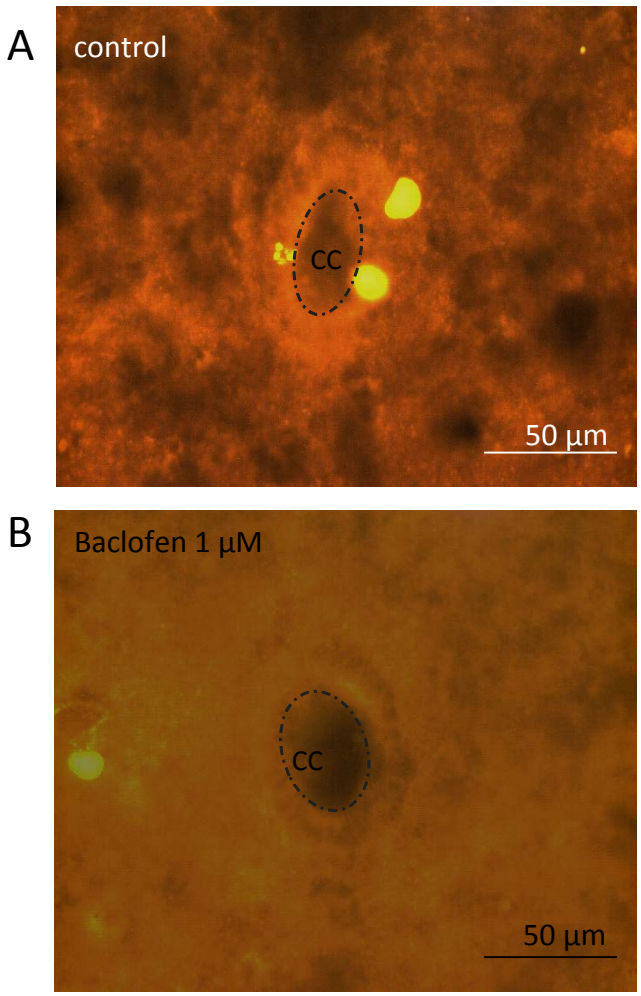


Figure 4-5 Fluorescent images of central canal of spinal cord show reduced numbers of EdU labelled cells after baclofen treatment

EdU-labelled cells were labelled using the streptavidin 555 and appeared red in colour and scattered throughout the whole area of spinal cord (A) but especially high in the central canal region. Sections treated with baclofen showed sparse to no EdU labelling in the central canal region (B). Images orientated with dorsal pole of the central canal (cc) at the top.

4.4 Discussion

4.4.1 $GABA_B$ receptors are expressed in neurogenic niche of spinal cord

In-situ hybridisation and immunohistochemistry in the rat revealed that $GABA_{B1}$ receptors were differentially distributed throughout spinal cord predominantly in laminae II and IX while $GABA_{B2}$ receptors were evenly distributed throughout the spinal cord including lamina X (Towers et al., 2000). In this chapter, we have shown that $GABA_{B1A}$ immunopositive structures form close appositions with ependymal cells, which are the putative neural stem cells in the central canal area. CD24, the marker used in this study is an ependymal cell surface marker (Pfenninger et al., 2011; Calaora et al., 1996). The $GABA_{B1A}$ immunopositive structures also seem to form close appositions near the stained GAD-GFP cells suggesting that $GABA_B$ receptors may influence CSFcCs, although this was not studied here. It would be possible to further confirm inputs onto ependymal cells using the nestin-GFP mouse described in chapter 5 to see if the $GABA_B$ receptors form close appositions with the nestin positive cells. Nestin is a marker of undifferentiated neural cells in both the developing and adult nervous systems but mostly indicate more immature ependymal cell phenotype (Hamilton et al., 2009). All the immunostaining results are not conclusive to show whether the immunofluorescent structures close to the cells are either in the membrane or in close appositions onto the membrane. So in future plans, a study using electron microscopy could be carried out, either using fixed sections as described here or even using cells filled with neurobiotin and then fixed to see whether those cells that respond to baclofen express the $GABA_B$ subunits at the electron microscopic level.

In this study, whole cell patch clamp recordings were made from ependymal cells in the central canal area in order to investigate GABAergic inhibition through $GABA_B$ receptor. The use of 1 and 5 μM doses of baclofen is to determine any discriminative responses to these concentrations of the agonist baclofen based on previous studies. In spinal cord slices, sympathetic preganglionic neurones showed 2 different responses to low concentrations (1 μM) of baclofen, with small amplitude hyperpolarisations being reduced in low Ca^{2+} solutions or cadmium, suggesting that the response was due to closure of calcium channels. Larger amplitude hyperpolarisations were observed in other cells that were mediated by opening of potassium channels (Wang, L. et al., 2010). They considered that these responses may be due to differences in the subunits of the receptors underlying these responses. This idea was feasible since baclofen at low concentrations (1 μM) is often used to stimulate receptors with high affinity such as presynaptic receptors (Brooks and Glaum, 1995) that act to close calcium channels and reduce the neurotransmitter release and these are most likely to express $GABA_{B1A}$ (Vigot et al., 2006). However baclofen at high concentration (5 μM) can alter postsynaptic membrane excitability in dorsolateral septal nucleus (Yamada et al., 1999) and these express $GABA_{B1A}$ (Vigot et al, 2006) and often involve opening of potassium channels. Ependymal cells however, did not respond differently to either concentration of baclofen unlike that observed in sympathetic preganglionic neurones (Wang, L. et al., 2010). In our observation, so far there are no cells that fail to respond to baclofen. The mean amplitude of baclofen effects in our study using 2 different concentrations showed no significant difference. In fact, both concentrations resulted in small hyperpolarisations, reminiscent of the responses of sympathetic preganglionic neurones that were mediated through closure of calcium channels and thus likely to involve $GABA_{B1A}$ receptors expressed in the postsynaptic membrane. This suggests that in ependymal cells, only $GABA_{B1A}$ containing receptors are present and this fits with our

immunofluorescence experiments showing $GABA_{B1A}$ immunopositive structures in the central canal area, although the presence of $GABA_{B1B}$ subunits was not tested due to a paucity of good antibodies. It is possible that the age of the mice used in these experiments affected these responses, since $GABA_{B1B}$ expression increases during the first postnatal month in the brain (Fritschy et al., 1999) while $GABA_{B1A}$ is predominant during embryonic period and at birth during first two postnatal weeks (Khoshdel-Sarkarizi et al., 2019). The immunofluorescence studies were done in adult mice, suggesting that the $GABA_{B1A}$ is still present into adulthood. However an explanation for only observing small responses in our electrophysiology experiments may be that the levels of $GABA_{B1B}$ subunits are very low compared to $GABA_{B1A}$ at the age of animals used and so only small responses were observed. It is also possible that the small response observed in the ependymal cells was due to hyperpolarisations of the coupled cells which were then conducted through gap junctions to hyperpolarise the recorded cell.

4.4.2 Responses to baclofen were antagonised by CGP 55845, a selective $GABA_B$ antagonist

As described in Chapter 3, bicuculline could not completely antagonise the GABAergic response, even at high concentration of 100 μM. This suggested that there was another receptor or transporter contributing to the mediation of GABAergic responses. Baclofen was applied in the bath solution and always caused a hyperpolarising response in ependymal cells tested. A small yet robust and reproducible hyperpolarising response to baclofen suggests that ependymal cells do express $GABA_B$ receptors.

CGP 55845 is a selective $GABA_B$ receptor antagonist. CGP 55845 was found to be the most potent $GABA_B$ receptor antagonist tested in a study using hemisected spinal

cord (Brugger et al., 1993) so this is convincing evidence that the $GABA_B$ receptor is present.

4.4.3 Activation of $GABA_B$ receptors affect levels of proliferation in central canal area

Since baclofen had effects on ependymal cells, we tested whether activation of $GABA_B$ receptors could affect proliferation. In the presence of baclofen, the numbers of proliferating cells in the area of central canal, identified using the EdU click chemistry method, were significantly lower than numbers in control slices. Once again, there was no significant difference between the two concentrations. Such a role for $GABA_B$ receptors has been suggested in other brain regions and cells (Giachino et al., 2014; Felice et al., 2012; Luyt et al., 2007; Magnaghi et al., 2004) and deserves further investigation.

4.4.4 $GABA_B$ receptor modulation of neurogenesis in other neurogenic niches

Felice et al. (2012) showed the evidence of modulation of $GABA_B$ receptors may affect adult neurogenesis in hippocampal dentate gyrus. Their work showed that chronic treatment of the $GABA_B$ antagonist CGP 52342 causes an increase in cell proliferation in the SGZ area without affecting cell survival. The neurogenic effects of $GABA_B$ receptor inhibition affect the ventral hippocampus which is responsible for stress and emotion regulation. This showed a link between antidepressant and neurogenic $GABA_B$ receptor blockade. A recent study was carried out by Giachino et al. (2014) where they used $GABA_B$ genetic deleted mice $GABA_{B1}^{-/-}$ or $GABA_{B2}^{-/-}$. Impairing $GABA_B$ signalling pharmacologically or in mice deficient for $GABA_{B1}$

receptor gene, increases the cell proliferation in dentate gyrus while activating $GABA_B$ receptors has the opposite effect (Giachino et al., 2014). Similar to Felice et al. (2012), cell survival was unaltered in $GABA_{B1}$ knockout mice. In $GABA_{B1}$ deficient mice, the ability to modulate GABA release may be altered. This suggests that activation of $GABA_B$ may synergistically work with $GABA_A$ receptor to mediate the antimitotic effects of GABA. The modulation of proliferation and differentiation by $GABA_B$ receptor may be an interesting future therapeutic target for conditions where there is over proliferation such as in spinal cord ependymomas or in conditions such as in multiple sclerosis where replacement cells may be useful to aid recovery.

4.4.5 $GABA_B$ receptor in neurotransmitter signalling.

$GABA_B$ couples to G proteins as evidenced in several studies. $GABA_B$ receptors predominately couple to $G_1\alpha$ and $G_o\alpha$-type G proteins. $GABA_B$ receptors were able to modulate opening of calcium channels at the cell membrane as well as intracellular calcium mobilisation in pyramidal neurons of postnatal rats (Gaiarsa et al., 1995). Other than coupling to Ca^{2+} channels, $GABA_B$ receptors also couple to K^+ channels and induce slow inhibitory postsynaptic currents through opening of GIRK channels. The physiological effect of GIRK is normally a K^+ efflux resulting in a hyperpolarisation. Presynaptic $GABA_B$ receptors seem to be coupled to GIRK channels in immature granule neurons of hippocampus and activation of GIRKs can induce changes in membrane excitability and affects cellular processes (Cabezas et al., 2012). $G_1\alpha$ and $G_o\alpha$-type G proteins inhibit adenylyl cyclase types I, III, V and VI (Simonds, 1999) while $G\beta\gamma$ stimulates adenylyl cyclase types II, IV and VII (Tang and Gilman, 1991) and the stimulations depend on the presence of $Gs\alpha$. Through cAMP signalling and adenylate-cyclase activity, $GABA_B$ receptors regulate neural development in perinatal period of pyramidal neuron progenitors (Bony et al., 2013).

Using ELISA immunoassay, and measurement of cAMP levels after bath application of $GABA_B$ receptor agonist or antagonist similar to that by Bony et al. (2013) would be beneficial to add on to our experiments to see any involvement of cAMP mediated signalling. The cAMP pathway also can be potentiated by $GABA_A$ receptors as in activation of $GABA_A$ receptor in SGZ neurogenic niche. Thus the adenylate-cyclase-cAMP pathway is a point where there is a crosstalk of $GABA_A$ and $GABA_B$ receptor in neurotransmitter signalling.

4.5 Conclusion

This study concludes that the ependymal cells surrounding the central canal of postnatal mammalian spinal cord express $GABA_B$ receptors and the pharmacological activation of $GABA_B$ receptors decreased neural stem cell proliferation. Our data indicate that signalling through $GABA_B$ receptors is an inhibitor of postnatal neurogenesis.

Chapter 5

Effects of serotonin on ependymal cells

5.1 Introduction

5-hydroxytryptamine, (5-HT) is a phylogenetically ancient neurotransmitter that appears early in development and extends widely throughout the brain. The distribution of 5-HT receptor subtypes in the spinal cord is heterogeneous. There is evidence for postnatal neurogenesis in the brainstem and central canal area of the spinal cord but whether 5HT influences this is not known.

5-HT has been reported to regulate adult neurogenesis (Banasr et al., 2004). The depletion of serotonin in the brain of adult rodents decreases neurogenesis in the hippocampal dentate gyrus (Brezun and Daszuta, 1999).

Previous research has highlighted 2 specific 5-HT receptors in the modulation of adult neurogenesis in the hippocampus; 5-HT_{1A} and 5-HT_{2} and this research is discussed in detail in section 1.9.2 of the Introduction. Research was carried out on the role of different receptors by using agonists, antagonists and partial agonists. Knockout mice were also used to investigate the involvement of 5-HT_{1A} receptors.

A previous report has shown that there is no effect of 5-HT on CSFcCs of spinal cords (Laura Corns, unpublished data), and in fact these cells were found not to express 5-

HT in the rat and mouse spinal cord, unlike the situation in lower vertebrates (Nagatsu et al., 1988).

Unpublished data from our lab has also shown that cisapride (5-HT_4 agonist) application in acute spinal cord slices resulted in higher numbers of proliferating cells in central canal of spinal cord compared to control.

Since 5-HT_{1A}, 5-HT_2 and 5-HT_4 receptors have been shown to have established effects on neurogenesis, this section will elaborate on the effects of activating or antagonising these receptors on ependymal cells since they are considered the neural stem cells of central canal neurogenic niche.

5.2 Hypothesis and aims

This chapter aims to determine whether activation of 5-HT receptors can depolarise or hyperpolarise ependymal cells of spinal cord. If the activation of these receptors does, they may have the potential to influence neurogenesis in spinal cord. The direct or indirect action of 5-HT on postnatal neurogenesis and the cascade involved to promote neurogenesis is still unclear. Since 5-HT expressing axons are known to project onto the supra-ependymal surface of lateral ventricles (Tong et al., 2014; Aghajanian, G.K. et al., 1975), we tested whether similar projections onto ependymal cells occur in the area of central canal of spinal cord. My first hypothesis was that 5-HT fibres would be located in close proximity to the ependymal cells. The second hypothesis was that activation of specific 5-HT receptors may influence the activity of these cells. The 5-HT_{1A} and 5-HT_2 receptors were chosen because the 5-HT_{1A} receptor agonist 8-OH-DPAT produces an increase in cell proliferation in the dentate gyrus (Arnold and Hagg, 2012; Klempin et al., 2010) and 5-HT_2 receptor antagonist cinanserin induces an increase in cell proliferation (Klempin et al., 2010). Cisapride,

a 5-HT_4 receptor agonist was also tested in the light of the changes in cell proliferation observed when this agonist was applied in acute slices.

5.3 Results

5.3.1 Close relationship between 5-HTergic fibres and nestin positive cells in spinal cord in nestin-GFP mouse

The relationship between neurogenic niches and 5-HTergic fibres is one of the factors that determine the likelihood of 5-HT modulating cell proliferation. To investigate this factor, 5-HT immunofluorescent staining was done on nestin-GFP (green fluorescent) spinal cord sections and the areas of close apposition noted. Nestin was used as a potential neural stem cell marker (Hendrickson et al., 2018; Wiese et al., 2004).

Immunohistochemistry using a specific antibody against 5-HT (1:1000; RA 2000; Neuromics) revealed 5-HT immunofluorescent terminals observed in close apposition to ependymal cells (Figure 5.1). The numbers of ependymal cells with close appositions from 5-HT immunoreactive terminals were not quantified but it was clear that not every ependymal cell had labelled close apposition. To investigate whether ependymal cell could respond to 5-HT, it was bath applied to spinal cord slices.

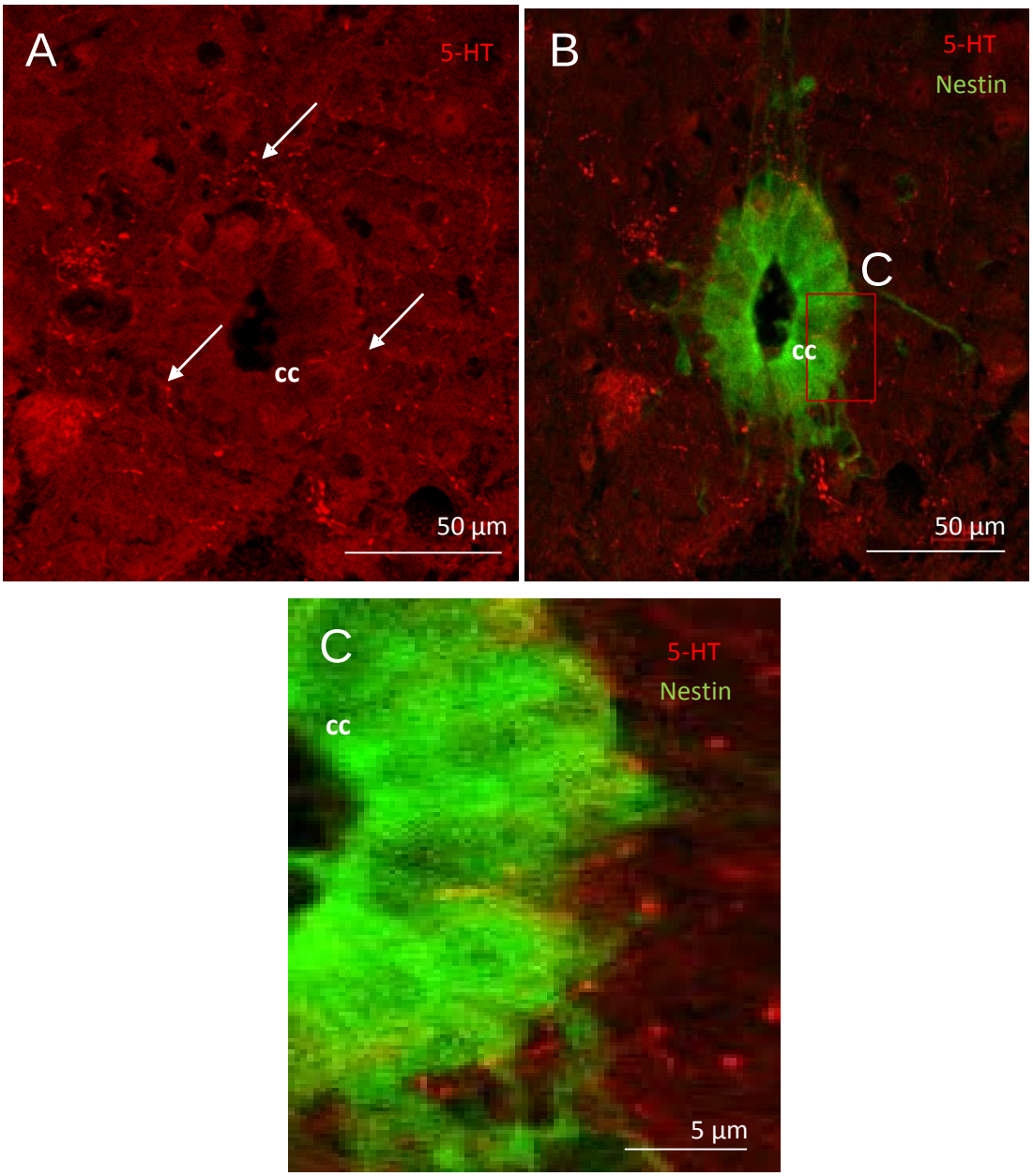


Figure 5-1 Relationship between 5-HTergic fibres and nestin-GFP cells in nestin-GFP mouse.

High power of fluorescent images of spinal cord showing 5-HT fibers in a spinal cord area in nestin-GFP mouse. 5-HTergic fibres (white arrows) are located near the central canal area (A). Nestin-positive neural stem cells (green) (B) 5-HTergic fibres closely apposed nestin-positive neural stem cells (red-squared boxes). Enlarged view of 5-HTergic fibres closely apposing nestin-positive neural stem cells (C), cc=central canal.

5.3.2 Ependymal responses to 5-HT

As ependymal cells responded to GABA, their ability to respond to other neurotransmitters was investigated. In particular, an interaction between ependymal cells and 5-HT was examined due to previous reports of 5-HT containing terminals in the area surrounding the central canal (Rajaofetra et al., 1989).

5.3.2.1 Responses to 5-HT are both depolarising and hyperpolarising

Whole cell patch clamp recordings on ependymal cells revealed that 5-HT at a concentration of 10 μM caused either depolarisation or hyperpolarisation (Figure 5-2). 18 of 27 cells tested responded to the application of 5-HT, the other 9 cells were not affected by this drug. The majority of responding cells (n = 13/18) were depolarised by 5-HT. The mean depolarisation was 1.71± 0.21 mV (n = 13) while the mean hyperpolarisation was -2.11 ± 0.56 mV (n = 5).

The locations of cells responding to 5-HT with either depolarisations or hyperpolarisations were plotted (see Figure 5-3) and it was clear that there was no correlation between location and response type (Figure 5-3) in the medial and ventral regions, however only depolarising responses were observed in the dorsal ependymal cell region.

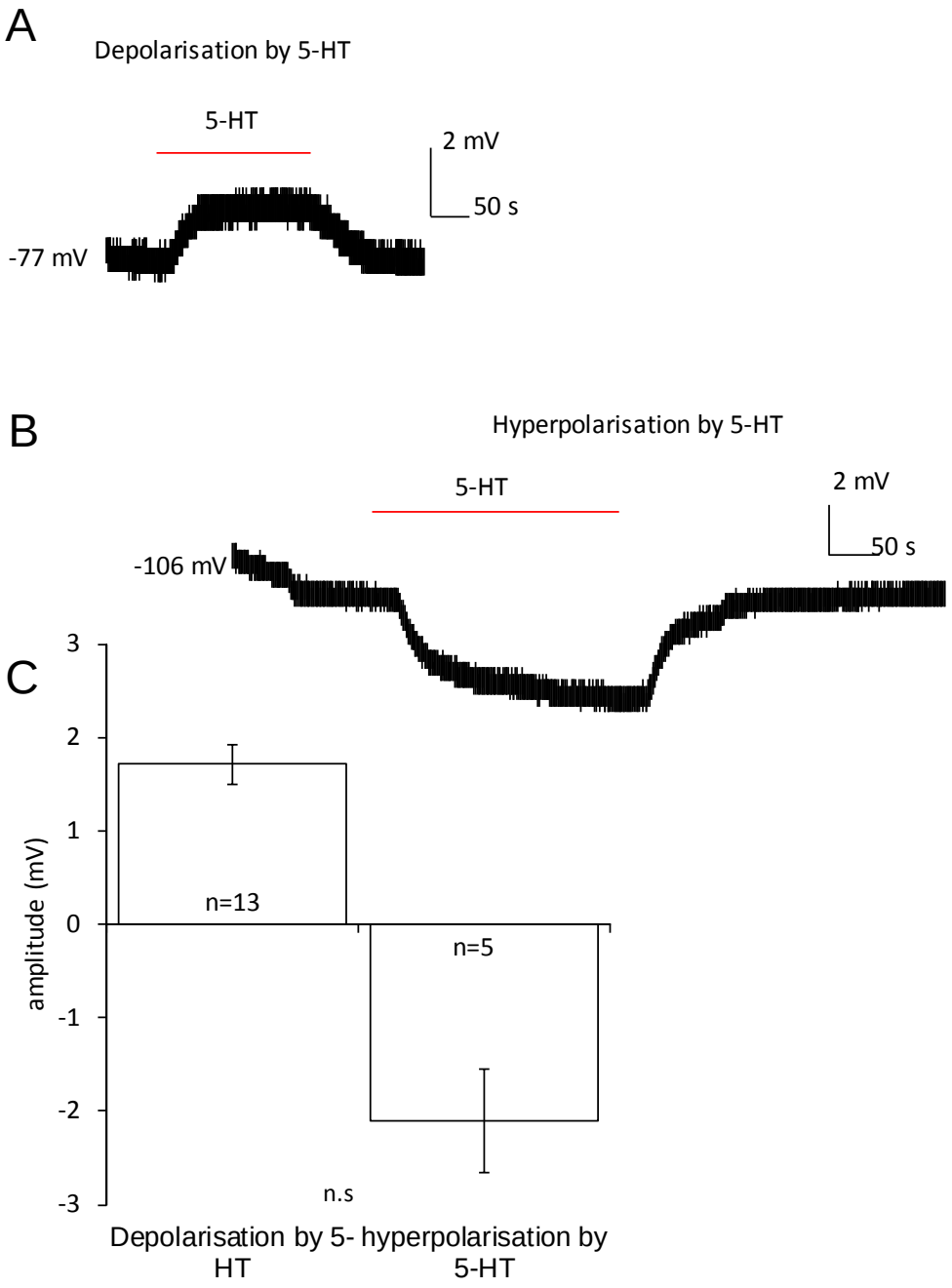


Figure 5-2 Responses of ependymal cells to 5-HT were either depolarisations or hyperpolarisations

Examples of whole cell patch clamp recording of ependymal cells which were (A) depolarised and (B) hyperpolarised by 10 μM 5-HT. From 27 cells, 13 cells were depolarised by 10 μM 5-HT, 5 cells were hyperpolarised and 9 cells showed no response to 10 μM 5-HT. Pooled data are shown in C showed the depolarisations and hyperpolarisations cell number with mean amplitude.

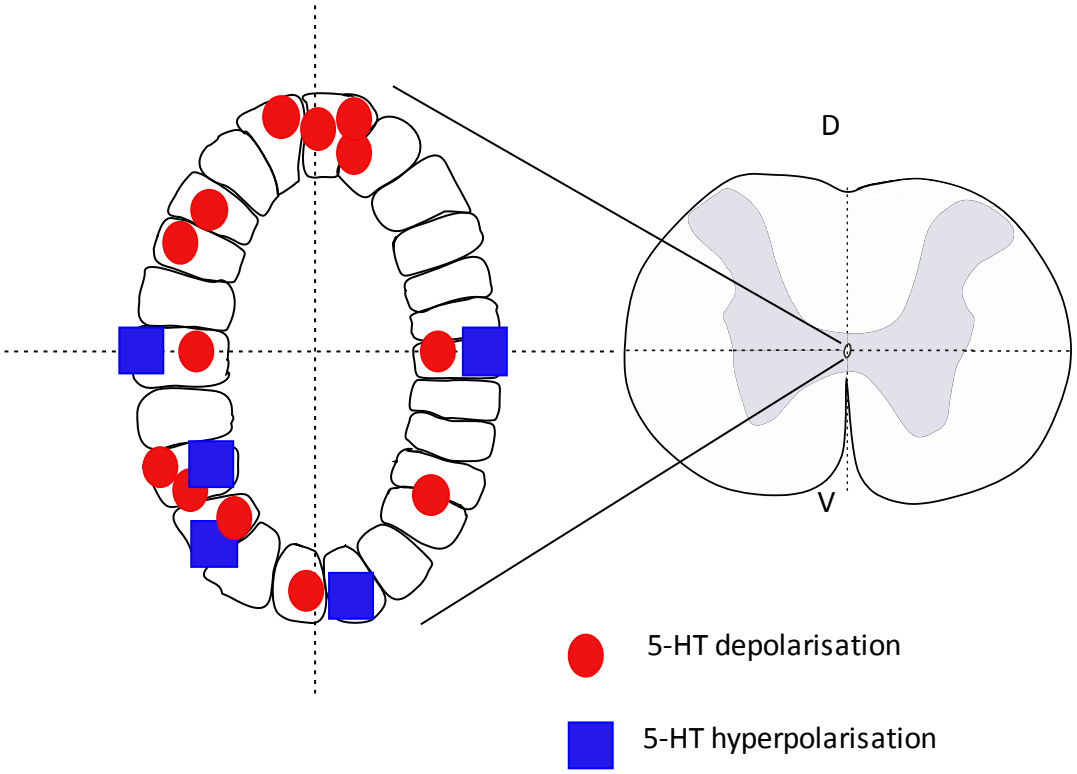


Figure 5-3 Location of ependymal cells exhibiting responses to 5-HT in central canal area

Location of ependymal cells exhibiting the different responses to 10 μM 5-HT. Whole cell patch clamp recordings were taken from throughout the area of central canal. 5-HT was applied using the bath solution.

5.3.3 Which 5-HT receptors were involved in responses observed in ependymal cells?

A number of agonists and antagonists were used to establish which receptors may be underlying the responses to 5-HT. Since 5-HT_2 receptors are known to be present in the spinal cord (Doly et al., 2004; Marlier et al., 1991), the effects of the 5-HT_2 antagonist; cinanserin on 5-HT induced depolarisations were determined on 8 cells that were depolarised by 5-HT. In seven of these cells, the 5-HT mediated depolarisations were significantly antagonised by 20 µM cinanserin, (1.63 ± 0.3 mV to 0.62 ± 0.2 mV, Figure 5-4).

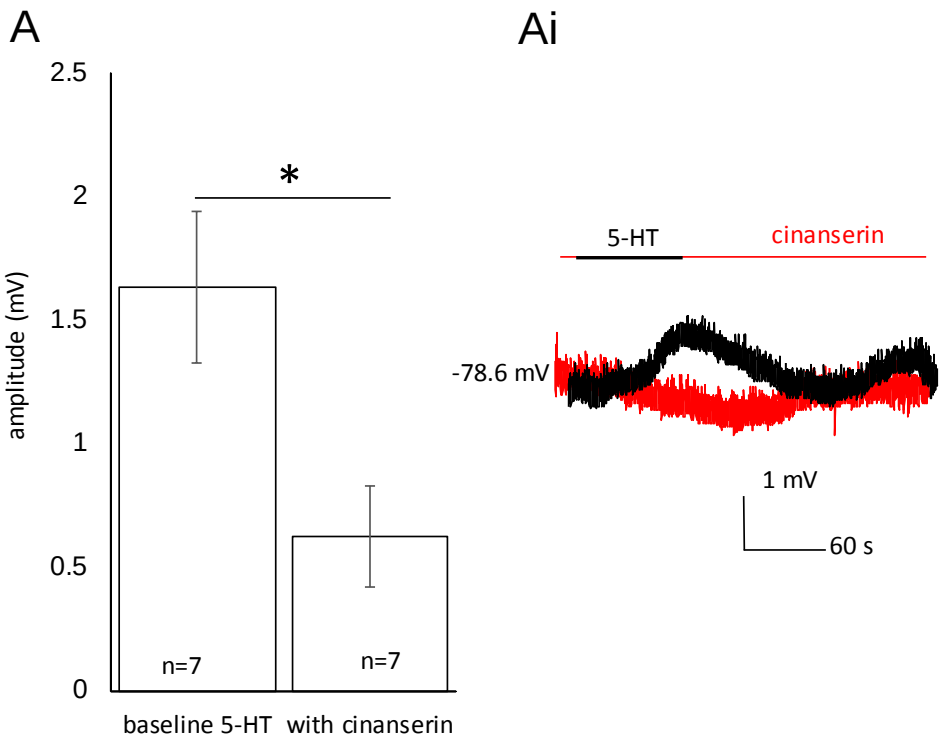


Figure 5-4 Depolarisations in response to 5-HT and were antagonised by cinanserin

An example of recording from whole cell patch clamp traces demonstrating that the depolarisations in response to 5-HT (10 µM) were antagonised by cinanserin (10-20 µM) ($p \leq 0.05$, n=7).

Since unpublished results of in vitro experiments from Deuchars Lab revealed that sections of spinal cord treated with a full agonist at 5-HTR_4 had higher numbers of proliferating cells, the effects of this drug on ependymal cell responses were determined. In slices treated with 5-HTR_4 agonist (cisapride), two different experiments were conducted with different concentrations. Only when using 10 μM of 5-HTR_4 agonist (cisapride) were there significantly more EdU positive cells in central canal area compared to control (3.6 ± 0.3 vs 2.59 ± 0.2; $p \leq 0.05$) (Ghani and Deuchars, unpublished observations). Therefore this concentration was used.

When proceeded with the whole cell patch clamp recordings on ependymal cells with cisapride 10 μM and of 5 cells tested, only 1 cell responded with a very small amplitude hyperpolarisation (0.8 mV, Figure 5-5 A).

The other receptor probed was 5-HT1A since Marlier et al. (1991) also reported that this receptor was expressed in the central region of the spinal cord. Of 6 cells tested with applications of the 5-HT 1A receptor agonist 8-OH-DPAT, only 2 responded with hyperpolarisations (Figure 5-5 B).The other traces showed no responses to 8-OH-DPAT.

This suggests that the major receptor mediating depolarisations is the 5-HT_2 receptor while small hyperpolarisations are mediated through either 5-HT_4 or 5-HT_{1A} receptors. The locations of all cells tested and their responses showed little correlation between types of response elicited and the location of the cell recorded (Figure 5-6).

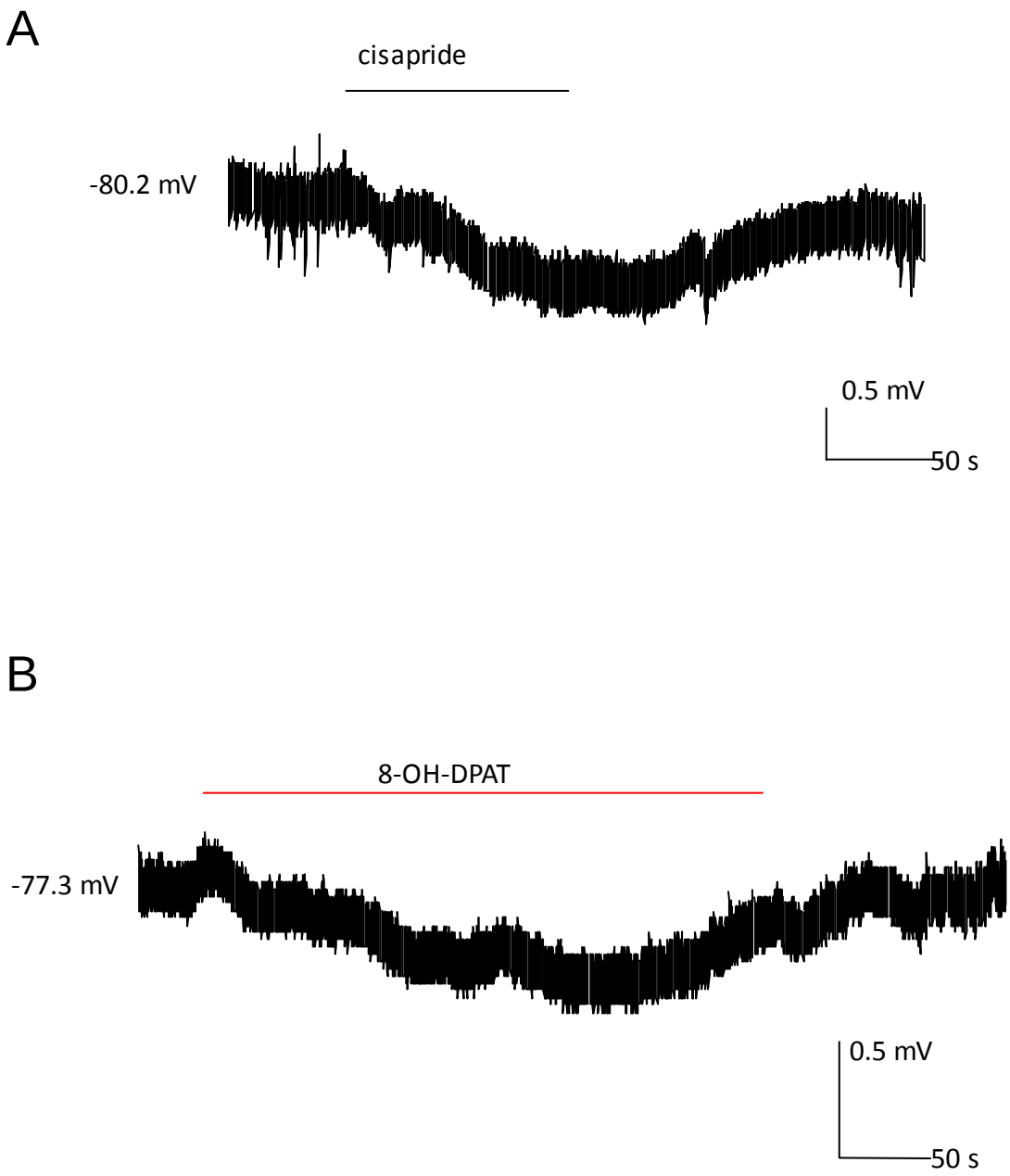


Figure 5-5 Responses of ependymal cells to cisapride and 8-OHDPAT

Whole cell patch clamp trace demonstrates hyperpolarisation in response to cisapride (10 μM) (A). Whole cell patch clamp trace demonstrates the hyperpolarisation in response to 8-OH-DPAT (40 μM) (B).

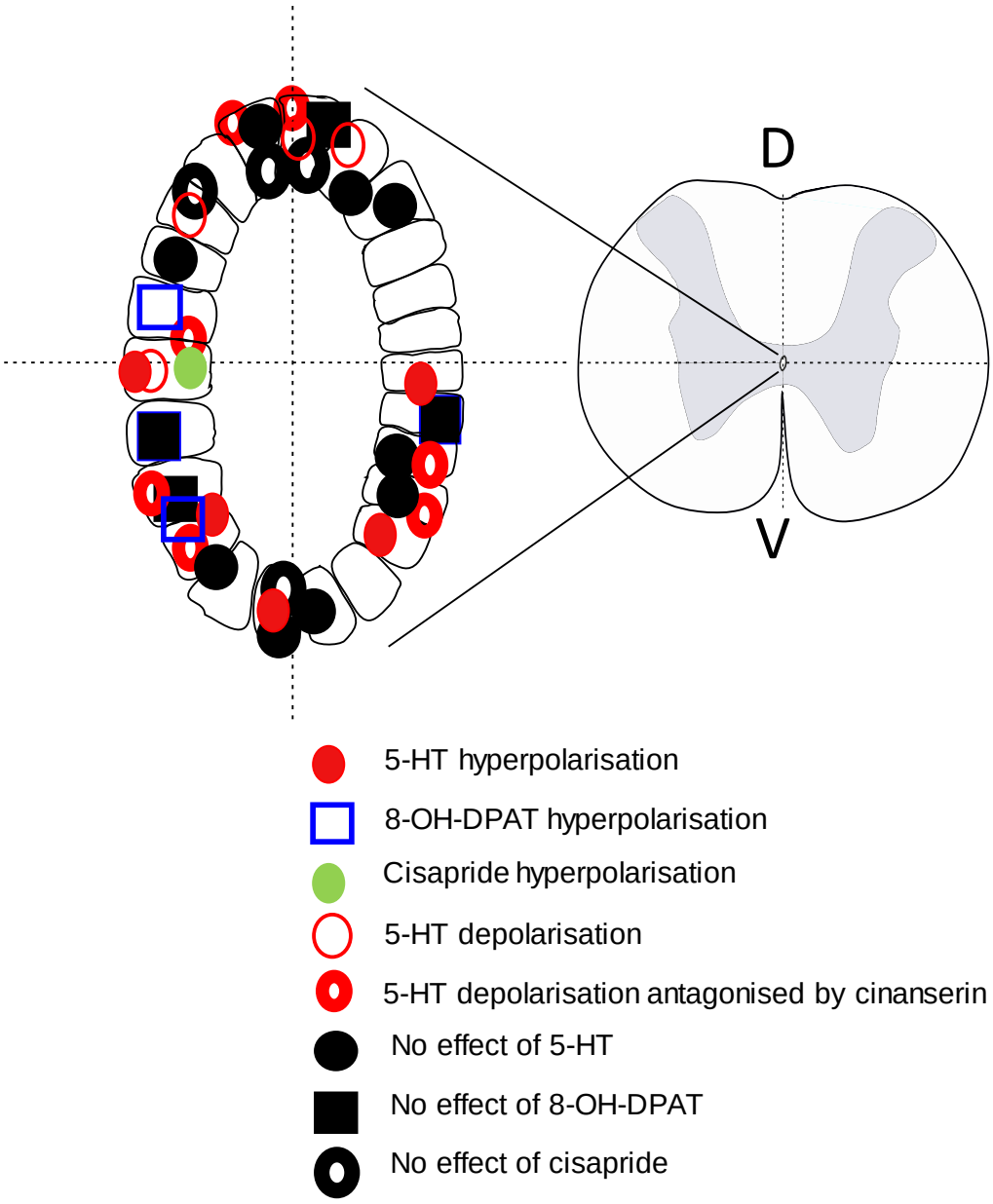


Figure 5-6 Location of ependymal cells exhibiting the different responses to 5-HT, agonist and antagonist.

Whole cell patch clamp recordings were taken from throughout the area of central canal. There is little correlation between specificity for the type of response observed and location of the cell.Dorsal pole cells showed depolarisation whereas ventral and midline cells showed hyperpolarisations. 5-HT were applied locally in the bath solution.

For summary, according to localisation of cell and the corresponding type of responses, hyperpolarisations occurred in the cells located mainly ventrally to the central canal and any effects of cisapride and 8-OH-DPAT were mainly in cells located around the midline of the central canal area (Figure 5-6).

5.4 Discussion

In this chapter, I have shown that 5-HT immunoreactive fibres form close appositions with the cells in the ependymal cell layer and furthermore that application of 5-HT can cause both depolarisations and hyperpolarisations of ependymal stem cells, which are mediated through activation of different 5-HT receptors.

5.4.1 The presence of 5-HT fibres close to ependymal cells suggest a possible neuromodulatory role

There is a close apposition of 5-HTergic fibres with Nestin-expressing neural stem cells localized at the central canal of spinal cord. Using nestin as neural stem cell marker that showed consistent results in a previous study (Hendrickson et al., 2018), nestin-GFP mice were used to identify the location of neural stem cells in the central canal of spinal cord. We observed that the nestin-GFP cells with high fluorescent intensity were confined to the central canal area. My findings suggest that 5-HT can influence the activity of nestin-positive neural stem cell as the position of these fibres is very close to the cell surface and by modulating 5-HT levels, we may be able to modify the degree of cell proliferation and cell differentiation at that area.

Serotonergic fibres, whose cell bodies reside in the raphe nuclei, travel down to all levels of spinal cord. The tracts are located at the dorsolateral funiculus then ramify

into the grey matter and establish the synaptic contacts with neurons that are involved in somatic outflow (Perrier and Cotel 2015). In addition, there are serotonergic inputs onto autonomic preganglionic neurones and interneurones (Deuchars, S. and Lall, 2015). 5HT fibres are also located in the dorsal horn of spinal cord (Croul et al., 1998; Thor et al., 1993). At the thoracolumbar level, the serotonergic fibres are concentrated in the intermediolateral part of spinal cord and then course medially to surround the central canal area. Detection of 5-HTergic distribution in nestin-GFP mice showed close apposition of 5-HTergic fibres with nestin-positive neural stem cell in central canal of spinal cord. This the first time that such innervation of potential stem cells has been observed in rodents but previous studies in turtle have shown a dense 5-HTergic plexus of fibres innervating central region and forming close appositions with radial glia and immature neurones, most probably the CSFcCs (Trujillo-Cenóz et al., 2007). They suggested that 5-HT may play a role in proliferation, migration or differentiation in turtle and this may also be the case in rodents.

5.4.2 Responses of ependymal cells to 5-HT

It is clear from the data that not all ependymal cells responded to either 5-HT or the specific agonists that were applied and this may be due to heterogeneity in this cell population with respect to the 5-HT receptor expression. The locations of cells that did respond to 5-HT indicated that there was a degree of region specific effect of 5-HT with cells at the dorsal pole only depolarised in response to 5-HT while those located at or ventral to the midline regions were more likely to be hyperpolarised. Furthermore, depolarisations were antagonised by the 5-HT_2 antagonist cinanserin. The majority of cells at the dorsal and ventral poles of the central canal are considered to be radial glia while the cells at the lateral regions are more likely to be cuboidal ependymal cells (see introduction). In response to injury, the majority of newly

proliferated cells are located at the dorsal and dorsolateral regions (Alfaro-Cervello et al., 2012). This suggests that these cells may be more responsive to perturbations and perhaps 5-HT acting at 5-HT_2 receptors on these dorsal cells may contribute to mediating this response. However, the 5-HT_2 receptor agonist α-methyl-5-HT (1 and 10 μM) (1.39 ± 0.3 vs 2.4 ± 0.3; 3.23 ± 0.3 vs 2.59 ± 0.2) did not affect levels of proliferating cells per 50 μm section in vitro when applied for 4 hours (Ghani et al unpublished observations). This is interesting since it suggests that activation of 5-HT2 receptors does not initiate short-term proliferative responses. This is similar to that observed by Banasr et al. (2004), who reported little effect of 5-$HT_{2A/C}$ agonists on hippocampal cell proliferation with short term treatment (one injection, administered 2 hours before BrdU and then the animals were sacrificed 2 hours after that). In contrast, longer term administration of the 5-HT_2 receptor agonist methyl-5-HT (for 1 or 7 days following BrdU injections) decreases hippocampal cell proliferation (Klempin et al., 2010). This therefore suggests that 5-HT may activate 5-HT_2 receptors on some classes of spinal cord ependymal cells and that long-term activation of these receptors may start to contribute to the proliferative responses. It would therefore be worthwhile testing the effects of 5-HT_2 receptor activation on numbers of proliferating cells in spinal cord slices maintaining in culture for up to 7 days. By monitoring the cells using time lapse microscopy, it may be possible to observe which cells in the different regions are most affected.

I also observed that there are no ependymal cells responsed to 5-HT_{1A} agonists at the dorsal and ventral poles of the central canal, although the numbers of cells tested in these regions was relatively small. Given the small responses of 8-OH-DPAT to the ependymal cells, maybe there are only low numbers of 5-HT_{1A} receptors located at the postsynaptic cell membrane. Further experiments could increase n numbers to establish whether this was indeed the case. In unpublished data from our laboratory, various antibodies to 5-HT_{1A} receptors were tested to look at expression patterns of

this receptor but the staining was not good and it seems that the established antibodies do not work well in the spinal cord. However, unpublished data from Deuchars Lab showed that 8-OH-DPAT applied to acute spinal cord slices (4 hours treatment, 30 μM concentration) did not significantly affect the numbers of proliferating cells in the central canal region (6.07 ± 0.5 vs 6.83 ± 0.5 cells per 50 μm section) so potentially, this receptor does not play a prominent role in modulation of proliferation in the spinal cord. This contrasts with other neurogenic niches (Klempin et al., 2010; Banasr et al., 2004).

Only one cell of the 5 tested, responded to cisapride with a small hyperpolarisation, the cell was located on the lateral point of the central canal. This is somewhat surprising since cisapride (at the same concentration used here, 10 μM) applied to spinal cord slices *in vitro* for 4 hours, caused significantly more EdU positive cells per 50 μm section in the central canal area compared to control (3.6 ± 0.3 vs 2.59 ± 0.2 cells ; $p \leq 0.05$). It is possible that responses to 8-OH-DPAT and cisapride were being missed in ependymal cells because the examined cells did not express the 5-HT_{1A} and 5-HT_4 receptors or potentially the G proteins necessary to mediate the response are being washed out by the intracellular solution from the patch pipette, although this does not occur with 5-HT alone.

There are a few other drug choices such as tegaserod that can be used in the experiments. Tegaserod maleate is a partial agonist for 5-HT_4 receptors. *In vivo* experiments showed that activation of 5-HTR_4 receptors using tegaserod maleate resulted in greater numbers of proliferating cells in mice gut of enteric system (Liu, M.-T. et al., 2009).

5.4.3 What could be the functional effects of 5-HT receptor activation on spinal cord plasticity and proliferation?

Altman (1969) suggested that cells proliferate and migrate from subependymal layer of lateral ventricle to olfactory bulb in normal conditions. This same process that occurs in brain areas might also apply to the spinal cord area. In an interesting study on proliferative responses to injury, Rusanescu and Mao (2015) suggested the migration of proliferating cells could occur from lamina X to the dorsal horn of spinal cord area after constriction injury of sciatic nerve in rats. Since neuropathic pain can stimulate an increase in the level of 5-HT (Rusanescu and Mao, 2015), it may be that this 5-HT then activates the ependymal cells to proliferate and migrate to the dorsal horn. To explore this further, the use of mice where ependymal cells are labelled with fluorescent markers for fate mapping (using a FoxJ1 promoter) would enable cells originating from the central canal to be visualised as they proliferate and migrate (Barnabé-Heider et al., 2010).

In many studies, acute application of serotonergic drugs showed lack of effects on neurogenesis. Malberg et al. (2000) found that ranylcypromine, reboxetine, fluoxetine and haloperidol only increased BrdU labeling after chronic (14 to 28 days), but not short-term (1 to 5 days) administration. This may be due to the fact that most of the drugs have long time courses for their therapeutic action. However, there is also evidence of acute effects on serotonin receptor activation observed in studies on selective serotonin reuptake inhibitors (SSRIs) (Klempin et al., 2010; Santarelli et al., 2003). Furthermore, in vivo, acute treatment with 8-OH-DPAT (2 h pre BrdU treatment) significantly increased precursor cell proliferation whereas 1 week of treatment had no significant effect (Klempin et al., 2010). Therefore it is clear that both the receptors are being activated and the timing of the drugs will influence

potential neurogenic effects and this must be carefully considered when carrying out experiments on spinal cord.

5.5 Conclusion

Despite uncertainty regarding the receptors responsible, the important observation of this study is it demonstrate that the ependymal cells are capable of responding to 5-HT. Molecular mechanisms of the 5-HT receptor mediated effects on spinal cord neurogenesis are yet to be determined.

Chapter 6

General discussion

6.1 Impact of this study

The region surrounding the central canal is proposed as a latent neural stem cell niche. This study observed the characteristics of ependymal cells, which are considered as a source of neural stem cells in the spinal cord, with an emphasis on a potential role of GABAergic signalling in controlling activity in these cells. This study has demonstrated the complexity of GABA mediated control of spinal cord ependymal cell activity, showing a potential role for multiple receptors and modulation sites on these cells. This study also included elaboration on the 5-HT receptor effects on ependymal cells as 5-HT receptors also have been shown to have influence on neurogenesis.

6.2 Applicability of these findings to neurogenesis

6.2.1 Ependymal cells may function as the neural stem cells of spinal cord

The identity of stem cells in the postnatal spinal cord has been difficult to establish and remains controversial. Although there are some studies which described the grey and white matter of the rat spinal cord as containing proliferating cells (Horner, P.J. et al., 2000), yet other studies showed the majority of stem cells reside within the central canal of spinal cord (Corns et al., 2015; Marichal et al., 2012; Trujillo-Cenóz et al., 2007) which is comprised of both ependymal cells and CSFcCs. The ependymal region is a complex structure consisting of cells arranged in lateral and dorsal-ventral domains (Marichal et al., 2012; Trujillo-Cenóz et al., 2007). Ciliated ependymal cells with basal processes mainly predominate in lateral domains of central canal while radial glial like cells are mainly concentrated in both polar region. In contrast, CSFcCs do not have specific locations and are not considered to be neural stem cells, at least in mammals. The concept of the ependymal cells being neural stem cells is therefore considered throughout this general discussion.

6.2.2 Relevance of gap junction coupling of ependymal cells within the spinal cord neurogenic niche

We used patch-clamp electrophysiology to assess passive and active electrical properties of spinal cord cells that are in the neurogenic niche region, namely ependymal cells and CSFcCs. Evidence from my experiments, it was clear that the

majority of ependymal cells exhibited extensive gap junction coupling but the role of this coupling is unclear, since it cannot fulfil the same role as that observed in neurones; i.e. to enable synchronised neuronal firing. It has been proposed that coupled ependymal cells can act as regulators of extracellular K^+ in the grey matter by taking up K^+ into their basal membranes, in regions where K^+ levels are high and then shunting K^+ through the gap junctions to the apical surface and from there into the CSF (Jarvis and Andrew, 1988). This has been suggested for ependymal cells in other regions, such as hypothalamus (Jarvis and Andrew 1988). Another suggestion is that the gap junctions enable synchronisation of the movements of the cilia, which also helps with movement of the CSF (Jiménez et al., 2014).

Another possible reason for ependymal cells to form gap junctions is to allow the control of cellular proliferation (Bittman et al., 1997). During development, the coupling occurs only between radial glia and neural precursor cells, and is cell cycle phase dependent; uncoupling the cells decreases the number of cells that enter the S (DNA replication phase) of the cell cycle (Bittman et al., 1997). It appears that it is the propagation of Ca^{2+} waves through gap junctions that influences this proliferation. This was demonstrated in the embryonic ventricular zone, as reducing the Ca^{2+} waves between neighbouring radial glia reduces the ventricular zone proliferation (Weissman et al., 2004).

This suggests that the propagation of Ca^{2+} waves through gap junctions is a common mechanism that influences proliferation in both the embryonic and postnatal brain. It is possible to suggest that this common mechanism could be extended to the postnatal spinal cord, with the propagation of Ca^{2+} waves between ependymal cells and other coupled cells (CSFcC Type 1), controlling the constant proliferation of ependymal cells that occurs to maintain the ependymal cell population.

6.2.3 GABAergic responses in ependymal cells may indicate that they are part of the spinal cord circuitry - implications for the neurogenic niche.

GABA is the key regulatory neurotransmitter in the developing brain as well as the main inhibitory neurotransmitter in the adult central nervous system. Extracellular GABA levels are regulated by high affinity sodium and chloride dependant GABA transporters (BORDEN, 1996) which efficiently removes GABA from synaptic clefts. There are possible endogenous sources of GABA which could influence ependymal cells and neighbouring cells.

The first source is from GABAergic terminals that are known to be present in the area surrounding the central canal. A comprehensive study of the GABAergic cells in the vicinity of the central canal suggested that at least a proportion of these may also co-release acetylcholine (Gotts et al., 2016), which is of interest since ependymal cells also respond to this neurotransmitter (Corns et al 2015). GABA may also be released from terminals associated with cells that are distant from the spinal cord, such bulbospinal cells originating in the rostral ventrolateral medulla identified by Deuchars, S.A. et al. (1997). Since in these experiments, I have always exogenously applied GABA, the possibility that GABA being released from distant terminal cannot be ruled out. The second source is from the CSFcCs. Many studies have shown that CSFcCs express GABA in rat and mouse (Corns et al., 2013; Marichal et al., 2009; Stoeckel et al., 2003; Barber et al., 1982) and also in other species such as turtle (Russo et al., 2004). These cells may therefore release GABA into the CSF region where it can activate receptors on the ependymal cells. Alternatively, the CSFcCs may release the GABA at different sites, potentially through GABA transporters on their membrane surface or from non-synaptic sites yet to be identified.

GABA binds to ionotropic receptors $GABA_A$ and $GABA_\rho$ which are ligand gated chloride channels, and metabotropic receptor $GABA_B$ to trigger downstream signalling events. So the presence of these receptors in the neural stem cells of spinal cord and the effects of their activation supports the idea that they modulate ependymal cell activity.

The effect of GABA depends on the type of receptor it binds to and on the intracellular Cl^- concentration. The subunit composition of the receptor determines the subcellular localisation, conduction, kinetics and pharmacology (Sibbe and Kulik, 2017). $GABA_A$ receptors are localized both synaptically and extrasynaptically. The former are activated by high GABA concentrations released into the synaptic cleft and generate fast inhibitory postsynaptic currents. Extrasynaptic $GABA_A$ receptors are present peri- and extrasynaptically. These receptors are activated by lower concentrations of GABA arising spilled over from nearby synapses.

GABA receptors containing α_1, α_2 and γ_2 subunits are often present at the synapse and mediate phasic inhibition, while receptors containing α5 and δ are often extrasynaptically localized and mediate tonic inhibition (Farrant and Nusser, 2005). Metabotropic $GABA_B$ receptors are coupled to guanosine triphosphate (GTP)-binding proteins (G proteins) and are responsible for mediating slow effects. GABA binding leads to a conformational change of the receptor, which is transmitted to the Gα subunit of the G protein, leading to the exchange of GDP for GTP (Ulrich and Bettler, 2007; Bettler et al., 2004). The GTP bound Gα subunit then dissociates from the other two subunits of the G protein, the β and γ subunit. Gα subunit can activate phospholipase C or inhibit the enzyme adenylyl cyclase, which reduces the levels of secondary messenger cyclic adenosine monophosphate (cAMP), this in turn leads to the inactivation of the cAMP dependent protein kinase A (PKA). The two pathways activated by Gα are some of the most important cellular signalling and can regulate

different processes, modulation of the activity of different ion channels (Terunuma, 2018).

The subunit composition of receptors on ependymal cells and the sensitivity of these to GABA may influence which of the receptors are activated by GABA from different sources.

6.2.4 DBI is expressed in ependymal cells and is responsible for modulation of $GABA_A$ receptors.

Previous work has shown that ependymal cells of spinal cord respond to GABA (Corns et al., 2013; Reali et al., 2011). There are important sites in the $GABA_A$ receptor that are responsible for modulating the effects of GABA. DBI is strongly and specifically expressed in all NSCs in the SVZ and in a population of transient amplifying cells (Alfonso et al., 2012). Since ependymal cells are considered as the spinal cord neural stem cell, I investigated modulation of these GABA receptor sites. The presence of DBI in stem cells from SVZ and SGZ suggests that the function of this protein might be related to the early process of neurogenesis (Dumitru et al., 2017; Alfonso et al., 2012) in adult brain, so maybe there would be a similar function of this protein in the spinal cord NSCs.

Modulation of GABA receptors by midazolam is likely to occur at dual sites, which are CBR and TSPO. The finding of this study showed that the response to GABA was inhibited by midazolam while the response to GABA was augmented by FGIN-1-27, an agonist at TSPO suggests that the effect at the CBR predominates with midazolam, causing an overall inhibition. Since my data indicate that DBI acts by modulating GABA signalling partly through the CBR, there is a potential of DBI to

have influences on regulating neurogenesis in the spinal cord. Future experiments could determine this and are described below in the section of future work.

The lack of effect of ODN on responses to GABA in my studies is in sharp contrast to the reduction in GABA responses induced by ODN in the subventricular zone (Alfonso et al., 2012). The reduction in GABA currents induced by ODN in their study was strongly diminished in gamma2 F77I mice which is an optimal mouse line to test whether DBI modulates neurogenesis via the $GABA_A$ receptor. I saw significant decreases in responses to GABA with midazolam. Therefore, using gamma2 F77I mice, would be useful to test if midazolam modulation of GABA on ependymal cells still occurs in the ependymal cells. The effects of ODN were intriguing, since overall no significant effect of ODN on GABA responses was observed, yet there was the strong immunopositive DBI and ODN staining around the whole of the central canal region. This suggests that there is further complexity to the role of ODN at the GABA receptors and potentially, we need to carry out correlated electrophysiology and immunohistochemical or mRNA-seq analysis of single cells to understand what GABA receptor subtypes are expressed in each cell and how they are affected by ODN. This idea is further developed in the section on future experiments.

Activation of TSPO may stimulate neurosteroidogenesis (Tokuda et al., 2010). The effects of application of a neurosteroid, allopregnanolone on responses of cells to GABA in our observations did not reach significance for both concentrations of allopregnanolone. The lack of response may be due to a high degree of variation in the amplitude of the responses of ependymal cells to GABA, both before and after allopregnanolone. Another possibility is that the types of receptor present on the ependymal cells vary and some are modulated differently by allopregnanolone. Increasing numbers of recorded cells may establish whether there are clear subclasses of cells with responses to GABA that are differentially modulated by this neurosteroid. Furthermore, further experiments using FGIN-1-27 in the presence of

finasteride, a blocker of neurosteroidogenesis could investigate whether in the spinal cord, the effects on GABA responses of activation of TSPO by FGIN-1-27 are indeed through stimulation of neurosteroidogenesis.

The potential sites of action of these modulators are summarised in Figure 6-1. DBI with/or ODN acts as positive and negative modulator at CBR. Other than CBR, there is also involvement of TSPO evidenced by modulation by midazolam and FGIN-1-27. The modulation of GABA receptor causes increases in neurosteroids production through neurosterodogenesis via TSPO. The neurosteroids; allopregnanolone: 1 μM and 10 nM may act both as allosteric modulators and directly at the $GABA_A$ receptor.

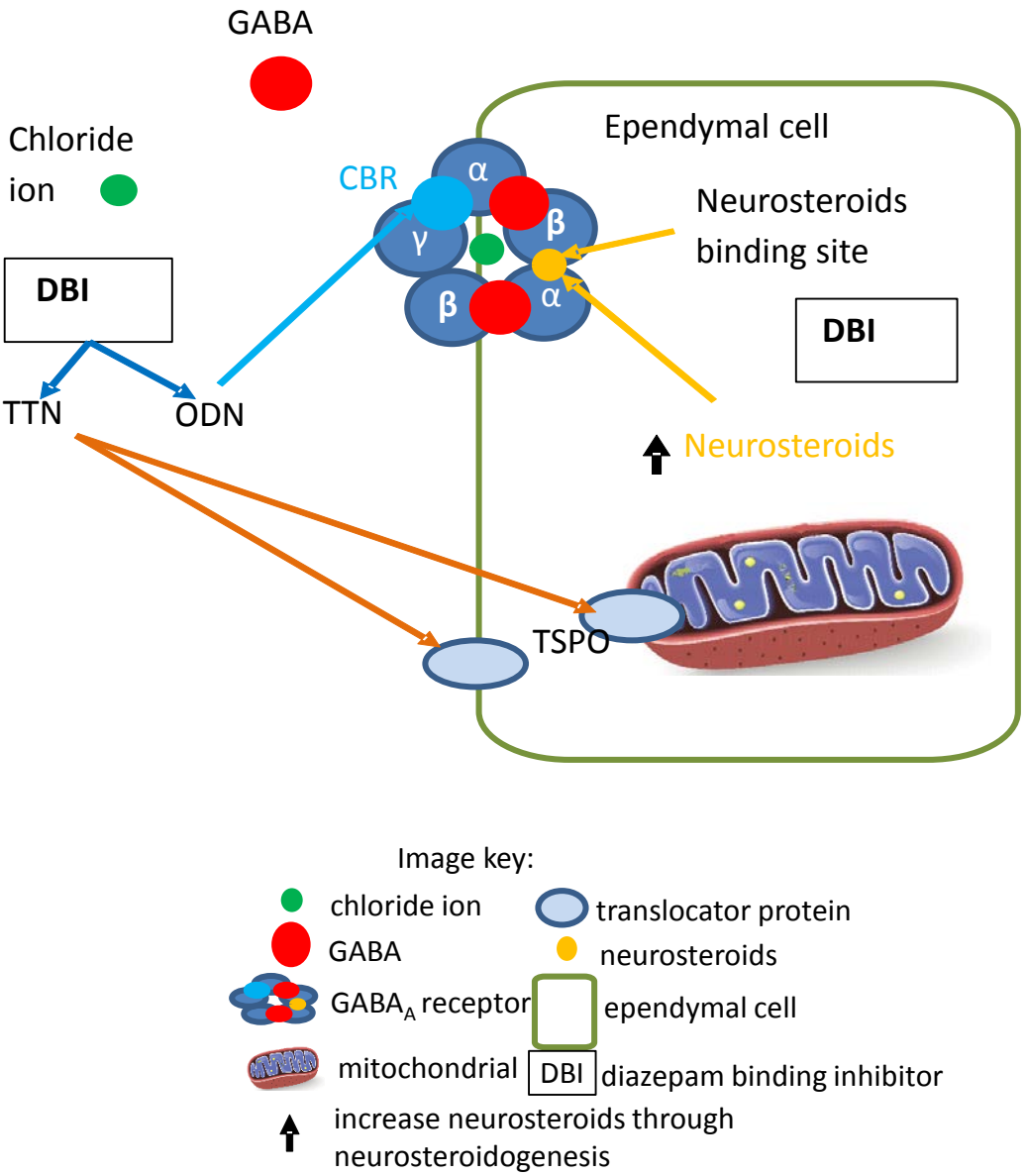


Figure 6-1 Potential mechanisms of action of DBI through CBR and TSPO in ependymal cell.

6.2.5 A reduction in proliferation of ependymal cells was observed, mediated by activation of $GABA_B$ receptors

My results on the role of $GABA_B$ receptors showed a direct activation of receptor with agonist baclofen, resulted in a hyperpolarising response. The hyperpolarisations were antagonised but not fully blocked with CGP 55845, a $GABA_B$ receptor antagonist. On using acute slices to see the effects of activating the $GABA_B$ receptor on ependymal cell proliferation, there was a clear and significant decrease in the numbers of proliferating cells in the central canal region. To further prove that this effect is through an action at $GABA_B$ receptors, CGP 55845 could be applied alongside baclofen to determine if the decreased proliferation was no longer observed.

My work suggests that ependymal cells have a diverse range of GABA receptors modulating their overall activity, with both hyperpolarising and depolarising responses observed. To dissect out the contribution of these different responses to the proliferative capabilities of the ependymal cells, it is important to consider further experiments using EdU labelling of dividing cells in combination with selective drugs used in this study that caused depolarising and hyperpolarising responses. If these experiments were carried out *in vivo*, then it would be possible to also investigate the fate of these newly proliferated cells. This would give a comprehensive picture of how GABA can modulate proliferation and differentiation of the neural stem cells in the spinal cord.

6.2.6 Can activation of the 5-HT receptor also contribute to the proliferative capabilities of the ependymal cells?

Results from this study also show that a population of ependymal cells can respond to 5-HT with both depolarisations and hyperpolarisations. Blockade of 5-HT receptor mediated depolarisations with cinanserin indicate the possible presence of 5-HT_2 receptors in the spinal cord. Furthermore, hyperpolarisations with 8-OH-DPAT indicate that 5-HT_{1A} receptors are also present. Since raphe nuclei neurones are the sole source of 5-HT terminals in the spinal cord (Deuchars, S. and Lall, 2015), it is likely that under normal circumstances, these receptors would be activated by release of 5-HT from these terminals. Given that antidepressant drugs can promote adult neurogenesis and new hippocampal neurons have been implicated in mediating some effects of antidepressants (Petrik et al., 2012), our findings are relevant to human disease. A role of both $5HT_2$ and $5HT_{1A}$ receptors in other neurogenic niches has been reported (Klempin et al., 2010). Therefore manipulating the receptors to induce proliferation and differentiation using specific drugs would be a valid next step to determine a potential role in spinal cord neurogenesis.

6.2.7 Ependymal cells contribute to increased cell division following spinal cord injury

How tissue injury can activate ependymal cells to trigger endogenous repair needs further clarification. It is not possible to infer that only ependymal cells are responsible for the proliferation of cells during spinal cord injury even though both mouse and rat with and without endogenous repair capabilities, respectively showed an increase in

the proliferation of ependymal cells (Meletis et al., 2008; Mothe and Tator, 2005). Some cells in the central canal still react to injury by proliferating and migrating toward the lesion (Mothe and Tator, 2005; Johansson et al., 1999) and some cells show proliferation and become astrocytes within the circle area of the scar (Meletis et al., 2008). Ependyma-derived cells seem to play a central role in the formation of the scar thereby limiting the extension of damage (Sabelström et al., 2013). Astrocyte-like and oligodendrocyte-like cells derived from the ependymal cells concentrate in the core of the scar and release growth factors that improve the survival of neurons around the lesion (Sabelström et al., 2013). However, Ren et al. (2017) questioned the actual contribution of ependymal cells to scar formation suggesting that more research is needed to understand the potential of the central canal area as a source for repair. Using FoxJ1 genetic knock-in cell fate mapping of ependymal cells, together with immunohistochemical labelling of astrocytes, they showed ependymal cell progeny contribute less than 2% of the total newly proliferated astrocytes. Furthermore they showed that ependymal cell contribution to other cell types after spinal cord injury is also minimal, ependymal progeny fail to migrate substantially into spinal cord injury lesions and remain primarily in the area of the damaged ependymal layer around the central canal. Finally, they indicate that ependymal progeny do not contribute any cells to nearby spinal cord injury lesions that do not directly damage the ependymal layer.

Two transgenically-targeted fate mapping strategies with a Nestin-CreERT line and a human FOXJ1-CreERT line used by previous studies (Sabelström et al., 2013; Barnabé-Heider et al., 2010; Meletis et al., 2008) differ in their findings from the study done by Ren et al. (2017). The difference may be due to recombination patterns or pattern of spinal cord injury used among them. After all the pattern of spinal injury may be the prominent cause of these differences because the extent of the injury would signify the involvement of ependymal layer or not.

6.3 Applicability of the findings regarding the neurogenic potential of ependymal cells to human

Cells in the adult human spinal cord ependymal region do not proliferate after injury at any time or distance from the lesion (Paniagua-Torija et al., 2018). Their findings seriously challenge the view of spinal cord ependymal region as a neurogenic niche in adult humans. However other studies show increases in nestin positive cells in the spinal cord region in post-mortem tissue from humans (Cawsey et al., 2015), so there are discrepancies in the literature and few studies have been possible of this nature. What is clear is that in humans, we have no knowledge of whether manipulating or modifying the activity of the ependymal cells with neurotransmitters can affect levels of proliferation.

6.3.1 Can ependymal cells and GABA levels contribute to increased cell division in ependymoma?

Ependymal tumours are a group of central nervous system tumours, where the tumour contains some cells that resemble ependymal cells. Ependymomas express cell surface markers CD133, Nestin, brain lipid-binding protein (BLBP) which are also expressed by neural stem cells (Hadjipanayis and Van Meir, 2009). Ependymomas also show most of the unique features of ependymal cells including lumen formation with junctional complexes, microvilli and cilia and molecular and genetic evidence shows that ependymomas arise from radial glia (Taylor et al., 2005). Indeed in a review on the cells that may contribute to the ependymoma, radial glial cells were considered as the candidate stem cells (Poppleton and Gilbertson, 2007) but it is still not clear whether this is also the case for spinal cord ependymomas. Ependymomas are different from other glial tumours, such as astroblastoma or chordoid glioma which focally displayed aspects of ependymal differentiation since in ependymomas, the cells remain in an undifferentiated state (Lehman, 2008).

In humans, laser microdissection of the ependymal layer and extraction of the mRNA enabled the gene expression profiles of this region to be studied (Garcia-Ovejero et al., 2015). They showed that the region had high levels of some genes associated with low grade or quiescent ependymomas and suggested that in humans, the potential for production of ependymomas was present but normally the cells were kept in a non-reactive state whereas in some cases ependymomas may arise (Paniagua-Torija et al., 2018; Garcia-Ovejero et al., 2015).

The main treatment for ependymomas has not changed for many years and involves resection of the tumour (Lehman 2008) but the ependymoma often recurs after surgery so a different approach is necessary to control recurrence. The discovery of ependymal like cancer stem cells has therefore provided researchers with a practical

point of focus for studying the postnatal cellular and molecular events of tumorigenesis. This is where my findings may be relevant as a potential avenue for exploration of modulating the activity of these ependymal stem cells.

Many central nervous system tumours show increased levels of DBI and TSPO (Alho, Hannu et al., 1995; Miettinen et al., 1995) and higher levels of TSPO are associated with a greater likelihood of malignancy and reduced survival (Janczar et al., 2015; Vlodavsky and Soustiel, 2007). Therefore my findings could suggest that modulating levels of the DBI or the GABA receptor itself may be a possible therapeutic avenue for exploration in reducing recurrence of ependymomas after surgery or reducing malignancy.

Considering their key roles in homeostatic control, it is not surprising that connexins and their channels are frequently targeted upon treatment due to impairment of this critical balance. Tumour cells generally display reduced gap junctional intercellular communication or coupling and numerous mechanisms underlie the loss of intercellular communication in carcinogenesis (Trosko, 2003). We do not know if the cells in ependymomas still express gap junctions, although it may be expected due to the high incidence of these in normal ependymal cells. However, epigenetic modifications, such as DNA hypermethylation could trigger silencing of connexin gene expression to see if this affects the extent of the ependymoma.

6.4 Technical consideration and limitations

Using selective agonists and antagonists to identify receptor or receptor subtype(s) on a specific cell is a popular method. By combining the effects of these drugs with patch clamp electrophysiology and immunohistochemistry the receptor types found in ependymal cell can be elucidated to a degree.

However, these methods can only provide incomplete evidence and therefore always need to be supported by other techniques which would provide more comprehensive confirmation e.g. integration of electrophysiological recordings with single cell RNA-seq that is Patch–seq, a method based on sequencing RNA aspirated from the soma of single patch-clamp-recorded neurons (Fuzik et al., 2016). In these experiments, it was possible to record from single cells then aspirate the complete contents of each cell into the recording electrode to then carry out full analysis of the transcriptome. This would be a very powerful way of exploring if responses that are observed electrophysiologically are directly correlated with what is expressed in that cell.

Whole cell patch clamp recordings are well suited to do recordings from small cells but perturbs intracellular ionic concentrations by dialysing cytoplasmic contents, so perforated patch recording is another option and can circumvent these limitations. We could perform patch recordings using the cation selective ionophore gramicidin which does not interfere with Cl^- concentrations. This would enable a better picture of how chloride channel opening and movement of chloride ions contribute to the responses observed. If we were to combine this with harvesting the recorded cells and then perform mRNA-seq (Fuzik et al 2016), this would provide information on the chloride transporters present in the potential subgroups of ependymal cells showing different responses to GABA.

The excitatory action of GABA may arise from the cellular injury due to slicing procedure, which leads to accumulation of intracellular Cl^- in injured cells and contributes to the depolarising responses observed. This problem is potentially reduced by taking recordings from the centre of the slice, which was 300 μm thick. The fact that ependymal cells are compact, with few processes is an advantage here but this issue of injury cannot be completely excluded. The use of 300 μm slices would also remove potential synaptic inputs onto these cells that may affect the activity in these cells. While there is little evidence in other studies of synaptic activity (Corns et al., 2015; Corns et al., 2013; Marichal et al., 2012), these studies also use slices. The extent of gap junction coupling may also be affected by removal of coupled cells in the slicing. However, recording from these cells *in vivo* would be an extremely technically demanding experiment and use of calcium or voltage indicators would also be prohibited due to the depth of the cells from the surface of the spinal cord. Therefore, the use of spinal cord slices at least maintains a degree of circuitry whilst enabling stable and prolonged recordings from the cells.

Due to the fact that there are unspecified subunits contributing to the receptors found in the central canal, there may be confounding effects at these uncharacterised receptors using agonists and antagonists even though some of the modulators are considered selective drugs. Even with the use of combining mRNA seq analysis of recorded cells, it is still not possible to elucidate the actual subunit composition of the different receptors so it is hard to know how this issue can be avoided at this stage of investigation.

The used of wild type/C57BL/6 in the study of patch clamp is the best animal for maintaining consistent normal activity compared to transgenic or knockout mouse because the animal represent the best normal environment. Using wild type mice allowed me to test the patch clamp methods in a C57BL/6 background without any genetic modifications that could influence the results. Used of acute slices possibly

not representing normal or in vivo physiology but this is the best way to do patch clamp as it will reach the cell specifically. The current result possibly showed the contribution from all cell type and factors that control GABAergic manipulations on cells proliferation in the spinal cord central canal niche and may not specifically represent the ependymal cell even though the recording were confirmed from the ependymal cell because of the remaining minimally disturbed environment of a cell and niche. The best animal with a genetically expressed FoxJ1 are the best animal model for ependymal cell investigation (see 6.5.3).

6.5 Future work

6.5.1 Extension of the existing experiments

The possibility that ODN activation in ependymal cells can result in an increase in intracellular Ca^{2+} was not investigated in this study, but it is undoubtedly an important future experiment, especially in the light that bath applications of ODN sometimes depolarised the cells. It is possible that activation of ODN receptors on the ependymal cell could induce increases in intracellular Ca^{2+} which could stimulate many of the neurogenesis processes. Propagation of Ca^{2+} waves through gap junctions as seen in radial glial cells during embryonic neurogenesis and neural progenitor cells in the postnatal SVZ (Weissman et al., 2004) may also contribute to a potential proliferative response in the spinal cord.

The relationships between the cells in the area surrounding the central canal would be very interesting to examine. A dual patch clamp electrophysiology could be used to investigate the relationship between ependymal cells and CSFcCs or between

ependymal cells and type 1 CSFcC. If a response is seen in one of the cells, different GABA receptor antagonists or modulators could be applied to see whether the response could be antagonised or enhanced. Another method would be to use breed mice in which a genetically encoded voltage indicator or a genetically encoded calcium indicator such as GCaMP is expressed solely in cells that express FoxJ1 so changes in fluorescence are only observed in the ependymal cells. Then changes in voltage or calcium influx to a specific activation of a receptor or application of a specific modulator can be monitored in all ependymal cells simultaneously.

6.5.2 Optogenetic activation of GABAergic CSFcCs or neighbouring neurones to investigate effects on ependymal cells

The identification and manipulation of different GABAergic inputs are important to understand the source of GABA that may be influencing neural stem cells in spinal cord circuits. Using an optogenetic approach based on expressing light-sensitive opsins in a given cell population would enable us to selectively activate these cells and investigate how this activation affects the responses of ependymal cells. CSFcCs express the ion channel PKD2L1 and this is not observed in other cell types in the spinal cord (Corns et al., 2015; Huang et al., 2006). If channel rhodopsin was expressed exclusively in PKD2L1 positive cells, light activation of these cells combined with patch clamp recordings from ependymal cells would allow investigation into whether this produced changes in membrane potential in the ependymal cells. Further pharmacological analysis with selective GABAergic antagonists or modulators would elucidate whether the effects were due to release of GABA from these CSFcCs. Similarly, channel rhodopsin could be expressed in parvalbumin expressing GABAergic interneurones in the spinal cord to see if

activation of these cells had any effect on ependymal cells. This may give further information about the source of the GABA.

6.5.3 Use of genetically modified animals to study the role of ependymal cells and their responses to GABA

The use of point mutation and gene knock-out techniques on animals will allow the contribution of receptor subunits to be studied separately, both on the responses of the cells to GABA and on potential proliferative responses. Receptor subunit differences may contribute to the wide array of observations on GABA receptor modulation. Specific GABA subunit null mutant mice have been developed and used for testing the contributions of these subunits e.g. for GABA α_1 and β_2 (Blednov, Y. et al., 2003) and ρ_1 (Blednov, Y.A. et al., 2014) but issues may arise when considering how much compensation occurs with such null mutants and what happens to the subunits composition of the remaining GABA receptors. Potentially use of conditional inactivation of a specific subunit gene in adults, such as that used by Earnheart et al. (2007) in their investigation into the role of γ_2 subunits in hippocampal neurogenesis would be the best way to examine each subunit.

Development of the spinal cord requires dynamic and tightly controlled expression of numerous transcription factors. Forkhead Box protein (Fox J1) is a transcription factor involved in the generation of cilia and is expressed in ependymal cells of adult central nervous system. In a study using Fox J1 fate mapping, Li, X. et al. (2018) observed that Fox J1 is also expressed by the progenitors of other neural subtypes during the developmental process but in adult, it is solely expressed by the ependymal cells in the spinal cord. Fox J1 deletion unsettled embryonic progenitor proliferation and cell fate determination and resulted in formation of adult ependymal cells having impaired stem cell potential and inability to respond to spinal cord injury. So by using these

transgenic mice with a specific Fox J1 cell deletion, we could investigate whether Fox J1 is critical in GABA/DBI mediated changes in ependymal cell proliferation.

Another approach is to study the role of the gap junctions in ependymal cells in the proliferative responses of spinal cord ependymal cells. In the third ventricle around the hypothalamus, tanycytes are considered the neuronal precursor cells and here, when coupling of tanycytes is genetically absent or pharmacologically inhibited, there are decreases in neural stem and neural precursors (Recabal et al., 2018). They demonstrated that Cx43 is the most abundant connexin expressed in rat tanycytes. Using wild-type, $Cx30^{-/-}$ and $Cx30^{-/-}$, $Cx43^{fl/fl}$: glial fibrillary acidic protein (GFAP)-Cre (double knockout, dKO) mouse lines, (Recabal et al., 2018) demonstrated that tanycytes are highly coupled to each other. In the dKO mouse line, there is absence of Cx43 expression, and they detected a significant decrease in the number of hypothalamic proliferative parenchymal cells. So it is relevant to suggest that the use of specific connexin knockout mice will determine the direct role of gap junctions in ependymal cells in the proliferative responses, either just in the intact spinal cord or in response to specific receptor activation or modulation.

Knock-out mice have been used to study the role of particular gene products in biochemical processes, in mediating the effects of neuropharmacological substances, and in complex behaviours. The advantage of using the knockout mouse line, is that there will be no interference of other recessive genetic variants, so the result will a specific and precise target. The disadvantages of using knock-out mice to study neurobiological problems is that often it is important to target the deletion or mutation to specific cell types and this requires complex cross-breeding of the different mouse lines to enable this. Injections of viruses that overexpress or knockdown proteins, such as those used by Dumitru et al. (2017); Alfonso et al. (2012) to specifically knockdown DBI and ODN in their transient amplifying cells would be a better approach.

6.5.4 Cultured slices and *in vivo* experiments to test the outcome of manipulation of GABA receptors on ependymal cell proliferation

Organotypic spinal cord slice cultures can provide many slices at one time to test for multiple conditions, so these experiments can help in identifying $GABA_A$ receptor compounds that modulate ependymal cell proliferation. EdU can be added to the culture medium along with the compounds to enable counting of proliferating cells. Furthermore, combining with immunofluorescence for specific markers of different cell types, such as neurones, oligodendrocytes and astrocytes, the fate of the proliferating cells can be established. Since agonists and antagonists can be co-applied, this experiments will enable to give pharmacological characterisation and subtypes of receptor involved which contribute to the cell proliferation. By using agonists and antagonist, an *in vivo* experiments also can be done by injecting the drugs for few days and after that the animal will be perfused and spinal cord will be processed for EdU immunohistochemistry to see the effects of proliferation.

6.6 Conclusions

This study has provided pharmacological characterisation of the responses to GABA and 5-HT of ependymal cells surrounding the central canal of spinal cord and how the GABA responses may be further modulated. The extent and diversity of modulation using the different modulator sites on ependymal cell demonstrate the complexity of the ependymal cell and highlights the need for further investigation. The pharmacological profile of responses to GABA in ependymal cells of spinal cord suggests atypical GABA receptors since it was not fully blocked by $GABA_A$ receptor antagonist; bicuculline, and showed evidence of a combination or coassembly of $GABA_A$ and ρ receptors due to low GABA responses were antagonised by TPMPA and further confirmation with an agonist of GABAρ (TACA) also showed changes with TPMPA and may be further significant with an additional increase in sample size.

Potential site of modulation of GABA receptors is through DBI breakdown product; ODN which acts as positive and negative modulator at CBR and TSPO. ODN is somehow directly activating the GABA receptors that contain ρ subunits since the hyperpolarising effects of ODN were significantly blocked by TPMPA. So there is an evidence there are atypical GABA receptors responsible for this action with ODN. From immunohistochemistry finding, there are evidences of DBI and ODN immunopositive staining and most likely they are in the cell membrane.

Baclofen (a selective $GABA_B$ receptor agonist) also hyperpolarised ependymal cells, an effect antagonised by $GABA_B$ antagonist; CGP 55845, indicating a further role for $GABA_B$ receptors on ependymal cells. The numbers of proliferative cells in the central canal region were significantly lower in baclofen treated slices compared with control. Further confirmation of the atypical or multiple receptors involvement can be further confirmed by Western blot study to detect and characterise the receptor proteins in

the ependymal cell using patch clamp cell isolation technique. Given that the ependymal cells are capable of responding to 5-HT, a further study of the synaptic contacts of 5-HT fibres onto GABAergic neurones can be done.

The balance of these $GABA_{A,}$ GABAρ and $GABA_B$ receptor modulation and 5-HT receptor may regulates the activity of ependymal cells as component of neural stem cells.

It is possible that the manipulation of one or all receptor types in spinal cord area could allow a greater control over the proliferation and differentiation of cells within this cell, with beneficial implications for the treatment of spinal cord injury or spinal cord pathologies such as ependymomas.

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