Cholinergic enhancement of cell proliferation and differentiation in the postnatal neurogenic niche of the intact and demyelinated spinal cord.

Norah Khaled Altuwaijri

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Abstract

Among the heterogeneous central canal (CC) cell population of the adult spinal cord are the normally quiescent endogenous neural stem cells; the ependymal cells (ECs). The naturally latent ECs are activated upon the occurrence of injury, and are capable of robust cell proliferation, migration and differentiation into neurons or glia. Furthermore, in the intact and injured adult spinal cord, oligodendrocyte precursor cells (OPCs) and astrocytes give rise to new progeny. Harnessing these endogenous resources to replace damaged or lost neurons and oligodendrocytes after spinal cord injury or other white matter disorders requires a better understanding of how the microenvironment they reside in modulates their proliferation and differentiation. This project investigates the role that the neurotransmitter acetylcholine (ACh) has on cell proliferation and differentiation in the adult mouse spinal cord. Enhancing the levels of ambient ACh using an acetylcholinesterase inhibitor (AChEI) donepezil decreased the number of proliferating cells (visualised using the thymidine analogue EdU) in the CC *in vivo* and *in vitro* compared to control. Potentiating the effect of ACh transmission on α7 nicotinic acetylcholine receptors (α7nAChR) using the positive allosteric modulator PNU-120596 in combination with donepezil resulted in higher numbers of EdU^+ cells in the CC, grey and white matter of the intact spinal cord *in vivo* compared to vehicle treated animals. There was also an increase in the proportion of EdU^+ cells that also expressed either the oligodendrocyte marker PanQKI in both the grey and white matter or the neuronal marker HUC/D in the spinal cord grey matter in PNU-120596 + donepezil treated animals. In a lysolecithin (LPC)-induced focal demyelination mouse model, treatment with either PNU-120596 alone or in combination with donepezil significantly enhanced cell proliferation and oligodendroglial differentiation compared to LPC alone treated animals. There was also a significant increase in the extent of remyelination post cholinergic treatment. Spinal cord slice cultures in the presence of donepezil alone or in combination with the muscarinic receptor inhibitor atropine and the non-α7nAChR inhibitor DHβE suggested that cell proliferation is mostly mediated through α7nAChRs. Finally, the calcineurin-nuclear factor of activated T cells (NFAT) calcium signalling pathway downstream of α7nAChR activation plays a role in cell proliferation *in vitro*. This study provides a potential therapeutic avenue for modulation of ACh through α7nAChRs to aid in spinal cord repair.

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Abbreviations

Arg-1- Arginase 1

AKT- Protein kinase B (PKB), also known as Akt, is a serine/threonine-specific protein kinase

AChE- Acetylcholinesterase

AChEI- Acetylcholinesterase inhibitor

Acetyl CoA- Acetyl Co-enzyme A

aCSF- Artificial cerebrospinal fluid

BrdU- 5-bromo-2'-deoxyuridine

BMP- Bone morphogenic protein

BDNF- Brain-derived neurotrophic factor

Bcl-2- B-cell lymphoma 2

Bad- Bcl-2-associated death promoter (BAD) protein; a pro-apoptotic member of the Bcl2 gene family

Bax- Apoptosis regulator BAX, also known as bcl-2-like protein 4

CC- Central canal

CAA- Central autonomic area

ChAT- Choline acetyltransferase

CSF- Cerebral spinal fluid

CC1- Anti-adenomatous polyposis coli (APC) clone CC1

CNS- Central nervous system

CNP- 2',3'-Cyclic nucleotide 3'-phosphodiesterase

CNTP- Ciliary neurotrophic factor

Cl^- - Chloride

Ca^{++}- Calcium

CCN- Central canal neuron

Cdk5- Known as neuronal CDC2-like kinase (NCLK); a member of the small serine/threonine cyclin-dependent kinase (CDK) family (Box 1).

cAMP- Cyclic adenosine monophosphate

CREB- cAMP response element binding protein; a nuclear factor that is regulated by protein kinase A phosphorylation

CO_2- Carbon dioxide

cDNA- Complementary strand of deoxyribonucleic acid

Cq- Quantitation cycle

CSPGs- Chondroitin sulfate proteoglycans

CRAC- Calcium release activated calcium channels

DiI- 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate

DCX- Doublecortin

DG- Dentate gyrus

DRG- Dorsal root ganglion

DHI- Dorsal horn interneuron

DHβE- Dihydro-β-erythroidine

DNA- Deoxyribonucleic acid

DON- Donepezil

EC- Ependymal cells

EGF- Epidermal growth factor

EAE- Experimental autoimmune encephalomyelitis

EM- Electron microscopy

EdU- 5-ethynyl-2'-deoxyuridine

Elisa- Enzyme-linked immunosorbent assay

Foxj1- Forkhead box protein J 1

FGF- Fibroblast growth factor

FGFR1- Fibroblast growth factor receptor 1

FKHRL1- Forkhead transcription factor 1

Fyn- Non-receptor tyrosine-protein kinase

FACS- Fluorescence-activated cell sorting

GFAP- Glial fibrillary acid protein

GC- Galactocerebroside

GABA- Gamma-Aminobutyric acid

GSK3- Glycogen synthase kinase 3 is a serine/threonine protein kinase

GFP- Green fluorescence protein

GalC- Galactocerebroside; major glycolipid in myelin

HMGB1- High mobility group box 1

HDB- horizontal diagonal band of Broca

IML- Intermediolateral cell column

IC- Intercalated nucleus

IHC- Immunohistochemistry

IL-PN- Intermediolateral preganglionic neuron

IM-PN- Intermediomedial preganglionic neuron

IIN- Inhibitory interneuron

IN- Inhibitory or excitatory interneuron

iNOS- Inducible nitric oxide synthase

IP3- Messenger molecule Inositol trisphosphate

IL-6- Interleukin 6

I.P.- Intraperitoneal injection

IGF-I- Insulin-like growth fator-1

ISX9- Isoxazole 9

JAK-2- Janus kinase 2; a non-receptor tyrosine kinase

Ki67- Protein present during all active phases of the cell cycle and encoded by MKI67

LDT- Laterodorsal tegmental nuclei

LPC- Lysophosphatidylcholine also known as lysolecithin

LIF- Leukaemia inhibitor factor

Lingo1- Leucine Rich Repeat and Ig Domain Containing 1

MS- Multiple sclerosis

MS- Medial septal

MBP- Myelin basic protein

MAG- Myelin-associated glycoprotein

MRI- Magnetic resonance imaging

MHC-I and II- Major histocompatibility complex I and II

Mg^{++}- Magnesium

Mash1- Mammalian achaete scute homolog-1

mAChR- Muscarinic acetylcholine receptor

MN- Motor neuron

MLA- Methyllycaconitine

MAP-kinase- Mitogen-activated protein kinase

mRNA- Messenger ribonucleic acid

MRI- Magnetic resonance imaging

MMSE- Mini-mental state examination

MSX1- Msh Homeobox 1

MMP-9- Metalloproteinases -9

NG2- Neuron-glial antigen 2, a chondroitin sulfate proteoglycan

NKx6.1- NK6 Homeobox 1 transcription factor

Nrg1- Neuregulin 1

nAChR- Nicotinic acetylcholine receptor

NSC- Neural stem cell

NFAT- Nuclear factor of activated T lymphocyte

N- Number of animals

n- Number of slice or section

Ngn2- Neurogenin 2

NPC- Neural precursor cell

NMDA- N-methyl-D-aspartate

NT-3- Neurotrophin-3

Nogo A- Neurite outgrowth inhibitor also known as Reticulon 4

Olig2- Oligodendrocyte transcription factor 2

Olig1- Oligodendrocyte transcription factor 1

OPC- Oligodendrocyte precursor cell

PDGFR- Platelet-derived growth factor

PECAM- Platelet endothelial cell adhesion molecule

PSA-NCAM- Polysialylated neuronal cell adhesion molecule

PLP- Myelin proteolipid protein

PPT- Pedunculopontine tegmental nuclei

PC- Partition cell

ProN- Projection interneuron

PI3K- Phosphoinositide 3-kinases

PKC- Protein kinase C enzyme

PAM2- Positive allosteric modulator type-2

PBS- Phosphate buffer saline

PB- Phosphate buffer

PFA- Paraformaldehyde

PBST- Phosphate buffered saline with 0.1 % triton

PFU- Plaque forming unit

PID- Post injection day

PLGA- Poly lactic co-glicolic acid

Pax6- Paired box protein -6

PI- Propidium iodide

qPCR- Quantitative polymerase chain reaction

Raf-1 kinase- Proto-oncogene serine/threonine-protein kinase, also known as proto-oncogene c-RAF or simply c-Raf or Raf-1

Rb- Retinoblastoma protein; a tumor suppressor

RFP- Red fluorescence protein

RNA- Ribonucleic acid

RCAN1-4- Regulator of calcineurin 1 isoform 4

SOCE- Store operated calcium entry

SEM- Standard error of mean

SFV- Semliki forest virus

SRC- Proto-oncogene tyrosine-protein kinase.

SGZ- Subgranular zone

SVZ- Subventricular zone

Shh- Sonic hedgehog

Sox2- Sex determining region Y-box 2

Sox3- SRY-related HMG-box transcription factor 3

Sox9- SRY-related HMG-box transcription factor 9

Sox10- SRY-related HMG-box transcription factor 10

S100β- Calcium binding protein b

TgF-β- Transforming growth factor

Tuj1- Neuron-specific Class III β-tubulin

TLR-3,4 and 9- Members of the toll-like receptor family of pattern recognition receptors of the innate immune system

TNF α- Tumor necrosis factor

TEM- Transmission electron microscopy

Tox- Thymocyte selection-associated high mobility group box protein

UK- United kingdom

VEGFR2- Vascular endothelial growth factor 2

VAChT- Vesicle acetylcholine transporter

%- Percent

ºC- Degree Celsius

Chapter 1

General introduction

1.1 Introduction

The global prevalence of spinal cord injuries is difficult to estimate. In the United Kingdom alone, 50,000 people currently suffer from a form of spinal cord injury with an approximate annual incidence of 2,500 newly injured each year (The Back-Up Trust, 2019). Most spinal cord injuries (up to 90% according to the world health organization) are caused by traumatic motor vehicle accidents or falls with some cases due to disease or degeneration. Demographically, younger males (20-29 years of age) and older males (70+ years) are more susceptible to injuries compared to females with a conservative ratio of 2:1 (World Health Organisation, 2019). A recent study however, reported a rise in the average age of spinal cord injury incidence from approximately 29 years of age in the 1970s to around 42 years old in the United States since 2010. This was attributed in part to an increase in activity in the older population (Geoffroy et al., 2017). Acute impact spinal cord injury involves primary and secondary mechanisms. Primary injury occurs after initial traumatic mechanical damage displaces bone or disc elements leading to disrupted blood flow and damaged blood vessels, immediate apoptosis of neuronal and glial cells and severance of axonal structures. This is usually followed by a delayed onset of secondary injury comprising vascular dysfunction, spinal cord inflammation, an extended period of ischemia and tissue necrosis as well as demyelination and astrogliosis reviewed in (McDonald and Belegu, 2006, Rowland et al., 2008, Lee and Thumbikat, 2015). It is common for these types of injuries to result in an incomplete transection of the spinal cord, thus sparing some axons near the injury site. However, often these axons are demyelinated or abnormally myelinated (Rowland et al., 2008). The therapeutic significance of these spared axons can be greatly enhanced by remyelinating therapeutic strategies since persistent demyelination leads to further axonal loss (Grigoriadis et al., 2004, Blight, 1983) and the adult central nervous system has a limited capacity to endogenously remyelinate (Franklin and Blakemore, 1995, Blight, 1983, Blakemore et al., 2000). The importance of myelin for axonal survival, metabolism and conductance is clear and will be discussed in section 1.6. Despite the variable types of injury, dependent on the segmental level involved and degree of neurological function loss, recovery is reliant on the number of axonal fibres preserved and / or regenerated. Following contusion spinal cord injuries, an improvement in axonal conduction in spared axons and

functional recovery after remyelination has been reported. These studies spanned the transplantation of neural precursor cells (Karimi-Abdolrezaee et al., 2006), human embryonic stem cell derived oligodendrocyte progenitor cells (Keirstead et al., 2005), hematopoietic stem cell and marrow stromal cells (Koda et al., 2005) among several others reviewed in (Wright et al., 2011, Mothe and Tator, 2013). To avoid the ethical and clinical complications associated with stem cell transplants however, an alternative method of remyelinating spared axons post injury would be to stimulate endogenous oligodendrocyte precursor cells or endogenous stem-like cells of the spinal cord to replenish lost neuronal or glial cells. The prevalence, heterogeneity and roles of oligodendrocyte precursor cells in myelination and remyelination, and the existence of an endogenous stem cell-like population in the adult mammalian spinal cord niche will be covered in this chapter.

Multiple sclerosis (MS) is another highly prevalent condition in need of remyelinating therapeutic strategies. It is an autoimmune disease primarily defined as an inflammatory demyelinating disorder characterized by loss of myelin with little sparing of axons (Thompson et al., 1997). The immune system attacks the myelin sheaths enwrapping axons in the central nervous system, disrupting messages travelling along these nerve fibres. An estimated 127,000 people are afflicted with multiple sclerosis in the UK alone with an incidence 2 to 3 times higher in females than males (National Health Service, 2018). There are two main types of multiple sclerosis; repeated individual relapses (relapsing- remitting MS) or cases in which the disease is in a gradual progression (progressive MS). According to the National Institute of Health in the UK, more than 80% of cases are of the relapse-remitting form of the disease and decades later, many of them develop into secondary progressive MS. There are currently no cures or treatments to slow the progression of primary or secondary progressive forms of the disease, however, a variety of immunomodulatory treatments is available to slow down the progression of relapse-remitting MS (Alajbegovic et al., 2012). Some of the most commonly prescribed treatments are beta-interferons which act through blocking the action of gamma-interferon to reduce inflammation (Alajbegovic et al., 2012, Melendez-Torres et al., 2018). Humanized immunoglobulin drugs that target $CD4^+$, $CD8^+$, $CD20^+$, $CD25^+$ and $CD52^+$ T lymphocytes are also widely prescribed (Goldenberg, 2012). Although demyelination can be seen in patients without

any associated neurological dysfunction (Mews et al., 1998), once axonal damage reaches a certain degree, MS is likely to progress from relapse-remitting to secondary progressive form (Trapp et al., 1999, Waxman, 1998). In both animal models and MS patients, axonal demyelination occurs at the initial stage of the disease and progressive and/or permanent axonal loss occurs secondarily due to chronic disease progression (Grigoriadis et al., 2004, Kornek et al., 2000). Axonal damage is also seen in normal appearing white matter adjacent to actively demyelinating lesions (Kornek et al., 2000, Jäkel et al., 2019) indicating a diffuse injury effect. Moreover, the degree of axonal damage in animal models has been shown to correlate with the severity of the disease (Wujek et al., 2002) indicating the need for early remyelinating therapeutic intervention. Interestingly, correlation between the degree of demyelination in patients and neurological dysfunction is sometimes absent suggesting additional potential contributing factors (Bodini et al., 2016, Jäkel et al., 2019).

This project focuses on evaluating the effects of cholinergic transmission modulation as a means to enhance plasticity and thus potentially help in recovery after injury in the adult mammalian spinal cord. It investigates the outcome of enhanced acetylcholine signalling on spinal cord proliferation and differentiation, and the potential influence of acetylcholine on cell proliferation and oligodendrocyte differentiation after focal white matter injury. Acetylcholine is known to influence cell proliferation and differentiation in other neurogenic niches of the central nervous system (section 1.9), thus we hypothesize a potential neurogenic and / or oligodendrogenic role in the adult murine spinal cord. The focus of this thesis is understanding adult neurogenesis in the spinal cord and the prospect to apply this knowledge to promote remyelination after spinal cord demyelination.

1.2 The spinal cord

The central nervous system can be divided into the brain, brainstem and spinal cord. The spinal cord extends caudally beginning at the end of the medulla oblongata and ending at the first lumbar vertebra in adult humans and most adult mammals, figure 1.1 A. Below the first lumbar vertebra are the lower lumbar and sacral nerves termed the cauda equina. The spinal cord can be divided rostrocaudally into four parts; the cervical (8 in humans, 7 in mice), thoracic (12 in humans, 13 in mice), lumbar (5 in humans, 6 in mice) and sacral (5 in humans, 4 in mice) segments, figure 1.1 A and B. At the median sagittal plane of the spinal cord are the anterior and posterior median fissure that divide the cord into symmetrical halves connected by the anterior and posterior commissures. The ventral and dorsal roots emerge from either side of the spinal cord at the anterior and posterior fissures forming the spinal nerves, figure 1.1 C. The spinal cord is enwrapped by three layers; the dura mater, the arachnoid and the inner pia mater collectively called the meninges figure 1.1 C. The cerebral spinal fluid is contained within the subarachnoid space (area between the arachnoid mater and pia). In contrast to the brain, the spinal cord grey matter is surrounded by the white matter, and at the centre of the spinal cord is the central canal figure 1.1 C. The proportion of the grey matter to the white matter varies between segments. The grey matter can be divided into the dorsal horn, intermediate grey area, ventral horn and central grey regions. The white matter is divided into the dorsal, dorsolateral, lateral, ventral and ventrolateral funiculi through which transmission of neural signals occur between the brain and the rest of the body or between spinal cord segments. The spinal nerves are composed of sensory fibres (cell bodies are located in the dorsal root ganglia) entering the spinal cord through the dorsal roots, and somatic motor and preganglionic autonomic nerve fibres exiting through the ventral root. The cell bodies of motor neurons are found in the ventral horn, while cell bodies of sympathetic preganglionic neurons are generally located in the intermediolateral column, figure 1.1.

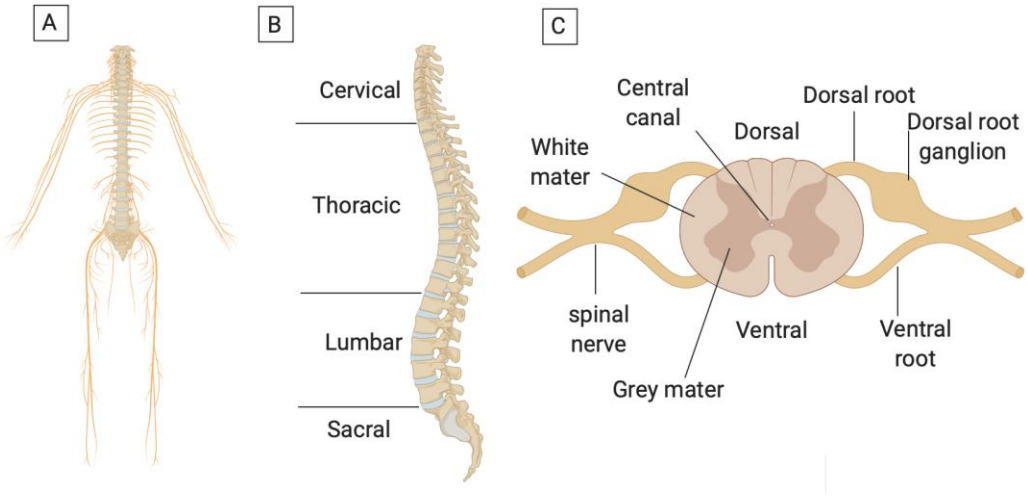

Figure 1.1 A schematic representation of the spinal cord.
Panel **A** demonstrates the vertebral column and spinal nerves exiting at their respective segmental level. Panel **B** depicts the segmental organization of the spinal cord which is divided into cervical, thoracic, lumbar and sacral regions. Panel **C** illustrates the basic anatomy of a cross section of the spinal cord. Created with Biorender.

1.3 The cytoarchitecture of the spinal cord

The grey matter of the spinal cord is composed of neuronal cell bodies organized into a well-accepted laminar distribution, while the white matter is made up of myelinated and unmyelinated axons making up the intrinsic, ascending and descending tracts.

The white matter consists of fibre tracts allowing the relay of messages through the central nervous system. The intrinsic pathways (propriospinal connections) connect different neuronal groups and segments as well as connect descending pathways with intrinsic neurons. In addition to the intrinsic spinal cord pathways, the spinal cord

connects to the rest of the central nervous system via well-defined ascending and descending tracts with species related differences.

The grey matter was first divided by Rexed into ten laminae (Rexed, 1954, Rexed, 1952). Lamina I is the most dorsal lamina located at the upper end of the dorsal horn Cell bodies in this area are less densely packed and are of variable sizes (Bennett et al., 1981). Lamina II is known to have a high neuronal density (substantia gelatinosa). The cell bodies here are smaller in size and variable in shape. The main cell types are the islet cells with a rostrocaudal orientation and stalked cells which have a dorsoventral dendritic tree. Other less numerous neuronal types have also been described in this area (Schoenen, 1982, Bennett et al., 1980). Lamina III has a lower neuronal density than lamina II and is characterized by the presence of intermediate sized neurons that are antenna-like and radial (Maxwell et al., 1983, Beal et al., 1988) with a simple dendritic morphology (Schoenen, 1982). The cell bodies of lamina IV are known as transverse cells with antenna-like dendrites spreading dorsally into laminae II and III (Schoenen, 1982). Laminae I-IV process sensory information through many propriospinal pathways within the dorsal horn (Petkó and Antal, 2012); this region processes external stimuli. Neurons in lamina V and VI have similar characteristics; medial neurons are fusiform and triangular while the lateral ones are not clearly separated from the dorsolateral funiculus. Laminae V and VI are concerned with proprioception; lamina V mainly processes sensory afferent stimuli from cutaneous, muscle, joint and visceral nociceptors and respond to many stimuli (involves a wide dynamic range of neurons). Lamina VI mainly receives input from large diameter fibres innervating muscles and joints. Lamina VII is located in the intermediate zone and is composed of medium sized multipolar neurons. This area contains two nuclei in the thoraco-lumbar segments; the intermediolateral nucleus (T1-L1) and the dorsal nucleus of Clarke (intermediomedial) (T1-L2). Sympathetic pre-ganglionic neurons (SPN) are only present in segments (C8-L3), and located predominantly in intermediolateral region of the spinal cord, some between the intermediolateral region (IML) and central canal (CC), (intercalated nucleus; IC) and some dorsal to the central canal (central autonomic area; CAA) (Deuchars and Lall, 2015, Chung et al., 1975, Strack et al., 1988). Their axons project through the ventral horn and synapse with sympathetic postganglionic neurons in ganglia close to the spinal cord that ultimately synapse with

target organs. These cells have been shown to express choline acetyltransferase and are found intermingled with other cholinergic cells in this region (Houser et al., 1983, Barber et al., 1984a). This intermediate zone is also the connection between the brain and muscle spindles. This region is known to contain various interneurons (Harrison et al., 1984); some are involved in sympathetic control (Deuchars, 2007, Deuchars and Lall, 2015) and some in motor control and function (Tillakaratne et al., 2014). More importantly, some interneurons in this region are cholinergic and may be involved in processing sensory input or involved in motor control (Barber et al., 1984b, Carr et al., 1995, Huang et al., 2000). Lamina VIII differs from lamina I-VII by having neurons with dorsoventral dendritic extensions and is home to motor interneurons. Lamina IX is composed of clusters of motor neurons making up motor nuclei. These neurons extend dendrites dorsally up to lamina VI. The α-motor neurons have the largest cell bodies in the spinal cord while γ-motor neurons are smaller. Rexed's classification (Rexed, 1954, Rexed, 1952) did not differentiate between motor neurons, however there are four separate nuclei in the human spinal cord; the ventromedial, ventrolateral, dorsolateral and central columns (Schoenen, 1982, Romanes, 1964). Laminae VIII and IX mainly contain motor neurons with axons that innervate skeletal muscle fibres. Lamina X is the grey matter around the central canal (substantia grisea centralis) and considered the main neurogenic niche in the spinal cord and will be discussed below.

1.4 The cerebral spinal fluid (CSF)

The cerebral spinal fluid is secreted by the cuboidal ependymal cells lining the choroid plexus present in all parts of the ventricular system. The lateral ventricles produce the majority of the CSF which flows through the intraventricular foramina to the third ventricle then the cerebral aqueduct to the fourth ventricle where it passes into the subarachnoid space via the spinal cord central canal, the median aperture and lateral apertures. It is mainly composed of sodium (Na^+), chloride (Cl^-) and bicarbonate (HCO_3) and smaller amounts of potassium (K^+), magnesium (Mg^{++}), calcium (Ca^{++}) and some vitamins (e.g. folate and thiamine), peptides and proteins (e.g. leptins, brain derived neurotrophic factor and insulin like growth factor) transferred from the blood and synthesized by the choroid plexus (see review (Spector et al., 2015)). The

continuous flow of the CSF is important to maintain the physical structure of the ventricular system and transport cells and factors throughout the central nervous system (Spector et al., 2015). It also serves as a shock absorber preventing mechanical injury to the brain, creates buoyancy allowing the brain to have high density, facilitates blood perfusion and prevents ischemia in tight regions of the brain by supplying less CSF there to decrease pressure, maintains homeostasis and clear metabolic waste. Therefore, the CSF is a composite fluid containing a variety of molecules with direct influence and contact with the central canal of the spinal cord.

1.5 Lamina X

The cell types present in this lamina can be divided into; ependymal cells (ECs), interneurons, sympathetic preganglionic neurons (SPNs) and cerebrospinal fluid contacting cells (CSFcCs). In addition to SPNs in the IML and IC, there are SPNs in the CAA, which corresponds to lamina X.

There are many subtypes of interneurons within lamina X, with two types being cholinergic and present throughout the rostrocaudal extent of the spinal cord; the partition cells (in the intermediate grey area partitioning the dorsal from the ventral region) and central canal cluster cells. The partition cells consist of lateral, intermediate and medial cells with only the medial ones being located in lamina X (Barber et al., 1984b). Choline acetyltransferase (ChAT) immunohistochemistry revealed that cholinergic interneurons in the central canal partition as central canal cluster cells have complex tree-like dendritic arborization and axons projecting ventrolaterally. Combining immunohistochemical labelling with retrograde tracing showed that these cells project rostrocaudally up to six spinal cord segments (Sherriff and Henderson, 1994). The central canal cluster cells are also ChAT positive with small longitudinal fascicles mostly ventral to the central canal (Barber et al., 1984a). They also project and extend up to six segments away from their cell body location (Sherriff and Henderson, 1994). Huang et al (2000) revealed that both partition cells and central canal cluster cells express the activity dependent transcription factor (c-fos) after fictive locomotion

(activating circuitry receptors without movement occurring) indicating an involvement of both cell subtypes in locomotion. Thus, fictive locomotion can be achieved in neural networks even when isolated from sensory inputs (Huang et al., 2000).

Cerebrospinal fluid contacting cells are present in the ependymal and subependymal layers of lamina X and are smaller than interneurons (Barber et al., 1984). They can be identified by processes extending dorsally from their cell body into the cerebral spinal fluid in the central canal (Shimosegawa et al., 1986). These cells can be divided into subtypes based on cell body shape or axon projection. They can be oval, polygonal or elongated with axons extending from the basal end of the cell body exactly opposing the process extension, originate from the same apical side as the CSF contacting process or from the lateral side of the cell body (Shimosegawa et al., 1986). The function of these cells is not well defined but it is proposed that they may function similarly to CSF contacting neurons in the periventricular regions of the brain (Vígh et al., 2004). These cells may be important in transmitting signals from the CSF to neural circuits through their CSF contacting dendrites and in some cases axons that form terminals bearing cilia (resembling sensory cells). They are thought to transmit these information to synaptic zones influencing the function of these areas (Vígh et al., 2004). Some of these cells (those located in the medulla oblongata and spinal cord central canal) may also be sensitive to flow pressure of the CSF (Vigh et al., 1977, Vigh et al., 1983, Vígh et al., 2004). In lamprey spinal cord, one subtype of CSF-contacting neurons responds to changes in CSF pH levels and mechanical stimulation (Jalalvand et al., 2016).

A subependymal zone (not in direct contact with canal lumen) constituting oligodendrocyte progenitors, astrocytes and neurons has been described (Hamilton et al., 2009). Astrocytes and CSF-contacting neurons of the subependymal zone are also in contact with the canal lumen (Hamilton et al., 2009, Vígh et al., 2004). Ependymal cells are present throughout the rostrocaudal extent of the spinal cord, however, electron microscopy of rat spinal cord revealed that the thickness of the cell layer varies depending on the segment (Bruni and Reddy, 1987). The thickest ependymal cell layer is found at the sacral segments (up to four layers), then the lumbar, then

cervical followed by the thoracic layer which has the thinnest (a single layer) layer (Bruni and Reddy, 1987).

Ependymal cells are the most abundant cell type in the central canal region of the spinal cord. They surround the central canal area forming the ependymal layer separating the CSF from the grey matter. These cells are of various subtypes, although researchers identify them differently. Ependymal cells are described as pseudostratified cuboidal or columnar cells with round nuclei located in the middle or basal part of the cell body (Bruni and Reddy, 1987). A more recent study in adult mice using transmission electron microscopy 3D reconstruction identified ependymal cells with two long motile cilia attached to a large basal body, binucleated ependymal cells with four cilia and cells with large basal bodies associated with one or three cilia (Alfaro-Cervello et al., 2012). Some identify these cells as two subtypes; ependymal cells and tanycytes (Mothe and Tator, 2005), others as cuboidal ependymal cells, radial ependymal cells (at the ventral and dorsal poles of the central canal) and tanycytes (Meletis et al., 2008). The study by Meletis and collegues identified two ependymal subpopulations that expressed CreER proteins from either Nestin-CreER or Foxj1-CreER (Meletis et al., 2008). These ependymal subpopulations were immunoreactive for the cilia marker (Crocc) and either transgenes (Nestin and Foxj1) and were strictly contacting the lumen of the central canal (Meletis et al., 2008). The structural morphology of these ciliated lumen contacting cells were either typical cuboidal ependymal cells (oval nucleus, light cytoplasm, 1-3 cilia and no process), of tanycyte morphology (irregular nucleus, dark cytoplasm, 1 cilia and processes), or the less abundant radial ependymal cells (binucleated with oval or irregular shaped nuclei, light cytoplasm, 1-3 cilia and basal processes similar to that described by (Alfaro-Cervello's research group (Alfaro-Cervello et al., 2012) and (Meletis et al., 2008), figure 1.2. Since there is such confusion regarding subtypes, in this thesis all these ependymal subtypes will be collectively referred to as ependymal cells. These cell express connexin proteins; a family of trans-membrane proteins that serve as structural components allowing intercellular communication through gap junctions. In rat spinal cord, connexin-43 was immunohistochemically localized in all laminae with the highest concentration found in the substantia gelatinosa and central canal (Ochalski et al., 1997). Ependymal cells of the central canal had apical cytoplasmic labelling of

connexin-43 including symmetrical labelling of gap junctions at their apex and asymmetrical labelling of gap junctions in non-labelled ependymal cells (presumably another ependymal subtype; tanycytes) (Ochalski et al., 1997). Ependymal cells of adult rats also express connexin-50, with a lower expression observed after spinal cord injury suggesting a role in modulation of cell fate post injury (Rodriguez-Jimenez et al., 2015). Interestingly, connexin-50 overexpression or endogenous high expression favors proliferating ependymal cells to differentiate down the glial lineage as opposed to the neuronal lineage in *in vitro* transplantation assays of cells derived from adult Sprague Dawley rats (Rodriguez-Jimenez et al., 2015).

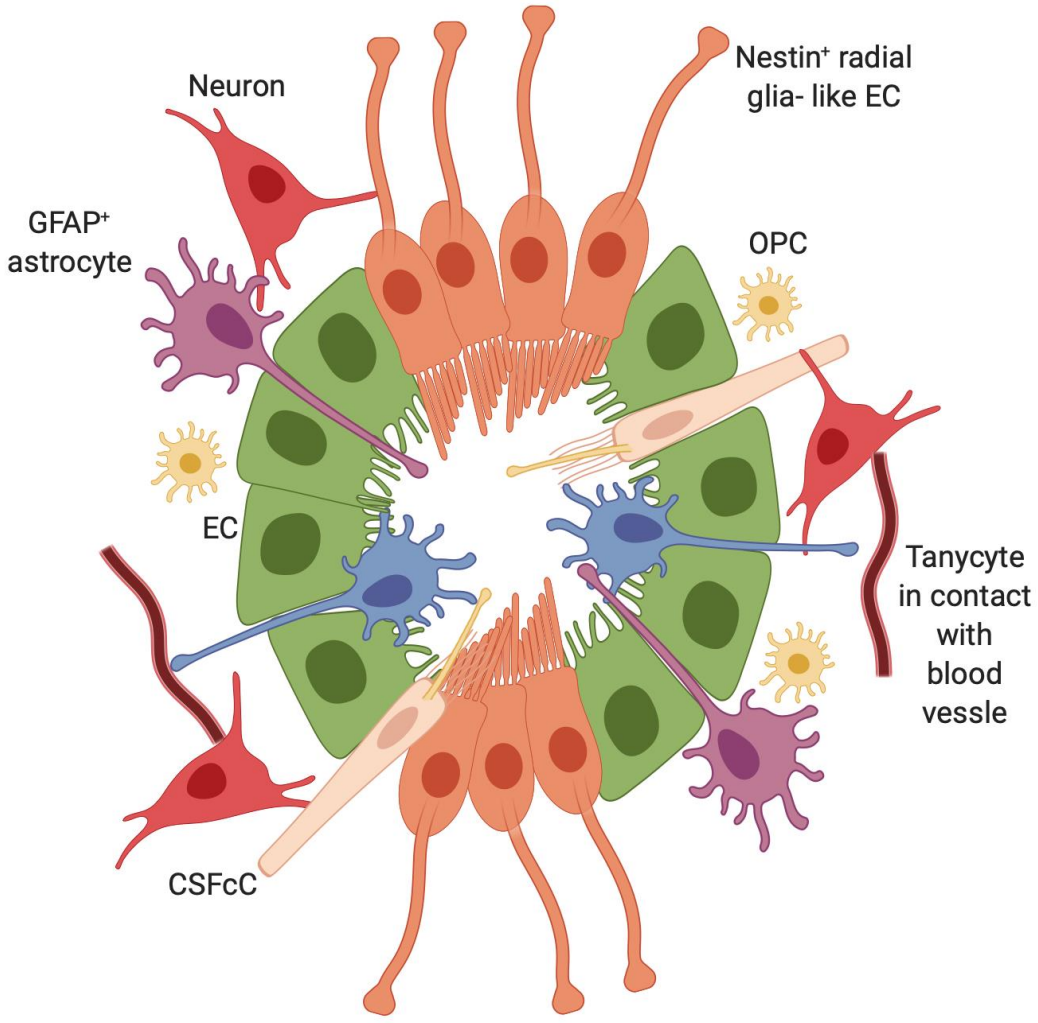

Figure 1.2 The heterogeneous cell population of the spinal cord central canal. The ependymal cells (ECs) are the main cell subpopulation lining the central canal. They are found intermingled with CSFcCs, tanycytes and the stem-like nestin^{+} radial glial ependymal cells located at the dorsal and ventral poles of the central canal. **OPC**: oligodendrocyte precursor cell. Created with Biorender.

1.6 Inputs into the central canal (lamina X)

Aside from the potential direct diffusion or exchange between the ependymal layer and CSF, the lamina X region receives input from various locally residing interneurons. Many ChAT negative cells within the ependymal layer of the central canal receive cholinergic fibres (some with terminal-like structures) (Barber et al., 1984b, Phelps et al., 1985). These possibly originate from the cholinergic interneurons and / or cholinergic partition cells previously described in section 1.4 (Barber et al., 1984b). There are also some GABA-ergic interneurons localized lateral to the central canal region (Barber et al., 1982) with presynaptic terminals in the ependymal layer (Barber et al., 1979). Additionally, a small cluster of $ChAT^+$ interneurons which have been shown to project to ventral motor neurons providing the only source of C-bouton inputs (Zagoraiou et al., 2009) may also have terminals near lamina X. Substance P fibres were immunohistochemically identified in regions ventral to the central canal of rat lumbar spinal cord (Barber et al., 1979). Furthermore, in lumbar rat spinal cord, substance P (neuropeptide), neurotensin (neuropeptide), cholecystokinin (a gastrointestinal peptide hormone) and methionine-enkephalin (an opioid growth factor) were seen in areas surrounding the central canal using the peroxidase anti-peroxidase method, each with a characteristic distribution (Gibson et al., 1981).

1.7 Are ependymal cells of lamina X the quiescent neural stem cells?

The spinal cord ependymal cells and cerebrospinal fluid contacting cells in lower vertebrates are known to play significant roles in spinal cord regeneration. They are immediately triggered by injury into proliferation and differentiation (Anderson et al., 1986, Ferretti et al., 2003, Bodega et al., 1994). In mammals however, these cells are not equally equipped to regenerate the spinal cord after injury. Reports of neural stem / progenitor cell or embryonic stem cell transplantation studies into the injured spinal cord show that they can contribute to some functional recovery in animal experimental models (Barnabé-Heider and Frisén, 2008, Hofstetter et al., 2005b). However, there are ethical and immunological implications associated with these experiments and the

exact mechanism of this recovery is not well understood. These studies endorse the idea that endogenous neural stem / progenitor cells exist. Moreover, it is interesting to consider what prevents endogenous stem cell proliferation in the intact spinal cord.

In adult mice, not all ependymal cells share the same molecular expression profile, although many of the markers are associated with immature or neural stem / progenitor cells such as the intermediate filaments nestin and vimentin, the typical neural stem cell marker CD133 / prominin-1, Musashi1, PDGFR-α, the transcription factors Sox2, Sox3 and Sox9 (Meletis et al., 2008) as well as the calcium binding protein S100β (Hamilton et al., 2009). Adult mouse ependymal cells homogenously express Sox9 and vimentin (Barnabé-Heider et al., 2010, Meletis et al., 2008) and Sox2 (Hamilton et al., 2009). Nestin; a marker for undifferentiated neural stem cells is only expressed by dorsal, ventral and a few lateral cells (Hamilton et al., 2009). The dorsally located nestin positive ependymal cells have long dorsally extending processes that are strongly immunoreactive to nestin. The ventrally located nestin positive cells had similar processes that are less strongly immunoreactive to nestin (Hamilton et al., 2009). Some report that none of the ependymal cells express the glial fibrillary acidic protein (GFAP) under normal conditions (Meletis et al., 2008, Mothe and Tator, 2005) while others find a dorsal subpopulation of cells to express GFAP with long dorsally extended processes (Hamilton et al., 2009).

1.7.1 Stem cell properties of ependymal cells both *in vivo* and *in vitro*

Adult mice ependymal cells exhibit neural stem cell properties *in vitro* (Meletis et al., 2008). This was observed by inducing recombination in Nestin-CreER and Foxj1-CreER mice to genetically label ependymal cells, then dissociating spinal cord cells for culturing and neurosphere formation. A significant percentage of neurospheres was derived from ependymal cells (roughly 76% from Nestin-CreER and 85% from Foxj1-CreER). Furthermore, 100% of recombined neurospheres were capable of being serially passaged for a minimum of 8 times surpassing the progenitor cell limited capacity for self-renewal, and exhibited multipotency by differentiating into neurons,

astrocytes and oligodendrocytes. The injection of a fluorescent lipophilic tracer (DiI) in the ventricular system of adult rats and mice to label ependymal cells uncovered their multipotent potential *in vitro* (Johansson et al., 1999). Culturing dissociated cells from the spinal cord in conditions known to support the growth of adult neural stem cells provided ependymal-derived neurospheres capable of self-renewal and differentiation (Johansson et al., 1999). An extended period of 5-bromo-2'-deoxyuridine (BrdU) administration in drinking water (2-6 weeks followed by 1-2 weeks washout period) showed that a large number of ependymal cells lining the spinal cord central canal are BrdU positive suggesting that these cells are proliferative in normal conditions, although their rate of proliferation is slow since the lipophilic label is not sufficiently diluted as those seen in the continuously dividing subventricular zone (Johansson et al., 1999).

Ependymal cells showed little cell proliferation or turnover *in vivo* (Meletis et al., 2008). A very small percentage of ependymal cells (roughly 4%) were BrdU positive with the majority of them seen in pairs following long term administration of BrdU in drinking water (Johansson et al., 1999). This suggests that they duplicate for self-renewal purposes. In the case of spinal cord injury however (incision in the dorsal funiculus), the extent of ependymal cell proliferation increased drastically and many recombined ependymal cells migrated to the site of injury. The migrated cells displayed a loss of Sox2 and Sox3 expression, maintained the expression of Sox9, acquired GFAP expression and exhibited astrocyte-like morphology contributing to glial scar formation. None of the recombined cells expressed neuronal markers, although olig2 positive recombined cells were observed within the lesion site during the first month post injury. Similarly, following bilateral needle incision into the lateral columns of the spinal cord, BrdU and lipophilic intraventricular labelling of ependymal cells revealed an 8.6 fold increase in ependymal cell proliferation and migration to the site of injury, an increase in nestin immunoreactivity and the presence of GFAP positive labelled ependymal cells at the site of injury; suggesting their multipotency and potential contribution to glial scar formation (Mothe and Tator, 2005). Ependymal cells of the spinal cord can be triggered into extensive proliferation (33.5 fold increase in BrdU positive cells around the central canal) after six days of basic fibroblast growth factor (FGF2) and epidermal growth factor (EGF) minipump infusion (Martens et al., 2002). A

small number of these cells had migrated away from the central canal to the white matter and differentiated into astrocytes and oligodendrocytes (Martens et al., 2002). Genetic fate mapping of oligodendrocyte progenitor cells, ependymal cells and astrocytes pre and post spinal cord injury in adult mice showed that in the intact cord, a similar proportion (4-5%) of Sox10 positive oligodendrocyte precursor cells and Sox9 positive ependymal cells incorporate BrdU with less than 1% of astrocytes labelled. 80% of proliferative cells in the intact cord were of the oligodendrocyte precursor cell lineage (Barnabé-Heider et al., 2010). Three different adult transgenic mouse lines (FoxJ1 for ependymal cells, Olig2 for oligodendrocytes and connexin 30 which is exclusively expressed by astrocytes) were used to determine the differentiated fates of proliferated cells. This resulted in two finding; the first is that the progeny of both ependymal cells and astrocytes were of the same lineage, however, oligodendrocyte precursor cells gave rise to immature and mature oligodendrocytes in normal conditions. Second, the number of recombined cells at 5 days and 4 months were similar for ependymal and astrocyte transgenic animals indicating that they proliferate and differentiate to maintain the size of their populations, whereas Olig2 transgenic animals had a significant increase in the total number of recombined cells at 4 months (some were NG2 positive immature cells and other CC1 positive mature oligodendrocytes). A transverse thoracic dorsal funiculus incision without the disruption of the grey matter in either a wild type mouse or any of the three transgenic mouse lines revealed that around 80% of neurospheres were ependymal cells derived in both cases. The *in vivo* response to a dorsal funiculus incision in the three transgenic lines showed a significant increase in the number of recombined cells at the site of injury; ependymal cells in the centre of the lesion, astrocytes in the perimeter and oligodendrocyte progenitor progeny throughout the injury site. Astrocytes and oligodendrocytes gave rise to progeny of their own lineage whereas ependymal cell progeny consisted all three cell types; neurons, oligodendrocytes and astrocytes (Barnabé-Heider et al., 2010). Juvenile ependymal cells (P21) give rise to more neurospheres and more oligodendrocytes than adult ependymal cells *in vitro*; with a ten-fold decrease between P10-P21 and adult ependymal cell neurospheres (Li et al., 2016). Furthermore, P21 ependymal cells contribute to glial scar formation after severe (dorsal hemisection) spinal cord injury and not mild injury (dorsal funiculi transection) as oppose to adult ependymal cells (Li et al., 2016). Analysis of the lesion area

indicated a greater response of glial cells and pericytes to injury in juvenile animals compared to adults (Li et al., 2016). By using a transgenic mouse line inhibiting ependymal cell cycle, lesions were more efficiently sealed in juveniles in an ependymal cell independent manner as opposed to adult animals which had a 50% cavity remaining unsealed (Li et al., 2016). Thus, the ependymal cells of juvenile animals exhibit greater stem cell potential, and regeneration and glial scar formation post injury is age dependent.

1.7.2 Are all ependymal cells neural stem cells?

Since ependymal cells vary morphologically and in their molecular expression, some sought to investigate the potential differences in cell turnover and proliferation between subpopulations. Immunohistochemistry for Ki67 (expressed by proliferating cells at all stages) and vimentin revealed that there was a dorsal to ventral gradient of cell proliferation in the central canal of adult mice (Hamilton et al., 2009). In the intact spinal cord, there were more (2-3 fold higher) proliferating cells in the dorsal ependymal zone than in the ventral pole (Hamilton et al., 2009). Immunohistochemistry for Ki67, vimentin and the endothelial cell marker platelet endothelial cell adhesion molecule (PECAM) showed that ependymal cell proliferation occurs in close proximity to ependymal layer projecting blood vessels, and proliferation was almost exclusively seen in doublets suggesting ependymal cells undergo proliferation to maintain their cell population in normal conditions (Hamilton et al., 2009). It is interesting to note that nestin immunoreactivity is present in dorsal and ventral ependymal cells and is also detected at the apical cytoplasm and basal processes of cells located in the dorsal pole of the central canal. The basal processes of dorsal cells display the highest immunoreactivity compared to ventral cells and can be seen extending from the dorsal ependymal layer up to the dorsal columns (Hamilton et al., 2009). Immunohistochemistry for GFAP, vimentin and Ki67 was also found localized in the dorsal pole of the central canal and possessed a lumen contacting apical process and a basal dorsally projecting process. These GFAP / vimentin / Sox2 positive cells however only constituted roughly 1% of cells in the ependymal layer with 82% located dorsally (Hamilton et al., 2009). There are also regional differences of proliferative activity in the ependymal layer of the adult rat central canal; immunohistochemistry for

BrdU or Ki67 showed that sacral / coccygeal levels had a significantly higher number of proliferating cells compared to rostral segments under normal conditions (Blasko et al., 2012). Thus, the distinct antigenic and morphologic heterogeneity in the rodent ependymal layer raises the possibility that a certain subpopulation (nestin positive) are normally in a stem cell state, and injury may be the requirement for the other subpopulations to enter a progenitor or stem like state.

1.7.3 Do human ependymal cells behave similarly?

The adult human spinal cord also bears neural stem / progenitor cells in the ependymal region of the central canal (Dromard et al., 2008, Mothe et al., 2011, Cawsey et al., 2015). Electron microscopy methods and immunohistochemical staining for nestin, Sox2, GFAP, Nkx6.1 and PSA-NCAM revealed the heterogeneous expression of all markers in the ependymal layer of non-pathological post-mortem adult human spinal cord (Dromard et al., 2008). Furthermore, the dissociation of spinal cord cells from non-pathological post-mortem adult subjects generated nestin and Sox2 positive neurospheres in culture that successfully differentiated into glial cells and neurons (Dromard et al., 2008). Neural stem / progenitor cells from the ependymal layer of human spinal cord central canal were isolated and successfully propagated and passaged *in vitro* in the presence of EGF and FGF enriched medium, generated nestin and Sox2 positive neurospheres and differentiated into glial and neural cells under differentiating conditions (Mothe et al., 2011). The transplantation of these human neural stem / progenitor cells into rat spinal cord after injury resulted in the survival of the xenograft and differentiation into glial and neural cells. Some researchers however, report that nestin immunoreactivity is only seen in preterm neonates and absent in early infancy (Sakakibara et al., 2007). A higher expression of nestin at the dorso-ventral midline of the central canal is seen in patients with amyotrophic lateral sclerosis and patients with tumours (Sakakibara et al., 2007). This potentially suggests that the pathological environment triggered the quiescent endogenous neural stem cells to activate. Nestin immunoreactivity was also used as a neural stem / progenitor cell marker to investigate potential differences in the ependymal layer of human controls and those who died as a result of trauma to the central nervous system (Cawsey et al., 2015). There was a positive correlation

between numbers of nestin immunoreactive cells and spinal cord injury. These cells were also located in the dorsal and ventral poles of the central canal with consistent morphology to those seen in rodents (apical cilia and long basal processes) (Cawsey et al., 2015). Taken together, these findings show that adult humans do harbour multipotent stem / progenitor cells in the spinal cord ependymal layer, and these cells are capable of responding to exogenous stimuli into proliferating and differentiating *in vitro* and once transplanted into an injury environment. Therefore, it is reasonable to investigate the response of ependymal cells to cholinergic modulation in normal conditions and after injury.

It is also important to note that there are discrepancies in literature regarding human ependymal cells. An example of such is the recent report by a research group led by Paniagua-Torija who argued against the existence of a spinal cord neurogenic niche and investigated the presence and contribution of the ependymal region in adult humans after spinal cord injury (Paniagua-Torija et al., 2018). Spinal cord tissue samples were acquired either post-mortem or by donation from the Miami Project to Cure Paralysis Biobank. Their study employed the use of two proliferative markers (Ki67 and MCM2) to evaluate the extent of cell proliferation in the ependymal region of spinal cord sections rostral and caudal to the lesion epicentre days or months after injury. They concluded that less than 0.08% of cells in the ependymal region out of 7607 cell nuclei were proliferative in SCI samples and show that only remnants of the central canal persist in adult humans compared to mammalian models.

1.7.4 Do ependymal cells respond to all forms of injury?

The expression of ependymal cells of neural stem cell behaviour after neurotransmitter modulation (discussed in section 1.9) or spinal cord injury makes them the prime target to modulate their contribution to the oligodendroglial progenitor pool in disorders where myelin and oligodendrocytes are the target of pathology, however, their response to inflammation and / or demyelination may differ to their response to injury. For instance, a recent study investigated the response of ependymal cells to spinal cord contusion injury, focal demyelination injury, and an

experimental autoimmune encephalomyelitis (EAE) model of multi-focal demyelination (Lacroix et al., 2014). Similar to other studies, proliferation of the central canal was induced following contusion injury to the spinal cord; they reported a robust peak in ependymal cell proliferation at days 3 and 14 post injury in the medial area of injury and in both areas rostral and caudal to the contusion. Surprisingly however, the ependymal zone in the focal demyelination model and in EAE mouse models showed no increase in the number of $Ki67^+$ proliferative cells compared to sham models. This suggests that ependymal cells do not contribute to remyelinating oligodendrocytes in these specific models. However, this raises important unanswered questions; does the degree or location of demyelination play a role in the ependymal cell response to this type of injury, and could neurotransmitter modulation following demyelination trigger the ependymal cells to respond to demyelination? Furthermore, a recent study comparing varying degrees and sites of spinal cord injuries suggested that ependymal cells of the central canal only respond to injury if it occurred near but not on the ependymal central canal region (Ren et al., 2017a). They showed that after a severe transverse crush injury across the whole length of the spinal cord; genetically fate mapped ependymal derived proliferation was very low to none and those failed to migrate to the site of injury. A stab injury lateral to the ependymal region of the central canal without reaching or damaging the ependymal layer also showed no contribution from ependymal cells. A radially oriented stab that did reach the ependymal layer however did result in a significant contribution of ependymal cell proliferation and differentiation into astrocytes (Ren et al., 2017a). This suggests that little endogenous neurogenesis occurs after demyelination injury. However, given that nicotine reduced inflammation in an EAE model and decelerated disease progression (Gao et al., 2014), it is essential to further investigate the effects of cholinergic modulation in an inflammatory demyelination model.

1.7.5 Proliferative capability of other immature cells

Interestingly, BrdU labelling prior to any injury in the spinal cord showed that roughly 50% of proliferating cells are nerve / glial antigen 2 (NG2) positive oligodendrocyte precursor cells (Horner and Gage, 2000). NG2 is a chondroitin sulphate proteoglycan that is expressed by immature glial cells (oligodendrocytes and

astrocytes) including pericytes associated with blood vessels and transiently by macrophages after injury (Dimou and Gallo, 2015). Similarly, 10 days of BrdU I.P. injections in adult rats revealed that most labelled cells were in the lateral regions of the spinal cord (mainly NG2 positive) with the rare event of proliferation occurring at the central canal (Horky et al., 2006). After a white matter injury (dorsal hemisection) of thoracic rat spinal cord and BrdU administration 24 hours post injury; the number of BrdU positive cells dropped significantly 1 day post injury, with most of the remaining cells being NG2 positive. One month after injury however, the total number of proliferative cells significantly increased surpassing normal levels, and the vast majority of these cells were also immunoreactive for NG2 (Horky et al., 2006). This suggests that although NG2 positive cells are vulnerable to injury, the remaining populations replenish the cell loss successfully. In these experiments, peak cell proliferation occurred 24 hours post injury and most cells were localized in the injury-adjacent grey matter suggesting they originate there with few derived from the white matter and even fewer from the ependyma. In animals that received a single injection of BrdU 24 hours post injury and analysed either 30 hours or 3 weeks after, none of the BrdU positive cells colocalised with neuronal markers. Many BrdU positive cells colocalised with S100β and nestin in the central canal region at both time points and very few S100β colocalised cells in the lesion site, while the most abundant phenotype in the parenchyma were BrdU positive NG2 positive cells (up to 50%) at both time points. Cells colocalising with the mature oligodendrocyte marker adenomatosis polyposis coli (APC / CC1) were only seen at 3 weeks post injury. At 1 week post injury, S100β colocalised cells constituted 2 % of all BrdU positive cells, and at 2 weeks they reached 12%. At 9 weeks post injury however, no S100β colocalised cells were found, and 15% of all BrdU positive cells were instead GFAP positive. This suggests that immature astrocytes could have originated from the ependymal zone or infiltrated from the periphery since S100β labels immature and mature astrocytes as well as Schwann cells (Bhattacharyya et al., 1992), and these immature cells matured to GFAP positive cells.

These experiments highlight the significant contributions of oligodendrocyte progenitor cells (OPCs) following white matter injury and point out potential differences

between ependymal cell proliferation (contribution to scar formation) and OPC cell proliferation (oligodendrogenesis) in this specific type of injury.

1.7.6 Other potential roles for ependymal cells

Although the ependymal layer of the spinal cord posesses neurogenic potential, the specific function(s) of ependymal cells is yet to be determined. Unlike the ependymal cells (tanycytes) lining the ventricles of the brain, little is known about those lining the central canal. They may have a role in transporting and modifying substances between the CSF and the perivascular and extracellular space (Rafols and Goshgarian, 1985). In cervical segments of adult rat spinal cord, ultrastructural analysis showed columnar shaped ependymal cells located within the ependyma with tail processes contacting blood vessels and flask shaped ependymal cells were seen subependymally with processes reaching the lumen of the canal. The apical surfaces of these ependymal cells were characterized by many microvilli and large cytoplasmic protrusions from the apical side of the soma into the lumen of the canal. Within the luminal surface, adjacent cells were seen laterally joined by tight punctate junctions containing fibrils and microtubules, while gap junctions were present at the basal end of the adjacent tail processes. These processes were intermingled together and had common boundaries with the basal lamina around the perivascular space (Rafols and Goshgarian, 1985). In zebrafish, the pan ependymal marker Foxj1 is expressed from the time of their birth in the embryonic neural tube and is required for the positioning of the central canal, proliferation and contribution to restoring the spinal cord structure (Ribeiro et al., 2017). Some of these functions may be conserved in higher vertebrates. The tanycytes of the third ventricle have deep extending processes into the hypothalamus and are thought to exchange chemical signals from the CSF to the central nervous system; possessing a potential neuroendocrine role in mice (Langlet et al., 2013, Müller-Fielitz et al., 2017). They are also thought to be gluco-sensitive and respond to feeding and arousal signals (Bolborea and Dale, 2013). Whether these functions are also carried out by ependymal cells of the central canal is still unknown.

1.8 The development of oligodendrocyte precursor cells and myelination

Neurons, astrocytes and oligodendrocytes are derived from the neuroepithelial cells of the neural tube, while microglia infiltrate from the hematopoietic system during development (Miller, 2002). Earlier studies by Altman and Bayer suggested that in regions of the central nervous system, neuronal cell types arise first followed by astrocytes then oligodendrocytes (Altman and Bayer, 1984). Most mature oligodendrocytes are found in the white matter, and oligodendrocyte precursor cells arise from specific regions of the neural tube (Miller, 2002). As early as the time of neural tube closure, the entire rostrocaudal extent of the spinal cord contains oligodendrocyte precursor cells (Warf et al., 1991). Conversely, after dorsal and ventral spinal cord regions separate during embryogenesis, oligodendrocyte precursor cells are only present in ventral regions of the spinal cord. This regional origin restriction is conserved among vertebrates including human, mouse and rat (Warf et al., 1991, Miller, 2002). Whether there are other sources in the spinal cord remains to be determined. The transient mesodermal structure; the notochord which is located ventral to the neural tube during embryogenesis and known to create the dorsal-ventral axis, is one mechanism influencing the dorsal origin of the oligodendrocyte precursor cells through a concentration dependent signalling of sonic hedgehog and activation or suppression of homeodomain transcription factors (Miller, 2002). The inhibition of sonic hedgehog signalling prior to the development of oligodendrocytes inhibits their formation altogether (with little effect on motor neurons), potentially due to lack of sonic hedgehog signals inducing oligodendrocyte specification transcription factors such as Olig genes (Miller, 2002, Lu et al., 2002, Zhou and Anderson, 2002). Bone morphogenetic proteins (BMPs) specifically BMP2 and BMP4, are oligodendrocyte inhibitors *in vitro,* although whether this inhibits oligodendrocyte production in ventral CNS regions *in vivo* is unknown (Miller, 2002). During development in the spinal cord, the emergence of oligodendrocyte precursor cells is dependent on several factors, one of which is neuregilin-1 which contributes to the induction of oligodendrocytes (Vartanian et al., 1999), and once committed to the lineage, they begin to express the cell surface marker A2B5. These cells are known as O-2A oligodendrocyte precursor cells that mainly differentiate into oligodendrocytes but can also give rise to astrocytes

in vitro under the right conditions (Miller, 2002); such as in the presence of ciliary neurotrophic factor (CNTF) in culture (Lillien et al., 1988). Once they mature into O4 expressing precursors, they lose the ability to differentiate into astrocytes (Miller, 2002). Oligodendrocyte precursor cells can be identified by the expression of PDGFA-α (Pringle et al., 1997), myelin associated proteins CNP, MBP and PLP and transcription factors such as Olig1 (Lu et al., 2002) and Olig2 (Zhou and Anderson, 2002). In general, precursor cells mature once they start expressing the major myelin glycolipid glactocerebroside (GC), exit cell cycle, become multiprocessed and have elevated levels of major myelin components (MBP and PLP) (Miller, 2002). Chemotaxis studies showed that PDGF may also be involved in promoting oligodendrocyte precursor cell migration in addition to promoting survival and proliferation (Fruttiger et al., 2000). Signals from adjacent axons are required for triggering oligodendrocyte precursor cell differentiation. These signals include FGFs, thyroid hormone, axonal cell surface markers L1, MAG, NCAM AND N-cadherin which may also regulate the formation of the myelin sheaths (Miller, 2002, Trapp et al., 1999). Axons also express neuregulins which promote oligodendrocyte maturation (Park et al., 2001). After development, the central nervous system is still significantly prevalent with oligodendrocyte precursor cells that are different in their antigenic profile and proliferative capacity than those during development (Wolswijk et al., 1991), figure 1.3.

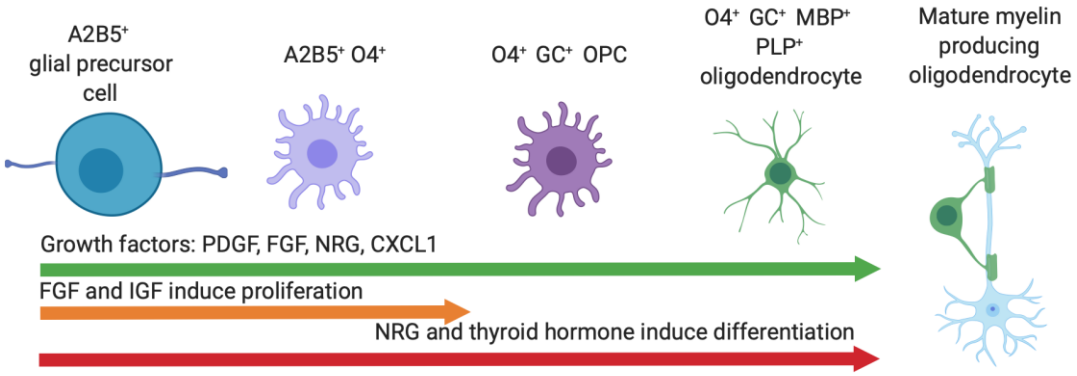

Figure 1.3 A schematic representation of the major developmental stages and growth factors involved in oligodendrocyte development.

The immature oligodendrocyte originates in distinct regions of the ventricular zone of the neural tube and quickly acquires the expression of the surface antigen A2B5. Various growth factors influence these bipolar glial precursor cells including: platelet derived growth factor **(PDGF)**, fibroblast growth factor **(FGF),** neuregulins **(NRG),** insulin-derived growth factor **(IGF)** and the chemokine factor **(CXCL1).** As they mature, they begin to express O4 and the major glycolipid in myelin galactocerebroside **(GC)** and lose proliferative responses to PDGF. Their full maturation is associated with the abundant expression of myelin components such as myelin basic protein **(MBP)** and proteolipid protein **(PLP)** enabling the formation of the myelin sheath. Created with Biorender.

Under normal conditions, most oligodendrocytes are located in the white matter forming myelin (Bunge, 1968). The myelin sheath is made of modified plasma membranes insulating axons and facilitating fast and efficient electrical conduction along axons (Bunge, 1968). A single oligodendrocyte is able to myelinate different axons at the same time (up to 60) based on the diameter of the axonal tract, they also form multiple sheaths (up to 30 or more) along the same axon as opposed to Schwann cells in the periphery (Salzer, 2015). Live imaging studies revealed that along the same axonal tract, different oligodendrocytes myelinate the axon in a sequential manner forming an expanding glial plasma membrane which ultimately compacts, and the longitudinal and radial expansion of the multilamellar myelin sheath occurs by

oligodendrocytes wrapping one axon then advancing around the apposed axon (Salzer and Zalc, 2016b). Electron microscopy studies show that compact myelin is composed of interperiod lines (extracellular leaflets of glial plasma membranes close together) alternating with major dense lines (cytoplasmic leaflets tightly close together) (Salzer and Zalc, 2016a). Myelin is a fatty tissue (Bunge, 1968) with a lipid content of roughly 70% composed of galactosphingolipids, some phospholipids, saturated long chain fatty acids and cholesterol which is necessary for myelin assembly (Salzer and Zalc, 2016a). The protein content of myelin in the central nervous system has some similarities to but also differences from that in the periphery while other organizational and structural proteins are still being discovered. In the central nervous system, the main two protein components of myelin are myelin basic protein (MBP) whose function is to bind and neutralize phospholipids on the inner plasma membrane leaflets and proteolipids protein (PLP) which spans four membrane segments. The myelin basic protein is also a major component of myelin in the peripheral nervous system. Myelin in the healthy adult is stable with little protein turn over (Salzer and Zalc, 2016a).

The nodes of Ranvier are gaps between the myelin segments on the axon body. They constitute less than 1% of the myelinated axon and are roughly 1μm long. Voltage gated sodium channels are located within these nodes. At either side of the nodes are paranodal junctions that are tightly packed between axons and the myelinating oligodendrocytes. At the electron microscopic level, these paranodal junctions can be seen as an evenly spaced series of invaginations. They function as membrane diffusion barriers promoting the accumulation of sodium channels at the node and separating them from potassium channels occupying the juxtaparanodal regions. In unmyelinated axons, these organizing structures are absent, and sodium and potassium channels are diffusely distributed among adhesion molecules (Salzer and Zalc, 2016a).

Myelin sheaths are crucial for axonal metabolism and speed of conduction. A minor change in the myelin thickness can cause significant alterations to conduction speed and affect neural circuitry (Gibson et al., 2014). The organization of the voltage gated ion channels on the axons and compact myelin allow for fast action potential

propagation. The compact myelin increases local resistance of axons and reduces membrane capacitance (the ratio of change in electrical charge to the change in electrical potential). The current flow is thus focused at the nodes of Ranvier where the sodium channels are mainly located (their presence is not prevalent between the nodes). The paranodes seal the electrical charge minimizing leakage under the myelin sheaths significantly increasing conduction speed compared to unmyelinated axons (Salzer and Zalc, 2016a). In the central nervous system, some axons are unmyelinated, however the signals dictating oligodendrocytes not to myelinate these axons are still unknown (Salzer and Zalc, 2016a). The myelinating oligodendrocytes support the axon metabolically through transferring energy metabolites such as lactate to axons via monocarboxylate transporters located on the inner membrane of the oligodendrocyte and on axons (Salzer and Zalc, 2016a).

1.8.1 Adult and adaptive myelination

Aside from developmental myelination, adaptive myelination can occur. A recent investigation employing optogenetic manipulation to activate neuronal circuitry in rodent cortex revealed that neuronal activity (cortical layer five projecting neurons) promoted oligodendrocyte precursor cell proliferation and differentiation which led to changes in the microstructure of myelin (Gibson et al., 2014). Furthermore, behavioural studies revealed that speed of forelimb swing increased at the correlate side (Gibson et al., 2014). Conversely, socially isolated mice exhibited impaired myelination and changes in behaviour as well as transcriptional and structural changes to oligodendrocytes of the prefrontal cortex, while social re-integration normalized behavioural and transcriptional alterations (Liu et al., 2012). In human brain, MRI imaging showed that learning triggered myelin structural changes although the cellular and molecular mechanisms underlying these events are not yet defined (Zatorre et al., 2012). Thus, oligodendrocyte precursor cells are capable of sensing and responding to neuronal activity. This occurs through the expression of various receptor subtypes (Karadottir and Attwell, 2007) and their posession of functional synapses with adjacent axons from early development throughout adulthood (Almeida and Lyons, 2014, Dimou and Gallo, 2015). Of relevance, the early studies of these precursor cells (O-2A) revealed that they express cholinergic receptors among other receptor types

(glutamatergic and GABA-ergic) (Bergles and Richardson, 2015); this suggests that the behaviour of precursor cells can be manipulated with cholinergic transmission. Oligodendrocyte precursor cells are exclusively post-synaptic and receive direct synapse input from axons and neurons. Interestingly, these precursor cells maintain their synaptic connections during cell division. This is done by fission-like division during which each daughter cell acquires half of the original processes and the mother cell repositions the processes continually on the axons while moving throughout the parenchyma (Bergles and Richardson, 2015).

A variety of factors have been implicated in promoting proliferation, differentiation and survival of these precursors including but not limited to; members of the transforming growth factor (TgF)-β / bone morphogenetic protein (BMPs), insulin-like growth factor-1, neuregulins, Wnt signaling, neurotrophin-3, chemokines, ciliary neurotrophic factor (CNTP), brain derived neurotrophic factor (BDNF), transferrin, thyroid hormone and others (Bergles and Richardson, 2015). Furthermore, additional roles for these precursor cells may involve maintaining potassium homeostasis in the extracellular matrix by being in contact with nodes of Ranvier and axonal / dendritic synapses or secretion of modulatory factors (Almeida and Lyons, 2017).

1.8.2 Remyelination in adulthood and pathological situations

It is still unknown whether oligodendrocytes of healthy individuals die, however, remyelination in adulthood differs from that occurring during development. There are distinct morphological changes in oligodendrocytes produced in adulthood with respect to length of internodal segments they form (shorter segments in adulthood) and thickness of myelin sheaths (less thick in adulthood or remyelination), slowing conduction velocity. Newly formed oligodendrocytes may also form new internodal segments from previously established ones (Young et al., 2013, Bergles and Richardson, 2015). The axonal diameter and molecular composition is also affected by neuronal activity and learning (Chéreau et al., 2017); axonal diameter increases with increased activity. Time-lapse super resolution microscopy and electrophysiological experiments showed axons growing transiently wider after firing high frequency action

potentials, while synaptic boutons grew transiently larger at a faster pace (Chéreau et al., 2017).

In cases of chronic relapse-remitting multiple sclerosis, remyelinating denuded axons is crucial to protect axons from further injury, neuronal death and irreversible neurological degeneration. Thus, remyelination may restore cell signalling between oligodendrocytes and axons and properly redistribute ion channels at the nodes of Ranvier for proper salutatory conduction. Although current treatments aim to reduce inflammation with immune modulatory drugs, a better understanding of innate immunity during myelination and remyelination after demyelination has occurred is necessary to identify therapeutic targets to promote and enhance remyelination. Although microglia and macrophages release cytotoxic molecules (proteinases, cytokines, reactive oxygen and nitrogen intermediates) (Banati et al., 1993) and are involved in myelin antigen presentation (Cash et al., 1993), they also provide myelin debris phagocytosis and oligodendrocyte precursor cell recruitment (Kotter et al., 2005). Induced focal demyelination in mouse corpus callosum revealed that 10 days post injury, a switch from an M1 (pro-inflammatory) phenotype of microglia to M2 (anti-inflammatory) phenotype. There were over 80% M1 type microglia at 3 days post injury and this significantly dropped to approximately 25% at 10 days, while M2 type microglia constituted roughly 5% at 3 days post injury and significantly increased to nearly 75% of all microglia at 10 days. This was analysed using state specific markers; the inducible nitric oxide synthase (iNOS) marker for M1 and arginase-1 (Arg-1) for M2 type microglia. The timing during which this had occurred (10 days) corresponds to the time of initiation of remyelination in this specific demyelination model (Keough et al., 2015). By 21 days post injury, a time during which remyelination is completed (Keough et al., 2015), there were mainly M1 type cells, and fewer M2 type microglia than seen at day 10 and a low percentage of unpolarized (iNOS and Arg-1 negative) cells. This polarization event was confirmed using different state specific markers and a different demyelination model employing ethidium bromide. It was also independent of peripherally derived macrophages since the switch in microglial phenotype still occurred in a knock out mouse model whose monocytes (from which macrophages derive) cannot migrate (Miron et al., 2013). The absence of macrophages in knockout mice did reduce the total number of M1 and M2 type microglia to roughly 70 % and

50% respectively. Furthermore, the exposure of oligodendrocyte precursor cells to M1 and M2 conditioned media enhanced precursor cell proliferation and chemotaxic migration. M2 alone conditioned media *in vitro* promoted the differentiation of these cells and prevented cell apoptosis while depletion of M2 *in vivo* using mannosylated clodronate liposomes (MCL) that bind to mannose receptors which are upregulated in M2 cells following polarization inhibits precursor cell differentiation. When M2 type microglia were increased in parabiosis (circulation sharing) experiments where the circulation of older mice was replaced by the circulation of younger mice and young monocytes can be recruited to demyelinated lesions in older mice; remyelination was enhanced (Miron et al., 2013). The oligodendrocyte precursor cell drive to differentiation by M2 microglia is suggested to be mediated by M2 microglia's contribution to the transforming growth factor-β (TGF)- β family member activin-A. M1 depletion *in vivo* however, did result in a lower number of proliferating oligodendrocyte precursor cells but did not affect cell differentiation or migration (Miron et al., 2013). These results define clear roles for the two microglial phenotypes during demyelination and remyelination. Regulatory or suppressor T cells are also implicated in enhancing developmental myelination and remyelination (Dombrowski et al., 2017). In a lysolecithin focal demyelination mouse model of either wild type mice, regulatory T cell depleted animals or transgenic mice lacking Foxp3-expressing regulatory T cells (implicated in tissue regeneration), they showed that regulatory T cells do not affect oligodendrocyte precursor cell proliferation, however, regulatory T cell$^-$ depleted animals had significantly lower numbers of mature CC1$^+$ Olig2$^+$ differentiated cells compared to wild type animals and those lacking Foxp3-expressing regulatory T cells. This was confirmed by electron microscopy, revealing significantly fewer myelinated axons in regulatory T cell$^-$ depleted animals. Injecting regulatory T cells harvested from wild type mice into regulatory T cell depleted animals resulted in rescued oligodendrocyte precursor cell differentiation (Dombrowski et al., 2017). Thus, T cells are critical for differentiation but not proliferation of OPCs in pathological conditions.

Recent research characterizing oligodendrocyte cell heterogeneity in health and disease revealed different oligodendrocyte and oligodendrocyte precursor cell subtypes with potential variable functions (Falcão et al., 2018, Jäkel et al., 2019). Experimental autoimmune encephalitis mouse models have distinct oligodendrocyte

and oligodendrocyte precursor cells with some expressing major histocompatibility complexes (MHC-I and II) that may be involved in antigen presentation, phagocytosis and CD4 positive T cell activation (Falcão et al., 2018). Furthermore, human multiple sclerosis brains contained unique oligodendrocyte cell subtypes exhibiting a molecular profile associated with multiple sclerosis genes (Falcão et al., 2018). Interestingly, similar markers of individual cell subtype or subcluster were seen in normal appearing white matter of post-mortem tissue of multiple sclerosis patients showing the diffuse effect of the disease (Jäkel et al., 2019). They also report the transcriptional heterogeneity of oligodendrocytes in human white matter of control and diseased individuals as well as some similarities between those in human white matter and mouse white matter (Jäkel et al., 2019).

This heterogeneity discovered between normal and pathological individuals can provide researchers with a better understanding of disease progression, while the similarities observed between humans and mice renders animal research more promising.

1.9 Can the adult spinal cord produce new neurons?

1.9.1 Other neurogenic niches of the CNS

The term neurogenesis is commonly used to describe the production of not only new neurons but also oligodendrocytes and astrocytes. However, when adult neurogenesis was first discovered by Altman and Das, they were considering only the production of new neurons (Altman and Das, 1965). They provided the first line of evidence for newly generated granule cells in the postnatal rat dentate gyrus. The use of a nucleotide analogue as a cell tracer (bromodeoxyuridine; BrdU) a few years later (Kuhn et al., 1996) propelled the field of adult neurogenesis even further, thus establishing the presence of the two adult neurogenic niches (SGZ and SVZ) in almost all mammals including humans (Eriksson et al., 1998). BrdU infusions into patients for tumour tracing purposes and post-mortem analysis concluded the presence of dividing cells in the dentate gyrus in the same location and similar numbers to that observed in

murine animals (Eriksson et al., 1998). Identifying these cells as stem / progenitor cells using immunohistochemistry was later done on post-mortem brains (Curtis et al., 2003, Boekhoorn et al., 2006) among others.

The formation of adult born functional neurons from adult neural precursors is generally referred to as postnatal or adult neurogenesis. This contributes to neural plasticity and improved cognition in adulthood. There have been major advances in the understanding of this phenomenon occurring in known mammalian neurogenic niches; the dentate gyrus subgranular zone of the hippocampus (SGZ) (Kempermann, 2012, Toni et al., 2008) and the subventricular zone of the lateral ventricle (SVZ) in recent decades (Lois and Alvarez-Buylla, 1994). The SVZ contains the largest pool of dividing neural cells in adult rodents (Goldman, 1998, Temple and Alvarez-Buylla, 1999). Neurogenesis in other regions is generally believed to be induced after injury (Gould, 2007, Chi et al., 2006, Danilov et al., 2006, Darian-Smith, 2009). Some suggest the occurrence of adult neurogenesis in the hypothalamus after the systemic administration of CNTF and BrdU (Kokoeva et al., 2005) and the neocortex (Cameron and Dayer, 2008). However, neocortical adult neurogenesis was disputed by Kornack and Rakic who argued that the BrdU positive cells in the cerebral wall and neocortex of adult primates were not neurons (Kornack and Rakic, 2001). This conclusion was based on the absence of double labelling with neuronal or glial markers, thus limiting neurogenesis to the two known regions. These neurogenic niches are complex structures that house a variety of different cell types including NSCs, glial cells, vascular cells, and neural progenitors. This environment's plasticity relies on cell-cell communication or extracellular signals from local and distant locations that reach the niche through the cerebrospinal fluid (CSF) or the vasculature to drive self-renewal and differentiation (Urban and Guillemot, 2014a). Although how all of these molecular cues communicate remain to be elucidated. A recent study estimated the total number of adult-born neurons in rat hippocampus to be around 9000 cells per day and about 2700 in mice (Sailor et al., 2017). Out of all these newly generated cells, roughly 30% of them would survive after 4 weeks to become mature neurons with integrated dendrites and axons.

The resident adult neural stem cells in each niche are thought to be distinct, along with the underlying neurogenic governing mechanisms. A recent comparison of the two neurogenic niches however revealed molecular morphogen signals persisting from embryogenesis to adulthood which may have important roles during adult neurogenesis (Alvarez-Buylla and Lim, 2004b). These morphogens include notch signalling, BMPs, ephrins, noggin and sonic hedgehog (Shh) (Alvarez-Buylla and Lim, 2004b). Adult subventricular neurogenesis, which occurs under normal physiological conditions consists of new neurons being generated in the subventricular zone then migrating through the rostral migratory stream (RMS) to the olfactory bulb where they differentiate and integrate as granule and periglomerular interneurons (Gage, 2000); this contributes to experience-dependent plasticity in the postnatal brain (Livneh and Mizrahi, 2011, Sakamoto et al., 2014, Livneh et al., 2014). In the SVZ, the radial glial astrocyte progenitors (type B cells) are the primary precursors that transform into rapidly dividing transit amplifying (type C cells) which then commit to migratory neuroblasts (type A cells) (Alvarez-Buylla and Lim, 2004b, Lim et al., 2000). It is widely accepted that CSF-contacting $GFAP^+$ $nestin^+$ $vimentin^+$ PSA-$NCAM^+$ and $Tuj1^+$ type B glial astrocyte progenitors of the lateral ventricle are the resident neural stem cells that produce the type A cell $Mash1^+$ transiently amplifying migrating progenitors (neuroblasts) that are now $GFAP^-$ and $vimentin^-$, but still express nestin, PSA-NCAM and Tuj1. These differentiate into DCX^+ neuroblasts which migrate to the olfactory bulb (Taupin, 2007).

Neurons in the dentate gyrus are born in the underlying subgranular zone and integrate into the dentate gyrus (Gage, 2000) contributing to enhanced memory processing (Kropff et al., 2015). In both these niches, resident radial glial astrocyte-like progenitor cells are thought to be the stem-like cells (Alvarez-Buylla and Lim, 2004b, Doetsch et al., 1999, Kempermann, 2012). Radial glial astrocyte progenitor cells from the SVZ are only capable of proliferation and interneuronal differentiation when transplanted into another SVZ and not when grafted into a non-neurogenic region (Gage, 2000, Lim et al., 2000, Alvarez-Buylla and Lim, 2004b). The SGZ radial glial astrocyte progenitors also only exhibit neurogenic potential in SGZ grafts which potentially implicates the extracellular microenvironment in enabling neurogenesis (Alvarez-Buylla and Lim, 2004b). Furthermore, the basal lamina in the SVZ, with which

the progenitors are in direct contact, contain abundant laminin and collagen-1 and are in close contact with perivascular connective tissue containing fibroblasts and macrophages (Mercier et al., 2002, Alvarez-Buylla and Lim, 2004b), figure 1.4. Proximity and contact with the basal lamina and perivascular tissue is suggested to potentially provide a permissive neurogenic environment (Mercier et al., 2002, Alvarez-Buylla and Lim, 2004b). In the SGZ, vascular endothelial growth factor receptor 2 (VEGFR2) colocalises with DCX and can potentially directly mediate neurogenesis in this region (Jin et al., 2002, Alvarez-Buylla and Lim, 2004b). Moreover, both the SVZ and SGZ astrocytes express receptors for the cell signalling molecule notch which is sometimes known to suppress differentiation and promote the precursor cell state. It has been also shown to enhance cell proliferation in the SVZ postnatally (Gaiano et al., 2000) and inhibit cell differentiation and migration to the olfactory bulb (Chambers et al., 2001, Alvarez-Buylla and Lim, 2004b). SVZ astrocytes also express the epidermal growth factor (EGF) activating receptor TGFα (Tropepe et al., 1997, Alvarez-Buylla and Lim, 2004a). Sonic hedgehog signalling plays a role in both the SVZ and SGZ neurogenic regions. Its overexpression increases cell proliferation in the SGZ *in vivo* and maintains the cells in a proliferative state in culture (Lai et al., 2003, Alvarez-Buylla and Lim, 2004b). Animals with a smoothened (the coreceptor for Shh) knockout had fewer proliferative cells in both the SVZ and SGZ (Machold et al., 2003, Alvarez-Buylla and Lim, 2004b). The bone morphogenetic proteins (BMPs) not only have a role in promoting astrocyte differentiation over neurons and oligodendrocytes during development but are also expressed (as well as their receptors) by the adult astrocytes in the SVZ and has been shown to promote these cells to remain in a stem cell-like state (Reynaud-Deonauth et al., 2002).

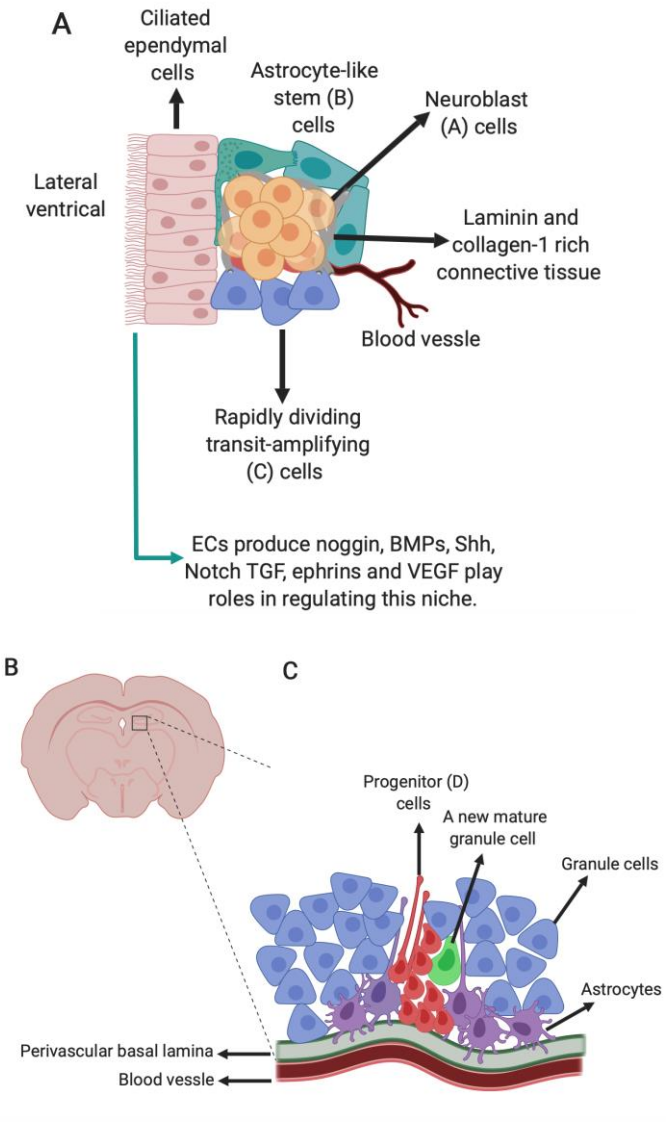

Figure 1.4 Adult SVZ and SGZ neurogenesis.

Panel **A** illustrates cell types involved in SVZ neurogenesis: astrocytes (B cells); the stem cells of this niche contact the ventricular lumen and give rise to rapidly dividing transit-amplifying cells (C cells). C cells give rise to neuroblasts (A cells) which migrate to the olfactory bulb. These cells are extensively intertwined with basal lamina (depicted in grey). The ciliated ependyma cells line the ventricle wall and produce factors known to regulate this niche. Panel **B** depicts a coronal section of an adult mouse brain at the level of the hippocampus. The dentate gyrus is shown in the enlarged panel **C**. Astrocytes give rise to progenitor (D) cells which mature into new granule cells. Created with Biorender.

1.9.2 Adult spinal cord production of new neurons

In the adult mammalian spinal cord, production of new neurons is known to exclusively occur after injury or neurotransmitter modulation. For instance, a study reported the occurrence of adult neurogenesis in the spinal cord after motor neuron degeneration in amyotrophic lateral sclerosis (ALS) (Chi et al., 2006), while newborn spinal cord neurons were observed in a rat model of multiple sclerosis (Danilov et al., 2006). After the induction of experimental autoimmune encephalomyelitis in these animals, Dil labelled ependymal cells proliferated and migrated to the inflamed region where they differentiated into NeuN and tuj1 positive neurons providing the first line of evidence for neurogenesis occurring in an inflammatory lesion. Electrophysiological assessment of these newly generated neurons confirmed their integration and functionality; exhibiting immature neuronal firing patterns (Danilov et al., 2006). Thus, the endogenous multipotent latent stem-like cells of the spinal cord can potentially contribute to some functional recovery after degeneration, although, these findings are yet to be reproduced and confirmed given the contradictory conclusions in later published studies (Ren et al., 2017a, Lacroix et al., 2014). Furthermore, contusion and transection spinal cord injuries result in robust ependymal cell proliferation, migration and multipotent differentiation as previously discussed in section 1.5 (Johansson et al., 1999, Mothe and Tator, 2005, Meletis et al., 2008, Barnabé-Heider et al., 2010, Lacroix et al., 2014), astrocytes and oligodendrocytes also contribute to self-renewal under these conditions (Barnabé-Heider et al., 2010), while demyelination injuries trigger oligodendrocyte precursor cells to proliferate, migrate and differentiate (see section 1.6).

In addition, several groups of researchers have recently identified a cell population residing in rat brain meninges that are capable of neural differentiation *in vitro* and after transplantation *in vivo* (Bifari et al., 2015). These cells also expressed neural precursor markers such as nestin, vimentin, Sox2, and doublecortin (Bifari et al., 2015). Radial-glial like cells in the meninges of peri-natal mouse brain have also been reported to migrate and differentiate into functionally integrated neurons in the neonatal cortex (Bifari et al., 2017). Given that the meninges are present throughout the entire central nervous system and the functional significance of this newly discovered cell

population is yet to be determined, these meningeal cells may contribute to spinal cord neurogenesis. Therefore, since our study investigates neurogenesis in the intact spinal cord of wild type mice without cell lineage tracing, it is plausible for meninge-residing neural stem cell-like cells to contribute to cell proliferation and / or differentiation.

This project focuses on adult spinal cord neurogenesis following acetylcholine signalling modulation and cholinergic modulation in a focal demyelination animal model. Cholinergic signalling and adult neurogenesis will be covered in the following section.

1.10 Acetylcholine (ACh)

The neurotransmitter ACh is synthesized from Acetyl CoA and choline via the catalytic enzyme choline acetyltransferase (ChAT). ACh is then transported into the presynaptic vesicle via the vesicle acetylcholine transporter (VAChT) where it is stored. After release of ACh to the synaptic cleft, it binds to specific receptors: ionotropic nicotinic acetylcholine receptors (nAChR) and /or metabotropic transmembrane muscarinic acetylcholine receptors which bind to G-coupled proteins (mAChR). Some of the ACh is hydrolyzed by the acetylcholinesterase (AChE) into choline and acetate, and the choline is then picked-up by a presynaptic high-affinity choline transporter (George J Siegel, 1999.), figure 1.5.

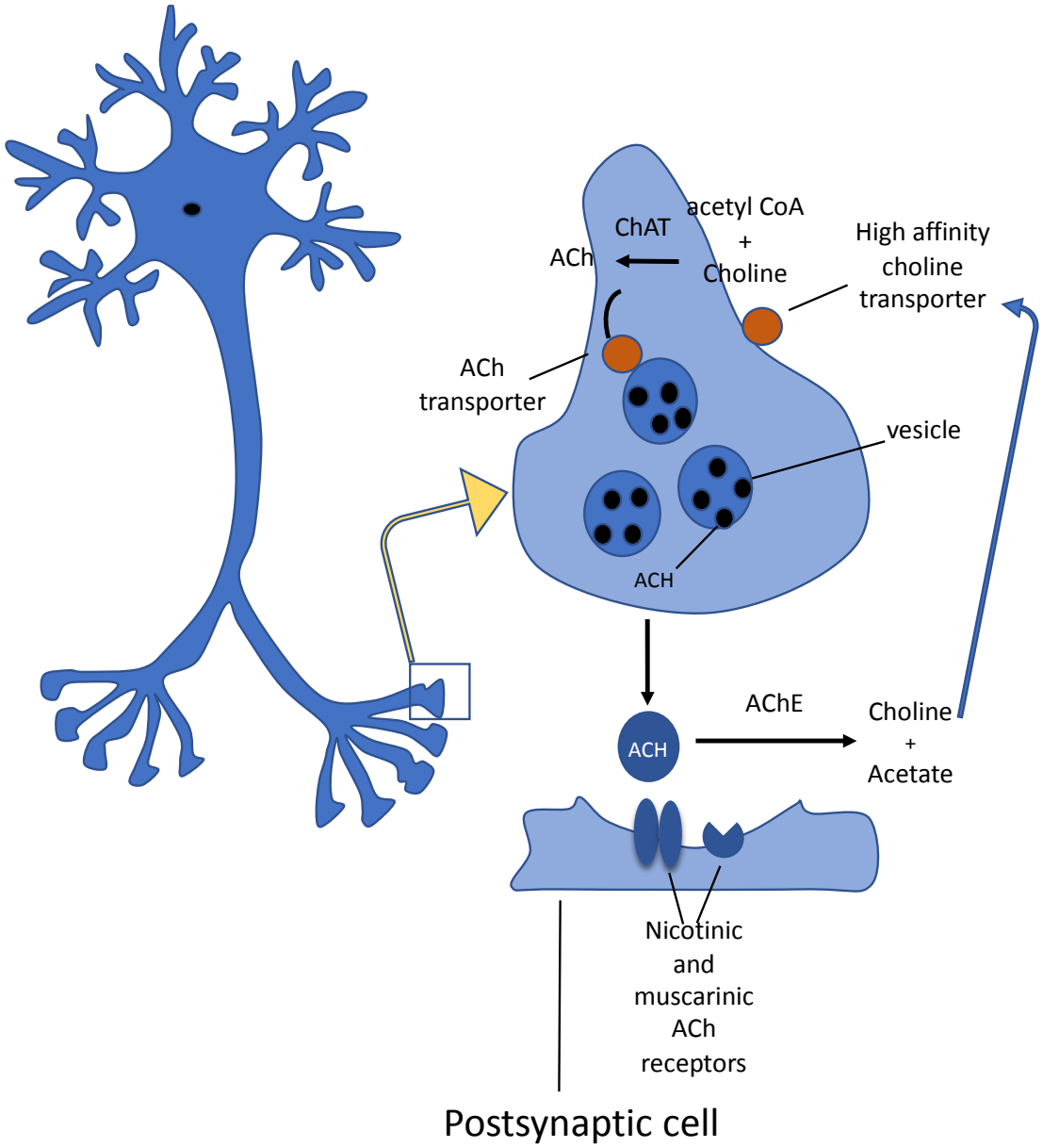

Figure 1.5 Acetylcholine synthesis and breakdown.

The presynaptic terminal of cholinergic neurons synthesize acetylcholine from both acetyl-CoA and choline. Choline acetyltransferase **(ChAT)** catalyzes the transfer of an acetyl group from acetyl-CoA to choline. Some acetylcholine will be stored in the vesicles and some will be released into the synaptic cleft. Postsynaptically, acetylcholine interacts with both nicotinic and muscarinic receptors. Acetylcholinesterase **(AChE)** then hydrolyses acetylcholine to choline and acetate to facilitate the reuptake by the high affinity choline transporter.

Acetylcholine neurotransmission occurs via either nicotinic or muscarinic receptors, collectively known as the cholinergic receptors. These receptors have been termed as such due to a study conducted in the 20th century using muscarine and nicotine alkaloids (Brown, 2006). Both the muscarinic and nicotinic receptors vary in subtype composition. The neuronal nicotinic receptors are pentameric structures composed of different combinations of α2 - α10 and β2 - β4 subunits. They are differentially distributed throughout the central nervous system (the distribution of identified receptors will be discussed below). On the other hand, the nicotinic receptors found on neuro-muscular junctions differ in composition. They are composed of β, α, γ, and δ subunits. These nicotinic subunits join and form a pore permeable to sodium, potassium, or calcium cations based on the receptor subunit composition. Neuronal nicotinic acetylcholine receptors (nAChRs) are of two kinds: the homomeric type– made up of a combination of five α2 - α9 subunits – and the heteromeric type – made up of a combination of different α2 - α10 and β2 - β4 subunits. In the central nervous system, most of the nicotinic acetylcholine receptors are of the α4β2 composition or α7 subunit composition (Romanelli et al., 2007). The nicotinic receptors may be found either pre or post-synaptically in both neuronal and non-neuronal areas such as the skin, lymphocytes, retina, and other regions (Gotti and Clementi, 2004). These receptors have different states; the active state, desensitized state, and the resting state. Brief exposure to the neurotransmitter in high concentrations activates the receptor by opening the cation permeable / selective pore which milliseconds later closes to become nonconductive, while prolonged exposure to the agonist nicotine at low concentrations causes desensitization of the receptor; keeping the receptor in a closed state and unresponsive to the agonist (Dani and Bertrand, 2007). The nicotinic acetylcholine receptors are widely distributed; they may be present on dendrites, axons and cell bodies, they also can be pre-synaptic (enhancing neurotransmitter release), post-synaptic and non-synaptic mediating the excitatory signals and modulating intracellular downstream signalling (Dani and Bertrand, 2007). Since not all cholinergic projections terminate with a synapse on cellular targets (e.g. a significant amount of diffuse cholinergic transmission occurs in hippocampal and cortical sites), the concentration of ACh in these regions would depend on the amount of AChE present (Dani and Bertrand, 2007). One area where AChE was not detected is the main

olfactory bulb of rats, however, a large number of cholinergic synapses are present there indicating the variable distribution of the AChE to ACh (Dani and Bertrand, 2007).

The nicotinic acetylcholine receptors are implicated in attention, learning and memory and developmental processes (Dani and Bertrand, 2007). The disruption of nicotinic acetylcholine receptor signalling is linked to various pathologies including Alzheimer's disease (Court et al., 2001), aging and dementia (Picciotto and Zoli, 2002) and epilepsy (Raggenbass and Bertrand, 2002) among other diseases (Gotti and Clementi, 2004).

The muscarinic acetylcholine receptors are also of multiple subtypes. The subtypes are termed M1, M2, M3, M4, and M5. The receptor contains seven different transmembrane domains and cytoplasmic and extracellular loops. The third inner cytoplasmic loop contacts the G protein. Upon activation, each distinct muscarinic acetylcholine receptor subunit may be involved in activating a specific signalling pathway and is implicated in distinct ion channel modulation and various physiological functions (Abreu-Villaça et al., 2011). Muscarinic receptor subtypes are variably distributed in the central nervous system. The use of highly selective antibodies to specific muscarinic receptor subtypes enabled analysis of muscarinic receptor distribution in rat brain models. The receptor subtypes showed high variability in their localization. The M1 subtype protein was found in the cortex and striatum cell bodies and neurites, the M2 subtype protein was prevalent in both cholinergic - the basal forebrain, striatal neurons, cranial motor nuclei, and mesopontine tegmentum- and non-cholinergic structures; the non-cholinergic cortical and subcortical areas implying postsynaptic modulation of other neurotransmitters (Levey et al., 1991).

The neuronal activity of some cholinergic populations consists of low-level, sustained activity patterns which results in the release of acetylcholine in low concentrations known as tonic firing patterns. Others have a more robust, synaptic and time-related activity known as phasic firing, occurring much faster (Parikh et al., 2008). Thus the type of firing and neurotransmitter release will affect the concentration of acetylcholine at the synaptic cleft and the rate of its breakdown by the acetylcholinesterase (Unal et al., 2012). The nicotinic acetylcholine receptors however

are known for their rapid desensitization after their activation by acetylcholine (Quick and Lester, 2002, Giniatullin et al., 2005) which results in short bursts of ACh release which in turn has a qualitatively different effect on the receiving cell compared to the continuous exposure to the neurotransmitter or its continuous absence. Muscarinic receptors have longer lasting currents. Moreover, activity-dependent synaptic release of ACh provides fast and easy access of high concentrations of ACh by the receiving receptors, while nearby spill over or bulk ACh release at non-synaptic sites results in low-level ACh diffusion into a larger space of receptors (Asrican et al., 2016). In neurons, the nicotinic and muscarinic acetylcholine receptors can have either opposing currents and divergent calcium signalling or complimentary currents activating the same intracellular signalling cascades (Asrican et al., 2016).

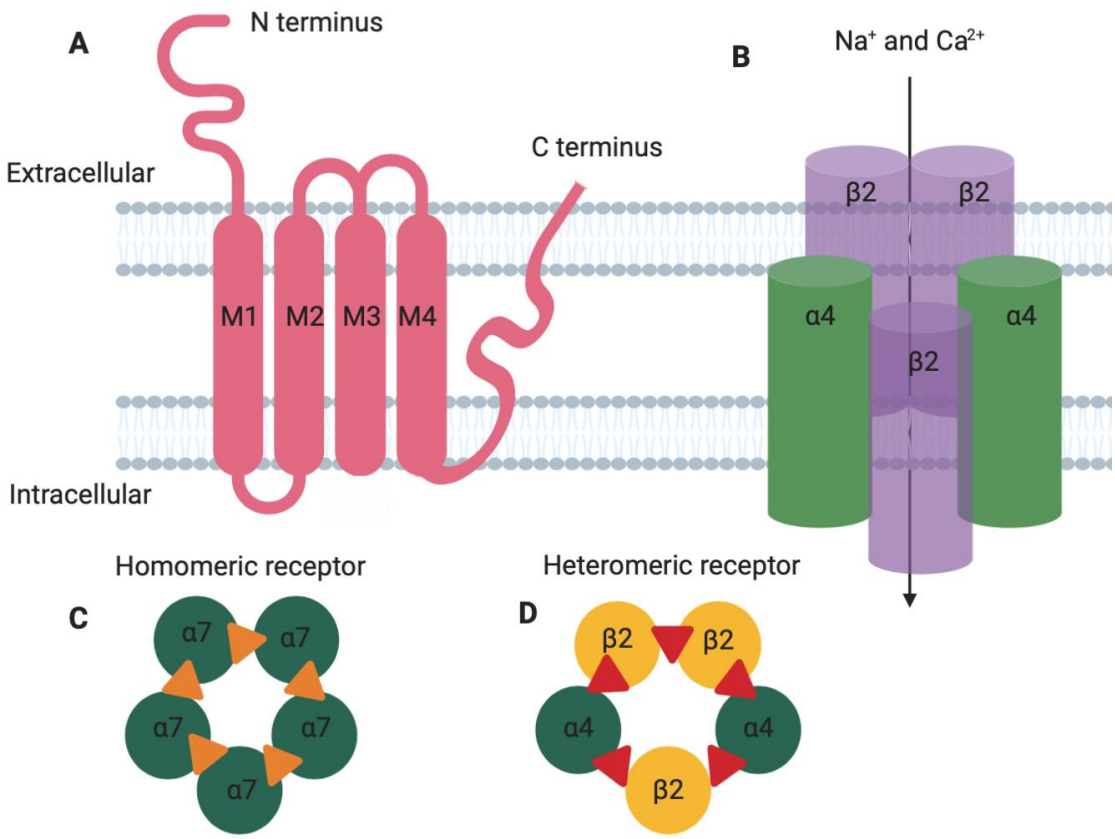

Figure 1.6 Nicotinic and muscarinic acetylcholine receptors.

These are schematic illustrations of neuronal acetylcholine receptors. **A)** Depicts a muscarinic metabotropic acetylcholine receptor (mAChR) with different transmembrane domains and cytoplasmic and extracellular loops. These metabotropic receptors are coupled to intracellular G proteins at the third inner cytoplasmic loop. They consist of five different subtypes (M1, M2, M3, M4 and M5). **B)** Illustrates a hetero-pentameric nicotinic acetylcholine receptor (nAChR). These receptors are made up of five subunits arranged in a circular pattern forming a central cation permeable pore. **C)** A schematic representing a homo-pentameric α7 nAChR. This specific receptor subtype contains five allosteric acetylcholine binding sites. **D)** A schematic representing a hetero-pentameric α4β2 nAChR. Hetero-pentameric receptors contain only two allosteric acetylcholine binding sites. Created with Biorender.

1.11 Cholinergic circuit involvement in adult neurogenesis

The cellular and molecular profile of resident neural stem cells in the SVZ and SGZ neurogenic niches are well characterized, however, the role of local neurotransmitter signalling in regulating this process is less understood.

Extracellular factors and cell-cell interactions have roles in regulating adult neurogenesis (Kriegstein and Alvarez-Buylla, 2009, Urban and Guillemot, 2014b). Neurotransmitters including GABA, serotonin, glutamate and dopamine also have regulatory roles in adult neurogenesis (Lennington et al., 2011, Young et al., 2011, Crowther and Song, 2014, Tong et al., 2014, Alenina and Klempin, 2015).

Cholinergic input into the brain and spinal cord include local cholinergic interneurons in the central cortex, hippocampus and striatum, as well as long projecting cholinergic neurons from the magnocellular basal nucleus, pontomesencephalic tegmentum, cranial nerve motor nuclei and motor neurons in the spinal cord, sympathetic preganglionic cholinergic neurons in the spinal cord and cholinergic neurons within the sympathetic nervous system (Bigl et al., 1982, Woolf et al., 1983, Mesulam et al., 1984, Nicoll, 1985, Smiley and Mesulam, 1999, Kasa, 1986). These cholinergic neurons in the brain are organized into groups or nuclei based on their originating anatomical location and projection (Asrican et al., 2016), (figure 1.6). These nuclei include: the nucleus basalis group consisting of nucleus basalis of Meynert and magnocellularis, substantia innominate and the horizontal diagonal band of Broca. The medial septal group consists of the medial septal nucleus and the vertical diagonal band. The pontine group in the upper brainstem consists of neurons of the pedunculopontine tegmental nuclei and laterodorsal tegmental nuclei (Asrican et al., 2016). The medial septum and diagonal band of Broca are thought to supply the greatest cholinergic innervation to the SGZ and thus may be the main cholinergic regulators of adult neurogenesis in that region (Asrican et al., 2016, Dutar et al., 1995, Frechette et al., 2009). Furthermore, cholinergic neurons have been identified in the subependymal layer of the LV (Paez-Gonzalez et al., 2014, Asrican et al., 2016).

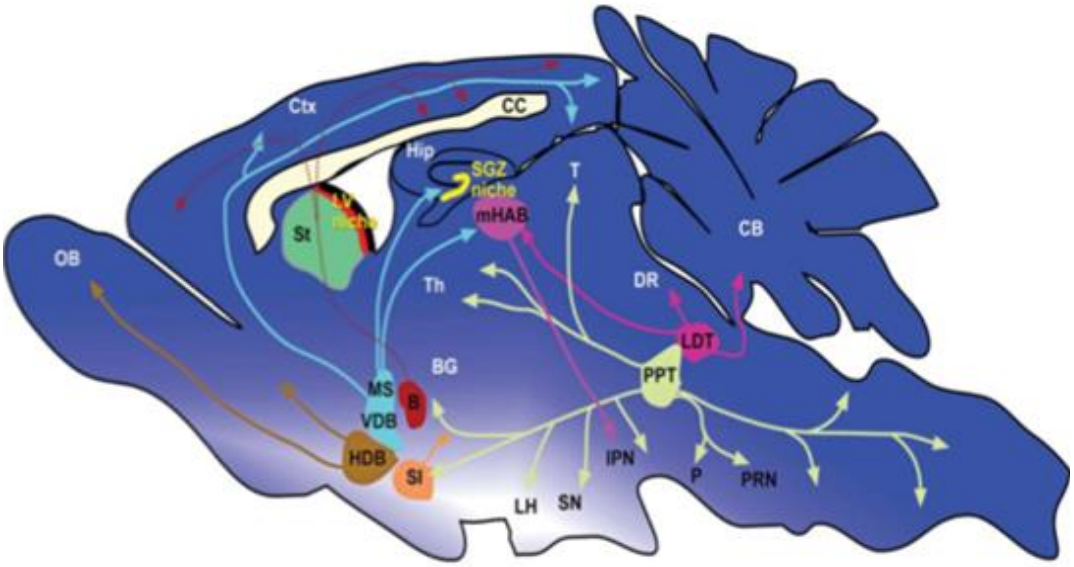

Figure 1.7 Cholinergic projections and nuclei in mouse brain SVZ and SGZ.

Adapted from (Asrican et al., 2016).

A sagittal representation of main cholinergic nuclei and their respective projections**.** The nuclei pertaining to the nucleus basalis group include: nucleus Basalis of Meynert magnocellularis **(B)**, horizontal diagonal band of Broca **(HDB)**, substantia innominate **(SI)**. The medial septal nuclei include: medial septal nucleus **(MS)**, vertical diagonal band **(VDB)**. The pontine cholinergic nuclei include: laterodorsal tegmental nuclei **(LDT)**, pedunculopontine tegmental nuclei **(PPT)**. Cholinergic neuronal groups are also present in: medial habenular nucleus **(mHAB)**, striatum **(St)** and subependymal zone **(SEZ)**. The main cholinergic projection targets include: basal ganglia **(BG)**, cerebellum **(CB)**, cortex **(CTx)**, dorsal raphae nucleus **(DR)**, hippocampus **(Hip)**, interpeduncular nucleus **(IPN)**, lateral hypothalamus **(LH)**, olfactory bulb **(OB)**, pons **(P)**, pontine reticular nucleus **(PRN)**, substantia nigra **(SN)**, thalamus **(Th)** and tectum **(T)**.

Interrupting basal forebrain cholinergic input by transecting the fimbria-fornix leads to a decrease in the number of BrdU positive neurons in the dentate gyrus suggesting the involvement of cholinergic signalling in SGZ neurogenesis (Fontana et al., 2006). Similarly, the elimination of cholinergic neurons in the basal forebrain, medial septum, nucleus basalis of Meynert and diagonal band of Broca by injecting the ribosome inactivating protein (192-saporin) also resulted in a decrease in SGZ neurogenesis. These cholinergic neurons were specifically targeted due to their expression of the p75 neurotrophic receptor which binds saporin (Frechette et al., 2009, Itou et al., 2011).

The employment of muscarinic agonists such as bethanechol, pilocarpine and oxotremorine increased the number of proliferative cells when administered *in vitro* with NSC cultures (Calza et al., 2003), hippocampal slices (Itou et al., 2011) and *in vivo* (Ma et al., 2004, Veena et al., 2011). The subcutaneous administration of the muscarinic antagonist scopolamine for 4 weeks in adult rats resulted in a decrease in the number of BrdU positive cells in the dentate gyrus, as opposed to animals treated with the acetylcholinesterase inhibitor donepezil which led to the enhanced survival of proliferative cells in the SGZ (Kotani et al., 2008). The stimulation of nicotinic acetylcholine receptors however produced variable results. Acute and intermittent nicotine administration to adult rats in vivo resulted in the enhancement of progenitor cell proliferation in the SVZ but not the SGZ. The enhanced proliferation of SVZ progenitors was reported to be supported by the presence of the growth factor (FGF-2); in in situ hybridization experiments, FGF-2 mRNA levels are upregulated in the SVZ whose progenitors express the growth factor's receptor, and that pre-treatment with the growth factor inhibitor attenuates nicotine-mediated proliferation (Mudo et al., 2007). Neural stem cells of the SVZ are reported to express α3 and α4 nicotinic acetylcholine receptor subunits (Paez-Gonzalez et al., 2014) similar to neurons of the rostral migratory stream which express α3β4 (Sharma, 2013). As opposed to the neural stem cells and neuroblasts, the Mash1^{+} transiently amplifying progenitors of the SVZ seem devoid of nicotinic acetylcholine receptor expression (Paez-Gonzalez et al., 2014). In the SGZ of the hippocampus, immunohistochemistry and functional analyses revealed the presence of M1, M4, α7 and β2 subunit expression by immature neurons (Kaneko

et al., 2006, John et al., 2015). Taken together, these results suggest that adult neurogenesis is responsive to acetylcholine, however, the variable results mediated by the nicotinic acetylcholine receptors may be due to the amount of acetylcholine release (acute verses chronic) and the cholinergic receptor subtypes that may mediate different effects.

1.12 The distribution of cholinergic neurons in the murine spinal cord

The identification of cholinergic neurons is generally done by using antibodies against the synthesizing enzyme choline acetyltransferase and / or the vesicular acetylcholine transporter or employing transgenic animals with promotors driving the expression of these proteins, as opposed to the older methods which entailed the identification of cholinergic neurons by detecting the presence or absence of the acetylcholinesterase enzyme which is present in both cholinergic and non-cholinergic neurons, (acetylcholinesterase will be discussed further in section 1.15). There are five subtypes of cholinergic neurons in the adult rat spinal cord (Barber et al., 1984b). The sympathetic preganglionic autonomic motor neurons are present in thoraco-lumbar segments only and located in the lateral funiculus and intermediolateral regions with ventral root axonal projections and a few within the spinal cord sometimes spanning several segments. The small scattered cells in laminae I-III of the dorsal horn are known as the dorsal horn cholinergic neurons. They are low in number and are presynaptic to sensory fibres and densely innervate the dorsal horn (d'Incamps et al., 2012). Partition cells and central canal cells are usually seen intermingled with sympathetic preganglionic neurons in thoraco-lumbar segments (d'Incamps et al., 2012, Barber et al., 1984b). These include somatic motor neurons, preganglionic autonomic motor neurons, small scattered cells in laminae I-III of the dorsal horn, partition cells and central canal cells. The somatic motor neurons are present in all segments of the spinal cord and located in laminae VIII and IX. Their axons mostly terminate on Renshaw cells and other motor neurons as collateral inputs (d'Incamps et al., 2012), (figure 1.7).

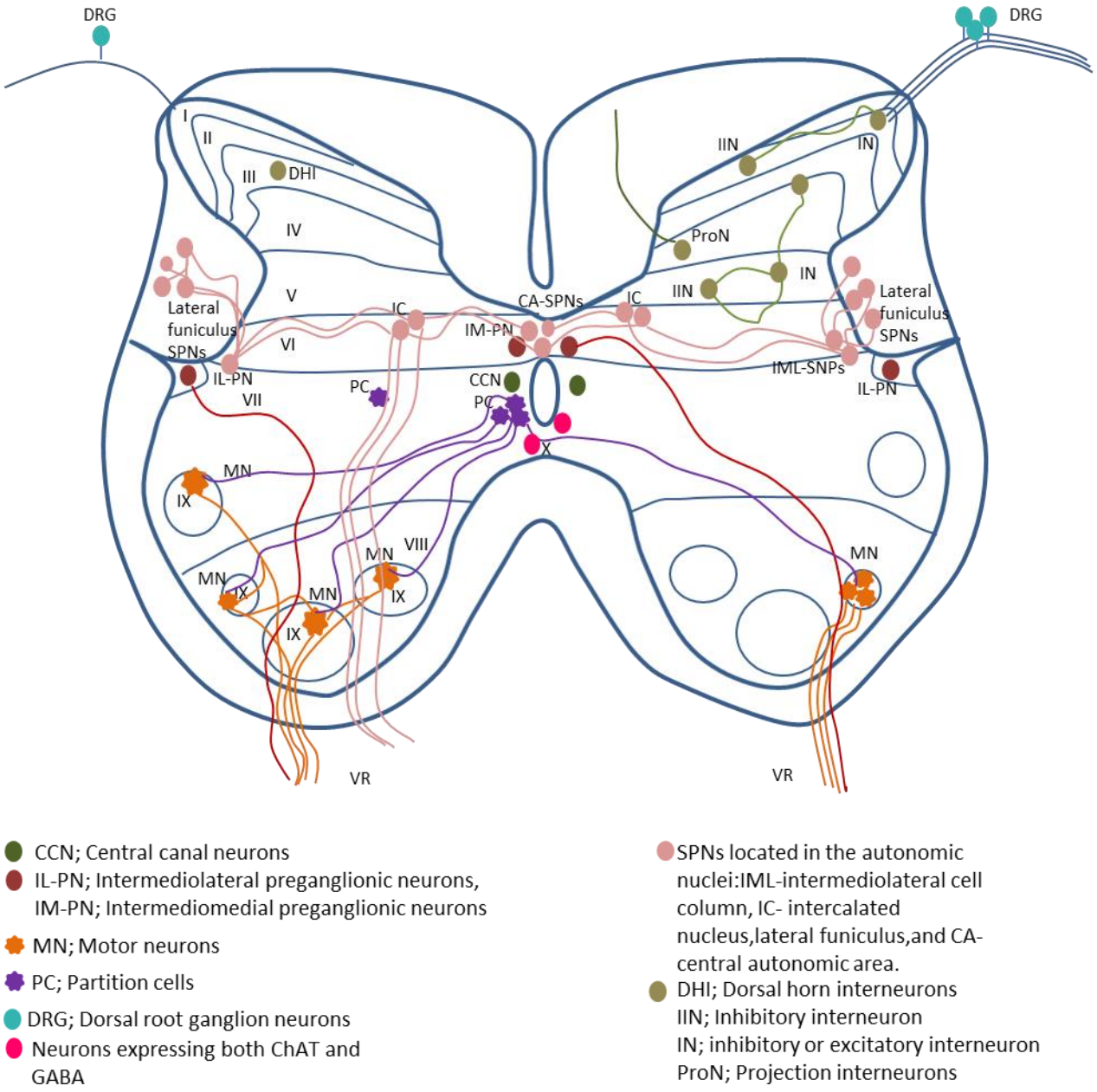

Figure 1.8 Schematic distribution of cholinergic neurons in the spinal cord circuitry.

In situ hybridization techniques and immunohistochemistry were carried out by researches to identify the distribution of nicotinic acetylcholine receptors in the spinal cord. These are summarized in the table below.

Receptor subtypes	Region identified in	Method used	Reference
α4	Marginal zone	In situ hybridization	(Wada et al., 1989)
α3, α4, β2	Substantia gelatinosa	In situ hybridization	(Wada et al., 1989)
α4, β2	Nucleus proprius, Intermediate grey, Intermediolateral column	In situ hybridization	(Wada et al., 1989)
α2, α3, α4, β2	Ventral horn, central grey region	In situ hybridization	(Wada et al., 1989)
α5	Substantia gelatinosa, ventral horn and intermediate grey	In situ hybridization	(Wada et al., 1990)
α2	Ventral horn	In situ hybridization	(Ishii et al., 2005)
No β4 signal detected	Spinal cord	In situ hybridization	(Dineley-Miller and Patrick, 1992)
α3, α4, α5, β2	Dorsal horn	PCR, IHC and western blot	(Khan et al., 2003)
α3, α5	Ventral horn	IHC	(Khan et al., 2003)
α3, α5, β2	Intermediolateral region of thoracic spinal cord	IHC	(Khan et al., 2003)
Colocalisation of anti GFAP and anti-α3, α5	Dorsal horn	IHC	(Khan et al., 2003)

Table 1.1 Nicotinic ACh receptor subtype distribution in the adult murine spinal cord.

In the adult brain, different regions contain different receptor subunit compositions. The receptor subtypes and the region they reside in are listed in the table below based on a review by (Gotti and Clementi, 2004, Romanelli et al., 2007).

Receptor subtypes	**Region identified in**
α7 and α4β2	The olfactory bulb
α7, α4β2 and α4α5β2	The cortex
α4β2, α7, α3β2 and α4α5β2	The hippocampus
α7, α4β2, α3β4 and α3β3β4	The medial habenula
α7, α3β4	The pineal gland
α4β2, α7, α3β2 and α3β4	The cerebellum
α3β4 and α6β2β3	The locus coeruleus
α4β2, α7, α2β2 and α3β3β4	The interpeduncular nucleus
α4β2	The thalamus
α4β2 and α7	The hypothalamus
α7 and α4β2	The amygdala
α4α5β2, α4β2, α6α4β2β3 and α6β2β3	The striatum

Table 1.2 Nicotinic ACh receptor subtype distribution in the adult murine brain.

1.13 The expression of α7nAChRs by progenitor cells

A great number of alpha 7 transcripts were identified in adult rat brain using in situ hybridization techniques (Seguela et al., 1993). These areas include the olfactory bulb, hippocampus, thalamus, amygdala and cerebral cortex. This receptor subtype has the highest permeability to calcium among all nicotinic acetylcholine receptors (Seguela et al., 1993). This is significant since calcium influx post receptor activation can activate

various downstream signalling cascades to promote survival, proliferation, migration, anti-inflammation among other functions (Resende and Adhikari, 2009a). It is thought to be the driving force behind cell proliferation (Resende and Adhikari, 2009a). However, whether signalling cascades downstream of calcium influx through the receptors play a role in cell proliferation in the context of neurogenesis is yet to be elucidated. The various downstream calcium signalling cascades induced by α7nAChRs activation will be discussed below.

Immunohistochemistry and electrophysiological recordings revealed the presence of functional α7nAChRs around immature granule cells in the germinal layer of the dentate gyrus SGZ of adult mice and rats (John et al., 2015). Mature granule cells however did not express α7nAChRs suggesting their potential role in supporting the maturation and / or integration of newborn granule cells into the dentate gyrus circuitry. The expression of α7nAChRs was also found on embryonic carcinoma stem cells *in vitro* (Resende et al., 2008).

The α7nAChRs are expressed by NG2 positive oligodendrocyte precursor cells of mouse hippocampus (Vélez-Fort et al., 2009) and rat corpus callosum (O2A) oligodendrocyte precursor cells (Rogers et al., 2001). Moreover, the expression of α7nAChRs by O2A oligodendrocyte precursors was not detected after the induction of differentiation (Sharma and Vijayaraghavan, 2002). Thus, the presence of this receptor on embryonic and immature cells suggests a possible developmental role in general, and potentially regulate migration and / or differentiation in oligodendrocyte precursors. The α7 nAChRs are also necessary for granule cell integration and function in the SGZ (Campbell et al., 2010). In α7knock out mice, newborn neurons have truncated and less complex dendrites than wild type mice. They also exhibit GABA-ergic immature kinetics (Campbell et al., 2010). Moreover, microglial cells also express α7nAChRs (De Simone et al., 2005) and the activation of these receptors promotes anti-inflammatory pathways (De Simone et al., 2005). The potential mechanisms by which α7nAChRs may mediate anti-inflammatory processes will be further discussed below.

In the spinal cord, immunohistological labelling of choline acetyltransferase (ChAT) revealed the presence of cholinergic structures closely apposing ependymal

cells of the spinal cord (Gotts et al., 2016). Given that ependymal cells of the central canal respond with large depolarising responses to ACh after potentiating α7nAChRs in electrophysiological settings (Corns et al., 2015), this suggests that ependymal cells express α7nAChRs, and that they potentially mediate a high proportion of the response. The α7nAChRs have also been shown to mediate the majority of the proliferative response of ependymal cells to the positive allosteric modulator PNU-120596 *in vivo* and *in vitro* (Corns et al., 2015). Moreover, effects of the pharmacological activation of α7nAChRs in the SVZ and SGZ postnatal neurogenic niches are also extensively described. In the dentate gyrus, not only are α7nAChRs the most abundant (Kaneko et al., 2006), but they are also necessary for neuronal survival, maturation and integration in the SGZ (Campbell et al., 2010). Furthermore, activating α7nAChRs lead to the activation of nuclear fibroblast growth factor receptor I (FGFR1) signalling promoting the differentiation of the neural stem / progenitor cells of the SVZ (Narla et al., 2013). Therefore, given that this receptor subtype is expressed during development and persists in almost all regions of the adult brain including the spinal cord (Broide et al., 1996, Vélez-Fort et al., 2009, Rogers et al., 2001, Gotti and Clementi, 2004, Romanelli et al., 2007, John et al., 2015); this suggests that this receptor subtype is important for development and adult neurogenesis.

Nicotinic acetylcholine receptors, especially α7nAChRs which predominate in the SGZ, alter hippocampal plasticity (Dani and Bertrand, 2007). Cholinergic modulation of plasticity in this region partly occurs through the presynaptic nAChRs increasing the release of GABA and glutamate of GABA-ergic interneurons and glutamatergic granule cells. The postsynaptic and perisynaptic nAChRs also influence plasticity by depolarizing and increasing intracellular Ca^{++} increase (Dani and Bertrand, 2007). The local administration of nicotinic antagonists such as methyllycaconitine (MLA); an α7nAChR antagonist or the α4 β2 antagonist dihydro- β - erythroidine (DHβE) into the amygdala or hippocampus impairs the working memory (memory with changing contents) of rats in a behavioral maze study (Levin et al., 2006). Conversely, acute and chronic administration of nicotine enhances working memory, while nicotinic agonists enhances learning and memory in humans and primates (Levin et al., 2006).

1.14 Calcium signalling downstream from α7nAChR activation

The signalling pathways that are activated downstream of α7nAChR activation may have roles in cellular signalling governing apoptosis / survival and proliferation (figure 1.8). Regarding an anti-apoptosis role, the α7nAChR can activate PI3-kinase through an src-family kinase (Fyn 1) which activates the anti-apoptotic kinase AKT. Activated AKT then phosphorylates the forkhead transcription factor FKHRL1 causing it to be retained in the cytoplasm with 14-3-3 blocking the expression of the apoptotic protein fas. The PI3K / AKt pathway has been proposed as a therapeutic target for cell protection against apoptosis triggered by amyloid beta in Alzheimer's disease (Shaw et al., 2002, Resende and Adhikari, 2009b). Interestingly, PI3K and Fyn (Src family of kinases) are physically associated with the α7nAChR and thus PI3k can be directly activated and lead to the phosphorylation of AKT through Fyn (Resende and Adhikari, 2009a). Furthermore, α7nAChRs can move Ca^{++} from ryanodine-sensitive intracellular stores and prevent apoptosis (Resende et al., 2008, Resende and Adhikari, 2009a). The mobilized intracellular Ca^{++} can activate BDNF which induces Cdk5 mediated neuroprotection through increased Bcl-2 expression (Resende and Adhikari, 2009a). Moreover, nicotine has been shown to promote survival of various cell types and produce anti-apoptotic effects through activating PCK, PKA and NF-kB, and downregulation of the tumor suppressor p53 (Zeidler et al., 2007, Mai et al., 2003)Resende and Adhikari, 2009a). The nicotinic phosphorylation of NF-kB triggers the phosphorylation of the apoptotic protein Bad (Bcl-2 antagonist of apoptosis) which inactivates it and prevents cell apoptosis. Other pathways including MEK and PI3K also lead to anti-apoptosis (Resende and Adhikari, 2009a). Nicotine can also induce phosphorylation of Bax through PKC and promote survival (Resende and Adhikari, 2009).

The activation of α7nAChR also enhances cell proliferation and promotes neurogenesis in both neurogenic niches; the SVZ and SGZ (Wang et al., 2017, John et al., 2015, Narla et al., 2013) including the intact spinal cord (Corns et al., 2015). However, the mechanisms mediating this proliferative effect is still unknown (Resende and Adhikari, 2009a). One possible way for α7nAChRs to promote proliferation following nicotine administration was shown to be through recruiting β-arrestin which

activates Src that binds Raf-1 kinase to Rb initiating cell cycle entry in human cell lung cancer (Dasgupta et al., 2006). These pathways are depicted in figure 1.8 based on a review by Resende and Adhikari (2009). Alternatively, or additionally, a newly implicated downstream calcium signalling pathway is the calcineurin / nuclear factor of activated T-lymphocytes (NFATs). It is a calcium / calmodulin dependent major pro-inflammatory pathway involved in the pathogenesis of various neurodegenerative diseases as a result of abnormal signalling, as well as neuronal or glial cell loss after traumatic brain or spinal cord injuries (Serrano-Perez et al., 2015, Furman et al., 2016, Gan and Silverman, 2015). Furthermore, NFAT deficient T cells in mice prevented the development of autoimmune encephalomyelitis (Reppert et al., 2015). Investigating the involvement of this particular signalling pathway in the context of cell proliferation in adult murine spinal cord is one focus of this study, and thus it will be covered in greater detail in chapter 5.

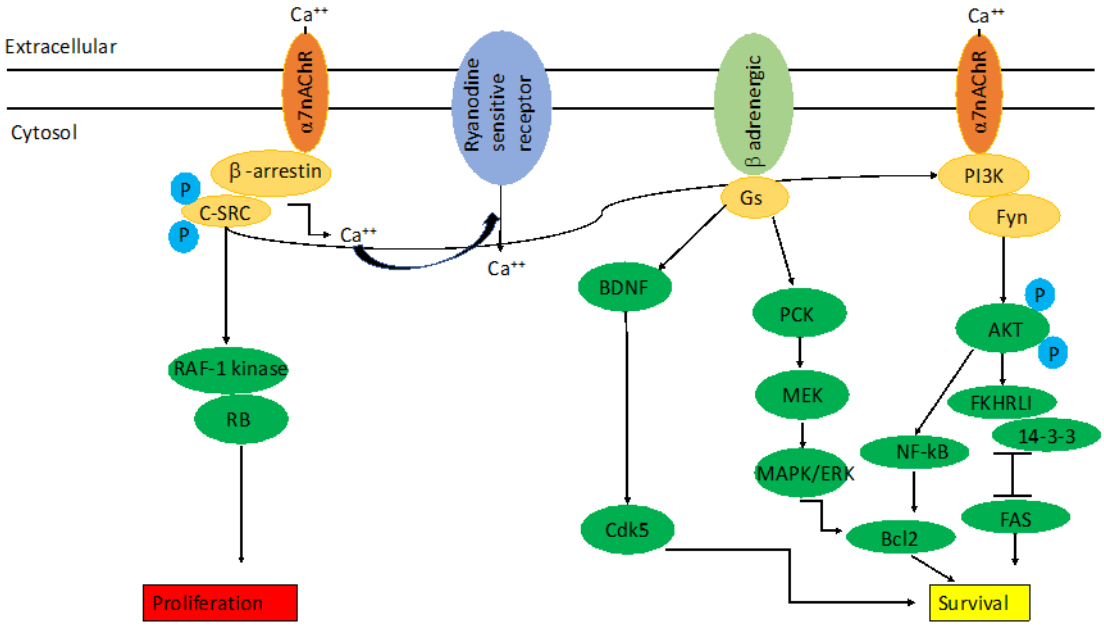

Figure 1.9 Calcium signalling downstream of α7nAChR activation.

A schematic representation of signaling pathways implicated in promoting cell survival and proliferation. α7nAChR activation leads to calcium entry and activation of the tyrosine kinase family members **(C-SRC),** the proto-oncogene protein kinase **(RAF-1)** and the retinoplasma protein **(RB)**. C-SRC activation can lead to the activation of phosphoinositide 3-kinase **(PI3K)**, the tyrosine protein kinase **(Fyn)**, protein kinase B known as **(AKT)** which activates either forkhead transcription factor **(FKHRL1)** associated with the protein **(14-3-3)**; a member of a family of conserved regulatory molecules expressed by all eukaryotic cells leading to the inhibition of the apoptotic protein **(FAS)** or by activating the nuclear factor kappa enhancer of activated B cells **(NF-kB)** which activates the pro-survival protein **(Bcl-2)**. Intracellular calcium can in turn activate brain-derived neurotrophic factor **(BDNF)** which acts on the signalling effector **(Cdk5)** promoting survival or alternatively activate protein kinase C **(PCK)** which acts on the serine / threonine kinase (MEK) that activates the microtubule associated protein kinases **(MAPK)** also known as extracellular signal-regulated kinases **(ERK)** which eventually activate Bcl2 promoting survival.

The α7nAChRs have also been shown to be involved in promoting anti-inflammatory processes. Thus, aside from being expressed by immature dente granule cells (John et al., 2015), hippocampal CA1 astrocytes (Shen and Yakel, 2012) and NG2 positive oligodendrocyte precursor cells (Vélez-Fort et al., 2009), it is also expressed by microglial cells (De Simone et al., 2005) within the central nervous system. They are also known to be expressed by cells of the immune system including monocytes (Hamano et al., 2006), dendritic cells (Sato et al., 1999), macrophages (Wang et al., 2003), T lymphocytes (De Rosa et al., 2009) and B lymphocytes (Skok et al., 2003). A variety of other cell types such as keratinocytes, adipocytes and cells involved in host pathogen defense and cell lines endogenously expressing this receptor subtype are reviewed in (Kalkman and Feuerbach, 2016).

What is interesting about α7nAChRs in neuronal cells is their substantial permeability to Ca^{++}, not only through receptor activated pore entry like the rest of nAChRs, but also when at resting or hyperpolarized membrane potentials (Fucile, 2004). It is yet to be determined if the same applies to immune cells. The anti-inflammatory intracellular signalling pathways downstream to α7nAChRs in microglial and lymphocyte cells involves calcium influx through the channel pore and occurrence of calcium induced calcium release from the ryanodine and IP3-dependent stores (Suzuki et al., 2006, Skok et al., 2003). The α7nAChRs can also act as metabotropic receptors; this can be seen in the context of receptor activation which leads to activation of adenylate cyclase-1 which increases cAMP levels which in turn stimulates protein kinase A (PKA) and may result in longer lasting signalling through CREB and GSK-β. Thus the anti-inflammatory effect is due to the inhibition of TLR-3, TLR-4 and TLR-9 gene transcription mediated through JAK-2 tyrosine phosphorylation of p85 subunit of PI3K, activation of AKT and CREB followed by the subsequent inhibition of NF-kB (figure 1.9). This specific pathway has been proposed by (Egea et al., 2015) to be the mechanism by which α7nAChR-expressing microglia mediate anti- inflammatory processes. An alternative pathway to NFkB can also be through JAK2 activating STAT3 (Shaw et al., 2002, Maldifassi et al., 2014). The activation of α7nAChRs can also inhibit p38 MAP-kinase which inhibits the release of the inflammatory mediators TNFα and HMGB1 (Shytle et al., 2004, De Simone et al., 2005, Suzuki et al., 2006) (figure 1.9). Pertinently, PNU-120596 (the positive allosteric modulator for α7nAChRs

used in our studies) has been shown attenuate the expression of the tumor necrosis factor (TNFα) and interlukin-6 (IL-6) in a murine model of inflammatory pain, and these results have been confirmed using a different positive allosteric modulator to α7nAChRs (PAM-2) (Pandya and Yakel, 2013, Bagdas et al., 2016). These positive allosteric modulators were administered in the form of injections (intraperitoneal or intrathecal) to adult mice or Sprague Dawley rats (Bagdas et al., 2016, Pandya and Yakel, 2013). Moreover, several studies have shown that positive allosteric modulators to α7nAChRs reduce the severity of brain injury after cerebral ischemia in rat models or traumatic brain injury and induce neuroprotective effects (Kalappa and Uteshev, 2013, Sun et al., 2013, Gatson et al., 2015). For instance, (Kalappa and Uteshev, 2013) employed complete oxygen and glucose deprivation on *ex vivo* hippocampal CA1 pyramidal neurons derived from Sprague Dawley rats or *in vivo* cerebral artery occlusion as ischemia models. Under electrophysiological settings, they show that choline in the presence of PNU-120596 significantly delayed anoxic depolarization injury of these hippocampal neurons which was blocked by the addition of methyllycaconitine (MLA); a selective α7nAChR antagonist. They also report a significant reduction in the infarct volume *in vivo* following the administration of PNU-120596 either pre- or post-ischemia (Kalappa and Uteshev, 2013). In a rat model of ischemic stroke (cerebral artery occlusion), intravenous injections of PNU-120596 6 hours post injury significantly reduced the infarct volume and attenuated neurological deficits compared to vehicle treated animals (Sun et al., 2013). Moreover, cortical impact rat models of brain injury were used to assess the effect(s) of PNU-120596 post traumatic brain injury (Gatson et al., 2015). They reported that subcutaneous administration of PNU-120596 5 minutes following injury significantly reduced the extent of reactive gliosis.

Furthermore, the activation of this receptor subtype induces a phenotypic switch of both macrophages and microglial cells from an M1 (pro-inflammatory) to M2 (anti-inflammatory) phenotype in microglial cultures (Carnevale et al., 2007) and human peripheral blood-derived mononuclear cells and monocytes (Hamano et al., 2006). In rat lipopolysaccharide (LPS) induced inflammation experiments conducted by (Park et al., 2007), nicotine and the α7nAChR selective antagonist α-bungarotoxin were employed to explore the anti-inflammatory effects mediated through α7nAChR. They

conducted the investigation by injecting nicotine (every 2 hours for 4 weeks) *in vivo* or by using tyrosine hydroxylase immune-positive substantia nigra-derived cells co-cultured with microglia. Pre-treatment with nicotine *in vivo* significantly decreased cell loss, while in culture; nicotine pre-treatment significantly reduced the amount of tumor necrosis factor (TNF-α) secreted by microglial cells along with the considerable reduction in cell apoptosis (Park et al., 2007). The addition of α-bungarotoxin in culture drastically reduced the anti-inflammatory effect implicating α7nAChRs as the main mediators of the anti-inflammatory process (Park et al., 2007). Thus the inhibition or dysfunction of this receptor would probably lead to a prolonged state of inflammation. Finally, the activation of α7nAChRs has been shown to mediate the nicotine-induced inhibitory proliferative response of lymphocytes (De Rosa et al., 2009). Here human peripheral lymphocytes were isolated and cultured in the presence of a mitogen (phytohemagglutinin; PHA) to induce lymphocytic proliferation (detected by ^{3}H-thymidine incorporation assays). The expression of α7nAChRs was assessed by reverse transcription-polymerase chain reaction (RT-PCR), flow cytometry and confocal laser microscopy. They show that PHA-induced lymphocytic proliferation resulted in the increased expression of α7nAChRs mRNA by (2.2 fold) compared to non-activated lymphocytes while nicotine inhibited the proliferative response in activated lymphocytes (De Rosa et al., 2009). Similarly, a study by (Nizri et al., 2009) identified the expression of α7nAChRs on $CD4^{+}$ T cells by means of flow cytometry and reported an upregulated expression following T cell activation through antigen representation. T cell proliferation was reduced in the presence of nicotine along with a reduction in inflammatory cytokine production. Nicotine has also been shown to inhibit microglial and macrophage activation; an effect mediated via α7nAChRs (Shytle et al., 2004, Park et al., 2007, Wang et al., 2003, Wang et al., 2004, Parrish et al., 2008). For instance, either ACh or nicotine pre-treatment inhibits the inflammation (LPS)-induced TNF-α release from cultured microglial cells derived from mice cerebral cortices. These cells expressed the α7nAChR subunit (detected by immunofluorescence, western blot and RT-PCR). The anti-inflammatory effects were attenuated in the presence of α-bungarotoxin (Shytle et al., 2004). Moreover, culture medium levels of the cytokine high mobility group protein 1 (HMGB1) released from human-derived macrophages is inhibited in the presence of either ACh or nicotine in a dose-dependent manner (Wang

et al., 2004). The anti-inflammatory effect was abolished in the absence of α7nAChRs (in cultures of specific oligonucleotide antisense-treated macrophages). They also report that intraperitoneal injections of nicotine 30 minutes prior to inducing inflammation with (LPS); serum levels of HMGB1 were lower than vehicle treated animals and animal survival rates were also significantly improved. Similarly, the intraperitoneal administration of choline prior to activating peritoneal macrophages with an endotoxin in a mouse model of endotoxemia, led to a significantly reduced level of systemic TNF levels compared to vehicle treated mice associated with the inhibition of NF-kB; the inflammatory nuclear factor kappa enhancer of activated B cells (Parrish et al., 2008). Conversely, choline failed to suppress TNF release in α7nAChR knockout mice.

Taken together, these studies suggest that promoting the activity of α7nAChRs can provide anti-inflammatory therapeutic potential in the treatment of multiple sclerosis which is characterized by neuroinflammation and immune cell activation. Indeed, in mouse models of multiple sclerosis, nicotine administration mitigated the severity of the disease; measured by reduced demyelination (Gao et al., 2014). After the induction of EAE, nicotine was administered through mini-osmotic pumps implanted subcutaneously for a continuous infusion over the course of either 14 or 28 days. Nicotine treated animals displayed a significantly lower symptomatic score compared to vehicle treated animals. Moreover, immunohistochemical labelling with fluoromyelin (which adheres to myelin proteins) indicated fewer and smaller foci of demyelinated lesions in nicotine treated animals compared to controls (Gao et al., 2014). In our studies, α7nAChR modulation with PNU-120596 was evaluated in the context of cell proliferation (oligodendrogenesis) and enhanced remyelination after focal white matter injury (refer to chapter 4) since the model used lacks the immune-infiltration aspect of multiple sclerosis.

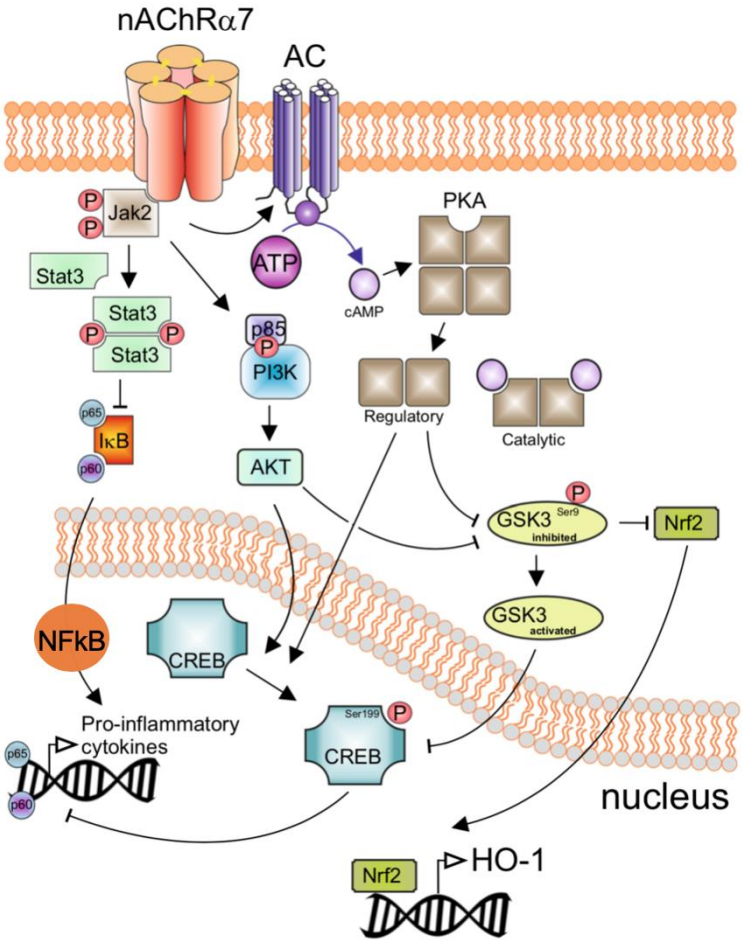

Figure 1.10 A schematic representation of anti-inflammatory signalling pathways downstream from α7nAChR activation promoting neuroprotective effects.

Adapted from (Kalkman-Feuerbach., 2016). α7nAChR activation activates the Janus kinases **(JAK2)** / signal transducer and activator of transcription factor proteins **(STAT3)** pathway which inhibits the pro-inflammatory nuclear factor kappa enhancer of activated B cells **(NF-kB).** It also inhibits glycogen synthase kinase 3 beta **(GSK3)** through the activation of phosphoinositide 3-kinase **(PI3K)** which acts on protein kinase B known as **(AKT)** leading to activation of response element-binding protein **(CREB)**. A different signalling pathway entails the activation of protein kinase A **(PKA)** via cyclic-adenosine 3'5' cyclic monophosphatase **(cAMP)** which in turn inhibits GSK3 leading to the activation of CREB and enabling the nuclear translocation of nuclear factor erythroid 2-related factor 2 **(Nrf2).**

1.15 The acetylcholinesterase enzyme (AChE); prevalence and therapeutic implications of increased ACh levels

Acetylcholinesterase is the primary cholinesterase in the body; an enzyme responsible for catalyzing the breakdown of acetylcholine (as well as other choline esters). It is mainly found at the neuronal synaptic terminals where it terminates synaptic transmission and neuromuscular junctions (Quinn, 1987). It is also present in the cytoplasm of many cholinergic and non-cholinergic cells, indicating its potentially diverse yet unidentified roles reviewed in (Layer, 1996, Darvesh and Hopkins, 2003, Darvesh et al., 2003).

Within the mammalian central nervous system, the AChE enzyme can be found pre and post synaptically. Immunohistochemistry for ChAT, VAChT and AChE in the ventral horn and intermediolateral nucleus of thoraco-lumbar and sacral segments of pig spinal cord revealed a variable distribution of AChE compared to cholinergic neurons expressing ChAT and VAChT (Calka et al., 2008). This study reported that only 64% of AChE positive cells in the imtermediolateral nucleus were cholinergic. The diversity in ChAT and AChE localization is corroborated by studies done on murine animals. The localiazation of ChAT and AChE in the rat brain (caudate putamen, nucleus basalis of Meynert, medial septum, nucleus of the diagonal band of Broca and zona incerta) showed that all cholinergic neurons were positive for AChE, however, some of the non-cholinergic neurons were AChE positive, and in some instances found intermingled with cholinergic neurons (Eckenstein and Sofroniew, 1983). Furthermore, in mouse spinal cord (both at P4 and P56), all cholinergic neurons are positive for AChE mRNA expression; indicating its presence pre-synaptically in the cell soma in addition to its known presence in the synaptic cleft of cholinergic neurons. The vast majority of the non-cholinergic neurons in the grey matter of spinal cord segments also expressed AChE mRNA (thus pre-synaptically alone) (Sengul et al., 2012). A study localizing AChE in human fetuses (8-10 weeks and 12-18 weeks) reported that AChE activity was lower at younger stages of development in the ventral and lateral horns compared to later stages. There were no AChE positive cells in the dorsal horn of younger fetuses however. At 12-18 weeks, more intense labelling and a higher number of cells were seen to express AChE in the ventral and lateral horn, including moderate

expression in the dorsal horn (Wolf et al., 1975). The functional significance of AChE in non-cholinergic cells is not yet been clearly elucidated.

AChE inhibition is a widely used therapeutic strategy in the treatment of Alzheimer's disease; a neurodegenerative condition partially characterized by cholinergic dysfunction (McGleenon et al., 1999). Since AChE enzymes terminate synaptic transmission in cholinergic systems, the inhibition of activity of this enzyme results in higher levels of its substrate acetylcholine and enhanced cholinergic signaling. Donepezil hydrochloride is one of the most widely prescribed central acting reversible AChEI to Azheimer's disease patients (Adlimoghaddam et al., 2018), and therefore, its safety and efficacy profile are well established.

In the dentate gyrus, long term administration of donepezil (orally for 4weeks) enhanced the survival of newborn neurons. This was mediated by nicotinic acetylcholine receptors since the group of animals receiving subcutaneous infusions of the muscarinic acetylcholine receptor blocker (scopolamine) at a dose known to inhibit cholinergic activity led to a decreased number of $BrdU^+$ cells compared to donepezil treated animals (Kotani et al., 2008). It was also reported that donepezil enhanced and scopolamine suppressed the phosphorylation of cyclic AMP response element binding protein (CREB) which is partly involved in cell survival in the dentate gyrus (Kotani et al., 2008). In a vascular dementia rat model (a bilateral common carotid artery occlusion), the administration of donepezil (orally for 3 weeks) 5 weeks after injury caused an increase in the numbers of $BrdU^+$ cells in the dentate gyrus and improved memory impairment and cognitive function (Kwon et al., 2014). These results indicate that donepezil enhances the survival of newborn neurons in the dentate gyrus of animals with vascular dementia. Here the authors examined the molecular mechanisms of donepezil mediated cholinergic activity in the dentate gyrus; they investigated the protein expression of phosphorylated CREB and BDNF using western blot analysis. The results indicated that animals with vascular dementia alone (without donepezil treatment) had a reduced CREB phosphorylation and BDNF expression as opposed to vascular dementia animals treated with donepezil which showed an elevated expression of both CREB and BDNF suggesting donepezil inhibited bilateral common carotid artery occlusion-mediated reduction of CREB and BDNF (Kwon et al.,

2014). Moreover, a recent study evaluated the effects of donepezil treatment with and without injury (Yu et al., 2015). They reported that in both a traumatic brain injury model and in a neurogenesis ablation model (using transgenic mice allowing for a temporally regulated ablation of adult neural stem cells); donepezil enhanced the survival of neurons and improved cognitive function independent of injury induced neurogenesis. Therefore, increasing the availability of ACh alone proved sufficient to promote adult hippocampal neurogenesis.

1.16 Hypothesis

The proliferation and differentiation of endogenous neural stem cells and precursor cells in the postnatal central nervous system has been extensively reported. However, a full understanding on how to generate specific cell types to replace damaged or lost neurons and oligodendrocytes after spinal cord injury or multiple sclerosis is still unclear. Evoking a neurogenic response in the spinal cord without the occurrence of injury is one of the objectives of this thesis. I aim to elaborate on and further extend findings reported by Corns and colleagues who provided evidence that ependymal cells of the spinal cord express nicotinic acetylcholine receptors, exhibit extensive proliferation in response to the positive allosteric modulator for α7nAChRs (PNU-120596) which potentiates the effect of ACh on these receptors both *in vivo* and *vitro* and identified a significant number of newly differentiated oligodendrocytes (Corns et al., 2015). Therefore, it is reasonable to hypothesize an increase in central canal cell proliferation by increasing the availability of ACh through donepezil administration or a combined treatment of PNU-120596 and donepezil.

The second objective of this study is to enhance specifically oligodendrocyte production and remyelination following focal white matter demyelination. I hypothesize that PNU-120596 treatment alone or in combination with donepezil can trigger oligodendrogenesis by enhancing the activity of α7nAChRs expressed by oligodendrocyte precursor cells (Vélez-Fort et al., 2009, Rogers et al., 2001) and / or awakening the normally dormant spinal cord neurogenic niche through enhancing ACh neurotransmission. This hypothesis was formed based on the significant number of oligodendrocytes produced in response to PNU-120596 treatment (Corns et al., 2015)

and results reported by Imamura who reported that donepezil administration in cultures of oligodendrocyte precursor cells enhanced the extent of their maturation into functional oligodendrocytes capable of myelin production compared to non-treated control conditions (Imamura et al., 2017). This effect was suggested to be mediated through nAChRs, however, the precise mechanism was not fully characterized. A previous study by Imamura showed that donepezil increased the number of neurons and oligodendrocytes produced by neural stem cell cultures derived from embryonic mouse brain (Imamura et al., 2015b). Therefore, it is reasonable to hypothesize that targeting α7nAChRs may enhance the production of oligodendrocytes in models of white matter injury.

The effects of acetylcholine in the brain neurogenic niches; the SGZ and SVZ have been reported to be mediated through both α7 and non α7nAChRs (Campbell et al., 2010, Paez-Gonzalez et al., 2014, Cooper-Kuhn et al., 2004). Similarly, in the spinal cord ependymal layer, the use of whole cell patch clamp on acute spinal cord slices and application of a selective non- α7nAChR antagonist and / or potentiating the activity of ACh on α7nAChRs using PNU-120596 revealed that both α7 and non α7nAChRs mediated the proliferative response (Corns et al., 2015). I therefore aim to test the extent of α7nAChR contribution to cell proliferation in organotypic spinal cord cultures using specific nAChR agonists and antagonists.

1.17 Aims

1. To determine how donepezil, which enhances ACh levels affects cell proliferation and differentiation in the intact spinal cord alone or in combination with the α7nAChR positive allosteric modulator PNU-120596.
2. To evaluate the extent of oligodendrogenesis and remyelination in a mouse model of focal demyelination in spinal cord white matter after potentiating the effect of ACh on α7nAChRs alone or in combination with donepezil.
3. To identify whether ependymal or oligodendrocyte cell proliferation in response to α7nAChR potentiation is mediated through subsequent activation of calcineurin and nuclear factor of activated T cells (NFATs) dependent gene transcription.

Chapter 2

General materials and methods

2.1 Animals

Animals were obtained from the Central Biological Services upon approval from the animal ethics committee. All experimental procedures were carried out under the regulations of the UK Home Office License and UK Animal Scientific Procedures Amended Act 1986. C57/BL6 mice of either sex between the ages of 9-15 days old were used for *in vitro* studies, and mice of 6-9 weeks old were used for *in vivo* experiments. Replacement, reduction of animal suffering and animal numbers, as well as the refinement of the experience of the animal were considered in all experimental measures. The number of sections used, number of animals, and ages used in each experiment are listed in tables below.

2.2 5-ethynyl-2-deoxyuridine (EdU) treatment

The classical way of monitoring novel DNA synthesis and cell proliferation is the incorporation and quantification of the nucleoside thymidine as cells enter S phase, however, this method is both expensive and very slow. Another well established method is the incorporation of EdU; a thymidine analogue which incorporates into the DNA during the S-phase of the cell cycle of cells undergoing division. The EdU protocol is a method of covalently binding an azide to an alkyne. It utilizes a copper catalyst and an azide modified fluorescent dye to form a stable triazole ring that can be seen under a fluorescent microscope, (figure 2.1). This method is relatively faster and independent of denaturing steps that are required when using other thymidine analogues such as in 5-bromo-2'deoxyuridine (BrdU) in order to access the DNA (Buck et al., 2008).

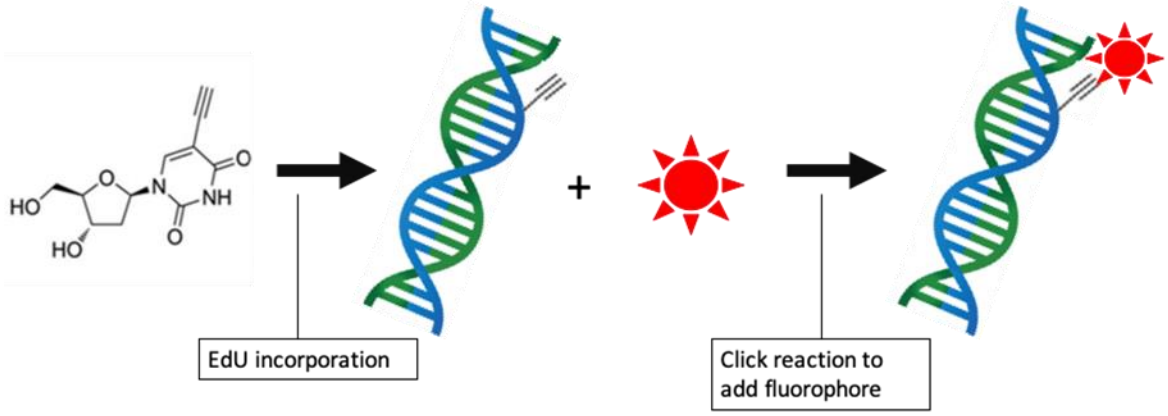

Figure 2.1 EdU cell proliferation assay principle.
Cells newly generated in the presence of EdU are detected by the incorporation of the thymidine analogue during S-phase. The Fluorophore labelled azide (in red) reacts with EdU to enable detection by fluorescence microscopy.

2.2.1 EdU detection in acute slices, organotypic slices and fixed sections.

To label and detect EdU positive proliferative cells, organotypic slices; 300 µm thick and re-sectioned acute slices 50 µm are first fixed with 4% para-formaldehyde in 0.1M phosphate buffer (PB) (4%PFA) for 1-2 hours. Fixed *in vivo* sections; 50 µm in thickness and *in vitro* slices are always free floating in their designated 24 well plates (CytoOne 24-well non-treated culture plate, cat # CC7672-7524) containing phosphate buffer saline (PBS) following fixation. The PBS solution is composed of (8 g/L sodium chloride, 0.2 g/L potassium chloride, 1.15 g/L disodium phosphate and 0.2 g/L monopotassium phosphate) at a pH of 7.4. The EdU protocol took place in room temperature.

First, cell membranes of cells in sections and slices are permeabilized through incubating in 0.2% triton (PBST) for 20 minutes. The PBST solution is composed of 8 g/L sodium chloride, 0.2 g/L potassium chloride, 1.15 g/L disodium phosphate and 0.2 g/L monopotassium phosphate and 2% Triton. This was followed by two 10-minute washes in 0.1M Tris buffer (Trizma base 0.695 g, Trizma hydrochloride 3.03 g, 500 ml

distilled water, pH 7.6). The tissue were then incubated in a series of solutions: 320 µl of distilled water, 25 µl of 2M Tris Buffer, 50 µl of either 5 mM or 10 mM of copper sulphate, 5 µl of 1mM picolyl biotin azide and 100 µl of Ascorbic acid for either 15 or 30 minutes depending on whether a fluorescence-sensitive antibody was used post EdU labelling to avoid potential fluorescence quenching by copper sulphate. From here forward, all histology plates were protected from light. The sections and slices were then washed with 0.1 M Tris followed by an incubation with Streptavidin Alexa ^{555} (Thermofisher), or Pacific blue Streptavidin Alexa $^{410/455}$ (Thermofisher); 1:1000 in PBS for 2 hours which fluorescently tags proliferative cells in red or blue respectively. All sections were then washed three times (10 minutes each) with PBS. Finally, the sections were mounted onto non-treated glass slides and covered with 4'6-diamino-2-phenylindole (DAPI) – (vectashield) and a glass cover slip. DAPI emits blue fluorescence which labels the nucleus of cells. A mounting medium free of DAPI (vectashield) was used in experiments containing Pacific blue Streptavidin Alexa $^{410/455}$ fluorescence.

2.3 Acute slice preparation

2.3.1 Introduction

Acute brain or spinal cord slices are routinely used for electrophysiology recording techniques. The acute slice preparation preserves cellular functionality and provides a mean by which neurophysiology of specific tissues can be assessed. For instance, this experimental *in vitro* model allowed the investigation of the effect of neurotoxic chemicals on the functional plasticity of hippocampal neurons (Lein et al., 2011). Given that brain or spinal cord tissue would undergo slicing; afferent inputs are severed. However, there are many advantages to this method such as the relatively preserved cytoarchitecture (Dissing-Olesen and MacVicar, 2015). The step-by-step protocol to obtain such slices is described below.

2.3.2 Animal perfusion and slice acquisition

C57/Bl6 mice (9-15 days old) were anaesthetized with an intraperitoneal injection (I.P. injection) of sodium pentobarbitone (60 mg/kg) then transcardially perfused through the left ventricle with15-25 ml ice cold artificial sucrose (aCSF) containing: sodium chloride, sodium bicarbonate, potassium chloride, magnesium sulphate heptahydrate, sodium phosphate monohydrate, glucose and calcium chloride at a pH of 7.4 and an osmolarity of 300 mOsm), table 2.1. The animal was then positioned with the dorsal side facing up, and a dorsal laminectomy was carried out to dissect out the spinal cord using fine spring scissors and fine forceps. The spinal cord was then placed into a 50 mm Petri dish containing ice cold sucrose. This was followed by meningeal (pia, arachnoid and dura mater) removal under a dissecting microscope using fine forceps. The thoracic and lumbar regions of the spinal cord were selected and transferred using a microspatula and embedded into 1.5-2% agar-containing weighing boat (Sigma-Aldrich, cat # W2751). Once the agar had solidified, longitudinal blocks of agar from the region containing the spinal cord segments were dissected out and glued onto the metal base of the vibrating microtome bath. Transverse thoracolumbar spinal cord slices were cut at 500 µm thickness using a vibrating microtome (Campden Instruments, integraslice 7550 PSDS) and collected into a holding chamber containing aCSF. The exact composition of artificial sucrose (aCSF) and aCSF is listed in the table below in table 2.1 and the number of animals / slices used are cited in table 2.2.

Component	Sucrose aCSF (at mM concentrations)	aCSF (at mM concentrations)
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

Table 2.1 The components of artificial sucrose (aCSF) and aCSF.

Experiment; ***In vitro*** **acute slices**	**Age** **9-15 days old**	**Number of animals** **N = 3**	**Total number of sections analysed** **n = 48**
Condition of treatment	**Number of slices each**		
	Control		n = 17
	Donepezil HCl		n = 23
	Donepezil HCl +atropine		n = 8

Table 2.2 Acute cholinergic manipulation of cell proliferation in the spinal cord central canal *in vitro*.

2.3.3 Incubation of slices with EdU and cholinergic drugs

Slices were distributed among four holding chambers all containing 4 ml of aCSF and 1µM EdU (Invitrogen, Paisley, UK) and either 5 µM donepezil or 5 µM donepezil and 5 µM atropine for one hour. The control chamber only received EdU and aCSF. The chambers were placed in a water bath and supplied with 95% oxygen and 5% carbon dioxide at 35-36°C. An hour later another dose of 1 µM EdU was added to all conditions and slices were allowed to incubate for an additional 4 hours, figure 2.2. The drugs and reagents used in these experiments are listed in table 2.3.

Drugs	**Final working concentration**	**Stock dissolved in**	**Action**
Donepezil HCL	5 µM	dH_2O	Acetylcholinesterase inhibitor
Atropine	5 µM	saline	Selective muscarinic receptor antagonist
EdU	1 µM	saline	Proliferation marker

Table 2.3 The cholinergic drugs and reagents used on acute slices.

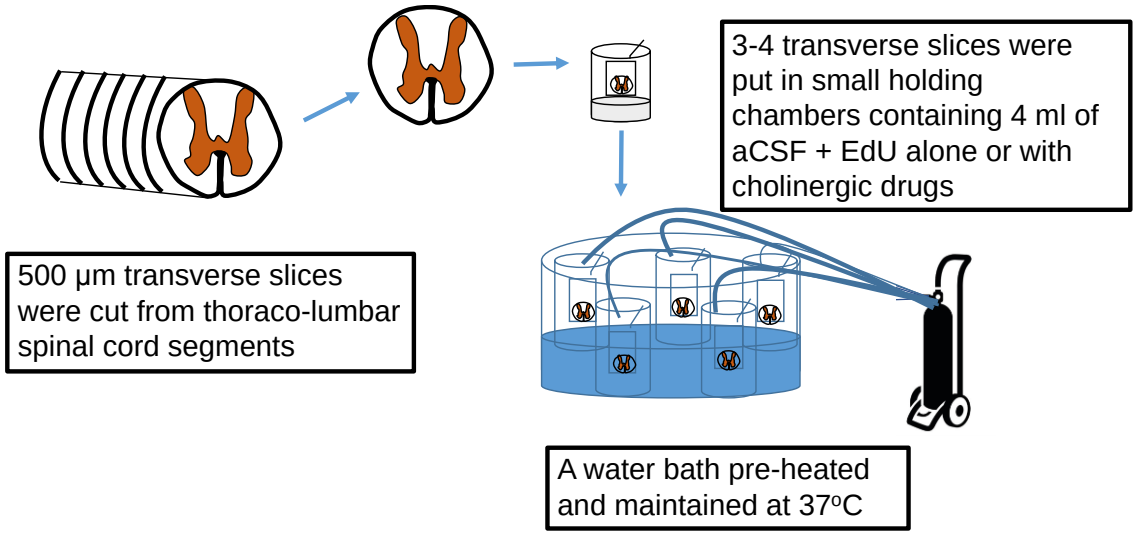

Figure 2.2 Schematic figure of acute slice incubation with EdU and cholinergic drugs.

2.3.4 Acute slices fixing and re-sectioning

Slices were then fixed for 1 hour or overnight in 4% PFA in 0.1M PB then embedded in 10% gelatin in distilled water (Sigma Aldrich- gelatin from porcine skin) and left at 4°C for 1 hour to solidify before fixing the gelatin with gelatin fixative (8% PFA in 0.2M PB) for 4 hours. Once the gelatin blocks were fixed, the sections were re-sectioned into 50 µm sections; using a vibrating microtome (Leica VT 1000S, Microsystems). The slices were then collected into 24 well histology plates containing PBS (1 ml). After that, all gelatin-embedded re-sectioned slices underwent EdU labelling (refer to section 2.2) for cell proliferation detection.

2.4 Organotypic slice culture

2.4.1 Introduction

Organotypic slice culturing of brain or spinal cord is a relatively common practice particularly because it provides an *in vitro* setting by which tracking physiological processes in tissues can be done over several days, weeks or even months, as opposed to acute slice preparations which usually lose neuronal viability within hours (Daviaud et al., 2013). Since this method involves sectioning of the tissue, severance of afferent and efferent connections occur, along with neuronal injury, glial activation and axonal / dendritic remodelling (Lein et al., 2011). However, the cytoarchitecture of the tissue is preserved, allowing for the exploration of cellular structures, drug screening and cellular development (Lein et al., 2011).

2.4.2 Animal perfusion and slice acquisition

Since we aimed to culture the tissue for several days, in order to maintain the viability and reduce chances to infection, all equipment and tools were autoclaved then sprayed with 70% ethanol prior to use.

C57/Bl6 (9-15 days old) were anaesthetized with an I.P. injection of sodium pentobarbitone (60 mg/kg) then transcardially perfused with 15-25 ml of ice-cold artificial sucrose (aCSF) through the left ventricle. The animal was then positioned with the dorsal side facing up, and a dorsal laminectomy was carried out to dissect out the spinal cord. This was followed by meningeal removal and transverse cutting of thoraco-lumbar spinal cord at 300 µm thick slices with use of a vibrating microtome (see section 2.3.2). All slices were collected into a falcon tube containing Dulbecco's modified eagles medium (DMEM) (Sigma-Aldrich) with 1% penicillin / streptomycin (Sigma-Aldrich) to prevent infection or contamination. The experimental conditions these slices were used for are listed in the table 2.4 and 2.5.

Experiment; **Organotypic cultured slices**	**Age** **9-15 days old**	**Number of animals** **N = 3**	**Total number of sections analysed** **n = 36**
Condition of treatment	**Number of slices each**		
	PNU-120596		n = 9
	PNU-120596 + FK 506		n = 9
	PNU-120596 + Cyclosporin A		n = 9

Table 2.4 The effect of downstream cell signalling inhibition after α7nAChR potentiation with PNU-120596 on cell proliferation *in vitro*.

Experiment; **Organotypic cultured slices**	**Age** **9-15 days old**	**Number of animals** **N = 3**	**Total number of sections analysed** **n = 47**
Condition of treatment	**Number of slices each**		
	Donepezil		n = 11
	Donepezil + Atropine		n = 12
	Donepezil + Atropine + DHβE		n = 12
	Cytisine + DHβE		n = 12

Table 2.5 The effect of muscarinic receptor antagonism with or without specific nicotinic receptor agonists on cell proliferation *in vitro*.

2.4.3 Transfer and plating of slices

Thoraco-lumbar spinal cord slices were transferred into a petri dish containing ice-cold Dulbecco's modified eagles medium under a laminar-flow hood. All slices were then transferred by aspirating individual slices into a sterile pipette tip with the end cut

off to limit the damage of the tissue during transfer. This also allowed for the slices to be placed flat onto Millicell organotypic filter inserts (Millipore, 0.4 µm, 30 mm diameter) which were placed in sterile six well plates with 1 ml of neurobasal A culture medium (Invitrogen), 1 % L-Glutamine (Sigma-Aldrich), 1 % Penicillin / streptomycin (Sigma-Aldrich) and 10 % Fetal bovine serum (Sigma-Aldrich) which had been pre-warmed in the incubator at 37 ºC. Each insert contained no more than 4 slices placed distantly from each other; this was done to ensure enough space for each slice to spread over the course of the experimental time (168 hours).

2.4.4 EdU and cholinergic drug treatment of organotypic slices

Slices were allowed to rest in neurobasal culture medium and serum for 48 hours at 37ºC under standard culture conditions; 100% humidity, 95% air, 5% CO_2 environment. After 24 hours, the culture medium was replaced with serum-free neurobasal medium (Invitrogen), 2% B27 supplement (Invitrogen) and 1% Penicillin / streptomycin (Sigma). The main reason that serum is omitted after 24 hours is to exclude any possible influence from unknown substances contained in the serum. After the 48 hour time frame, 1 µM EdU (Invitrogen, Paisley, UK) was added into the medium along with 1µM PNU-120596 alone or with either 100 nM FK-506 or 100 nM Cyclosporin A while some slices of the same animal were treated as controls containing only 1 µM EdU. Slices were kept in culture for a total of 168 hours. EdU and drug administration was given directly by addition into the culture medium, (figure 2.3). The cholinergic drugs and reagents used in culture experiments are listed in table 2.6.

Drugs	Final working concentration	Action
PNU-120596	1 µM	Positive allosteric modulator for αnAChRs
FK-506	100 nM	Impairment of gene expression in target cells; inhibits calcineurin phosphatase and prevents phosphorylation of nuclear factor of activated T cells (NFATs)
Cyclosporin A	100 nM	Impairment of gene expression in target cells; inhibits calcineurin phosphatase and prevents phosphorylation of nuclear factor of activated T cells (NFATs)
Atropine	5 µM	Selective muscarinic receptor antagonist
Cytisine	20 µM	Partial nicotinic receptor agonist
Donepezil HCL	5 µM	Acetylcholinesterase inhibitor
EdU	1 µM	Proliferation marker

Table 2.6 The cholinergic drugs and reagents used on organotypic cultured slices.

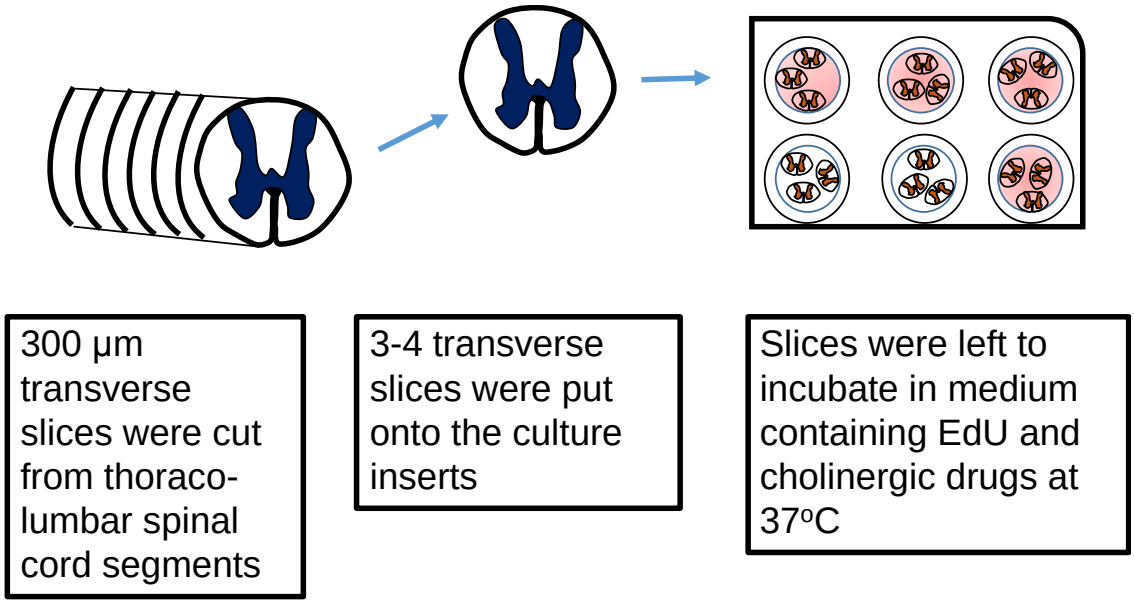

Figure 2.3 Schematic representation of organotypic slice culturing.

2.4.5 Fixing and EdU labelling of organotypic slices

After 7 days (168 hours), the filter inserts were removed from the 6 well plates and placed in a new 6 well plate containing 1 ml of 4% PFA then a small amount (250-500 µl) of 4% PFA was added carefully onto the slices to avoid damaging the slices while submerging them completely. Slices were left to fix for 1-2 hours, then carefully removed with a paint brush to avoid slice breakage into a 24 well plate containing PBS and left in a free-floating condition from here on. All slices were then treated with EdU click chemistry (based on the protocol in section 2.2) with the exception of placing the slice containing plates on the shaker. This was omitted due to the fragility of the slices.

2.5 Immunohistochemistry

Immunohistochemistry is a technique based on antibodies binding to antigens and proteins through the principle of antibody-antigen interaction. These antibodies require a histological or fluorescent tag in order to be visualised.

For the purpose of experiments listed above, the indirect method of antibody detection is used. This method entails the use of an unlabelled primary antibody which binds to the antigen or protein of interest followed by the use of a secondary antibody containing a fluorescent conjugate to enable visualisation, (figure 2.4). This method is useful since it amplifies relatively weak antigen signals in the tissue given that many secondary antibodies can bind to different antigenic sites of the primary antibody.

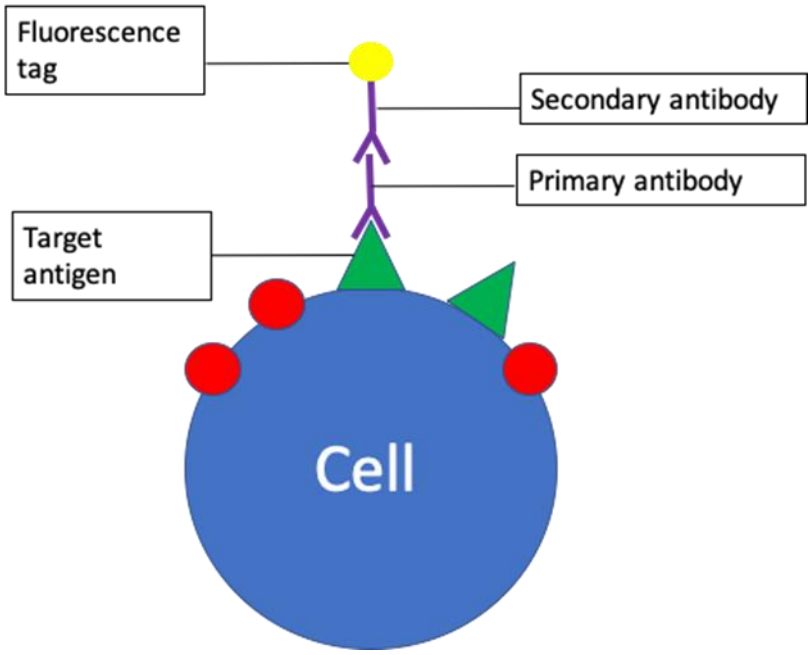

Figure 2.4 Indirect immunohistochemistry assay principle.
This method uses an antibody against an antigen and a secondary tagged antibody against the first.

2.6 *In vivo* experiments

Tables 2.7-2.11 summarise the number of animals and sections used in each *in vivo* experimental condition conducted in this thesis. The tables also include the drugs and vehicle used, length of administration and age of the animals. Each table entails information pertaining to a single *in vivo* experiment.

Experiment	**Age**	**Number of animals**	**Number of sections analysed**		
In vivo; I.P. injection of saline+ EdU or Donepezil HCl + EdU for 7 days	6-8 weeks old	4 control	**Thoracic** n = 65	**Dentate gyrus** n = 12	
		4 donepezil HCl	**Thoracic** n = 88	**Dentate gyrus** n = 12	
In vivo; I.P. injection of saline+ 10% DMSO and EdU or Donepezil HCl + PNU-120596 + EdU for 7 days	6-8 weeks old	3 control	**Thoracic** n = 82	**Lumbar** n = 92	**Dentate gyrus** n = 79
		3 donepezil HCl + PNU-120596	**Thoracic** n = 80	**Lumbar** n = 94	**Dentate gyrus** n = 132

Table 2.7 Cholinergic manipulation of cell proliferation in the central canal, grey matter and white matter of the intact spinal cord and dentate gyrus of the hippocampus.

The age, numbers of animals and sections evaluated.

Experiment	Age	Number of animals	Number of sections analysed		
			Thoracic	Lumbar	dentate gyrus
In vivo; labelled with EdU and S100β	6- 8 weeks old	3 control	n = 19	n = 24	n = 18
		3 donepezil HCl + PNU-120596	n = 18	n = 27	n = 18
In vivo; labelled with EdU and PanQKI	6- 8 weeks old	3 control	n = 31	n = 25	n = 18
		3 donepezil HCl + PNU-120596	n = 19	n = 23	n = 18
In vivo; labelled with EdU and HUC/D	6- 8 weeks old	3 control	n = 16	n = 20	---
		3 donepezil HCl + PNU-120596	n = 26	n = 23	---

Table 2.8 Lineage identification of newly proliferated cells in the intact spinal cord and dentate gyrus.

The age, numbers of animals and sections evaluated.

Experiment ***In vivo*; LPC surgical focal dorsal lumbar demyelination.**	**Age** **6 -8 weeks old**	**Number of animals per group**			**Number of sections analysed per group**		
		LPC + EdU	LPC + EdU + PNU	LPC + EdU + PNU + Don	LPC + EdU	LPC + EdU + PNU	LPC + EdU + PNU + Don
Time point of sacrifice	**Post surgery day 4 (PSD)**	N = 2	---	N = 2	n = 5	---	n = 6
	Post surgery day 14 (PSD)	N = 4	N = 4	N = 4	n = 24	n = 21	n = 22
	Post surgery day 21 (PSD)	N = 3	N = 3	N = 3	n = 19	n = 18	n = 20

Table 2.9 Enhancing oligodendrogenesis post focal lumbar dorsal demyelination in C57/BL6 mice.

Experiment ***In vivo*; LPC surgical focal dorsal lumbar demyelination.**	**Age** **6 -8 weeks old**	**Number of animals per group**	**Number of sections analysed per group**
Time point of sacrifice	**Post surgery day 14 (PSD)**	LPC alone N = 4	LPC + PNU + Don N = 5

Table 2.10 Evaluating the extent of endogenous remyelination and remyelination post focal white matter demyelination and cholinergic treatment.

Experiment ***In vivo*; Semliki forest virus I.P. injection in 9 week old C57/BL6 mice**	**Post injection day of sacrifice (PID)**	**Number of animals fixed (4% PFA) for immunocytochemistry**	**Number of animals perfused with 0.1M PB for qPCR**	**Tissue analysed**
	Day 4	N = 2	N = 1	Entire brain and spinal cord; brain was cut into 50 µm transverse sections while spinal cord was longitudinally sectioned (50 µm).
	Day 7	N = 2	N = 1	
	Day 10	N = 2	N = 1	
	Day 14	N = 2	N = 1	
	Day 21	N = 2	N = 1	

Table 2.11 The pilot experiment in generating central nervous system inflammation and multi-focal demyelination using semliki forest virus in C57/BL6 mice.

2.6.1 *In vivo* tissue preparation for immunohistochemistry

Animals were terminally anaesthetised with an intraperitoneal injection (I.P.) of 60 mg/kg of sodium pentobarbitone (Pentoject animal care). Once the absence of the corneal reflex and all pedal withdrawal reflexes was confirmed, the animal was considered properly anaesthetised for the procedure. In order to transcardially perfuse the animals, the chest was first opened to expose the heart, then a small incision made into the right atrium to release pressure prior to injecting a syringe into the left ventricle where the perfusion was performed. First, 20-50 ml of 0.1M of PB was infused to flush out the blood from the animal's system. This was followed by 50-200 ml of 4% PFA solution made up in 0.1M PB. Once the animal was sufficiently fixed, the skin and limbs were separated, and the brain and vertebral column of each animal was then placed in 35-40 ml of 4% PFA at 4^{o}C overnight for postfixation. Then, the whole brain and vertebral column was excised using fine spring scissors. Finally, the pia and dura mater were dissected from the cord under a dissecting microscope using fine forceps. At this stage the tissue was ready for sectioning using a vibrating microtome (Leica VT100S, Microsystems, Milton Keynes, UK).

2.6.2 Immunohistochemistry on fixed tissue

In order to section the spinal cord transversely, the appropriate region(s) of the spinal cord were first separated from the entire spinal column (thoracic and lumbar). Then, approximately 300 µm of spinal cord tissue was cut transversely using a scalpel blade and glued on a metal plate with super glue. Once the tissue adhered, the plate was screwed into the bath chamber of the vibrating microtome. The bath contained 0.1M PB in which the tissue was submerged to avoid dryness or dehydration. A sharp razorblade was secured onto the microtome to cut the tissue to the required thickness. All spinal cord tissue was transversely sectioned at 50 µm thickness.

To acquire transverse 50 µm thick dentate gyrus sections, the entire brain was securely glued upright onto the metal plate. All sections were collected and placed into their designated 24 well plate containing 0.1M phosphate buffered saline (PBS).

The sections were then blocked with the appropriate blocking serum at 10% in 0.1 % PBST for 30 minutes to assist in membrane permeabilisation. Then sections were

washed three times with 0.1M PBS; 10 minutes each. After that, the sections incubated with the appropriate antibody (table 2.12) at respective dilutions in 0.1M PBST for a minimum of 18 hours on a shaking plate at 4^{o}C. After sufficient incubation in primary antibodies, sections were washed three times with 0.1M PBS for 10 minutes each, followed by a two hour incubation in appropriate Alexa-conjugated secondary antibodies 1:1000 PBS, (table 2.13). Once fluorescent secondary antibodies were added, all tissue was protected from light to avoid photo-bleaching or fading of samples. Finally, sections were washed three times with 0.1M PBS for 10 minutes each, mounted onto glass slides, and covered in vectashield (with or without DAPI) before being coverslipped with a glass coverslip and secured with nail polish. A summarised list of primary and secondary antibodies used in *in vivo* experiments described in this thesis are provided in tables 2.12 and 2.13 respectively.

Antibody	Raised in	Dilution	Specificity	Source
S100β	Rabbit	1:750 in 0.1 PBST	Used to identify mature astrocytes (Wang et al.,2008)	Abcam; ab52642
PanQKI	Mouse	1:100 in 0.1 PBST	Used to identify Oligodendrocytes (Hardy et al., 1998).	UC Davis/NIH Neuromab Facilities, Lot # 443.2ks.51.
HUC/D	Rabbit	1:1000 in 0.1 PBST	Used to identify neurons Akamatsu et al., 2005).	Proteintech; Cat# 130321AP
Olig 2	Rabbit	1:200 in 0.1 PBST	Used to identify Oligodendrocytes (Liu et al., 2007)	Abcam ab109186
Fluoromyelin	----	1:300 in dH_2O	A lipid stain for subcellular myelin (Aoyama et al., 2006).	Thermofisher Scientific F34651
MBP	Chicken	1:200 in 0.1 PBST	Used to identify myelin (Achiron et al., 2007)	Abcam ab134018
RFP	Rabbit	1:250 in 0.1 PBST	Targets fusion proteins (Daadi et al., 2013)	MBL International PM005 Lot 041
DCX	Rabbit	1:1000 in 0.1 PBST	Used to identify immature neuronal cells (Payne et al., 2019)	Abcam ab 18723

Table 2.12 Primary antibodies used on fixed tissue sections.

This tables summarises the primary antibodies used in *in vivo* experiments throughout the thesis.

Antibody	Raised in	Dilution	Specificity	Source
AlexaFluor 488	Donkey	Mouse	1:1000 in PBS	Invitrogen
AlexaFluor 488	Donkey	Rabbit	1:1000 in PBS	Invitrogen
AlexaFluor 488	Goat	Chicken	1:1000 in PBS	Invitrogen
AlexaFluor 555	Donkey	Rabbit	1:1000 in PBS	Invitrogen

Table 2.13 Secondary antibodies used on fixed tissue sections.

A summarised list of secondary antibodies employed in *in vivo* experiments thoughout the thesis.

2.6.3 Previously established primary antibody specificity

Anti-S100β

S100β is a calcium binding protein localised in the cytoplasm and nucleus of a variety of cells. It is mainly produced or expressed by a subtype of mature astrocytes around blood vessels and NG2 expressing cells (Wang and Bordey, 2008). It has a role in promoting vascular inflammation and is secreted by injured astrocytes into the extracellular space. It is blood-brain barrier permeable and its overexpression in the central nervous system can be linked to neurodegenerative disorders (Michetti et al., 2012). The antibody employed here is a monoclonal primary antibody raised in rabbit. It is reactive with mouse, rat, goat, human, zebrafish and macaque monkey tissue and is suitable to use in western blot, immunofluorescence and immunohistochemistry (Abcam, 2019d).

Anti-PanQkI

QKI is also known as protein quaking or HQkI. It is predominantly found in the nucleus with lower levels found in the cytoplasm. It is an RNA binding protein which is both regulated by and regulates pre-mRNA splicing (UniProtKB, 2019). It also has a role in mRNA export, stability, protein translation, regulates oligodendrocyte differentiation and maturation in the brain and has a prominent role in myelination and

nuclear export of myelin basic protein (MBP) mRNA. In the central nervous system, it is expressed by oligodendrocytes and some astrocytes and highly expressed by myelinating cells. It is also expressed by Schwann cells of the peripheral nervous system (Ebersole et al., 1996, Hardy, 1998). The antibody used here is a monoclonal primary antibody raised in mouse. It is reactive against human, mouse and rat tissue. The application of this antibody includes western blots and immunohistochemistry (Gong et al., 2016, Neuromab, 2018). It has not been validated in a knockout mouse, however, it has been shown to specifically target QkI with a cross reactivity to Qki-5, QkI-6, QkI-7 and QkI-7b (Antibodies incorporated, 2019).

Anti-MBP

In the central and peripheral nervous systems there are seven myelin basic proteins which are involved in the multi-layered glial cell membrane; the myelin sheath. Certain proteins regulate oligodendrocyte maturation before myelin formation. The myelin protein is usually located in the cytoplasmic part of membrane, and in pathological conditions such as multiple sclerosis, myelin proteins have weakened attachments to the membrane. Certain MBP proteins have a higher affinity to major histocompatibility complexes (MHC) produced by multiple sclerosis patients and are considered targets of autoantigens (Achiron and Miron, 2007). Anti-myelin basic protein is a chicken polyclonal antibody suitable for immunocytochemistry and immunohistochemistry applications. The positive control for this antibody is the labelling of white matter tracts adjacent to the mouse hippocampal formation (Abcam, 2019b) and has been referenced in (Beyer et al., 2017). It has not been validated in a knockout model.

Anti-Olig2

Oligodendrocyte transcription factor 2 (Olig2) is a lineage specific transcription factor. Oligodendrocyte specification is linked to the expression of Olig2, Sox10 and Nkx2.2 in mice in a dosage dependant manner, and is required to drive neuronal progenitors into oligodendrocyte differentiation (Liu et al., 2007). The increased expression of Olig2 was related to an increase in the extent of oligodendrocyte

differentiation, enhanced migration rates and myelination of the mouse central nervous system (Wegener et al., 2015). For the purpose of our studies, anti-olig2 was used to label pre-myelinating oligodendrocytes. It is monoclonal primary antibody raised in rabbit, suitable for western blot analysis, fluorescence immunocytochemistry and immunohistochemistry. The positive control for this antibody is oligodendroglioma lysate and human glioma tissue which exhibited high specificity to the oligodendrocyte lineage (Abcam, 2019c).

Fluoromyelin

This is a specific lipophilic stain that binds to the high lipid content of myelin. Its specificity to label myelin sheaths has been demonstrated in wild type and transgenic knockout mouse corpus callosum (Aoyama et al., 2005). Here FluoroMyelin Green fluorescent myelin stain (479/598 nm) was used to detect the presence or absence of demyelinating lesions in the semliki forest virus experimental model.

Anti-HUC/D

HUC/D is a pan-neuronal marker, also known as Elav-like protein 4, which is in a class of RNA-binding proteins. These proteins are exclusive to neurons and have a critical presence in neurons during brain development and plasticity (Perrone-Bizzozero and Bolognani, 2002). Anti-HU antibodies specifically label neuronal Elav family proteins. These proteins are mainly expressed in immature progenitor neurons, and have been shown to promote neuronal differentiation (Akamatsu et al., 2005). The primary antibody used here is a polyclonal antibody raised in rabbit. It is reactive and specific to human, mouse, rat and zebrafish tissue. It is detected in human and mouse cerebellum tissue, with mouse brain tissue as the positive control for immunofluorescence experiments (Proteintech, 2019).

Anti-RFP

Anti-red fluorescence protein is a primary polyclonal antibody that is primarily used to detect vectors containing a protein and a tag protein. It is applicable in western

blots, immunohistochemistry and immunocytochemistry techniques. It is raised in rabbit and reacts with DsRed, mRFP1, mCherry, mOrange, mPlum, tdTomato and mStrawberry (MBL International Corporation, 2019). This particular antibody was only used to detect the presence or absence of the semliki forest virus in the inflammatory viral mouse model experiment.

Anti-DCX

Doublecortin (DCX) is a protein associated to microtubules expressed by neural precursor cells of both embryonic and adult cortical brain regions. The neuronal expression of doublecortin emerges when cells undergo active division and neuronal daughter cells continue to express it up to 3 weeks old. After that, the expression starts downregulating and coincides with the start of NeuN expression; a mature neuronal marker (Brown et al., 2003). Anti-DCX is a rabbit polyclonal antibody which is reactive in mouse, rat, chicken, human, cynomolgus monkey (crab-eating macaque), quail and rhesus monkey. It is suitable for western blots, free floating or paraffin embedded immunohistochemistry and fluorescence immunocytochemistry. This antibody is localised in the cytoplasm of neural cells and it is highly specific to neuronal cells in the cortical plate, intermediate zone and ventricular zone of foetal brain, and in the frontal lobe of adult brain. It is also slightly expressed in other regions of the brain (Abcam, 2019a).

2.6.4 Controls for immunohistolabelling

Immunohistochemical techniques carried out in these experiments consisted of labelling with primary and secondary antibodies. To ensure that secondary antibodies are providing specific labelling, a control section from each experiment had the secondary antibody added without the addition of its appropriate primary antibody. All secondary antibodies showed no non-specific labelling; only some faint background fluorescence was seen. Tissue sections that were double labelled did not exhibit any cross reactivity of the secondary antibody with the incorrect primary antibody. The secondary antibody was used on a tissue section with its respective primary antibody omitted; only faint background fluorescence was present (data not represented).

2.7 Dorsal focal white matter demyelination surgery

In these experiments, C57/Bl6 mice (7-9 weeks of age) of either sex were used. These experiments took place in a surgical theatre under sterile conditions. Every mouse was operated on a continuously warmed surgical table to avoid hypothermia. Optical emulsion was applied on the eyes to avoid drying throughout the procedure.

First, the mice were intraperitoneally anaesthetised with 0.2 ml/10 g of 5 mg/ml ketamine (Ketavet, Zoetis) and 1 mg/ml meditomidine (Domitor, Orion Pharma). Once the animal was properly and sufficiently under anaesthesia (which was judged based on the absence of pedal reflexes), the dorsal side of the animal was shaved and disinfected with 70% ethanol. The animal was then placed in a stereotactic frame with the dorsal side elevated upwards, and a dorsal midline incision from T3/T4 down to approximately L4 was made to visualise the vertebrae overlying the spinal cord. During the first few experiments, gentle lateral scrapes were made to remove the dura mater between vertebrae at levels L3 and L4 and expose the spinal cord for injection, and the pia mater was cauterised. However, a less invasive method of injection was carried out eventually, in which scraping the dura and cauterisation of the spinal cord were avoided completely. Instead, the injection was administered through the gaps present between individual vertebra; providing a better chance of recovery, and avoiding any additional damage to the white matter that may be caused by cauterisation. Once the injection site was determined- usually between L3 and L4, the dura and pia mater were gently teased off using very sharp forceps. A thick-walled glass electrode (inner diameter; 0.94 mm and outer diameter; 1.24 mm) with a tip diameter of (~4-5 µm) was filled with 1 µl of Lysophosphatidylcholine (LPC) (L4129-100MG, Sigma) and attached onto the stereotactic frame for a controlled delivery. The injecting electrode was carefully lowered into the exposed white matter: a depth of approximately 0.3 mm determined by the Z-direction stereotactic arm. Once the injection was delivered, the electrode was allowed to rest for 5 minutes before elevating the electrode out of the tissue. This ensured the uptake of the injected material by the tissue and avoided any possible spillage by prompt removal of the electrode. The animals then had interrupted sutures applied (Vicryl sutures absorbable 17MM ½ circle taper point, Cat # W9105) to close the surgical site, first by suturing the adipose and muscle layers then the skin.

Antiseptic wound powder (Tosylchloramide Sodium 2.0%) was applied on the incision site to avoid infections. Animals then received an intraperitoneal injection of 0.5 ml saline to keep them hydrated post operatively, followed by an intraperitoneal injection of 0.05 ml of vetergesic (0.3 mg/ml buprenorphine hydrochloride, Ceva Animal Health)- an analgesic medication to ameliorate post-operative pain, and a subcutaneous injection of reversal anesthesia; 0.1 ml/10 g antisedan, 5mg/ml, Orion Pharma. Two more doses of analgesia (one later the same day of surgery, the other on the following day) were also given at the same dose.

2.7.1 Cholinergic drug and EdU treatment post LPC injection

There were four different dorsal focal demyelination experiments which terminate at different time points. All experiments had the same surgical procedure done, however, the days of EdU and cholinergic drug administration as well as the day of sacrifice were different. The first experiment entailed establishing demyelination in the dorsal white matter; site of injection, and comparing the number of proliferative cells in animals which received the LPC injection alone compared to those which received the LPC injection then cholinergic modulation with donepezil and PNU-120596. As a stock solution, donepezil was first dissolved in distilled water at a stock concentration of 10 mM then diluted in saline to a final working concentration of 480.827 µM equating to 1mg/kg for *in vivo* administration. PNU-120596 was first dissolved in dimethyl sulphoxide (DMSO) at stock concentration of 10 mM then diluted in saline to a final working concentration of 1mM. Thus, control animals in these experiments received vehicle injections composed of either saline alone or saline and 10% DMSO.

Here N = 2 animals were administered 0.1 ml EdU (10 µM) alone and N = 2 were given 0.1 ml EdU (10 µM) in combination with 0.1 ml donepezil HCl (10mg/kg) and 0.1 ml PNU-120596 (1 µM) at days 1,2 and 3 post-surgery and sacrificed on day 5. Day 1 was considered the next day after surgery, as the surgical day was considered day 0. Here demyelination was assessed through immunohistochemical labelling with MBP and Olig2.

The second experiment aimed to investigate the numbers of proliferative cells post-demyelination and the extent of oligodendrogenesis present at the site of injury in different conditions. Here the mice were left to recover from surgery for 3 days; this is the time during which demyelination occurs (Keough et al., 2015). There were three experimental groups; N = 4 animals were administered 0.1 ml EdU (10 µM) alone, N = 4 animals were administered 0.1 ml EdU (10 µM) and 0.1 ml PNU-120596 (1 µM) and N = 4 were given 0.1 ml EdU (10 µM) in combination with 0.1 ml (10mg/kg) donepezil HCl and 0.1 ml PNU-120596 (1 µM) at days 4,5,6 and 7 post-surgery and sacrificed on day 14. Day 1 was considered the next day after surgery, as the surgical day was considered day 0, figure 2.5. Here proliferation and oligodendrogenesis were assessed through click chemistry with EdU and immunohistochemical labelling with Olig2 respectively.

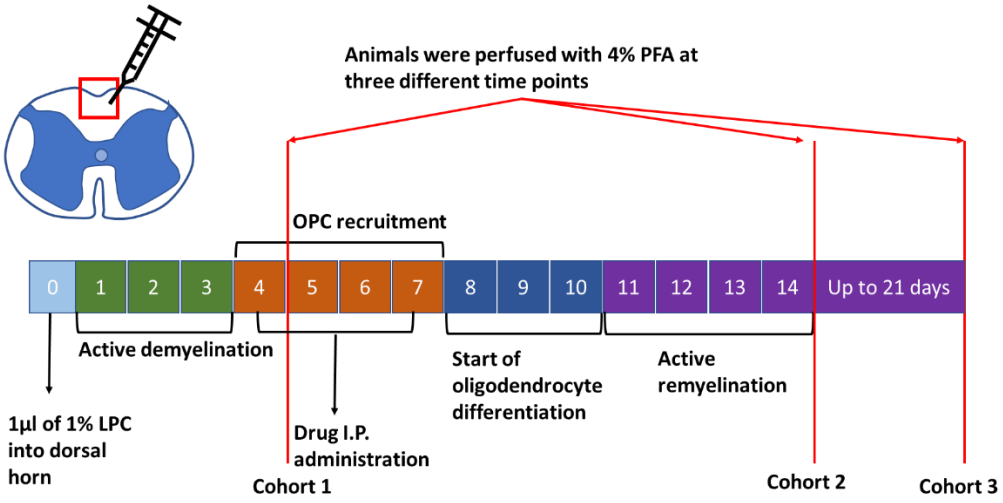

Figure 2.5 LPC experimental timeline.

This schematic depicts the three different timepoints at which the experiments took place, the days of cholinergic drug administration and the established course of the LPC demyelination injury.

The third experiment aimed to determine the difference in oligodendrogenesis and remyelination among three experimental groups. N = 3 animals were administered 0.1 ml EdU (10 µM) alone, N = 3 animals were administered 0.1 ml EdU (10 µM) and 0.1 ml PNU-120596 (1 µM) and N = 3 were given 0.1 ml EdU (10 µM) in combination with 0.1 ml (10mg/kg) donepezil HCl and 0.1 ml PNU-120596 (1 µM) at days 4,5,6 and

7 post-surgery and sacrificed on day 21. Day 1 was considered the next day after surgery, as the surgical day was considered day 0. Here proliferation and oligodendrogenesis were assessed through click chemistry with EdU and immunohistochemical labelling with Olig2 respectively. In all three experiments, the method of EdU labelling and immunohistochemistry were identical to the method employed in other experiments, detailed in sections (2.2 and 2.6).

The fourth cohort of animals had undergone the exact same surgical procedure and were administered either LPC alone or LPC + PNU + DON for 4 days; similar to the first three experiments. They were sacrificed at day 14 similar to the second cohort of animals described earlier. Since these animals were intended for electron microscopy analysis to assess the extent of remyelination, they were perfused with 4% PFA + 2% Glutaraldehyde.

2.8 Electron microscopy (EM)

Electron microscopy is a favourable technique for obtaining high resolution images of biological samples. It is mainly used to visualise the detailed structure of tissues, cells or macromolecules. High resolution images are acquired through the use of a beam of accelerated electrons which have a characteristically short wavelength to illuminate the sample once transmitted through it. The image is formed from the interaction of the electrons with the sample. Since the wavelength of an electron can be 100,000 times shorter than visible light photons, the resolving power of electron microscopes surpasses that of the light microscope. The imaging device associated with electron microscopes is capable of magnifying images at a significantly higher magnification than other microscopes (Bogner et al., 2007). Electron microscopy can include various methods of sample preparations such as sectioning and staining among other modifications. For the purpose of our focal demyelination experiments, we employed transmission electron microscopy (TEM). Our step-by-step protocol is as follows.

2.8.1 Tissue acquisition

At post surgery day 14, animals were transferred from the animal housing unit to the laboratory for perfusion. They were first terminally anaesthetised with an I.P. injection of 60mg/kg pentobarbitone (Pentoject animal care) and assessed for the absence of the corneal reflex and all pedal withdrawal reflexes prior to starting the perfusion. They were then transcardially perfused (similar to the method described in section 2,6.2) with 20-50 ml of 0.1M of PB to flush out the blood then with 50-200 ml of 4% PFA + 2.5 % glutaraldehyde (Gurr microscopy materials, 50% solution cat# 2810) solution made up in 0.1M PB (pH 7.4). Once the animal was sufficiently fixed, the skin and limbs were separated, and the brain and vertebral column of each animal was excised using fine spring scissors. The pia and dura mater were dissected from the cord under a dissecting microscope using fine forceps and the spinal cord tissue containing the demyelinated lesion was sectioned at 100 µm thick sections using a vibrating microtome (Leica VT100S, Microsystems, Milton Keynes, UK). All sectioned spinal cord tissue were collected into 4% PFA + 2.5 % glutaraldehyde containing 24 well plates for transfer to the EM facility at the University of Leeds for analysis.

2.8.2 Sample preparation

All spinal cord sections were further fixed for TEM analysis as follows:

They were first fixed with 2.5% glutaraldehyde in 0.1 M PB for 2.5 hours. This was followed by two washes 20 minutes each with 0.1 M PB. They were then post-fixed with osmium tetroxide in 0.1 M PB for 1 hour followed by two washes 20 minutes each with 0.1 M PB. The spinal cord sections were then dehydrated using an ascending alcohol series (20%, 40%, 60%, 80%, 100% X2) for 20 minutes at each concentration. After that the samples were embedded in epoxy resin as follows: sections were submerged in 50% propylene oxide and 50% Araldite overnight followed by 75% - 25% Araldite-propylene oxide for 3 hours. Next, the sections were submerged in neat Araldite for 3 hours before their transfer into embedding moulds with fresh Araldite and polymerise for 24 hours at 60 ^{o}C. The spinal cord sections were then re-sectioned into ultra-thin sections (80-100 nm) and picked up on 3.05 mm grids and stained with

saturated Uranyl acetate for 5-120 minutes and Reynolds Lead citrate for 5-30 minutes.

2.8.3 Image acquisition and analysis

The ultra-thin spinal cord sections were images using Jeol Jem 1400 TEM (80 KV) and all sections were analysed using the Carl Zeiss Zen software 2017. The aim of this experiment was to measure the G-ratio (the inner axonal diameter to the total outer fibre diameter) of axons to assess the extent of endogenous axonal remyelination in animals that received LPC alone 14 days post demyelination and compare that to those that received LPC followed by treatment with PNU-120596 and donepezil. Therefore, the inner and outer diameter of individual axons (located within the demyelinated-remyelinated region) were measured using a distance measuring tool in the Carl Zeiss Zen software 2017. This semi-automated method was employed to obtain accurate measurements. The value of each measurement was added to an Excel spread sheet before being transferred onto Graph Pad Prism 7 for statistical analysis. Throughout the data analysis stage, all samples were re-labelled by a member at the EM facility in order for me to conduct the data analysis blinded and avoid any experimental bias. A student T test was employed to compare both experimental data sets. Measuring the G-ratio of axons is considered the gold standard method to evaluate the extent of myelination and the microstructure of axons both in animal models of white matter disorders (Rittchen et al., 2015) and in human brain axonal studies (Mohammadi et al., 2015) which employed high fidelity magnetization transfer imaging and single shell diffusion MRI to measure axonal fibres of 37 healthy individuals. Measuring the G-ratio of axons can also be done by tracing the axonal circumference and the whole fibre circumference and divide the two values. This method was employed by Rittchen and colleagues using a graphics pad and pen (Rittchen et al., 2015).

2.9 The generation of viral inflammation and multi-focal demyelination with the semliki forest virus (SFV)

The semliki forest virus is a positive single stranded RNA historically used to study the molecular biology of virus replication (Strauss and Strauss, 1994). It was originally isolated from mosquitos in Uganda in 1944 (C. Smithburn and J. Haddow, 1944). The original viral strain is termed L10 and is neurologically virulent in mice; leading to lethal encephalitis, while the subsequent strain named A7, also isolated from mosquitos in Mozambique is avirulent in rodents and causes no phenotypic symptoms (Atkins et al., 1999). This particular strain of virus has been used to produce acute encephalomyelitis in mouse models for many years to investigate the pathogenesis of white matter diseases like multiple sclerosis through infecting the central nervous system (Fazakerley et al., 1983, Subak-Sharpe et al., 1993), although, various strains of mice were used other than C57Bl/6 mice. This avirulent A7 (74) strain is the one used in our experiment to establish central nervous system inflammation and multi-focal demyelination. It was fluorescently tagged with mCherry.

2.9.1 Virus administration and tissue acquisition

These experiments were carried out at the University of Leeds St James's University Hospital Campus – Wellcome Trust Brenner Building and the SBS unit. All animal handling occurred in a quarantine room of level 2 (CL2) containment and the supervision of Dr. Clive McKimmie (University of Leeds St James's University Hospital Campus). We administered the virus intraperitoneally to C57/Bl6 male mice, aged 9 weeks. They received a single dose of the virus (100,000 PFU; plaque forming units) each at the start of the experiment; day 0. This experiment was the first one of its kind in our lab, thus in order to establish this model, we first aimed to determine the amount of time needed for the virus to enter the nervous system and replicate for inflammation to occur in the central nervous system and for any demyelinating lesions to appear in the brain and/or spinal cord. The dose of the virus was determined by a collaborator (Dr.McKimmie, University of Leeds St James's University Hospital Campus) who has previously worked with this strain *in vitro*.

Our experiment included a total of N = 15 male mice, all of which received the virus on the same day. These mice were then separated into 5 groups, each group consisted of N = 3. From each group of mice, N = 2 had undergone a perfusion with 20-50 ml 4% PFA- identical to other *in vivo* perfusions and tissue extraction for immunohistochemistry, while N = 1 of each group was perfused with 20-50 ml 0.1 M PB only for quantitative polymerase chain reaction (qPCR) analysis. Furthermore, each group was sacrificed at a specific time point; group 1 on post injection day (PID) 4, group 2 on PID7, group 3 on PID10, group 4 on PID14 and group 5 on PID21.

2.9.2 Immunohistochemistry

Once N = 2 animals of each group at each time point was perfused with 4% PFA, the brain and spinal cords were dissected out in an identical fashion to the method of fixed tissue extraction in section 2.6.1. As soon as the brain and spinal cord of each animal were extracted, they were allowed to post-fix in falcon tubes containing 35-40 ml of 4% PFA overnight. After proceeding with tissue extraction, the pia mater was gently removed from the spinal cord, and tissue sectioning of the brain and spinal cord was done using a vibrating microtome (Leica VT100S, Microsystems, Milton Keynes, UK). The brain was sectioned transversely into 50 µm sections, while the cervical, thoracic and lumbar regions of the spinal cord of each animal were separated and sectioned longitudinally into 50 µm sections; to allow for the visualization of axonal fibers. All sections of the brain and spinal cord were then collected into 6 well and 24 well histology plates respectively, permeabilised with 0.1% PBST for 30 minutes, washed with PBS 3 times (10 minutes each), blocked with 10% goat serum in 0.1% PBST for 30 minutes and finally immunohistolabelled with both anti-RFP and anti-MBP or fluoromyelin primary antibodies overnight. After that, the primary antibodies were washed off with PBS and secondary fluorescent antibodies were added. The sections were then mounted onto glass slides and examined for the presence of the virus in the brain or spinal cord sections by looking for mCherry fluorescence, and any demyelinated lesions throughout the central nervous system by examining the pattern of myelin sheaths visualized by the use of anti-MBP or fluoromyelin.

At each time point, N = 1 of each group was perfused with 0.1M PB and the tissue was extracted in the same way fixed tissue was. Once the brain and spinal cord were dissected out, they were put into RNA later solution and kept in -80 ºC for at least 24 hours. RNA later is an aqueous solution used for storing tissue prior to qPCR analysis. It stabilises and protects RNA in cells of fresh frozen tissue.

2.9.3 qPCR of SFV brain and spinal cord tissue

For viral gene expression analysis, the brain and spinal cord tissue were removed from the RNA later and RNA extraction and purification was done. This process was carried out by submerging the tissue in lysing (TissueLyser LT (Qiagen)) and homogenizing buffers (Life tech (Ambion) Trizol plus kit); this removes the particulate debris of tissue and separates the RNA from tissue prior to RNA purification. The purification of RNA is usually performed by centrifugation and separating the RNA supernatant before treatment with ethanol (95-100%) and further centrifugation. Since RNA columns are generally filtered at the bottom, the flow through during centrifugation is repeatedly discarded during the process of purification. RNA was then treated with wash buffers before it was analysed or stored at ≤-70 ºC.

In this experiment, the steps were as follows;

- Tissue was placed in 2 ml tube with a metal bead (Cat. 69989, Qiagen) and 1 ml Trizol to shake for 10 minutes at 50Hz.
- Once homogenised, the liquid was transferred to a 1.5 ml tube with 200 µl chloroform.
- Tubes were centrifuged for 15 minutes at 12,000 g at 4^{o}C.
- 500 µl of top clear aqueous phase was removed and placed in 1.5 ml tubes.
- An equal volume of 70% ethanol and RNAase-free water were added by vortexing
- 700 µl volume were added to PureLink column to purify RNA as per manufacturer's instructions (ThermoFisher scientific, 2019b).

Prior to the qPCR reaction, the RNA was first used to synthesize complementary DNA (cDNA) strands using one of many kits available to purchase. The total amount of

RNA was assessed using the nanodrop. cDNA was synthesised from total RNA by reverse transcription (RT) using Life tech (ABI) High capacity kit (cat # 4387406), as per manufacturer's instructions. Up to 2 ug total RNA in 9 µl volume was used, and added to this was 1 µl enzyme mix and 10 µl 2 X buffer to incubate for an hour at 37^{o}C on the PCR machine with a short denaturing step at 95^{o}C for 45 minutes. Samples were then reverse transcribed using the same master mix.

All primers were designed using Primer3 Software available online (ThermoFisher scientific, 2019). A standard DNA template for each gene assayed was generated by taking the PCR product generated using the "outer" primers, purifying the PCR product on a PCR purification column as per manufacturer's instructions (Qiagen QIAquick columns, or the Ambion PureLink PCR purification kit). The master mix made by Quanta, supplied by VWR in the UK: Cat # 733-1386 FastMix™, 1250×20 µl reactions (10×1.25 ml) was used. For primer sequences please refer to table 2.14. Each sample / standard was assayed in quadruplicate on the QPCR ABI machine. cDNA samples were diluted either 1 in 5 or 1 in 10 in nuclease free water and used as template for QPCR. Finally, housekeeping genes were used to normalise cDNA samples.

The qPCR reaction involves the use of sequence specific primers in a master mix containing a fluorescent DNA dye which reports the increase of the amount of DNA with a proportional increase in the fluorescence signal, nucleotides and enzymes. The steps involved in a qPCR reaction are: first, the transcription of RNA into cDNA by reverse transcriptase enzyme via reverse transcription reaction from total RNA or messenger RNA (mRNA); the steps include pre-denaturation, primer extension and finally cDNA synthesis. Then, the cDNA is the template used for qPCR. The basic principle of qPCR involves the amplification of the cDNA with fluorescently tagged targets that are monitored during the entire process. The cycle in which the fluorescence can be detected is called the quantitation cycle (Cq); lower Cq values mean higher initial copies. Given that the SFV experiment was done in collaboration with Dr.McKimmie and colleagues, for further detail on primer design please refer to (Pingen et al., 2016).

SFV genes and standards	Product size	Sequence
SFV E1, forward	173	CGCATCACCTTCTTTTGTG
SFV E1, reverse		CCAGACCACCCGAGATTTT
SFV E1 standard, forward	446	AAGTGAAGACAGCAGGTAAGGTG
SFV E1 standard, reverse		TATGAGTTGCCCCGAGTTTC
SFV 18S, forward	124	GACTCAACACGGGAAACCTC
SFV 18S, reverse		TAACCAGACAAATCGCTCCAC
SFV 18S standard, forward	443	CGTAGTTCCGACCATAAACGA
SFV 18S standard, reverse		ACATCTAAGGGCATCACAGACC

Table 2.14 SFV gene primers.

The table lists the primers used to amplify the virus gene E1 and the housekeeping gene 18S. The size of individual sequences are also provided.

2.10 Image acquisition and analysis

2.10.1 Fluorescence microscopy

All sections were first visualised under a fluorescent Nikon Eclipse E600 microscope to confirm proper labelling with immunohistological antibodies and EdU.

2.10.2 Cell counting

In vitro counts

All acute slices were manually counted under the fluorescent Nikon Eclipse E600 microscope. Manual counts were achieved by identifying individual cells through 20X and 40X objectives. Central canal, grey matter and white matter cell counts for acute slices were obtained and statistically evaluated separately. In organotypic culture

slices, the software Image J was used to conduct automated counting. The central canal was counted separately, however, the grey matter and white matter of each slice were counted together to yield a total number of EdU positive cells outside of the central canal.

The automated counts using the software Fiji or Image J entailed opening an individual file containing a maximum intensity image of a single slice that was previously tile scanned at 20X and Z-stacked to include all of the cells across the different focal planes of the slice. The file was then converted to a 16-bit file then processed to subtract background. After that, the threshold of the image was adjusted and finally the particles were analysed. An example of each of these steps was captured as detailed in figure 2.6.

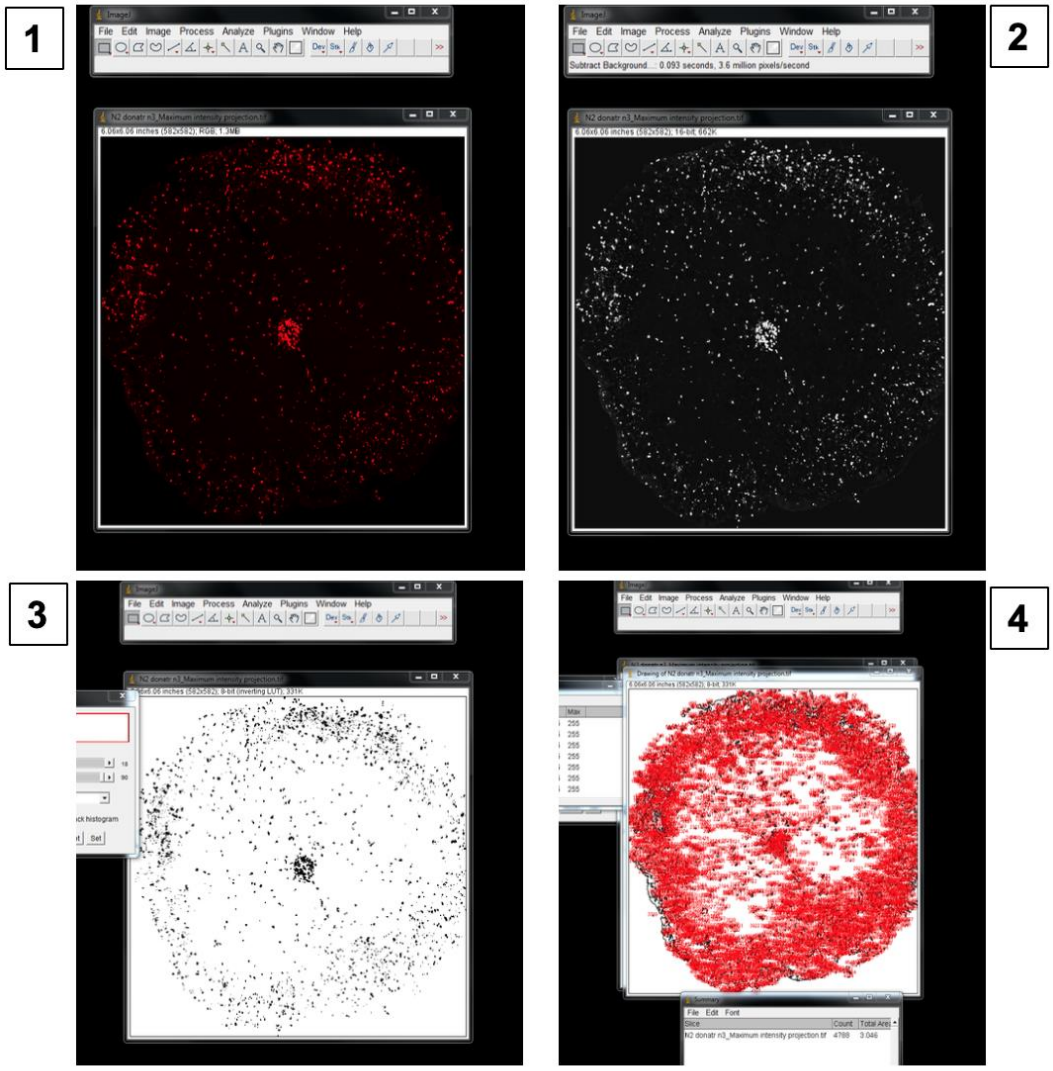

Figure 2.6 A representative sample analysed using image J.

Panel **1** depicts an individual open file which was then converted to a 16-bit image as shown in panel **2** followd by background subtraction in panel **3**. The threshold of the image was adjusted to choose the particles to be analysed. Finally, the chosen particles were analysed and a window with the total number of particles is provided as shown in panel **4**.

In vivo cell counts

In all *in vivo* experiments, slides containing tissue sections had labels removed and re-labelled by another researcher to avoid experimental bias. *In vivo* experiments comparing control levels of proliferation to donepezil treated animals or donepezil and PNU-120596 treatment were all evaluated using the fluorescent microscope. Thoraco-lumbar and dentate gyrus sections were all evaluated. The number of EdU positive proliferative cells and cells colocalised with differentiation markers were counted manually given the unavailability of a properly functioning Airyscan laser scanning microscope.

In *in vivo* dorsal lumbar focal demyelination experiments, all sections were first captured as a Z-stack image in order to analyse the entirety of each section using a Zeiss LSM880 upright or inverted Confocal microscope + Airyscan laser scanning microscope equipped with argon and He-Ne lasers using both 20X and 40X objectives. This type of image produces a high-resolution image with increased signal to noise ratio and eliminated out-of-focus signal at each stack or plane. These Z-stack images were then compressed into a single Maximum intensity projection image to view as a single plane section. EdU positive and differentiated colocalised cells were then counted using the Zeiss software tool ‘Events’; a selection tool which enables the selection, automated counting and marking of each individual cell. This semi-automated method ensures that each cell is counted without running the risk of missing cells or accounting for one cell twice. The *in vivo* and *in vitro* captured images were adjusted for brightness and contrast on both Carl Zeiss Zen software and Coreldraw 17 software for presentation in figures. The images presented are either single plane or Z-stacks with maximum intensity fluorescence projection.

2.11 Statistical analysis

All data are expressed as means ± the standard error of the mean. All manually or semi-automated cell counts were first entered into a Microsoft Excel spreadsheet then exported into GraphPad Prism 7 for statistical analysis of the mean, standard error and

standard deviation of individual regions of thoraco-lumbar spinal cord slices, spinal cord sections or dentate gyrus sections. For differentiation analysis, percent colocalisation was also analysed. In all experiments described in this thesis, N = animal and n = section or slice. To determine the statistical significance between control and treated slices in terms of proliferation *in vitro*, a One-Way Anova test followed by post-hoc Brown-Forsythe (a test for equal population variances) and its suitable alternative Bartlett's tests (equal variances) were employed. The normality of each test was also assessed. P values of calculated data were considered significant when $P<0.05$ (*); $P<0.01$(**); $P<0.001$ (***); $P<0.0001$ (****).

In order to determine the statistical significance between control and treated *in vivo* thoraco-lumbar spinal cord or hippocampal dentate gyrus sections in terms of both proliferation and differentiation; an Independent T-test was performed to calculate the number of proliferating cells and percentage of colocalisation. The normality of each test was also evaluated. P values of calculated data were considered significant when $P<0.05$ (*); $P<0.01$(**); $P<0.001$ (***); $P<0.0001$ (****).

Chapter 3

Cholinergic enhancement of cell proliferation in thoraco-lumbar segments of the spinal cord and dentate gyrus of the hippocampus

3.1 Introduction

The influence of several neurotransmitters on neurogenesis in the developing spinal cord have been elucidated, although postnatal spinal cord neurogenesis is widely less described. The effects of increasing endogenous levels of acetylcholine and potentiating the activation of α7nAChRs on postnatal neurogenesis in the murine spinal cord is the focus of this study. The neurogenic niche of the spinal cord is comprised of morphologically distinct and anatomically organised cells with a dorso-ventral subpopulation exhibiting stem cell - like behaviour and regenerative potential, as previously covered in chapter 1 (sections 1.4 - 1.6).

The significant increase in the extent of central canal cell proliferation following PNU-120596 administration both *in vivo* and *in vitro* (Corns et al., 2015) which encouraged our focused investigation on α7nAChR activation in the intact spinal cord. The ependymal cell response to acetylcholine in the presence of a selective non-α7nAChR antagonist (DHβE) was substantially potentiated by PNU-120596 under electrophysiological settings which suggested the presence of α7nAChRs on ependymal cells and highlighted their potential importance in cell proliferation (Corns et al., 2015). Furthermore, Corns demonstrated by means of immunohistochemistry the presence of $ChAT^+$ cholinergic cells and terminals in close proximity to ependymal cells in the central canal displaying a potential source of endogenous acetylcholine (Corns et al., 2015). This is of pertinence since the studies conducted in this chapter entail the employment of an AChEI (donepezil) in order to increase endogenous levels of ACh. Therapeutic cognitive effects as a result of enhanced cholinergic function and cell proliferation in the adult hippocampal niche following the systemic administration of donepezil is well established. However, this study is the first to test whether donepezil alone or in combination with PNU-120596 can promote the normally dormant spinal cord neurogenic niche to proliferate under normal conditions, while the dentate gyrus of the hippocampus from each animal was evaluated as a positive control.

3.1.1 Hypothesis

Since ependymal cells exhibit stem cell potential, depolarise in response to acetylcholine and are triggered to proliferate following PNU-120596 administration; it would be reasonable to hypothesise a greater proliferative response to the modulation of cholinergic transmission using both donepezil and PNU-120596 in combination. Furthermore, based on the consensus of enhanced hippocampal cell proliferation as a result of systemic donepezil administration, we can also hypothesise an increase in cell proliferation compared to control conditions.

3.1.2 Aims

The aim of the studies conducted in this chapter is to examine the effects of an acetylcholinesterase inhibitor (donepezil HCL) alone or in conjunction with an α7nAChR positive allosteric modulator (PNU-120596) on ependymal cell proliferation and differentiation in the postnatal mammalian spinal cord. These experiments were also compared to cell proliferation and / or differentiation in the dentate gyrus of the hippocampus. Donepezil was administered as an intraperitoneal injection (alone or in combination with PNU-120596 *in vivo* or applied in the aCSF incubating solution containing free floating acute (500 µm thick) spinal cord slices *in vitro*, refer to chapter 2 (sections 2.3.3 and 2.4.4) for the experimental method.

To detect the occurrence of cell proliferation, the thymidine analogue EdU was used in both *in vivo* and *in vitro* experiments either by direct addition to the aCSF incubating solutions or through intraperitoneal injection. *In vivo* experimental tissue was then immunohistochemically labelled using specific markers to identify differentiated cells. Three monoclonal antibodies were employed to detect neurons, astrocytes and oligodendrocytes: anti-HUC/D, anti-S100β and anti-PanQKI respectively. *In vitro* tissue was only utilized to assess cell proliferation.

3.2 Results

3.2.1 Spinal cord slices treated with an acetylcholinesterase inhibitor exhibited lower levels of proliferation in the central canal

Acute application of donepezil (5 μM) *in vitro* resulted in significantly lower numbers of EdU positive cells in the central canal; P= 0.002, mean 3.9 ± 0.3 SEM, N=3, n=23 when compared to control slices; mean 5.1 ± 1.5 SEM, N=3, n=17, (figure 3.1). A significant difference (P=0.0453) was also observed between donepezil and atropine treated slices (mean 3.0 ± 0.4 SEM, N=3, n=8 in comparison to control conditions.

There was no change however in the overall number of EdU positive cells between control and either of the treatment conditions.

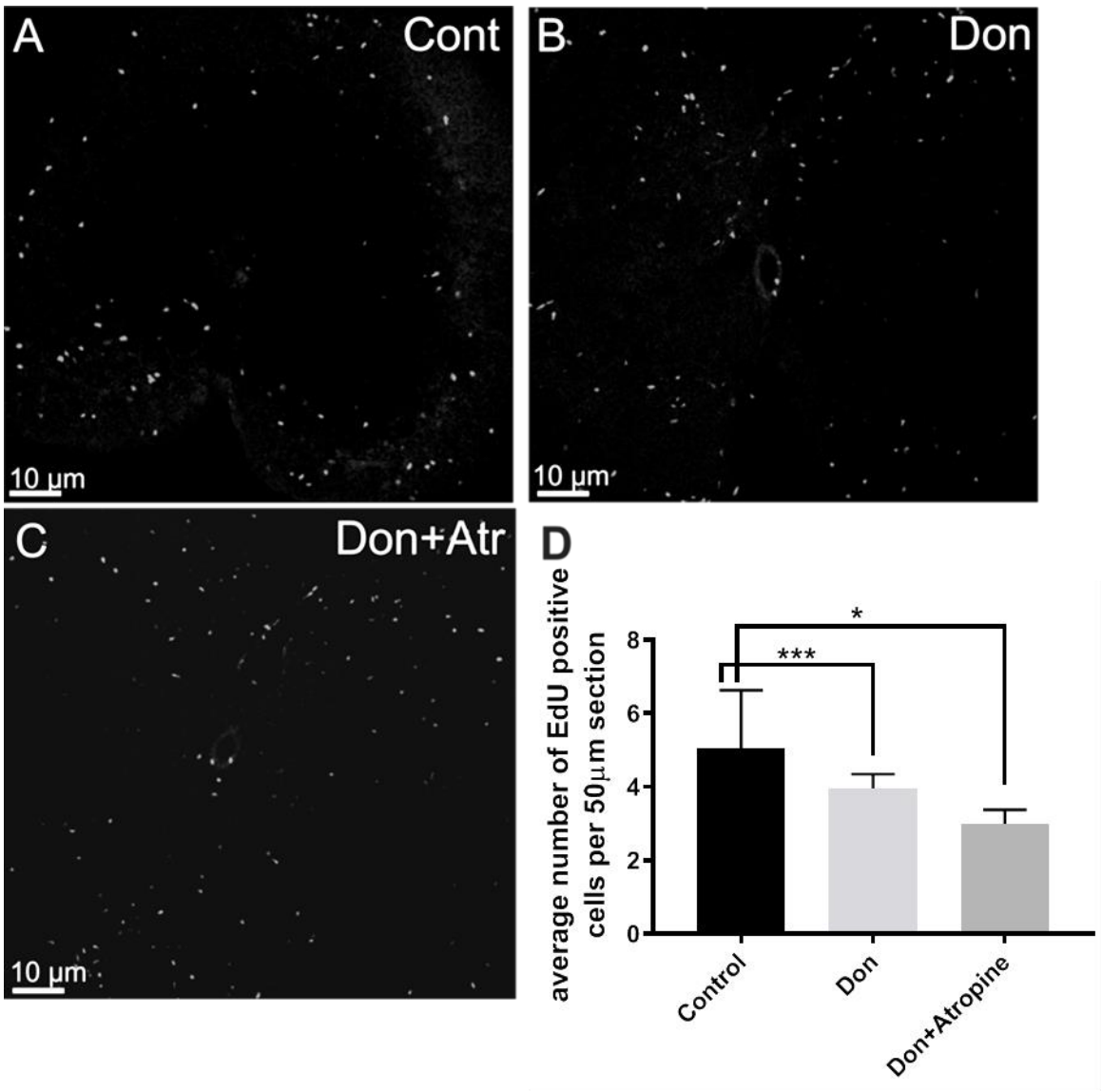

Figure 3.1 Donepezil treated slices showed lower levels of cell proliferation in the central canal of the spinal cord compared to control.

A) Representative images of EdU positive cells in a control slice. **B)** Representative images of EdU positive cells in a 5 µM donepezil-treated slices. **C)** Representative images of EdU positive cells in a 5 µM donepezil + 5 µM atropine-treated slices. **D)** Number of EdU labelled cells for each of the three conditions. Data are shown as mean number of EdU labelled cells per 50 µm section ± SEM of: N=3 animals and n=17 slices for Control, N=3 animals, n=23 slices for Donepezil and N=3 animals and n=8 slices for Donepezil + atropine. ***; P= 0.002 and *; P=0.0453.

3.2.2 Inhibiting acetylcholinesterase in the spinal cord niche results in lower levels of proliferation at the central canal *in vivo*

This experiment entailed the administration of donepezil intraperitoneally for seven consecutive days prior to perfusion with 4% PFA, figure 3.2 A. Acetylcholinesterase inhibition in the thoracic segment of the spinal cord did not stimulate proliferation. Instead, in the central canal region control animals had a significantly higher number of EdU positive cells mean 3.9 ± SEM 0.3, N = 4 animals, n = 65 sections compared to donepezil treated animals, (figure 3.2, Ci, Cii, Di). However, at the whole spinal cord level (combining the white matter and grey matter regions excluding the central canal) there was no significant difference in the number of EdU positive cells in donepezil treated animals; mean 50.8 ± SEM 3.0, N = 4 animals, n = 88 sections compared to control sections; mean 54.5 ± SEM 3.4, N = 4 animals, n = 65 sections, (figure 3.2, Bi, Bii, Dii).

Since donepezil was known from previous studies to cause an increase cell proliferation in hippocampal regions (Kaneko et al., 2006, Imamura et al., 2015b), the effects of donepezil in the dentate gyrus were also tested here as a positive control. The number of EdU positive cells in animals administered donepezil were significantly higher; mean 43.5 ± 12.6 SEM, N =4 animals, n=12 sections compared to control animals (mean 4.1 ± 1.2 SEM, N = 4 animals, n = 12.); P = 0.0054, (figure 3.3).

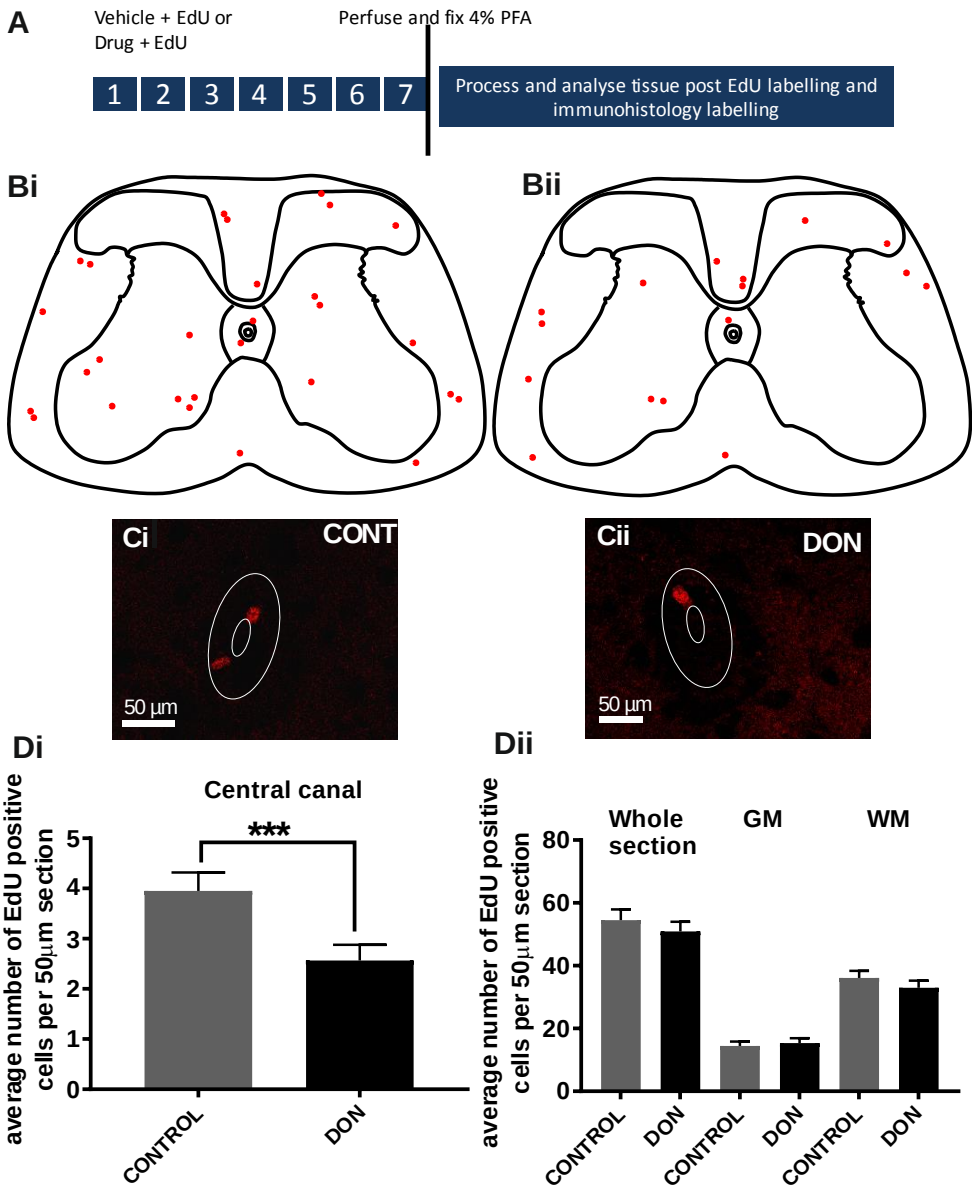

Figure 3.2 Animals administered donepezil had lower numbers of EdU positive cells at the central canal but there was no change in numbers in the white and grey matter *in vivo.*

A) Experimental timeline. **Bi)** A Schematic illustrating the distribution of EdU$^+$ cells in control. **Bii)** Representative schematic figure illustrating the distribution of EdU$^+$ cells in treated animals**. Ci)** EdU$^+$ cells within the central canal region of control animals. **Cii)** EdU$^+$ cells within the central canal region of treated animals. **Di-Dii)** Number of EdU$^+$ cells in each condition. Data are shown as mean number of EdU$^+$ cells per 50 µm section ± SEM of: N = 4 animals, n = 88 sections in treated animals verses N = 4 animals, n = 65 sections in control animals. ***; P= 0.004 in the central canal.

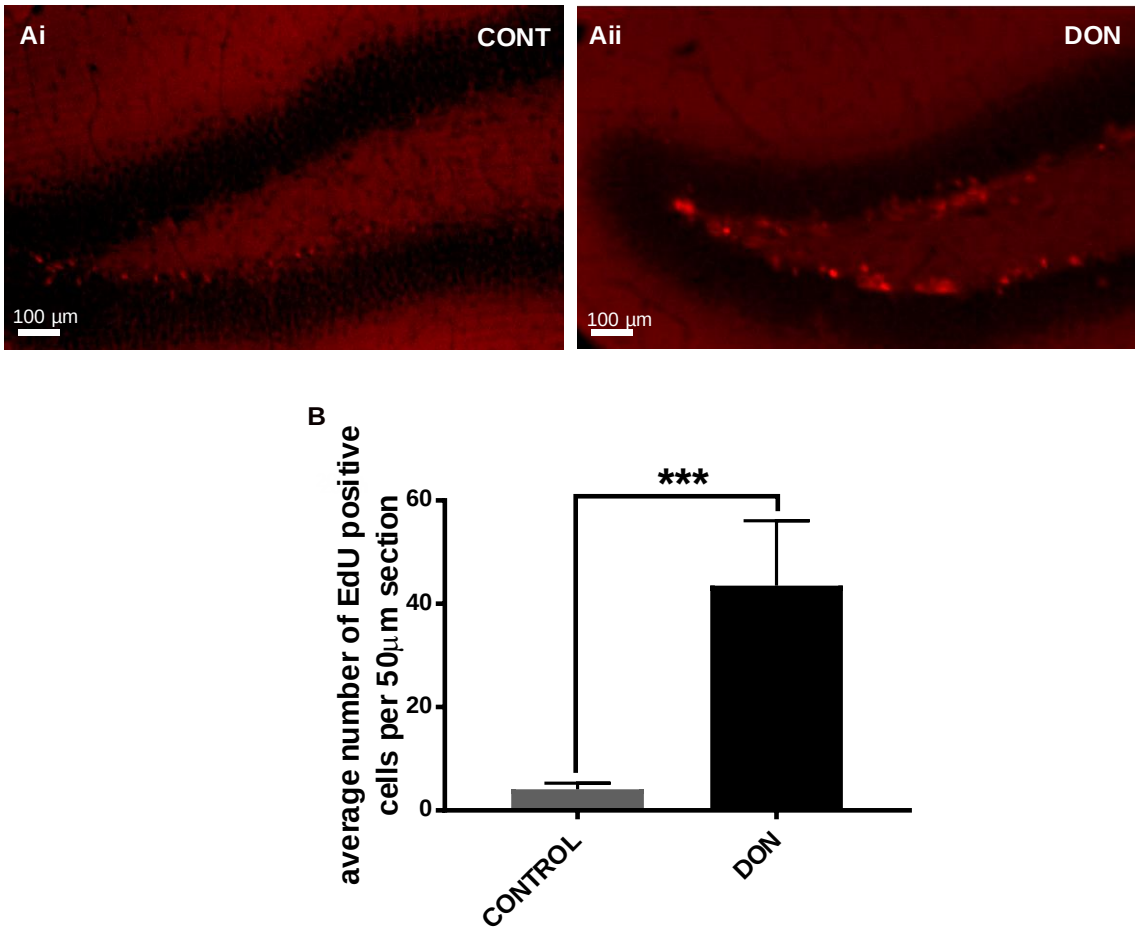

Figure 3.3 *In vivo* administration of donepezil results in higher numbers of newly proliferated cells in the dentate gyrus compared to control levels of cell proliferation.

Ai) Representative images of EdU positive cells in control animals. **Aii)** Representative images of EdU positive cells in donepezil treated animals. **B)** Number of EdU labelled cells in each condition. Data are shown as mean number of EdU labelled cells per 50 µm section ± SEM of: N = 4 animals, n = 12 sections in the dentate gyrus of treated animals verse control conditions (N = 4 animals, n = 12 sections). ***; P= 0.005.

3.2.3 Increased endogenous acetylcholine levels and potentiating α7nAChRs in combination enhances cell proliferation *in vivo*

Since there are various different nAChR subtypes which may be mediating the increase in cell proliferation when increasing the levels of ambient acetylcholine, the contribution of α7nAChRs specifically in cell proliferation was investigated by administering the selective modulator PNU-120596 in conjunction with donepezil. Both the thoracic and the lumbar regions of the spinal cord were analyzed along with the dentate gyrus of hippocampus; used for comparison. At the spinal cord level, all regions had significantly higher numbers of EdU positive cells when compared to vehicle treated animals ($P < 0.0001$). This was the case for all regions tested- central canal, grey matter and white matter (figure 3.4). Exact values of numbers of EdU positive cells for each condition and region are given in (table 3.1). Using the dentate gyrus once again as a positive control, animals treated with the combination of donepezil and PNU-120596 had significantly higher numbers of EdU positive cells (mean 139.8 ± 4.8, N =3, n =132) compared to control animals; mean 108.5 ± 3.8, N = 3, n =79, (figure 3.5).

Spinal cord region	**Central canal** *		**Grey matter** *		**White matter** *	
	Control	**DON + PNU**	**Control**	**DON + PNU**	**Control**	**DON + PNU**
Thoracic	4.5 ± 0.4 (n=82)	7.5 ± 0.4 (n=80)	41.1 ± 2.9 (n=82)	91.8±5.1 (n=80)	107.1±4.5 (n=82)	159.6±7.9 (n=80)
Lumbar	5.7 ± 0.3 (n=92)	10.8±0.5 (n=94)	49.5 ± 3.1 (n=92)	88.4±5.7 (n=94)	107.3±5.4 (n=92)	191 ± 10 (n=94)

Table 3.1 The numbers of EdU positive cells post treatment with donepezil and PNU-120596 in the spinal cord.

This table summarises the exact number of sections used per condition when the number of animals used per each condition is N=3.

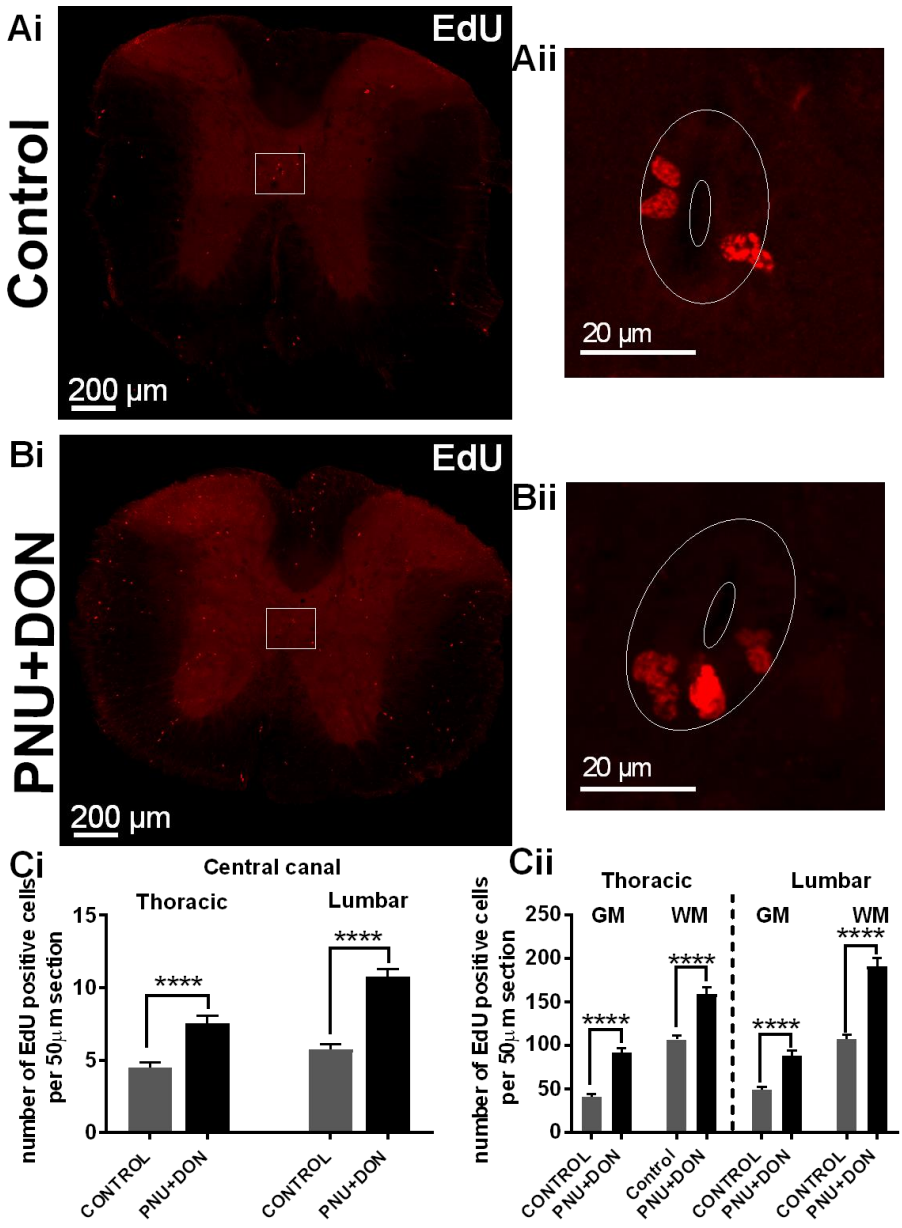

Figure 3.4 Animals treated with donepezil + PNU-120596 exhibited higher levels of proliferation in all regions of the spinal cord compared to control.

Ai) Representative image of EdU^+ cells in a control section. **Aii)** Magnified central canal area. **Bi)** EdU^+ cells in donepezil+ PNU treated sections. **Bii)** Magnified central canal area**. Ci-Cii)** Number of EdU^+ cells in treated animals versus control animals in the entire section **(Ci)** and in the central canal, grey matter and white matter **(Cii).** Data are shown as mean number per 50 µm section ± SEM of: N=3 animals and n= 92 sections for control animals and N=3 animals, n= 94 sections for treated animals. ****; P<0.0001.

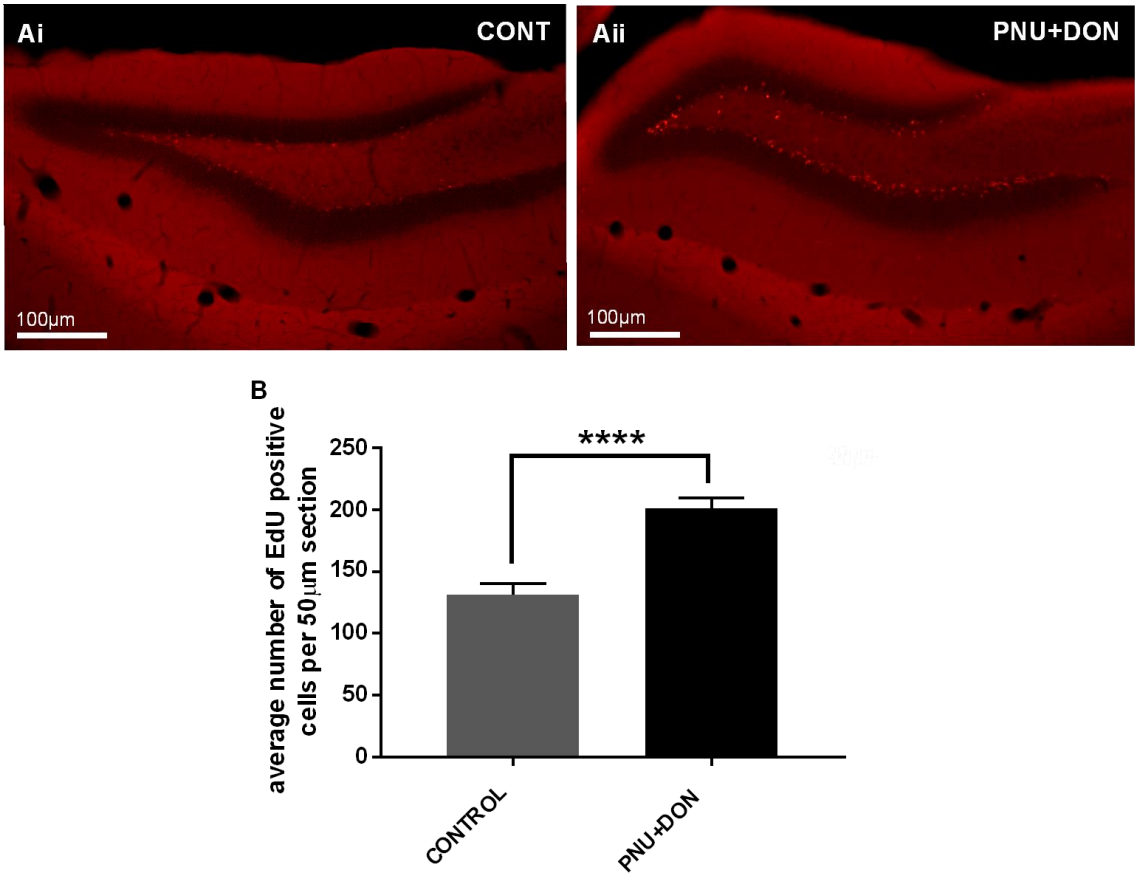

Figure 3.5 *In vivo* administration of donepezil with PNU-120596 causes higher levels of proliferation in the dentate gyrus of the hippocampus compared to control.

Ai) Representative images of EdU labelled cells in control sections.

Aii) Representative images of EdU labelled cells in animals treated with donepezil+ PNU-120596. **B)** Number of EdU labelled cells in each condition. Data are shown as mean number of EdU labelled cells per 50 µm section ± SEM of: N =3 animals, n =79 sections for control animals compared to N =3 animals, n =132s sections in PNU+donepezil treated animals. ****; P< 0.0001.

3.2.4 Increasing endogenous acetylcholine levels and potentiating α7nAChRs promotes oligodendroglial differentiation of newly proliferated cells

To determine the identity of the newly proliferated EdU postive cells in the spinal cord, sections were double labelled with various immunohistochemical markers to identify their lineage of differentiation. Oligodendroglial differentiation was assessed using the antibody against the RNA-binding QKI protein which is essential in myelination, known as hQKI or PanQKI (Neuromab, 2018, Gong et al., 2016).

Although animals treated with donepezil in conjunction with PNU-120596 had significantly ($P<0.0001$) higher levels of EdU positive cells in the central canal region compared to control levels, none of the ependymal cells in either control or treated animals were immunoreactive for any of the markers; HUC/D- a neuronal marker, PanQKI- the oligodendroglial marker, or S100β- an astrocytic marker. This suggests that ependymal cells are triggered to proliferate and either migrate away from the central canal then undergo differentiation, or simply stimulated into proliferation and differentiation down their own cellular lineage.

In both the grey matter and white matter of treated animals, the percent colocalisation of the oligodendroglial marker PanQKI immunoreactivity with EdU positive cells was significantly higher ($P<0.0001$) in both thoracic and lumbar regions of the spinal cord in comparison to control animals, (figure 3.6). Specific values of the changes are given in table 3.2.

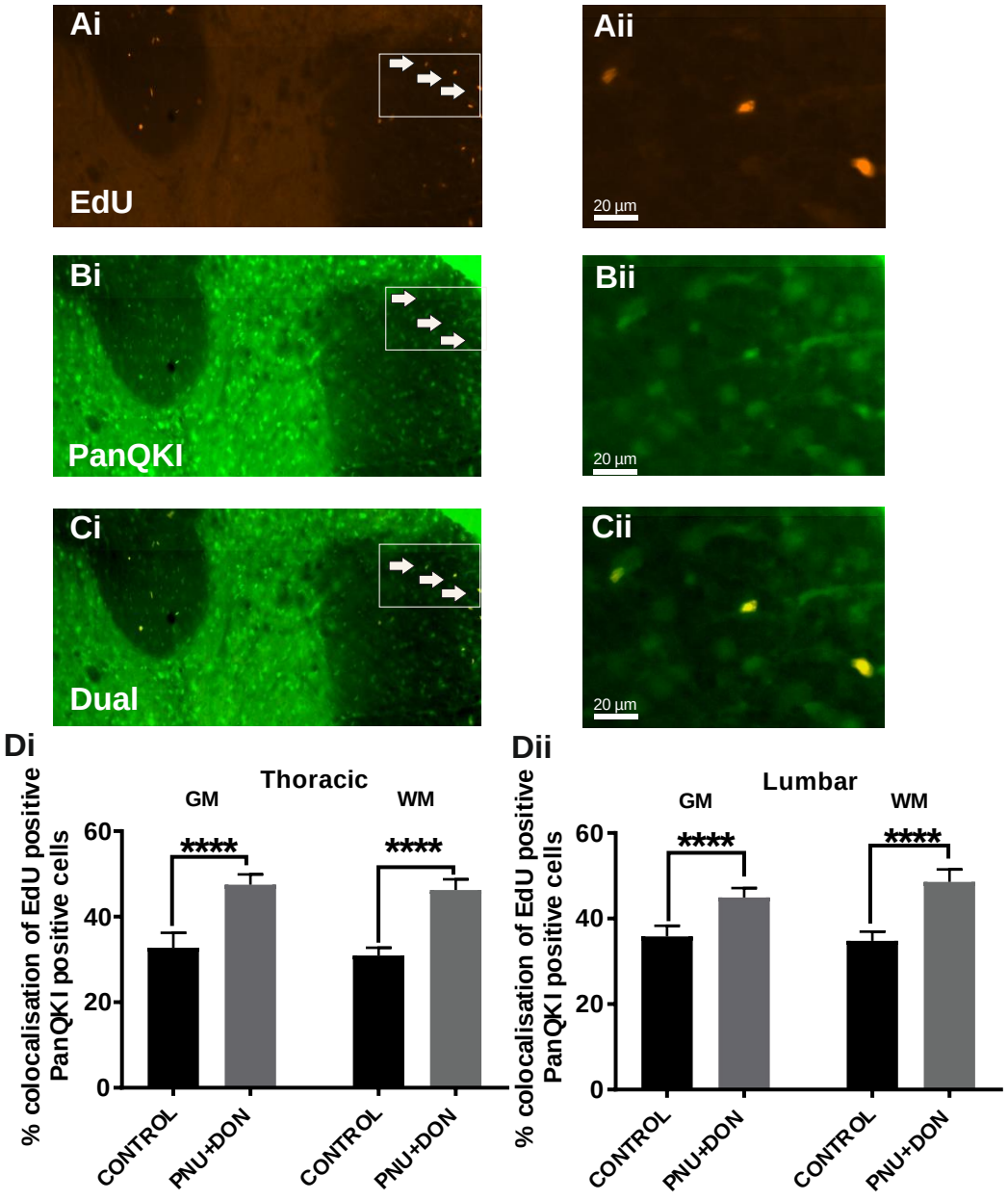

Figure 3.6 *In vivo* administration of donepezil in combination with PNU-120596 showed higher percentages of colocalisation of EdU with PanQKI in the grey and white matter of the spinal cord.

Ai) Representative images of EdU labelled cells. **Aii)** Magnified image of the boxed area in (Ai). **Bi)** EdU cells colocalised with PanQKI. **Bii)** Magnified image of the boxed area in (Bi). **Di,Dii)** Percent colocalization in each condition. Data are shown as mean number of percent colocalisation per 50 µm section ± SEM of: N=3 animals, n=42 sections versus control conditions N=3 animals, n= 56 sections. ****; P<0.0001 in the grey matter and white matter.

3.2.5 Animals treated with PNU-120596 + donepezil had higher numbers of EdU positive cells in the grey matter that differentiated down the neuronal lineage

The spinal cord sections of both control animals and treated animals were subjected to neuronal immunohistochemical labelling to explore whether any proliferated cells differentiated towards a neuronal lineage within the allotted period of time.

In order to correctly identify these cells, a marker (anti-HUC/D) which binds to specific and exclusive neuronal proteins was used. HU antigens are RNA-binding proteins that belong to a family of highly conserved drosophila to mammal embryonic lethal abnormal visual system (ELAV) proteins (Akamatsu et al., 2005, Rogulja-Ortmann et al., 2014). These proteins are important in the developmental transformation of immature neurons to mature neuronal cells, their maintenance in a mature state, and they also play a role in neuronal metabolism (Rogulja-Ortmann et al., 2014, Akamatsu et al., 2005).

In the grey matter of thoracic and lumbar spinal cord of treated animals, the percentage of EdU positive cells that were immunoreactive for the neuronal marker HUC/D was significantly higher; $P<0.0001$ than the percentage of colocalised cells in control sections, (figure 3.7), although it must be noted that almost none of the EdU positive cells were HUC/D positive in control tissue. Specific values of the changes are given in table 3.2.

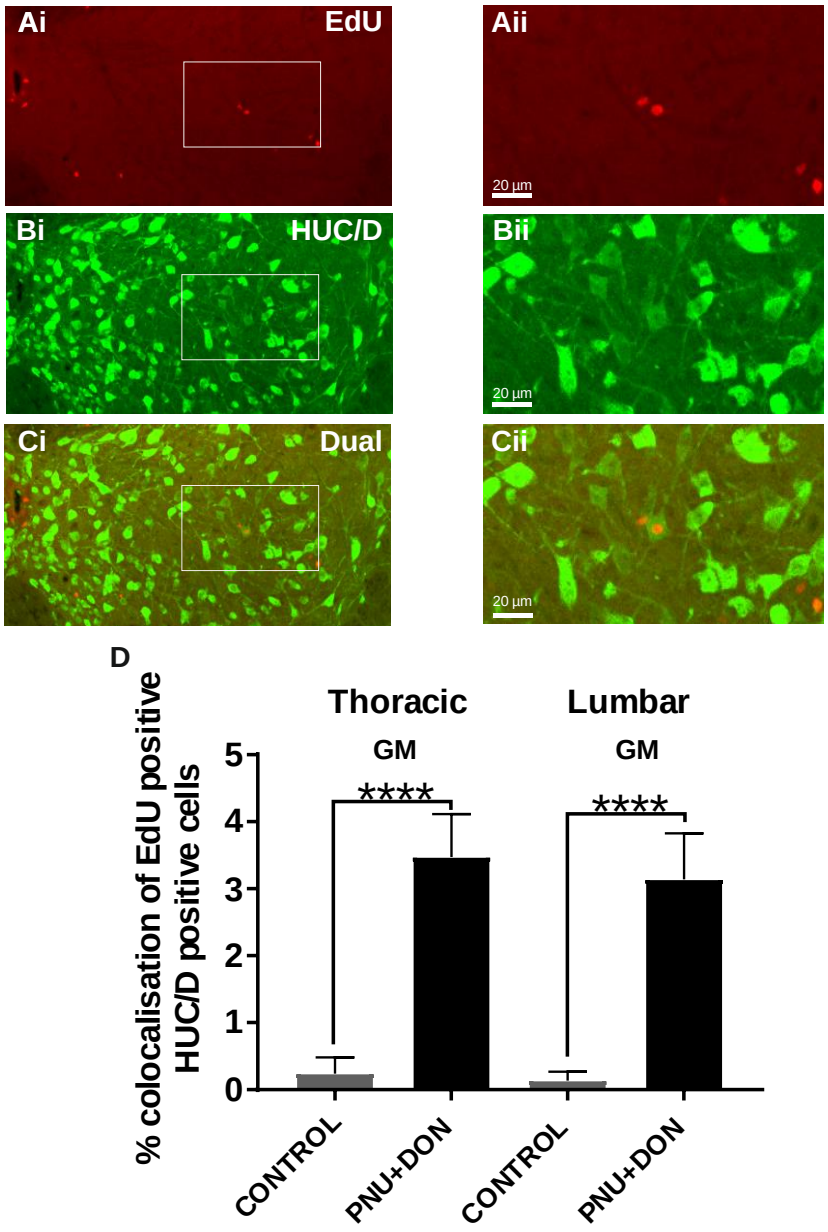

Figure 3.7 A significantly higher number of EdU positive cells colocalised with the neuronal marker HUC/D in the spinal cord grey matter of animals treated with donepezil in combination with PNU-120596.

Ai) Representative image of EdU^{+} cells. **Aii)** Magnified image of boxed area in (Ai). **Bi)** HUC/D+ cells. **Bii)** Magnified image of boxed area in (Bi). **Ci)** colabelled cells in the grey matter. **Cii)** Magnified image of boxed area in (Ci). **D)** Percent colocalization in each condition. Data are shown as mean number of percent colocalisation per 50 µm section ± SEM of N= 3 animals, n=49 sections versus control conditions N=3 animals, n=36 sections. ****; P<0.0001 in the grey matter.

Spinal cord region	Mean % coloc of HUC/D in the Grey matter *		Mean % coloc of PanQKI in the Grey matter *		Mean %c oloc of PanQKI in the White matter *	
	Control	DON + PNU	Control	DON + PNU	Control	DON + PNU
Thoracic	0.2 ± 0.2 (n = 16)	3.4 ± 0.6 (n = 26)	32.7 ± 3.4 (n = 31)	47.4 ± 2.4 (n = 19)	30.9 ± 1.7 (n = 31)	46.2 ± 2.5 (n = 19)
Lumbar	0.1 ± 0.1 (n = 20)	3.1 ± 0.6 (n = 23)	35.8 ± 2.4 (n = 25)	44.9 ± 2.2 (n = 23)	34.7 ± 2.1 (n = 25)	48.5 ± 2.9 (n = 23)

Table 3.2 The average percentage of cells that are both EdU positive and immunoreactive to either HUC/D or PanQKI in animals treated with donepezil and PNU-120596 in the spinal cord compared to control animals.

3.2.6 Treatment with PNU-120596 and donepezil does not result in significant astrocytic differentiation

Thoracic and lumbar spinal cord sections of both control animals and treated animals were immunohistochemically labelled with the astrocytic marker anti- S100β to investigate whether any newly proliferated EdU positive cells differentiated toward the astrocytic lineage.

The glial-specific marker S100β is expressed by mature astrocytes and NG2-expressing cells. S100β is one of 13 S100 genes present in the cytoplasm and nucleus of various cells; they partake in numerous cell processes (Wang et al., 2008). There was no significant change in the number of EdU positive cells colocalising with the astrocytic marker among groups; control and treatment (figure 3.8); exact values are listed in table 3.3.

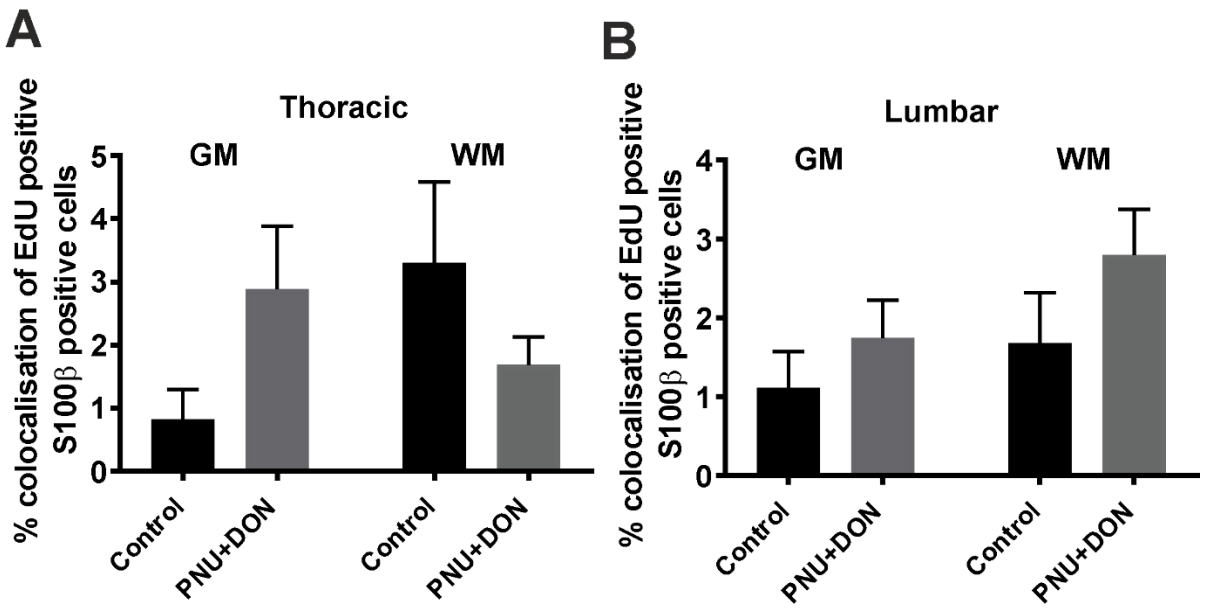

Figure 3.8 *In vivo* administration of donepezil in combination with PNU-120596 did not change the extent of astrocytic differentiation.

A-B) Percent colocalization in each condition. Data are shown as mean number of percent colocalisation per 50 µm section ± SEM of treated animals N=3 animals, n=45 sections versus control conditions N=3 animals, n=43 sections in the grey and white matter.

Spinal cord region	**Mean % coloc of S100β in the Grey matter**		**Mean % coloc of S100β in the White matter**	
	Control	**DON + PNU**	**Control**	**DON + PNU**
Thoracic	0.8 ± 0.4 (n = 19)	2.9 ± 0.9 (n = 18)	3.3 ± 1.2 (n = 19)	1.6 ± 0.4 (n = 18)
Lumbar	1.1 ± 0.4 (n = 24)	1.7 ± 0.4 (n = 27)	1.6 ± 0.6 (n = 24)	2.8 ± 0.5 (n = 27)

Table 3.3 The average percentage of cells that are both EdU positive and immunoreactive to S100β in animals treated with donepezil and PNU-120596 compared to control animals.

This table summarises the exact number of sections used per condition when N=3 animals was employed per condition.

3.2.7 Treatment with PNU-120596 and donepezil does not result in significant oligodendrocytic or astrocytic differentiation in the dentate gyrus of the hippocampus

In the dentate gyrus, the number of EdU positive cells post treatment with donepezil and PNU-120596 was significantly higher in treated animals compared to control animals (figure 3.5), thus we aimed to establish if any of these cells differentiated into neuronal or glial lineages.

In both control animals and treated animals, very few EdU positive cells were co-labelled with the astrocytic marker anti- S100β or the oligodendrocyte marker anti-PanQKI, (figure 3.9). This could be due to the fact that the developmental stages that EdU positive cells go through span immature glial-like cells to mature neuronal granule cells which express different antigens and markers. Since DCX has been shown to overlap with the expression of PSA-NCAM (Kempermann et al., 2015) during hippocampal neurogenesis delineating early post-mitotic maturation phase, it is possible for newly generated cells to have differentiated mainly into immature neurons which has not been labelled for in this thesis.

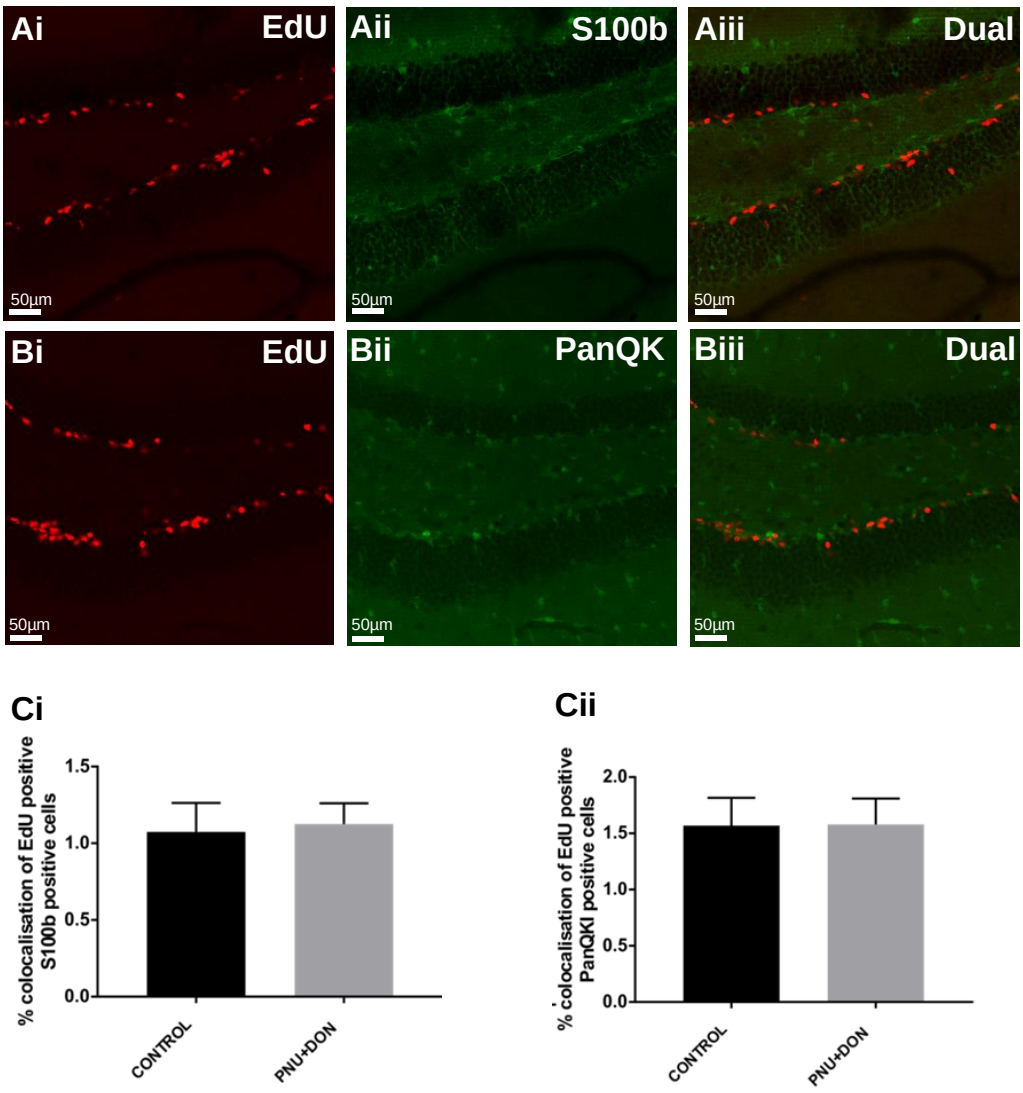

Figure 3.9 *In vivo* administration of donepezil in combination with PNU-120596 exhibits no change in the proportion of glial differentiated cells in the dentate gyrus.

Ai) Representative image of EdU labelled cells. **Aii)** Representative image of S100β positive cells. **Aiii)** Representative image of lack of EdU^+ $S100\beta^+$ cells in the dentate gyrus. **Bi)** Representative image of EdU labelled cells. **Bii)** Representative image of $PanQKI^+$ cells. **Aiii)** Representative image of lack of EdU^+ $PanQKI^+$ cells in the dentate gyrus. **Ci-Cii)** Percent colocalization in each condition. Data are shown as mean number of percent colocalisation per 50 µm section ± SEM of N=3 animals and n=36 sections were analysed per condition (vehicle and treated conditions).

3.3 Discussion

This study shows that modulating the levels of acetylcholine provides a mean by which the relatively quiescent stem-like ependymal cells of the spinal cord and the stem-like astrocyte population of the dentate gyrus can be stimulated into significant levels of proliferation. Significant numbers of these proliferative cells in the spinal cord differentiated down the neuronal and oligodendroglial lineage; this presents exciting potential therapeutic avenues in the treatment of spinal cord injury and / or degenerative diseases.

3.3.1 The potential source(s) of endogenous acetylcholine in the spinal cord

The experiments described above rely on the presence of endogenous acetylcholine to exert proliferation and / or differentiation effects, however, in the spinal cord niche the source of acetylcholine is yet to be elucidated.

Since there is no evidence for descending cholinergic pathways (d'Incamps et al., 2012), one possible source may be the presence of low concentrations of both acetylcholine and its breakdown product choline in the cerebral spinal fluid (CSF). This is feasible since researchers have shown that these are present in the cerebrospinal fluid and indeed change in pathological conditions (Jia et al., 2004). Given that cholinergic neurons are either damaged or impaired in Alzheimer's disease, these researchers set out to explore whether the same occurred in patients of vascular dementia and to correlate those events with the extent of their cognitive impairments (Jia et al., 2004). Using high performance liquid chromatography, acetylcholine levels in the lumbar cerebrospinal fluid of Alzheimer's disease subjects and vascular dementia subjects were shown to be significantly lower than control subjects. These decreased acetylcholine levels were also positively correlated with decreased cognitive performance in mini-mental state examinations. The levels of choline in the cerebrospinal fluid of subjects with Alzheimer's disease did not vary greatly from

controls, however, choline levels in vascular dementia patients were significantly higher than controls. Thus, the cerebrospinal fluid is one potential source.

The other possible source may be the cholinergic terminals of various cholinergic cells in the spinal cord (Barber et al., 1984b). Cholinergic neurons have been divided into five groups in adult rat spinal cord: somatic motor neurons, preganglionic autonomic motor neurons, small to medium neurons in layers I-III of the dorsal horn, central canal cells, and partition cells in the intermediate grey area (Barber et al., 1984b). Moreover, the subpopulation of ChAT positive interneurons with C-bouton projections to motor neurons may also be a viable source of ACh in lamina X (Zagoraiou et al., 2009). The identity of cholinergic cells giving rise to the cholinergic terminals that innervate ependymal cells are yet to be defined yet it is clear that there are extensive cholinergic positive structures in close appositions with ependymal cells (Corns et al., 2015). Since cholinergic interneurons are present within the central canal region, they are strong candidates to be a source of the acetylcholine released to activate cholinergic receptors present on ependymal cells.

3.3.2 How can we interpret the inhibitory effect on proliferation that donepezil elicits in the spinal cord central canal?

Given that acetylcholine does depolarize ependymal cells (Corns et al., 2015) suggesting the presence of cholinergic receptors, it was imperative to examine their response to the neurotransmitter and attempt to activate the dormant plastic environment of the central canal niche. We first sought to increase endogenous acetylcholine levels by inhibiting acetylcholinesterase using donepezil and evaluate the response of the spinal cord niche to this modulation.

Acetylcholinesterase inhibitors include a wide variety of earlier drugs such as tacrine, physostigmine, and velnacrine, as well as later developed ones such as rivastigmine, metrifonate, and the widely used donepezil which exhibit high selectivity for acetylcholinesterase inhibition (McGleenon et al., 1999). Donepezil has become a staple drug in the treatment of people with Alzheimer's disease and is commercially

available. Multiple studies have administered donepezil *in vivo* and *in vitro* and investigated its effects in the brain. For instance, a recent study administered donepezil orally in intact rat models (Imamura et al., 2015) and revealed an enhanced survival of new born neurons in the dentate gyrus while scopolamine, a muscarinic acetylcholine receptor blocker, suppresses the survival of these neurons. Another study which administered donepezil intraperitoneally to physiologically healthy mice showed a significant enhancement of new born neuronal survival, however reported no change in the proliferation of neural progenitor cells in either the dentate gyrus of the hippocampus or subventricular zone of these mice (Kaneko et al., 2006). Indeed, the numbers of BrdU positive cells were not significantly different in the dentate gyrus, subventricular zone, rostral migratory stream, and olfactory bulb between control and donepezil treated groups when BrdU was administered at the completion of donepezil treatment. In contrast, when BrdU was administered at the beginning of donepezil treatment, the numbers of BrdU positive cells in the dentate gyrus significantly increased after 1 week treatment.

This suggests that inhibiting acetylcholinesterase with use of donepezil does not promote proliferation, and instead simply enhances the survival of pre-existing neurons in two active neurogenic regions, as opposed to the dormant neurogenic niche of the spinal cord. This may be an explanation as to why an increase in spinal cord proliferation was not observed in donepezil treated animals, but still does not explain why there was a significant decrease in the numbers of proliferating cells.

The positive control to studies presented in this chapter; the dentate gyrus, receives cholinergic fibres from the basal forebrain medial septal nucleus and diagonal band nuclei in addition to the presence of local cholinergic neurons within interneuron subpopulations (Ballinger et al., 2016). The immature neurons and mature granule cells in the dentate gyrus and olfactory bulb are in close contact to cholinergic fibres and express multiple subtypes of cholinergic receptors (α7nAChR, β2nAChR, M1, M2) (Kaneko et al., 2006). Furthermore, the cholinergic receptor subtype population and the process of postnatal / adult neurogenesis in the dentate gyrus are well characterized.

In the spinal cord, cholinergic neurons are widely spread throughout the rostrocaudal extent of the spinal cord (Gotts et al., 2016) and immunohistological labelling of choline acetyltransferase (ChAT) revealed the presence of cholinergic structures closely opposing ependymal cells of the spinal cord (Gotts et al., 2016). However, the identity and role of each receptor subtypes involved in mediating proliferation and / or differentiation in both physiological conditions and post injury is yet to be tested. Moreover, the complex cholinergic neural circuitry and receptors of the spinal cord can be exemplified by the variable and sometimes contradictory results of cholinergic receptor activation. The dose of cholinergic agonist used and length of application may activate different receptor subtypes, which in turn can mediate different responses in terms of proliferation in neurogenic niches. For instance, administering chronic high doses of nicotine- a known cholinergic agonist- *in vivo* decreased cell proliferation in the sub-granular zone (SGZ) (Abrous et al., 2002), while low doses of nicotine over a period of 28 days *in vivo* resulted in increased cell proliferation in the central canal of EAE mouse models (Gao et al., 2015). This may mean that different receptors are being activated with donepezil that act to inhibit proliferative responses in this condition. Thus the receptor expression patterns and cholinergic organization, all potentially contribute to decreased proliferation with donepezil in the spinal cord niche when compared to hippocampus. It must be noted that there were no significant changes in the numbers of EdU positive cells in the white and grey matter of donepezil treated animals compared to control. One reason that may explain this lack of effect is that the EdU positive cells in the white and grey matter are from a number of different sources, not just the ependymal cells, and these different sources may express different cholinergic receptors that affect the overall response to donepezil. As discussed in the general introduction, another potential source of stem cells is present in the meninges and these cells may not express cholinergic receptors. Furthermore, some of the oligodendrocytes may be generated from OPCs, which are known to express a diverse cholinergic receptor profile. Thus, overall donepezil has no effect on cell proliferation in these regions.

Another reason for the differences in effects of donepezil in hippocampus compared to spinal cord may be due to actions of this drug on other cell types that somehow in turn release growth factors in the hippocampus. For example, one study

attributed improved cognition and increased cell survival and proliferation in the dentate gyrus to stimulation of peripheral sensory neurons, through a mechanism separate from donepezil's action as an AChE inhibitor since tacrine did not mimic this effect (Narimatsu et al., 2009). They suggested that donepezil caused opening of TRPV1 channels in these sensory afferents which in turn led to activation of dorsal horn neurons. This somehow indirectly increased hippocampal levels of CGRP, increasing production of insulin-like growth factor I (IGF-I) which lead to increased neurogenesis and angiogenesis in the dentate gyrus. Brain-derived neurotrophic factor (BDNF); a member of the neurotrophin family, plays important roles in cell survival and maturation during development and in post developmental neurogenesis. It has been shown to promote long term survival of neurons when administered *in vitro* to rat sub-ventricular zone (SVZ) derived neuroblasts (Kirschenbaum and Goldman, 1995). In the olfactory bulb, infusion of BDNF lead to doubling in the number of neurons *in vivo* (Zigova et al., 1998). More importantly, long term clinical (15 month) oral administration of donepezil to Alzheimer's patients revealed a significant increase in BDNF serum levels compared to pre-treatment with donepezil (Leyhe et al., 2008). Thus, donepezil's neuroprotective effects in the brain encompasses more than cholinergic modulation and may further explain the differences between effects in hippocampus and spinal cord.

3.3.3 Enhancement of α7nAChRs in combination with donepezil was effective in increasing the proliferative response

Since donepezil acts as a central global reversible acetylcholinesterase inhibitor, modulating a specific receptor subtype would be difficult to achieve. In the preliminary *in vitro* experiment, some slices were treated with donepezil and the selective muscarinic antagonist atropine in an attempt to isolate the nicotinic effects. The numbers of proliferative cells in the central canal in these slices compared to donepezil alone were significantly different, potentially suggesting that muscarinic receptors do contribute to the effects of donepezil. This observation is not in line with what was reported by Corns; atropine (5 mM) in the presence of acetylcholine had no significant effect on the response of ependymal cells to ACh, which suggests that most of the response is mediated by nicotinic acetylcholine receptors (Corns et al., 2015).

Consequently, we pursued cholinergic modulation along with targeting a specific receptor subtype; α7nAChRs. The α7 subtype of nAChRs is present in various CNS niches and implicated in adult neurogenesis. The pharmacological activation of α7nAChRs in the sub-ventricular zone and dentate gyrus postnatal neurogenic niches is also extensively described. In the dentate gyrus, not only are α7nAChRs the most abundant (Kaneko et al., 2006), but they are also necessary for neuronal survival, maturation and integration in the dentate gyrus (Campbell et al., 2010). A recent study (John et al., 2015) reported that α7, α4 and β2 nAChRs are present in the highest abundance in the postnatal dentate gyrus, and functional α7 nAChRs are specifically located on immature granule cells of both rats and mice. Furthermore, activating α7nAChRs led to the activation of nuclear fibroblast growth factor receptor I (FGFR1) signalling promoting the differentiation of the neural stem / progenitor cells of the sub-ventricular zone (Narla et al., 2013). The central canal of the spinal cord (lamina X) has been reported to contain α2, α3, α4, α7 and β2 nAChRs by means of in-situ hybridisation techniques (Wada et al., 1989). Potentiating α7nAChRs in addition to elevating endogenous levels of acetylcholine by donepezil resulted in a significant increase in the number of EdU^+ proliferative cells in both the dentate gyrus of treated animals and in all three regions of the spinal cord; central canal, grey matter and white matter of donepezil and PNU-120596 treated animals *in vivo* compared to control levels of proliferation ($P<0.0001$). This is also consistent with previous results using PNU-120596 alone of enhanced spinal cord proliferation *in vivo* and in organotypic spinal cord cultures by (Corns et al., 2015). They also showed ependymal cells of the central canal responding with large depolarising responses to acetylcholine after potentiating α7nAChRs in electrophysiological settings; this suggests that ependymal cells express α7nAChRs, and that they potentially mediate a high proportion of the response. In addition, the acetylcholine breakdown product choline has been shown to be a selective agonist for α7nAChRs in rat brain (Alkondon et al., 1997); thus this may be exerting some of the effects observed here. This means that the combination of donepezil and PNU-120596 enables the excitatory response through α7nAChRs to predominate. Since α7nAChRs are highly permeable to calcium and lead to an increase in intracellular calcium levels upon activation (Bertrand et al., 2015) it would be of great interest to investigate the downstream pathways of α7nAChRs activation. Thus it is important to note that since oligodendrogenesis is somehow enhanced by

PNU-120596 treatment either alone or in conjunction with cholinergic modulation in intact healthy murine spinal cords, this suggests that α7nAChRs are candidate therapeutic targets to investigate for the potential treatment of spinal cord injuries and neurodegenerative diseases in which the primary loss is the neuronal and / or oligodendrocytic pool of cells.

PNU-120596 treatment alone in mouse spinal cord has previously been reported to result in a significant number of cells differentiating into oligodendrocytes after enhancing proliferation *in vivo* and *ex-vivo* (Corns et al., 2015). Here we see similar results in the spinal cord after treatment with PNU-120596 and donepezil *in vivo*. The mechanism in which this occurs still remains uninvestigated. What's more, PNU-120596 and donepezil treatment also resulted in a significant increase in the number of cells differentiating into neurons; this is seen in the grey matter of both thoracic and lumbar regions of the spinal cord ($P<0.0001$).

Finally, based on the fact that acetylcholine does depolarize ependymal cells (Corns et al., 2015) and the significant increase in the number of EdU^+ proliferative cells around the central canal in (PNU-120596 and donepezil) treated animals, it would be reasonable to assume that the enhanced proliferative response observed in these experiments includes ependymal cell proliferation and potentially migration and differentiation. In the case of oligodendrocytic differentiation, another possible scenario is plausible based on the presence of α7nAChRs on oligodendrocytes themselves (Vélez-Fort et al., 2009) suggesting that oligodendrocytes may respond directly to acetylcholine modulation and α7nAChR potentiation. However, since these experiments were conducted in wild type mice without any cell lineage tracing, it would be impossible to conclusively determine which one or more cell types contributed to the cholinergic modulation.

Chapter 4

Cholinergic modulation of neurogenesis in LPC-induced focal demyelination in mouse dorsal lumbar spinal cord

4.1 Introduction

4.1.1 Axonal demyelination underlies some CNS pathologies

Axonal demyelination occurs in various neuropathological diseases or physical injuries leading to loss of proper signal transduction and neuronal survival. Spinal cord injuries for instance often result in irreversible axonal damage and improper remyelination (Plemel et al., 2014). Once injury occurs, the first inhibitor to axonal regrowth is the glial scar formed by astrocyte infiltration and proliferation (Frisen et al., 1995); which acts as a barrier surrounding the lesion site and provides an inhibitory environment to axonal sprouting (Yiu and He, 2006). The absence of a regenerative response after axonal severance is primarily attributed to inhibitory molecules and proteoglycans present in the glial scar environment (McKeon et al., 1991, Chen et al., 2000). Myelin associated inhibitors such as myelin associated glycoprotein (MAG) and Nogo A produced by oligodendrocytes and myelin debris present in the lesion site also hinder axonal regrowth (Chen et al., 2000, McKerracher et al., 1994). Interestingly, glial scar forming astrocytes post mouse spinal cord injury have been shown to be derived from both astrocyte progenitors and ependymal cells, while oligodendrocytes give rise to cells of their own lineage found scattered around the lesion site (Barnabé-Heider et al., 2010, Meletis et al., 2008).

More importantly, spinal cord contusion injuries induce demyelination which is followed by abnormally thin remyelination at the injury site. One of the first elaborate studies on demyelination post spinal cord injury by (Blight, 1985) demonstrated the extensive damage of axons occurring in the lesion epicentre, and the delayed demyelination of non-injured axons a week after injury at the perimeter of the lesion leading up to additional loss of axons. This indicates the importance of early oligodendrogenesis post injury. A recent study looked at the timing of remyelination after spinal cord contusion injury and found that new myelin begins to appear between months 1-3 (Powers et al., 2013). Thus, if demyelinated axons are linked to an increase in the potential of secondary damage then when injured axons are swiftly remyelinated, secondary damage that occur days or weeks after injury can be omitted.

Furthermore, oligodendrocytes can be the target of immune attacks such as in multiple sclerosis causing demyelination. Multiple sclerosis is an inflammatory neurodegenerative disorder of the central nervous system characterized mainly by oligodendrocyte apoptosis which leads to myelin loss, axon degeneration, and eventually loss of axonal conductivity (Plemel et al., 2017). In early stages of the disease, endogenous remyelination occurs producing new myelin sheaths from oligodendrocyte progenitor cell (OPC) pools upon differentiating into more mature myelinating cells. Since multiple sclerosis is a progressively degenerative disorder with many suffering from relapse-remitting episodes, repeated endogenous remyelinating responses tend to deplete the oligodendrocyte progenitor pool in afflicted individuals (Plemel et al., 2017). In both humans and mice; multiple sclerosis and in experimental encephalomyelitis mouse models, demyelination has been shown to be mediated primarily by $CD4^+$ and / or $CD8^+$ T cell infiltration (Fletcher et al., 2010). Increasingly however, studies report the possible implication of B lymphocytes in the pathogenesis of the disease (Oliver et al., 2003). A recent humanized antibody against the cell surface glycoprotein CD52 expressed by both T and B cell lymphocytes was approved as a first-line treatment for relapse-remitting multiple sclerosis (Gallo et al., 2017). All available current treatments aim for immunomodulation which slow the disease progression but do not directly enhance remyelination. Since complete and proper remyelination is required for adequate axonal conduction to be restored following spinal cord injury or multiple sclerosis among various demyelinating neuropathologies, it is essential to promote oligodendrogenesis and enhance the process of remyelination.

4.1.2 Prevalence and function of oligodendrocyte precursor cells

Oligodendrocyte precursor cell populations are present throughout the central nervous system constituting the major dividing cell population post development (Dawson et al., 2003, Gensert and Goldman, 1997). These precursor cells are present in both the white matter and grey matter with a higher density (1.5 times) found in the white matter of the central nervous system; hippocampus, cervical and thoracic spinal cord, corpus callosum, the cerebral cortical grey matter and the granular and molecular layers of the cerebellum (Dawson et al., 2003). They reportedly possess different

migratory, proliferative and differentiating capacities. For instance, brain white matter derived oligodendrocyte precursor cells are capable of faster and more efficient differentiation into mature myelinating oligodendrocytes than grey matter derived precursor cells when transplanted into the white matter of the cerebral cortex (Viganò et al., 2013). This suggests a possible favourable environment for oligodendrocyte maturation and differentiation in the white matter. Moreover, white matter oligodendrocyte precursor cells of the corpus callosum have a shorter replication cycle (10 days in P60 mouse) compared to those in the grey matter (36 days at P60) (Young et al., 2013).

It is possible for oligodendrocyte precursor cells to have functional differences. Three separate fate mapping studies reported that adult oligodendrocyte precursor cells have an exclusive function throughout life; the generation of oligodendrocytes (Dimou and Gallo, 2015, Kang et al., 2010, Dimou et al., 2008, Zhu et al., 2011). On the other hand, (Richardson et al., 2011) also employed fate mapping methods and concluded that oligodendrocyte precursor cells are multipotent; capable of giving rise to astrocytes and neurons as well. Moreover, oligodendrocyte precursor cells in the cortex employ their ramified processes to assess their environment and maintain an even distribution of their population using self-repulsion mechanisms; this phenomena was captured with live imaging (Hughes et al., 2013).

The factors underlying continuous oligodendrogenesis, and whether the function(s) of oligodendrocyte precursor cells are conserved among species are in need of further elucidation. A recent study by (Yeung et al., 2014) revealed that oligodendrocyte turnover in humans is roughly 0.3% compared to 36% in mice. Furthermore, they suggested that human oligodendrocytes may be able to remodel the myelin sheath without the need for generating new oligodendrocytes. This study used tissue from post-mortem tissue of humans aged 0.2-92 years and revealed that a large proportion of oligodendrocytes were not newly generated, instead they were predominantly mature and were $Sox10^{+}$ and $CC1^{+}$. Furthermore, there was an initial significant increase in myelin volume (from around 2 months to 5 years) followed by a

gradual increase through adolescence (reaching a maximum by 17 years) which slowly declined with age.

4.1.3 Implications for cholinergic signalling on myelination

Most importantly for this study, cholinergic signalling has long been implicated in various white matter disorders with symptoms including demyelination and cognitive impairment, however the exact role of acetylcholine signalling in modulating maturation of the oligodendrocyte precursor cells and myelination is not well understood.

Histochemical analysis of acetylcholinesterase revealed some activity at the node of Ranvier of frog nerve fibres including less intense staining in internodal regions (Brzin and Dettbarn, 1967). Acetylcholinesterase activity was also found in peripheral nerve and dorsal root ganglia axons (Novikoff et al., 1966); here specific acetylcholinesterase activity was localized in the perikaryon cytoplasm and the axoplasmic surface of myelinated fibres of dorsal root ganglia by staining with acetylcholine iodide. This study revealed that cholinesterase activity in rat is present in all perikarya at various levels, but not present in axoplasmic surfaces of all myelinated nerves. Interestingly, the sciatic nerve proximal to crush injuries showed increased activity of acetylcholinesterase. Acetylcholinesterase activity was also identified in myelinating Schwann cells of peripheral nerves during development (Bogusch, 1991).

Furthermore, choline acetyltransferase (ChAT) immunohistochemistry in young adult gerbils revealed that some oligodendrocytes and glioblasts (oligodendrocyte precursors) of the external cuneate nucleus of medulla oblongata in brainstem are ChAT positive (Lan et al., 1996). Oligodendrocytes also variably express all five muscarinic acetylcholine receptors based on their subtype specificity, regional and developmental state (De Angelis et al., 2012, Ragheb et al., 2001). What's more, various cell culture studies suggested muscarinic acetylcholine receptor activation plays a role in oligodendrocyte precursor cell proliferation, differentiation and survival depending on receptor subtype activation and their developmental stage (He and McCarthy, 1994). For example, MacDonald reported that GalC neurospheres express

the acetylcholine transporter, and showed that culturing these neurospheres in the presence of the muscarinic antagonist atropine reduced their number suggesting a role for muscarinic receptors in oligodendrocyte development (MacDonald et al., 2002). Moreover, recent studies also report the expression and involvement of M1, M3 and M4 receptor subtypes in oligodendrocyte precursor cell differentiation where antagonism of muscarinic receptors using benztropine and clemastine promoted oligodendrocyte differentiation (Mei et al., 2014b, Deshmukh et al., 2013). These studies entailed high throughput screening of over 1000 compounds using cultures of purified OPCs and oligodendrocytes from postnatal rats and mice on fabricated microarrays (Mei et al., 2014b), while (Deshmukh et al., 2013) conducted a high content imaging assay based on the induction of MBP expression in rat optic nerve derived OPC cultures and *in vivo* benztropine administration. In culture, benztropine induces the differentiation of OPCs and their subsequent expression of MBP upon maturation, while *in vivo* administration to EAE rodent models of multiple sclerosis dramatically decreased the severity of the disease (Deshmukh et al., 2013). This suggests that cholinergic signalling may be involved in the maintenance of oligodendrocyte precursor cells in an immature state. Oligodendrocyte precursor cells have also been shown to express nicotinic acetylcholine receptors. Immunohistochemistry and RT-PCR analysis of rat corpus callosum revealed that oligodendrocyte precursor cells express α3, α4, α5, α7, β2 and β4 nicotinic acetylcholine receptor subunits (Rogers et al., 2001). Immunolabelling, patch-clamp recordings and calcium imaging indicated the presence of functional nicotinic acetylcholine receptors on NG2 positive oligodendrocyte precursor cells in the hippocampus of mice during their second postnatal week (Vélez-Fort et al., 2009). Pharmacological studies showed that oligodendrocyte precursor cells expressed α7nAChRs (Vélez-Fort et al., 2009). These two studies collectively show that a large number of oligodendrocyte precursor cells increase cellular calcium in response to nicotine application and these influxes were sensitive to the calcium channel antagonist suggesting that nicotinic receptors may play a role in myelination.

Interestingly, acetylcholinesterase inhibition using donepezil induced oligodendrocyte differentiation from neural stem cell-derived oligodendrocyte precursor cells and increased myelin associated glycoprotein (MAG) and myelin basic protein

(MBP) activity indicating an increase in myelin gene expression (Imamura et al., 2015b). Acetylcholinesterase inhibition treatment in rat models of experimental auto-immune encephalomyelitis showed an improvement in cognitive deficits (D'Intino et al., 2005), and similar results were observed in chronic cerebral hypoperfusion (Wang et al., 2010). Collectively, these studies suggest the involvement of cholinergic signalling in remyelination and oligodendrocyte development, however, given the complexity of cholinergic signalling, there is still a need to distinguish the heterogeneity of acetylcholine receptor expression on oligodendrocytes and oligodendrocyte precursor cells and understand the functional implication of each receptor in its given microenvironment.

4.1.4 Hypothesis

Since α7nAChRs are expressed by oligodendrocyte precursor cells alongside other nicotinic acetylcholine receptor subtypes (Vélez-Fort et al., 2009, Rogers et al., 2001) and donepezil induced oligodendrocyte differentiation and increased myelin gene expression (Imamura et al., 2015b); it is reasonable to hypothesize an enhancement of oligodendrocyte differentiation as a result of PNU-120596 and donepezil administration or PNU-120596 alone in comparison to baseline remyelination in untreated injured animals. This would also be in line with the previous results generated in the intact spinal cord experiment (chapter 3) which could have been in part mediated through oligodendrocyte precursor cell proliferation and differentiation.

4.1.5 Aims

Since demyelination is common and widespread causing substantial disability, and oligodendrogenesis is of the most relevance to both immunologic and traumatic central nervous system disorders; here we aim to test our drugs; PNU-120596 alone or in combination with donepezil on cell proliferation and oligodendrocyte differentiation after focal white matter demeyelination injury. We sought to compare endogenous baseline remyelination after focal dorsal lumbar demyelination to the outcome in remyelination after systemic cholinergic treatment.

Here we induced white matter demyelination by focal injections of a demyelinating agent; lysolecithin (LPC) into the dorsal region of lumbar spinal cord in C57/Bl6 mice. We then quantified the number of EdU^+ proliferative cells and labelled for oligodendrocyte differentiated cells within the region of injury with and without modulation of cholinergic transmission.

It was essential to properly select the appropriate experimental model to effectively evaluate the key objective of the study. The fundamental question of our specific experiment is to explore whether our combination of drugs; donepezil and PNU-120596 or PNU-120596 alone can provide sufficient numbers of mature myelinating oligodendrocytes compared to baseline spontaneous remyelination in control injuries excluding the immune component of the disease and the complex environment of multiple sclerosis. Thus, we carefully considered the focal demyelination model using the lysolecithin since it provides the required environment; white matter degeneration and demyelination to test our hypothesis.

The method employed in our demyelination experiments entails treatment with lysophosphatidylcholine or lysolecithin (LPC), an endogenous phospholipid which has been widely used in literature to mediate precise temporal demyelination (Plemel et al., 2018, Keough et al., 2015, Ghasemlou et al., 2007, HALL, 1972) which is followed by almost complete remyelination (Ghasemlou et al., 2007) is the method employed in our demyelination experiments. This model of demyelination induces robust remyelination (Keough et al., 2015). This procedure is thought to cause demyelination within the first 3 days post LPC injection; during which macrophage and microglial cells infiltrate and activate, axonal injury occurs and astrogliosis arises. This is then followed by oligodendrocyte progenitor cell recruitment between days 4 and 7 post injury. Subsequently, these progenitors begin their differentiation phase starting at day 10 up to day 21 post-surgery (Keough et al., 2015). This chemically induced focal demyelination model is a particularly favourable one since the regenerated myelin sheaths after injury occur as early as 7 days post chemical injection and remyelination is generally complete by 10-21 days post demyelination (Fancy et al., 2009) as opposed to spinal cord contusion injuries (Powers et al., 2013).

The experiments conducted here included three different time points (figure 4.1 A); the first experiment entailed establishing demyelination in the dorsal white matter (site of injection), and comparing the number of proliferative cells in animals which received the LPC injection alone to those which received the LPC injection followed by treatment with cholinergic drugs. Here the mice were left to recover from surgery for 3 days only; this is the time during which demyelination occurs (Keough et al., 2015). The second and third experiments aimed at investigating the potential difference in the number of proliferative cells post-demyelination, the number of oligodendrocytes present at the site of demyelination between experimental groups at days 14 and 21 respectively, figure 4.1 A. To properly detect demyelinated axons and quantify the thickness of myelin sheaths post cholinergic treatment; the white matter of LPC alone and PNU-120596 + donepezil treated animals 14 days post injury were assessed by electron microscopy.

4.2 Results

4.2.1 Demyelination can be seen 4 days after focal injection of LPC into the dorsal white matter

The timeline of each experimental cohort of animals as well as the course of demyelination and reyelination in each experiment is sumnmarised in figure 4.1 A. The occurrence of demyelination was visualized by using the immuno-label myelin basic protein (MBP). The absence of myelin labelling or presence of myelin debris can be seen under a fluorescent microscope. Since the injured lumbar spinal cord of each animal was sectioned transversely, an example of (MBP) labelled myelinated axons in areas distal to lesion is shown in (figure 4.1B). When the spinal cord is transversely sectioned, axons are severed and myelin can be seen as rings surrounding axonal tracts in the white matter, as opposed to the absence of myelin labelling seen in our experiments (figures 4.1 C and 4.2).

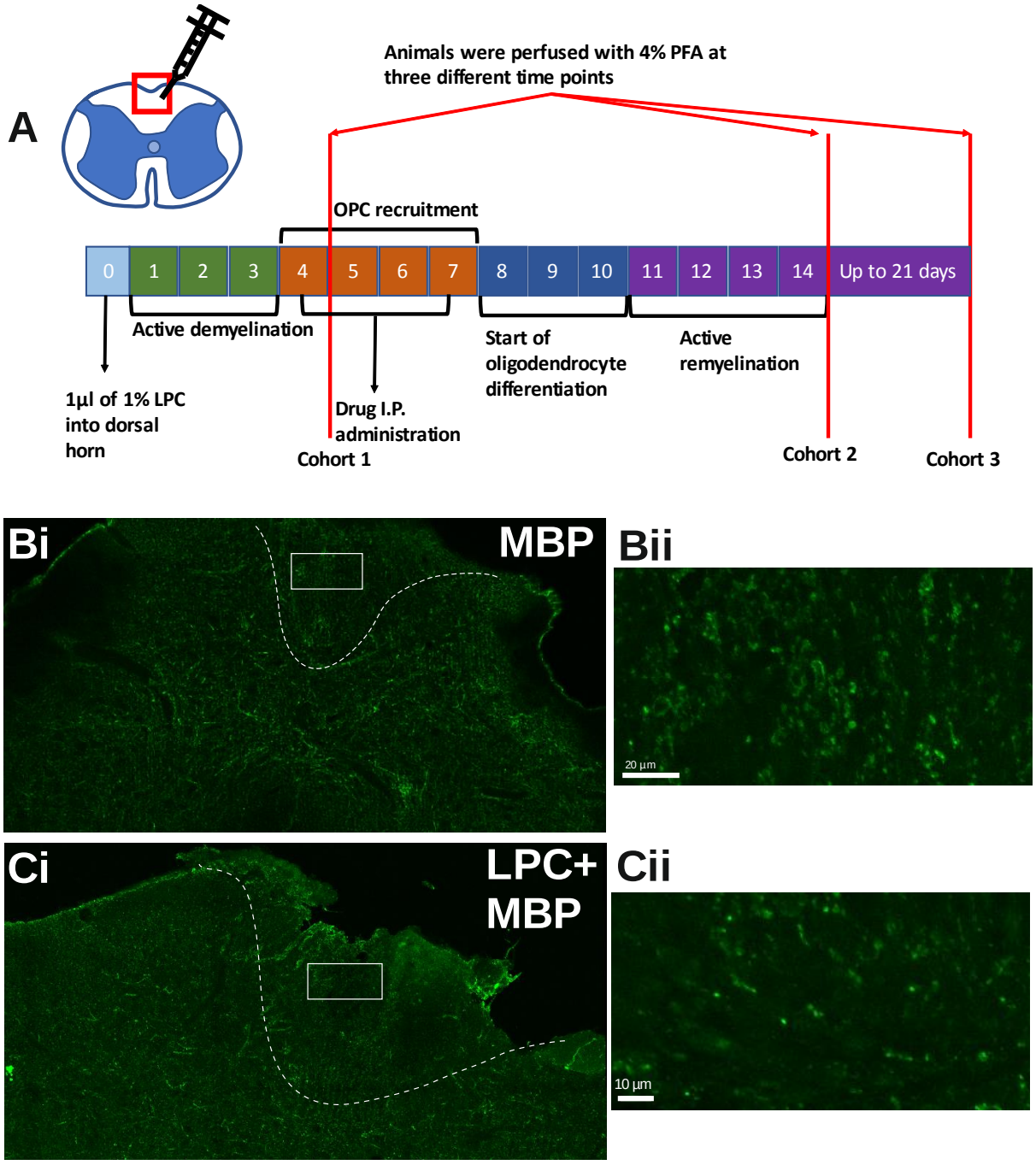

Figure 4.1 Myelin basic protein (MBP) labelling with and without LPC-induced demyelination.

A) Experimental timeline illustrating the course of injury, drug administration and experimental cohorts. **Bi**) Represents a myelinated lumbar spinal cord section which did not undergo treatment with LPC. **Bii)** Depicts a few examples of myelinated axons appearing as rings in a transverse section. **Ci)** Shows the occurrence of demyelination after the administration of LPC as a dorsal injection into the lumbar spinal cord. **Cii)** depicts the absence of tight concentric myelin around axons at 4 days post injury.

4.2.2 Cell proliferation is higher after acetylcholine modulation at 4 days post LPC-induced focal demyelination compared to LPC alone

The numbers of EdU positive cells in response to demyelination between LPC treated animals (Figure 4.2) and those treated with both donepezil and PNU-120596 after LPC injection (Figure 4.3) were significantly different; P= 0.0015. At 4 days post injection, LPC treated animals had a mean cell count of 144.6 ± 8.4 per 50 μm section, n = 5 compared to donepezil and PNU-120596 treated animals (mean cell count 229.5 ± 15.4 per 50 μm section, n = 6, Figure 4.4). There was no significant difference however in the number of proliferated cells that differentiated along the oligodendrocyte lineage between the two groups (Figure 4.4). Note the low extent of colocalisation for both conditions.

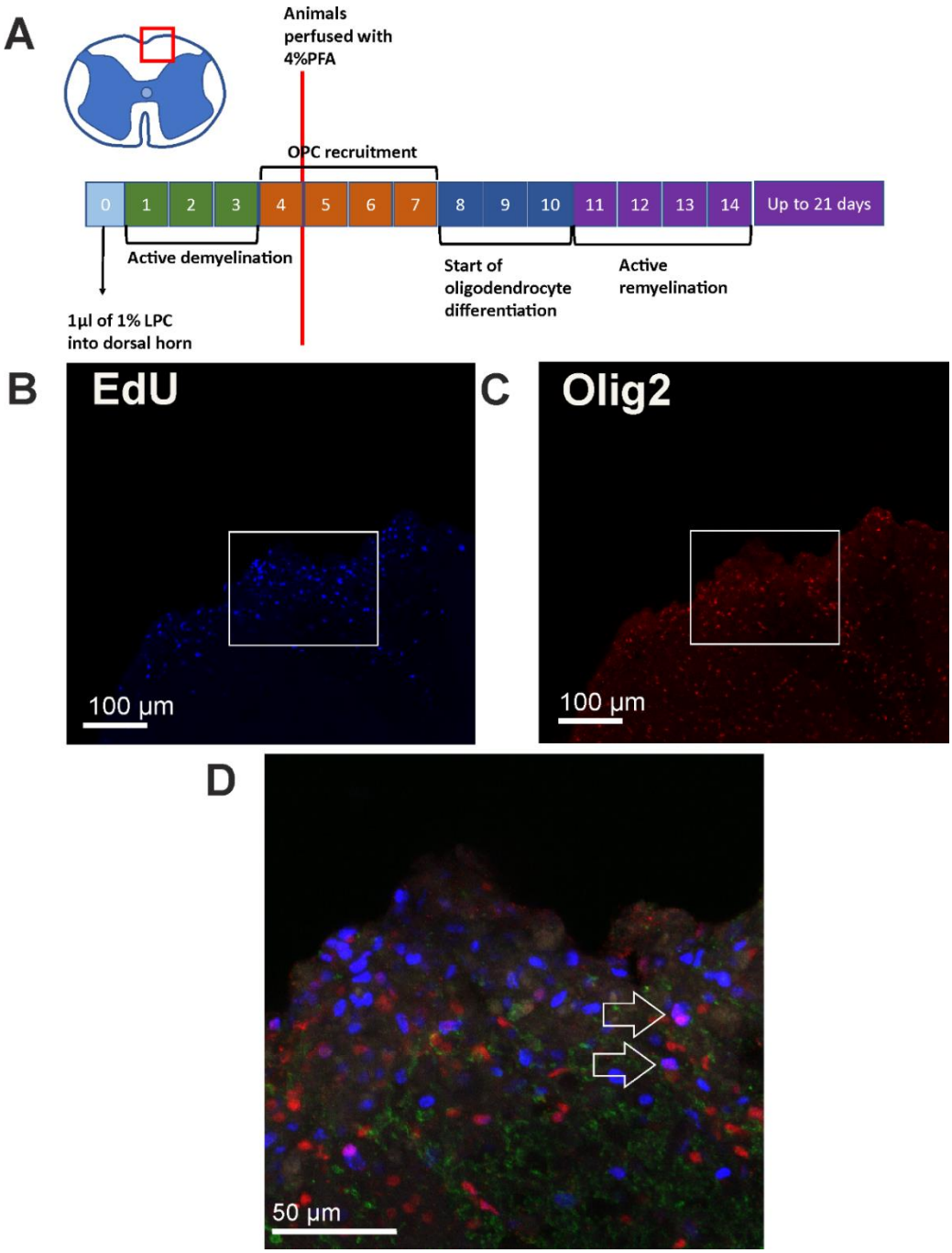

Figure 4.2 Cell proliferation is confined to the injury site after LPC-induced demyelination.

A) Experiment timeline. **B)** Representative image of EdU labelled cells**. C)** Representative image of Olig 2 labelled cells. **D)** A merged image with both channels, where MBP is also shown in green**.** Arrows represent a few examples of co-labelled cells.

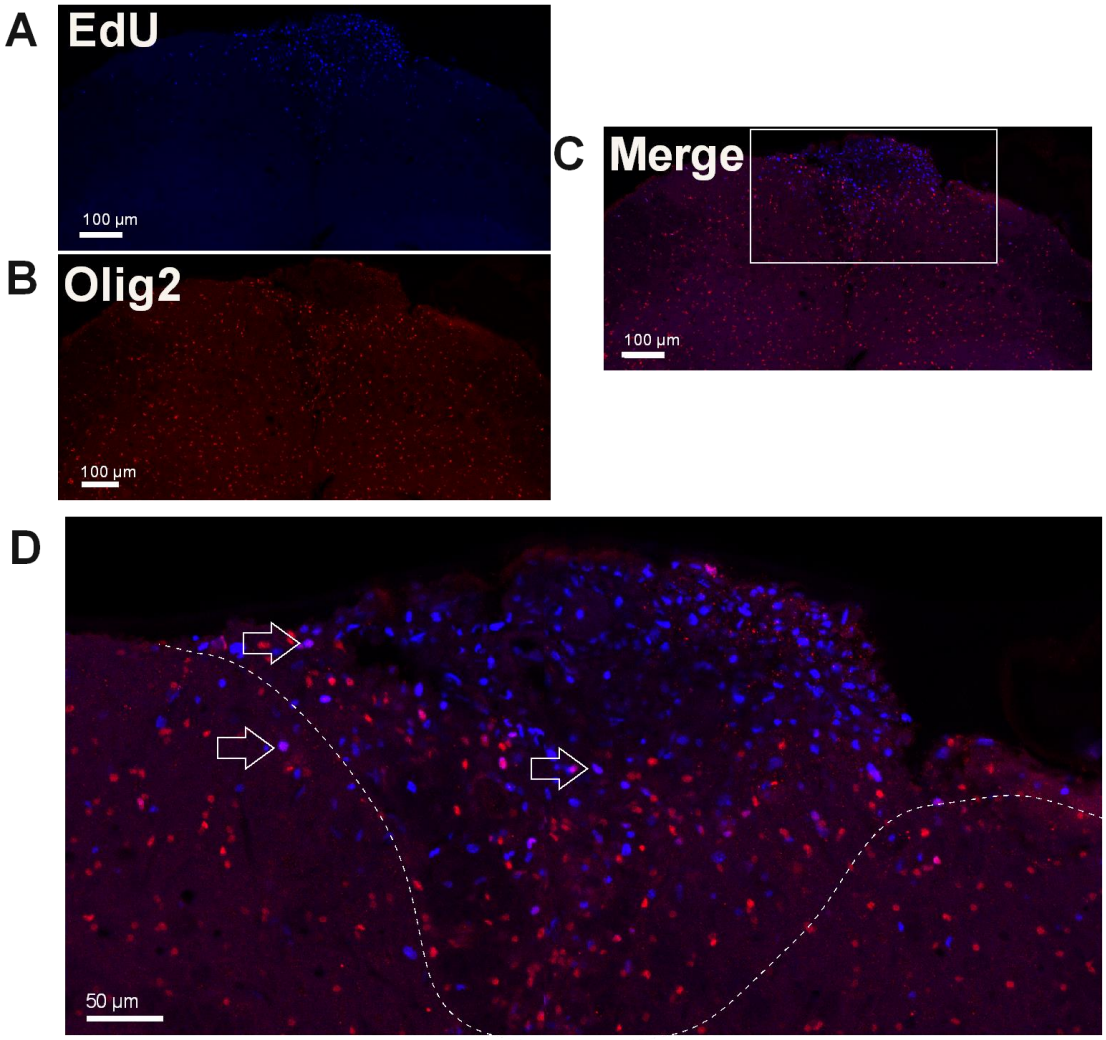

Figure 4.3 Example of proliferation occurring after LPC-induced demyelination in a mouse treated with PNU-120596 + donepezil.

A) Representative image of EdU labelled cells. **B)** Representative image of Olig 2 labelled cells. **C)** A merged image with both channels. **D)** A magnified image of the selected area in panel (C). Arrows represent a few examples of co-lablled cells.

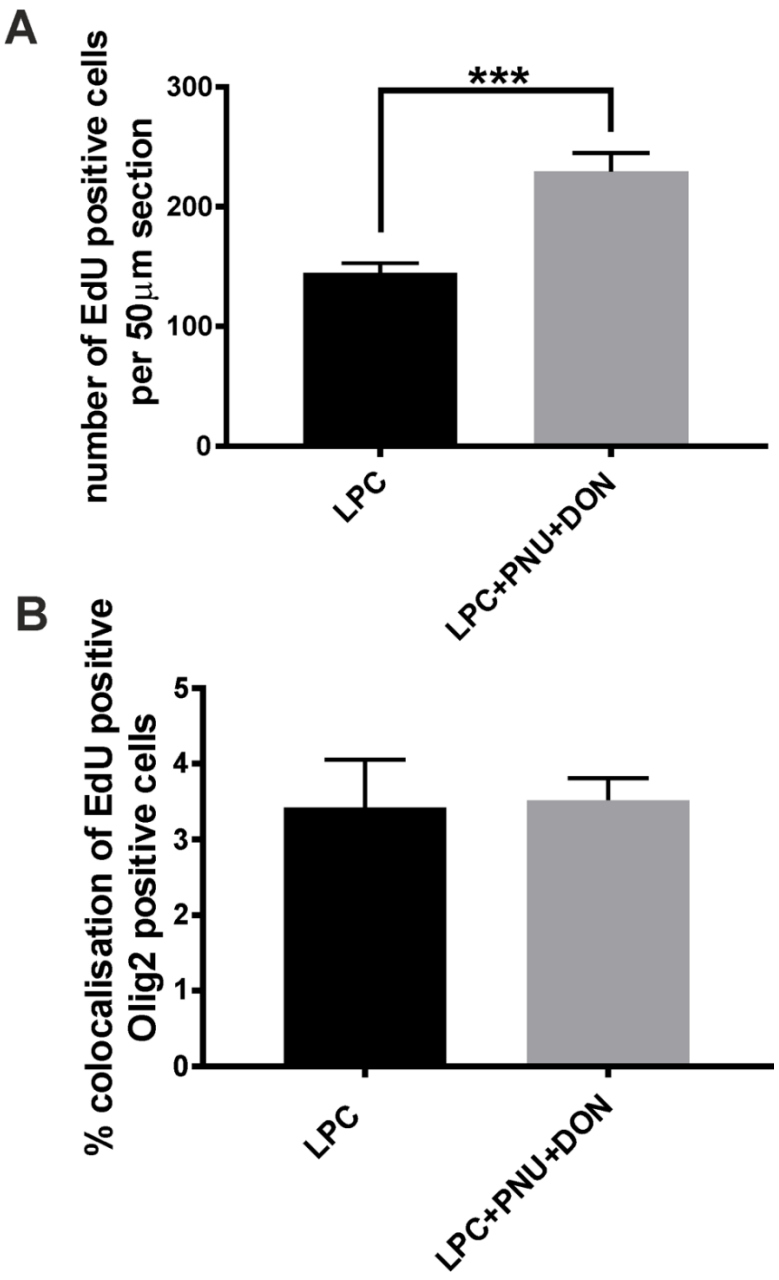

Figure 4.4 Cholinergic treatment enhanced cell proliferation in the site of injury. **A)** Number of EdU labelled cells for each of the conditions. Data are shown as mean number of EdU labelled cells per 50 µm section ± SEM. **B)** Percent colocalisation of EdU positive / Olig2 positive cells per 50 µm section ± SEM in the dorsal white matter of the spinal cord of: N= 2 animals, n=5 sections in LPC alone treated animals and N=2 animals, n=6 sections in LPC+PNU+Don treated animals.***; P = 0.0015.

4.2.3 Robust cell proliferation and oligodendroglial differentiation 14 days post LPC-induced focal demyelination and acetylcholine modulation

In order to determine whether our drug combination has a pro-oligodendroglial and / or myelinating effect, we injected LPC into the dorsal white matter of lumbar segments of the spinal cord, then quantified and compared the number of proliferative cells in animals which received the LPC injection alone to those administered PNU-120596 alone or PNU-120596 and donepezil, figure 4.5 A.

Since this experiment terminated at post-injection day 14 (PID14); it was expected for OPC recruitment to have ended by PID10 and for oligodendrocyte maturation to have commenced. Therefore, we immunolabelled with an antibody against Olig2 to detect pre-myelinating / myelinating oligodendrocytes and quantified the percentage of EdU positive cells that were also immunoreactive for Olig2 within the region of injury with and without modulation of cholinergic transmission, figure 4.5 B-D.

At the site of injury, these were 329.8 ± 30.1 EdU positive cells per 50 μm, (N = 4, n = 24) in animals subjected to LPC injections without treatment. The number of EdU positive cells were significantly higher (P=0.0057) in animals which received PNU-120596 alone and (P<0.0001) in animals treated with PNU-120596 and donepezil when compared to baseline proliferation, figures 4.5-4.8.

Moreover, the percentage of EdU^+ cells that were colocalised with Olig2 in PNU-120596 treated animals (figures 4.6 and 4.8) was significantly higher (24.1 ± 3.3 %, n = 21; P<0.0001) compared to LPC alone; (6.6 ± 0.4 %, n=24, figure 4.5). A significantly higher (P=0.0037) extent of colocalisation was also seen in animals administered PNU-120596 and donepezil; 20.6 ± 4.2 %, n = 21 (Figure 4.7) in comparison to those subjected to LPC alone, (figures 4.8). Regions rostral and caudal to the site of injury were examined, however, proliferation was restricted to the site of injury only.

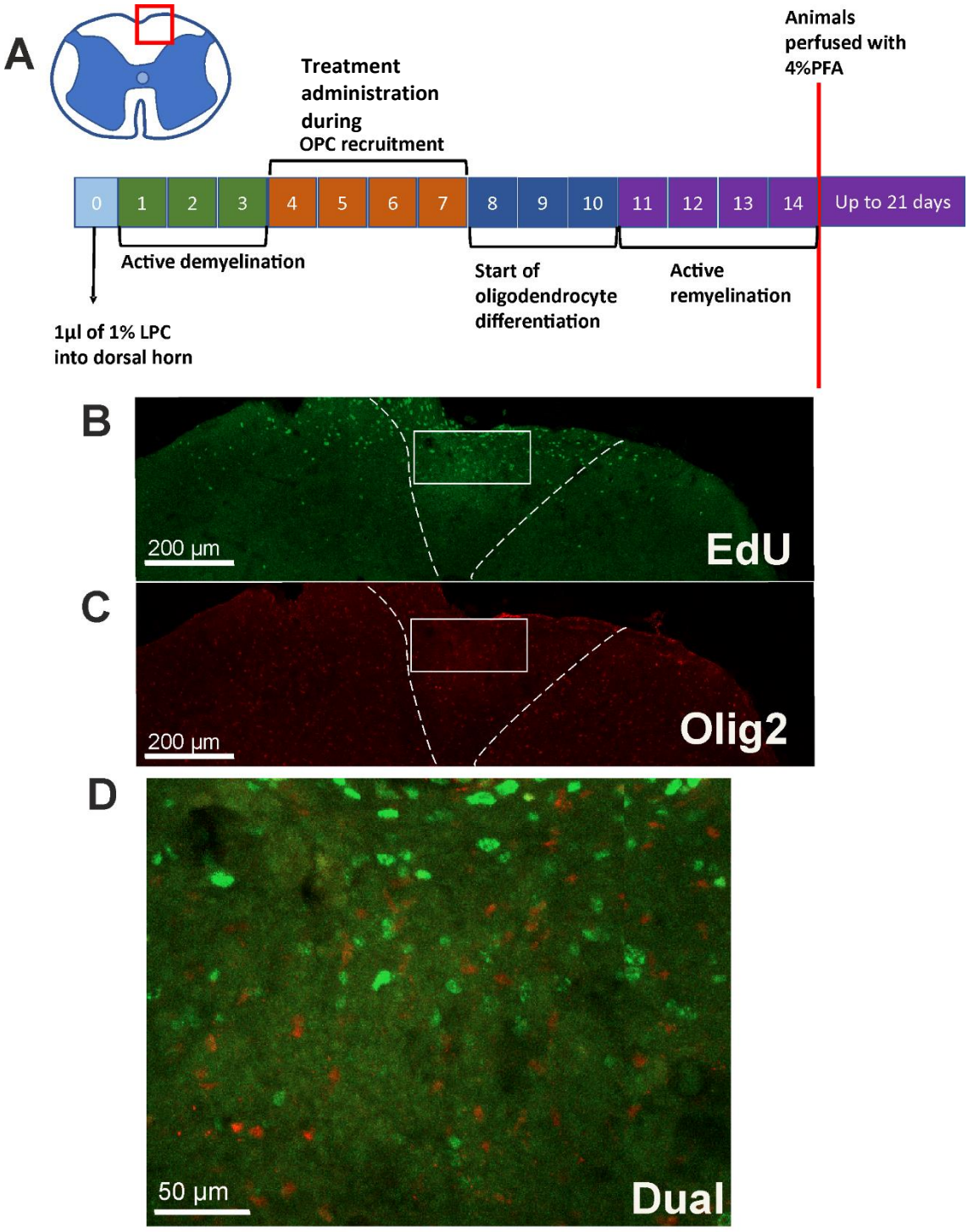

Figure 4.5 Cell proliferation and oligodendrocyte differentiation in animals treated with LPC alone.

A) Experiment timeline. **B)** Representative image of EdU labelled cells. **C)** Representative image of Olig 2 labelled cells. **D)** A merged image with both channels.

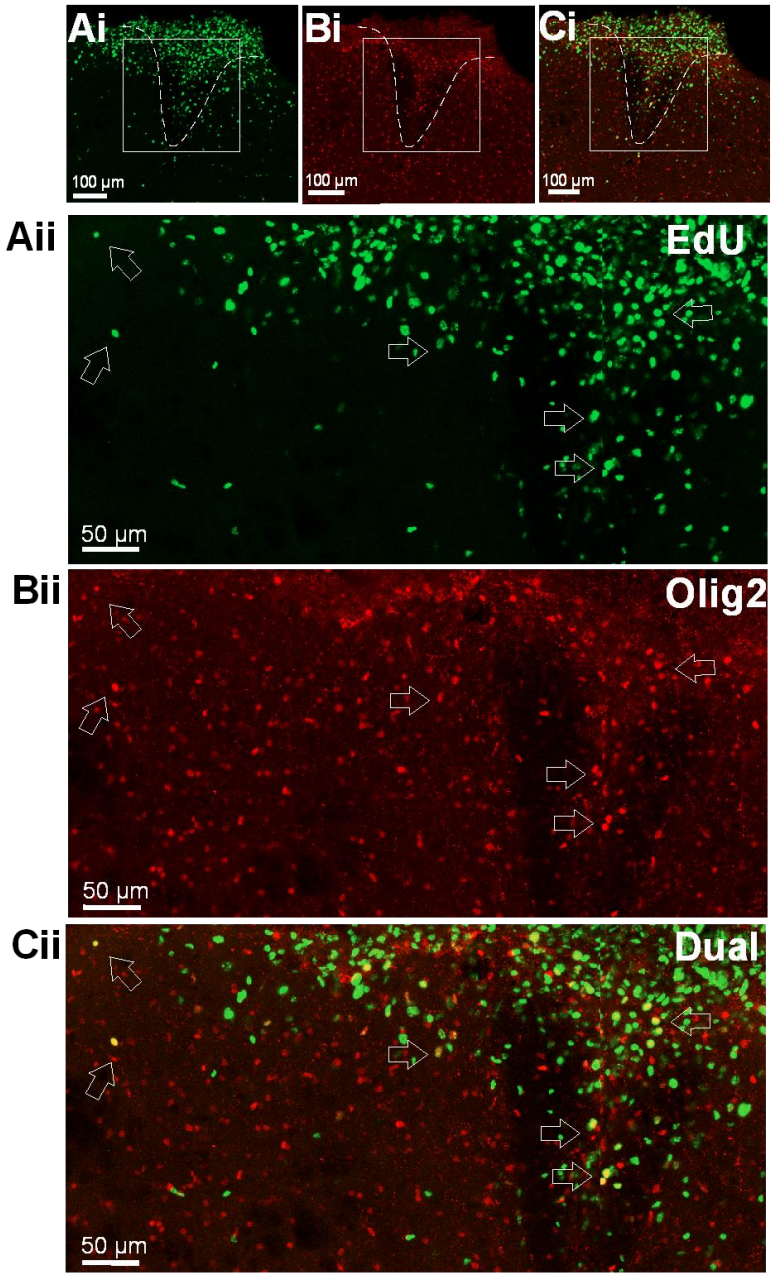

Figure 4.6 Example images of cell proliferation and oligodendrogenesis in animals treated with PNU-120596 after focal lumbar LPC injection.

Ai-Cii) Representative image of a lumbar spinal cord after LPC injection into the dorsal white matter followed by treatment with PNU-120596. **Ai)** EdU labelled cells. Bi) Olig 2 labelled cells. **Ci)** A merged image. Panels **(Aii-Cii)** are magnified images of the selected areas in panels **(Ai-Ci)** showing EdU^+ $Olig2^+$ colocalised cells. Arrows in panel **(Cii)** point out only a few examples of colocalised cells.

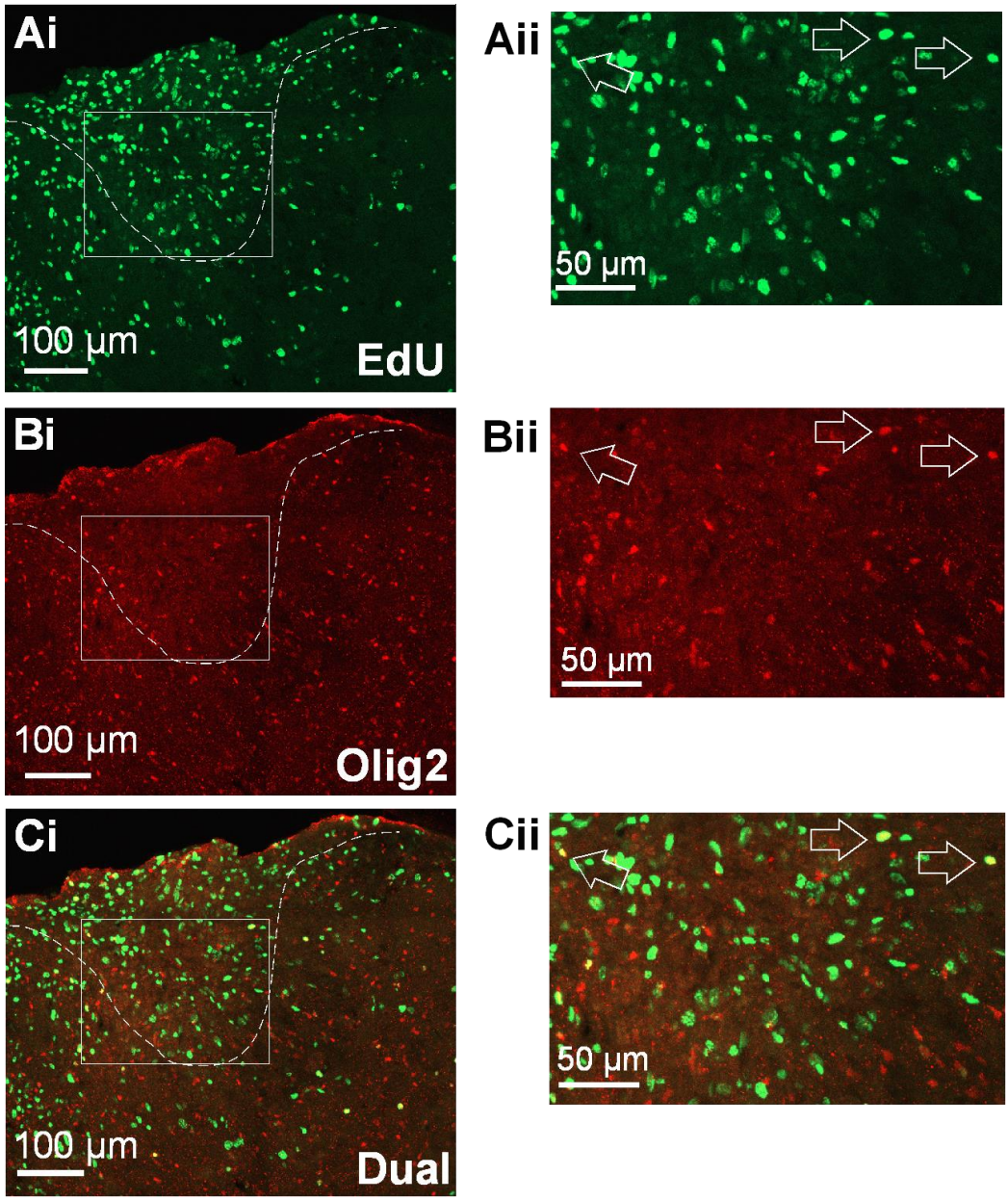

Figure 4.7 Example images of cell proliferation and oligodendrogenesis in animals treated with PNU-120596 in combination with donepezil after focal lumbar LPC injection.

Ai-Cii) Representative images of a lumbar spinal cord after LPC injection into the dorsal white matter followed by treatment with PNU-120596+ donepezil. **Ai)** EdU labelled cells. **Bi)** Olig 2 labelled cells. **Ci)** A merged image with both channels. Panels **(Aii-Cii)** are magnified images of the selected areas in panels **(Ai-Ci)** showing EdU^+ $Olig2^+$ colocalised cells. Arrows in panel **(Cii)** point out only a few examples of colocalised cells.

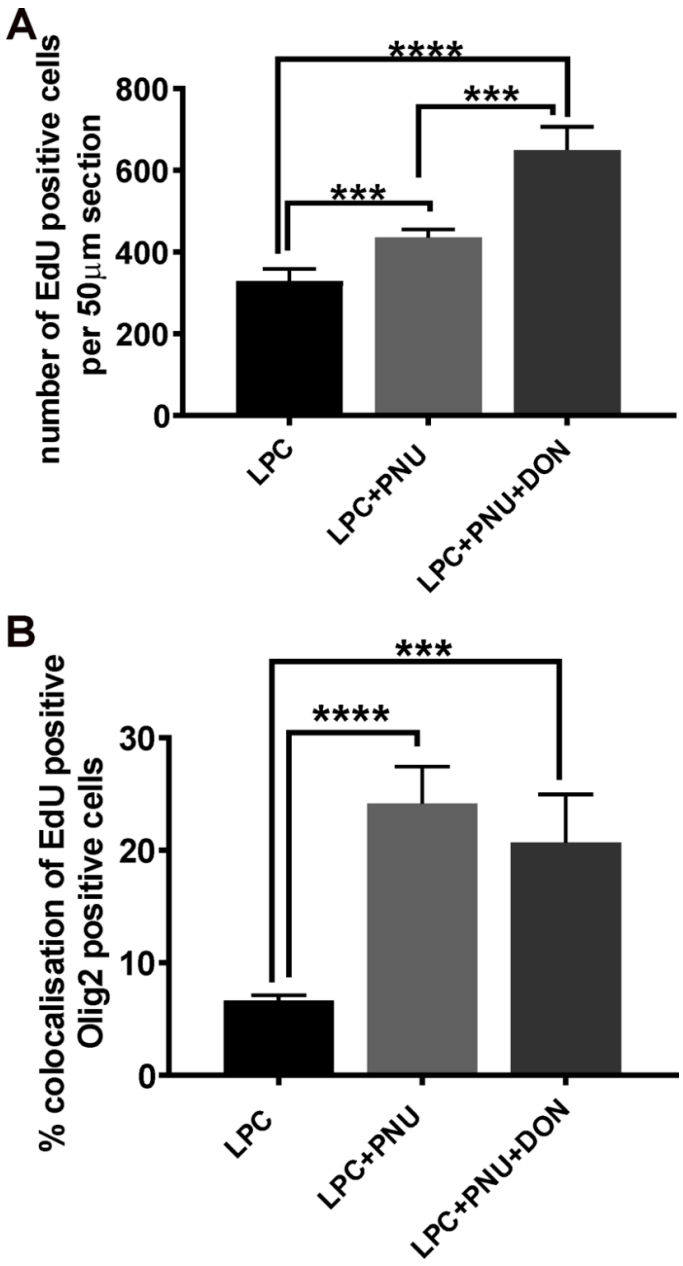

Figure 4.8 *In vivo* cholinergic modulation after dorsal white matter demyelination stimulates significant proliferation and oligodendrogenesis.

A) Number of EdU labelled cells for each of the conditions. Data are shown as mean number of EdU labelled cells per 50 μm section ± SEM. **B)** Percent colocalisation of: N=4 animals, n= 24 sections in LPC alone treated animals, N=4 animals, n=21 sections in LPC+PNU treated animals and N=4 animals, n=22 sections in LPC+PNU+Don treated animals. ***; P=0.0057, ***; P=0.0037, ***; P=0.0016, ****; P<0.0001.

4.2.4 Cholinergic modulation after LPC-induced focal demyelination enhances cell proliferation and oligodendrogenic differentiation at 21 days post injury

Given that the experiment terminated at post-injection day 21 (PID21), figure 4.9 A; it was expected for oligodendrocyte maturation to have taken place after recruitment at the site of injury, figure 4.9 A. The immunolabel Olig2 was used to detect myelinating oligodendrocytes. Here we quantified the percentage of the mean number of EdU positive cells immunoreactive for Olig2 within the region of injury with and without cholinergic modulation. Consistent results with the previous time point (PID14) were seen at the dorsal site of injury; there were 285 ± 17.1 EdU positive cells per 50 μm section, (N = 3, n = 19) in animals which were subjected to LPC injections without subsequent treatment; (figures 4.9, 4.10 and 4.11). The number of EdU positive cells was significantly higher ($P<0.0001$) in animals which received PNU-120596 alone; 465 ± 12.7, n = 18 and ($P=0.0003$) in animals treated with PNU-120596 and donepezil; 499 ± 47.1, n =20 when compared to baseline proliferation; (figure 4.12). Moreover, the mean percentage of EdU^+ cells that were also $Olig2^+$ was significantly higher ($P<0.0001$) compared to baseline; 13.46 ± 0.78 %, n = 18 in animals treated with PNU-120596 alone compared to 6.53 ± 0.59 %, n = 19 in LPC alone treated animals. A significant increase ($P=0.0014$) was also seen in animals administered PNU-120596 and donepezil; 17.55 ± 2.98 %, n = 20 in comparison to those subjected to LPC alone, (figures 4.12). Regions rostral and caudal to the site of injury were examined, however, proliferation was restricted to the site of injury alone.

It is important to note that in some animals of this experiment, LPC injections were administered slightly deeper than intended; this created an injury that was in close proximity to the central canal. As a result, some proliferation was seen in the central canal area including EdU^+ cell proliferation starting at the central canal and spreading vertically spanning the area ventral to lamina X, (figure 4.13). This observation is reminiscent of the results obtained by (Lacroix et al., 2014) which revealed central canal proliferation in demyelination injuries only occur if the central canal itself was injured.

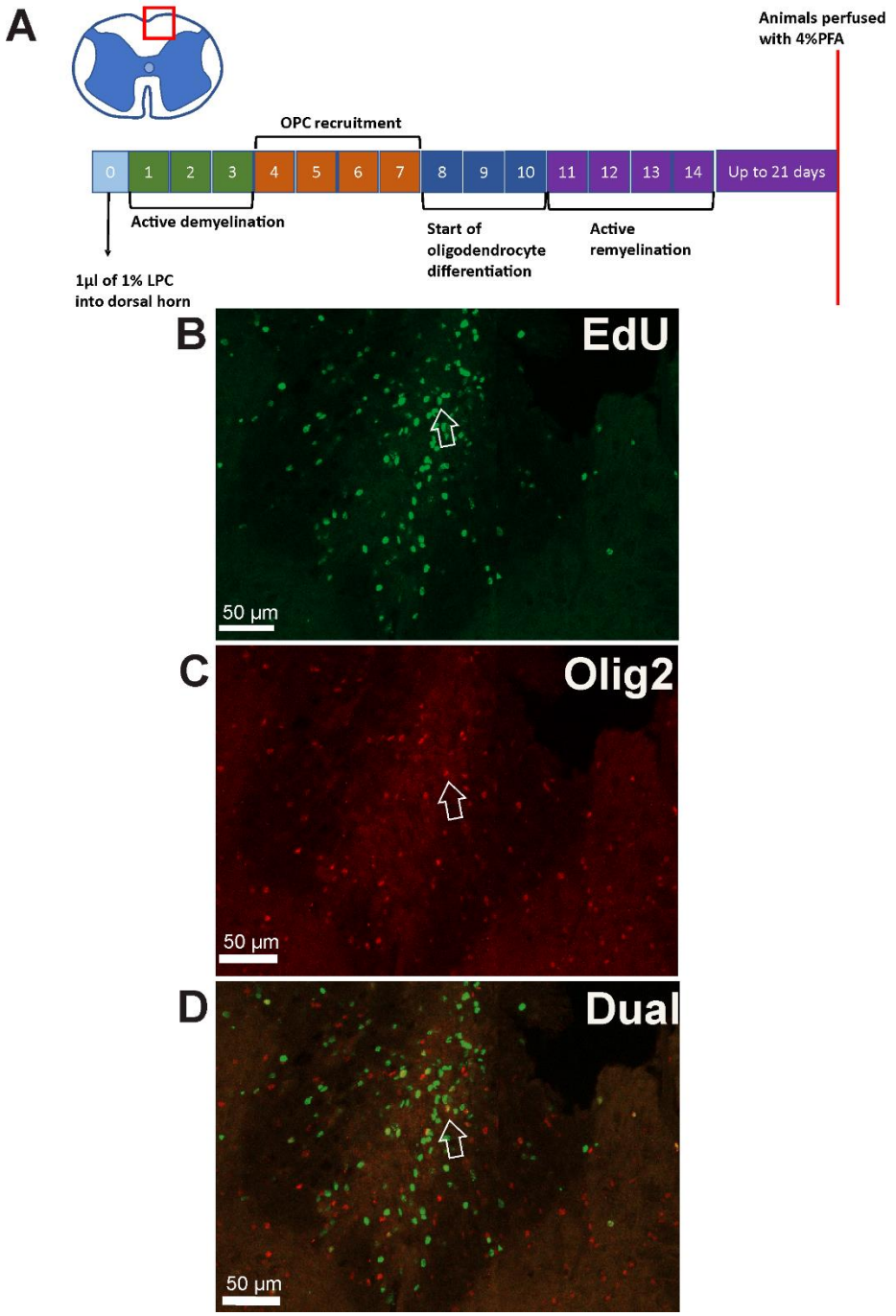

Figure 4.9 Cell proliferation and oligodendrocyte differentiation 21 days post treatment with LPC alone.

A) Experiment timeline**. B)** Representative image of EdU labelled cells. **C)** Representative image of Olig 2 labelled cells. **D)** A merged image with both merged channels. Arrow points out only an example of a colocalised cell.

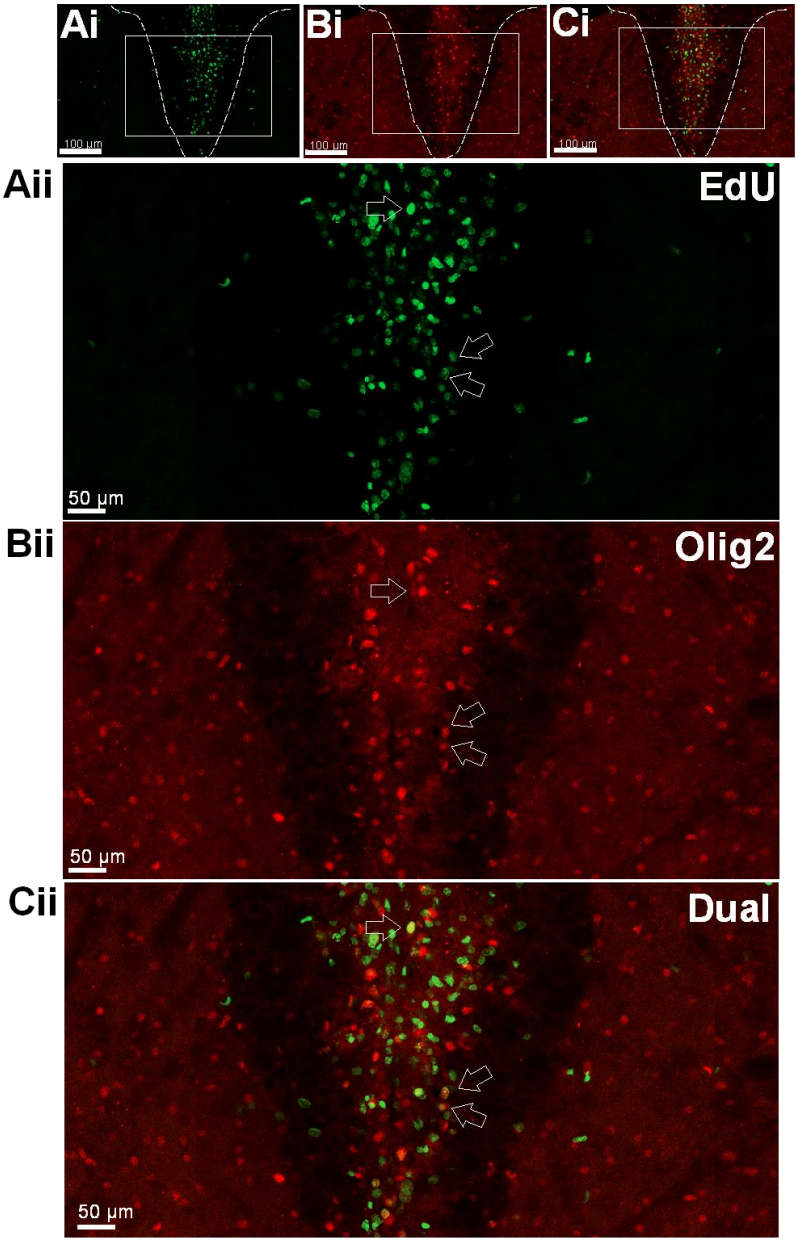

Figure 4.10 Proliferation and oligodendrogenesis after LPC injury and subsequent treatment with PNU-120596 alone.

Ai-Cii) Representative images of a lumbar spinal cord after LPC injection into the dorsal white matter followed by treatment with PNU-120596. **Aii)** EdU labelled cells. **Bii)** Olig 2 labelled cells. **Cii)** A merged image with both channels. Panels **(Aii-Cii)** are magnified images of the selected areas in panels (**Ai-Ci)** showing EdU^+ $Olig2^+$ colocalised cells. Arrows in panel **(Cii)** point out only a few examples of colocalised cells (Yellow).

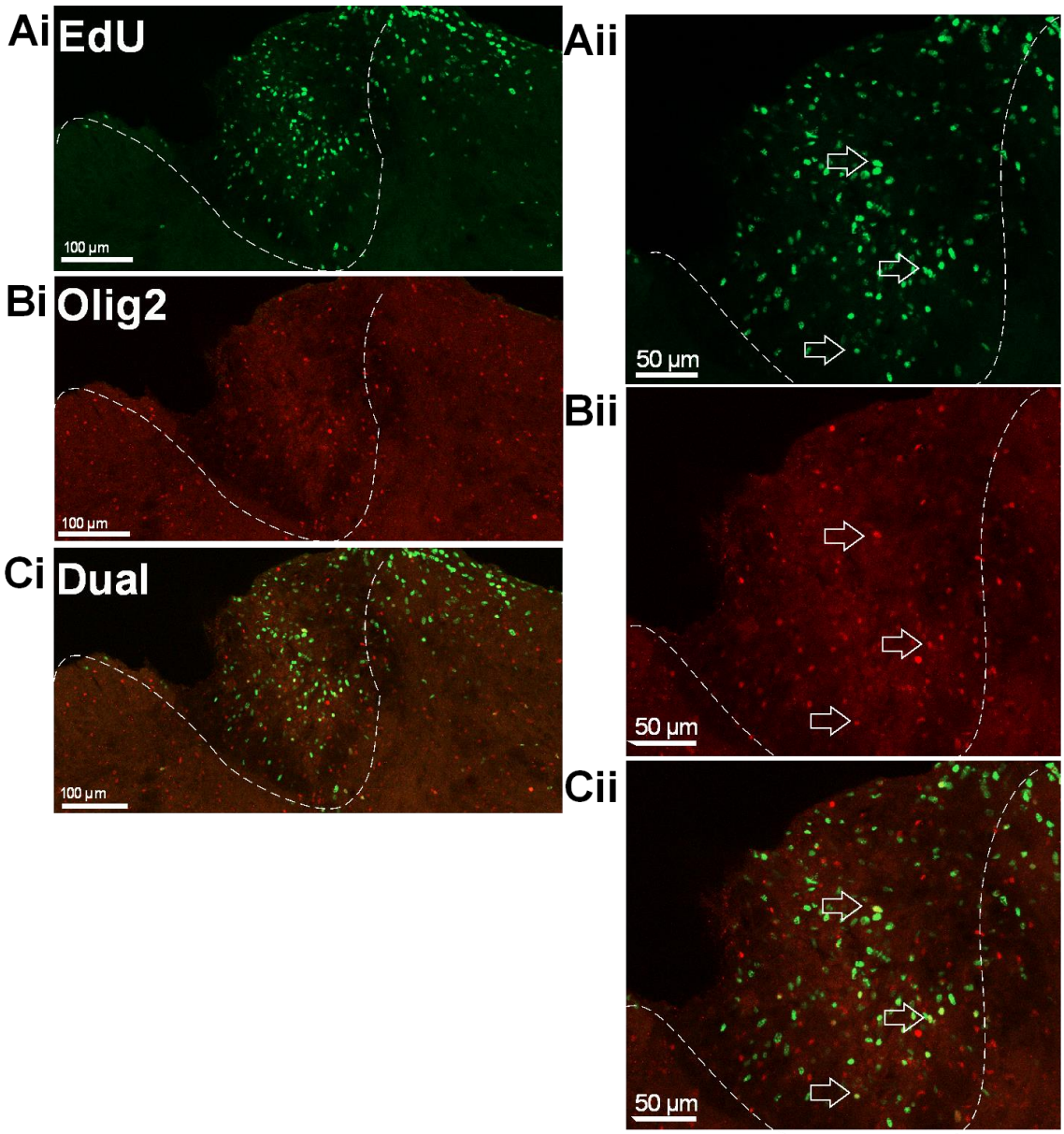

Figure 4.11 Proliferation and oligodendrogenesis after LPC demyelination and subsequent treatment with PNU-120596 and donepezil.

Ai-Cii) Representative images of a lumbar spinal cord after LPC injection into the dorsal white matter followed by treatment with PNU-120596+ donepezil. **Ai)** EdU labelled cells. **Bi)** Olig 2 labelled cells. **Ci)** A merged image with both channels showing EdU^{+} $Olig2^{+}$ colocalised cells. Panels **(Aii-Cii)** are magnified images of the selected areas in panels (**Ai-Ci)** Arrows point out only a few examples of colocalised cells in (Yellow).

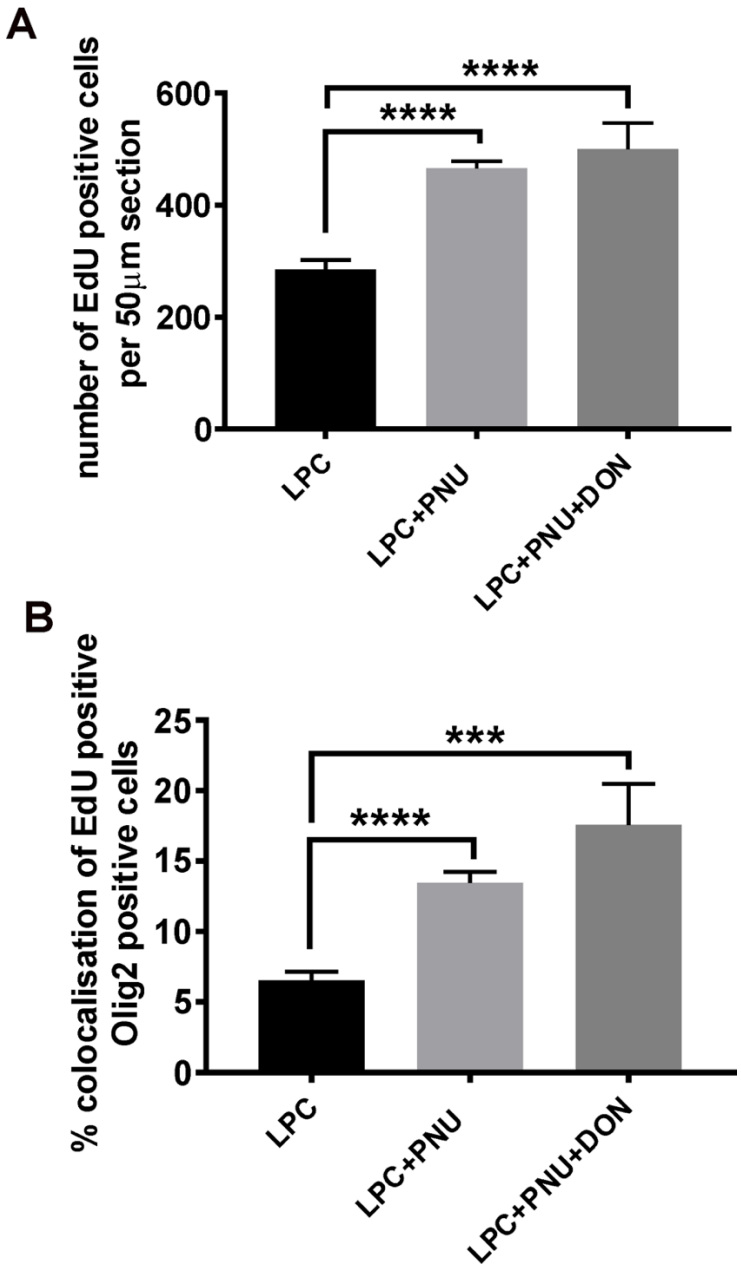

Figure 4.12 *In vivo* cholinergic modulation in dorsal white matter demyelination stimulates significant proliferation and oligodendrogenesis 21 days post injury.

A) Number of EdU labelled cells for each of the conditions. Data are shown as mean number of EdU labelled cells per 50 µm section ± SEM. **B)** Percent colocalisation of: N=3, n=19 in animals subjected to LPC alone, N=3, n=18 in animals treated with LPC+PNU and N=3, n=20 in LPC+PNU+Don treated animals. ***; P=0.0014, ****; P<0.0001, ****; P=0.0003.

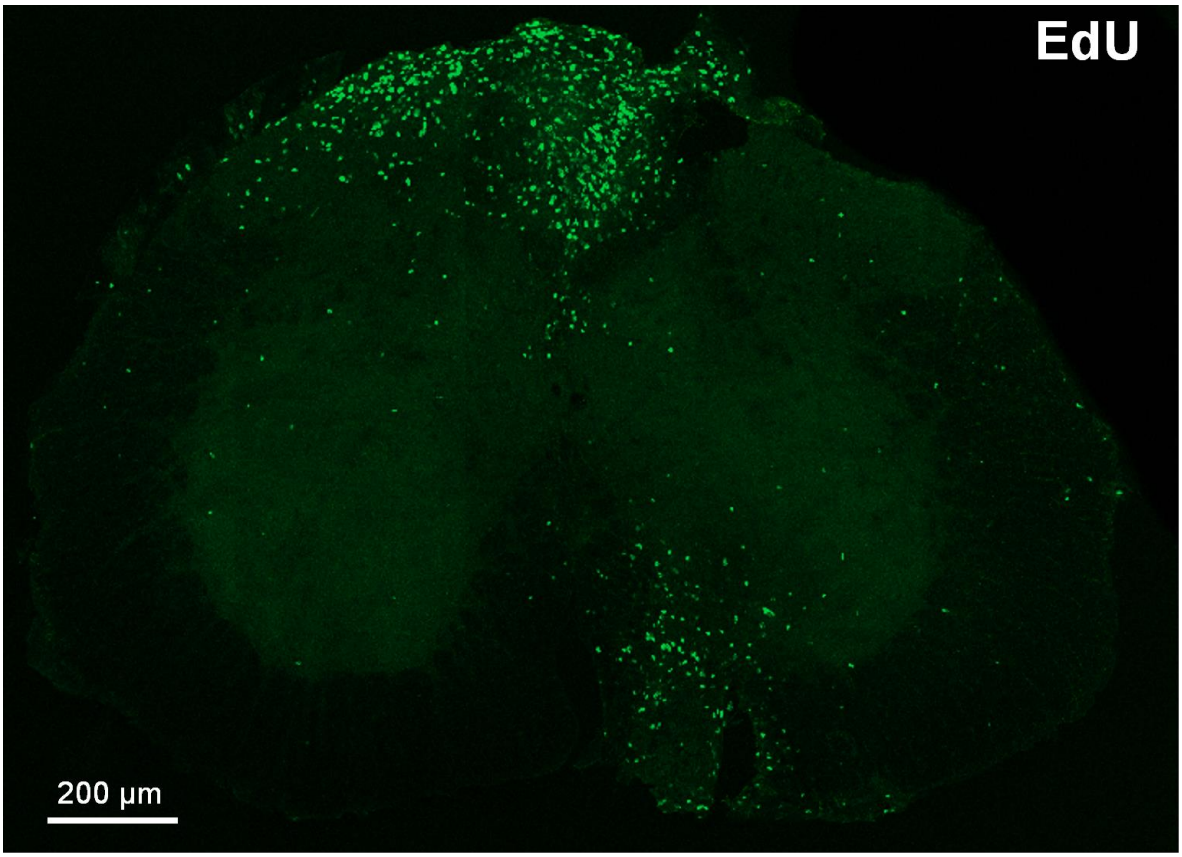

Figure 4.13 A representative image of proliferation after LPC injections reach close proximity to central canal.

EdU positive cells are seen at site of injection, at the central canal and in the ventral horn.

4.2.5 There was a significant increase in the extent of remyelination after cholinergic treatment 14 days post LPC injection

In order to compare the amount of newly generated myelin enwrapping axons 14 days after LPC induced demyelination alone (endogenous remyelination) and remyelination after treatment with PNU-120596 and donepezil, the G-ratio of individual axons localised within the demyelinated lesion of both animal groups was calculated. Given that the size of individual axons can vary greatly between individual animals, axons of similar sizes were selected for comparison (axons with a diameter of 1,200 pixels or less: 1 pixel= 264.58 µm). This was done in order to avoid any variabilities that may affect the value of the G-ratio.

The mean difference between the inner axonal diameter and outer axonal dimeter (G-ratio) of control animals representing endogenous remyelination was 0.80 ± 0.01 nm, N = 4, n = 40 compared to 0.76 ± 0.001 nm, N = 5, n = 40 in PNU-120596 + donepezil treated animals 14 days following LPC injection, figure 4.14, 4.15. A higher ratio value indicates lower extent of myelination. Thus, there was a significant (P = 0.0119) difference in the amount of myelin present between control animals and PNU-120596 and donepezil treated animals at 14 days following focal demyelination, figure 4.16.

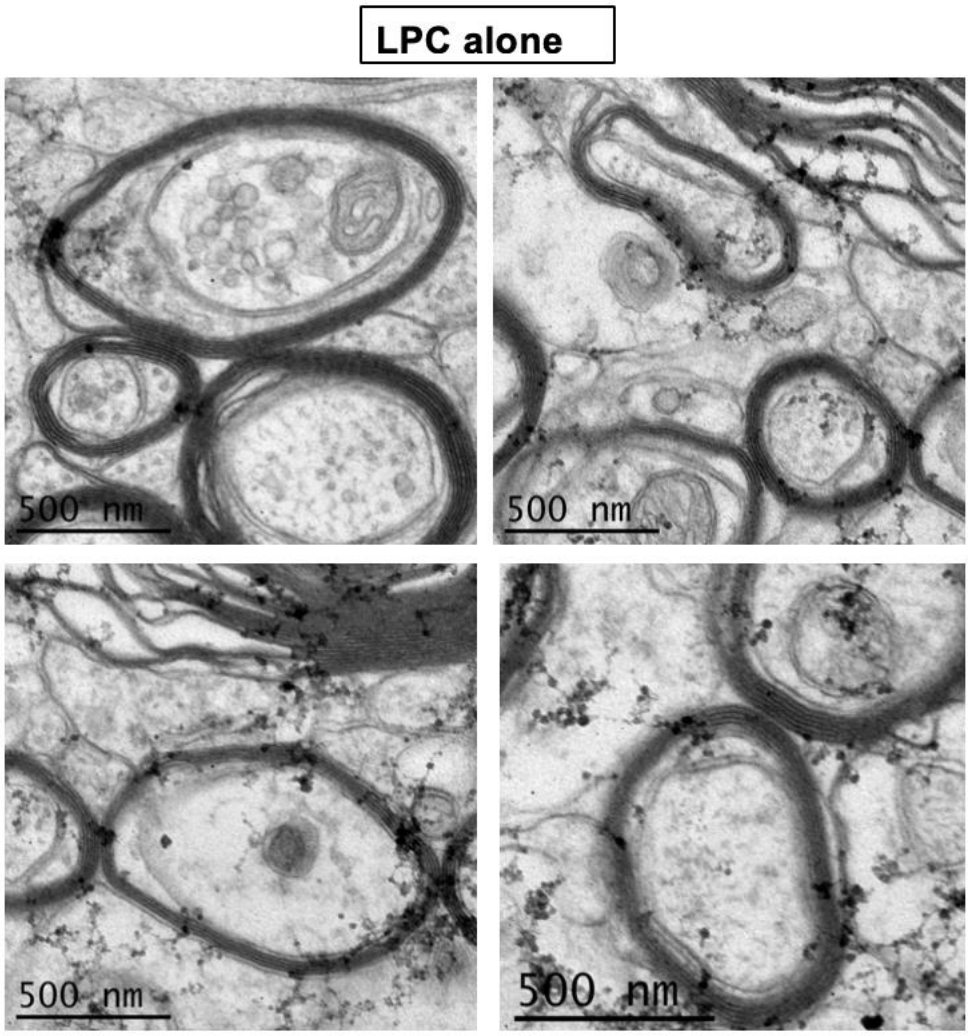

Figure 4.14 Representative images of endogenous remyelination 14 days following LPC-induced demyelination.

Electron micrographs (80 nm thin sections) of remyelinated axons within the LPC demyelinated lesion of vehicle treated animals.

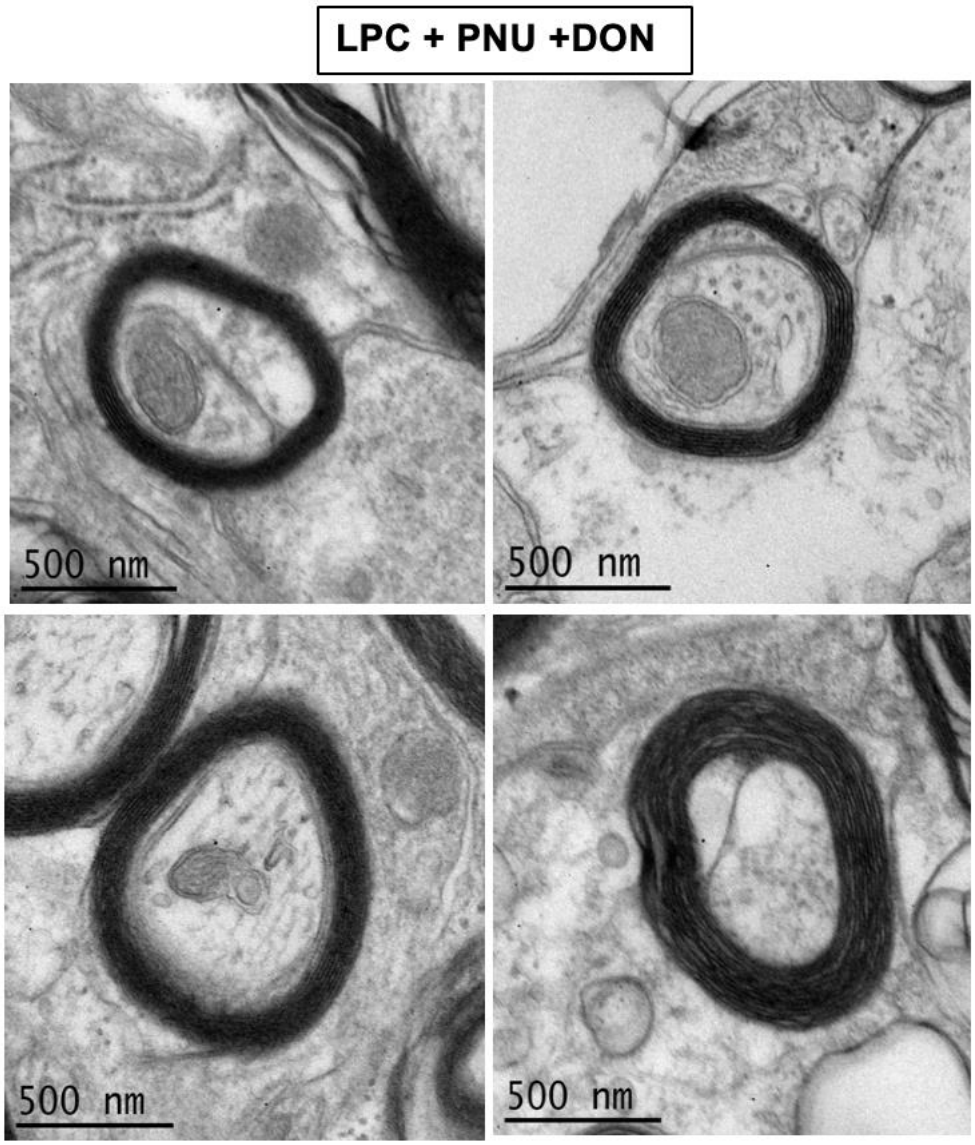

Figure 4.15 Representative images of remyelination post cholinergic treatment 14 days following LPC-induced demyelination.

Electron micrographs (80 nm thin sections) of remyelinated axons within the LPC demyelinated lesion in animals treated with PNU-120596 and donepezil.

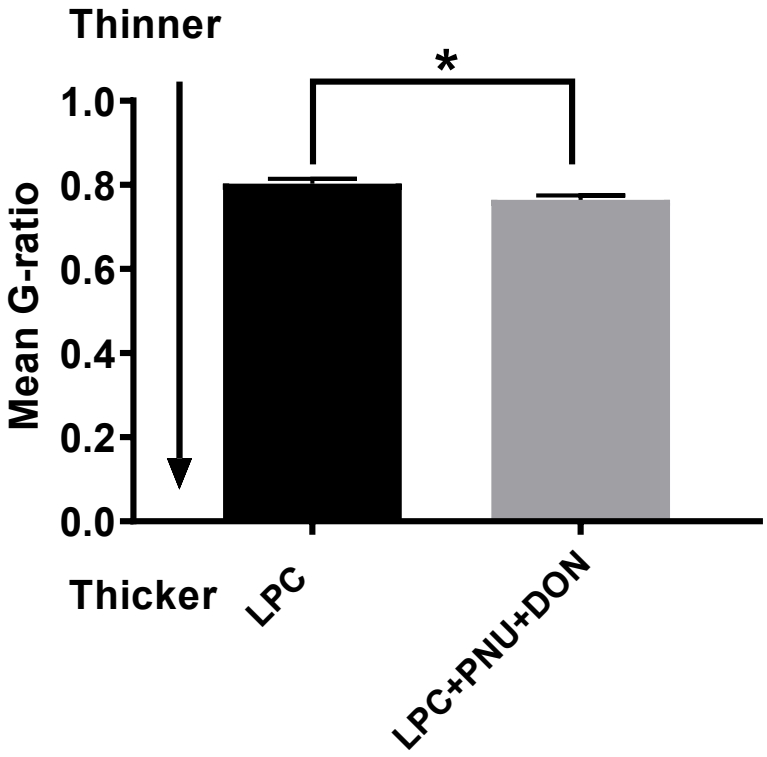

Figure 4.16 There was a significant increase in the extent of remyelination following treatment with PNU-120596 + donepezil 14 days following demyelination.

The mean G-ratio (axon diametre / myelinated fibre diametre) was significantly lower (thicker myelin) in PNU-120596 + donepezil treated animals (N= 5 animals, n=40 axons) compared to LPC and vehicle treated animals (N=4 animals, n=40 axons). Mean G-ratio per axon ± SEM. *; P = 0.0119.

4.2.6 The semliki forest virus was not detected immunohistochemically in the central nervous system of C57BL/6 mice

In an attempt to generate an inflammatory model of demyelination in mice, replicates of two reference genes of the semliki forest virus was detected in the spinal cord of animals at post injection days 4, 7, 10 and 14, and in the brain at days 4 and 7, (figure 4.14). The virus was injected intraperitoneally, for details refer to section 2.9.1. Peak replication for this strain of virus was expected at day 7 post injection with a start of demyelination at day 10, however, the highest number of copies observed here was at day 4 post injection in both the brain and spinal cord samples. More importantly, immunohistochemistry against m-Cherrry failed to detect the presence of the virus in the entire central nervous system of all animals; indicating a lack of infection and any subsequent demyelination.

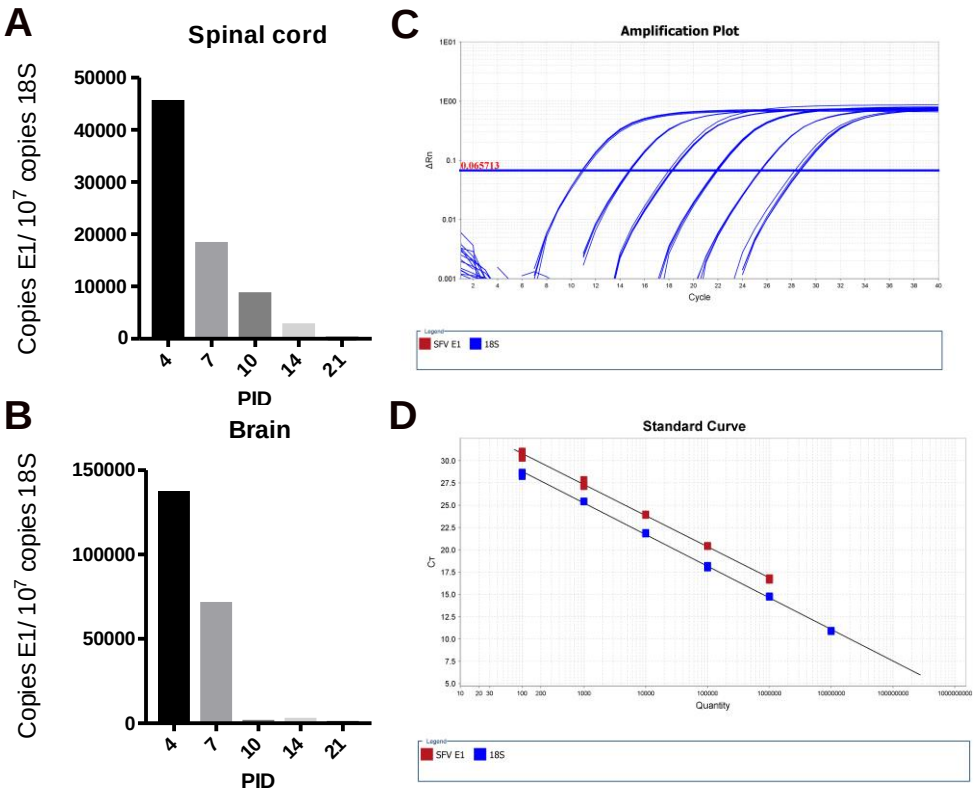

Figure 4.17 qPCR analysis of virus replication in the brain and spinal cord of semliki forest virus inoculated animals.

These graphs show the number of viral RNA replicates.

4.3 Discussion

Manipulating acetylcholine signalling after focal white matter demyelination significantly enhances cell proliferation, oligodendrogenic differentiation and remyelination. These results are potentially mediated through cholinergic receptor activity of oligodendrocyte precursor cells, however, our current understanding of the full functional consequences of cholinergic signalling in the context of oligodendrogenesis and myelination is minimal.

4.3.1 The therapeutic potential of manipulating acetylcholine to promote remyelination

Pharmacological manipulation of acetylcholine signalling to promote remyelination is a promising future treatment in white matter disorders, however, there still remain many basic yet critical mechanistic questions that need to be answered. The two most important questions that need to be addressed are; the source of acetylcholine in the spinal cord and the functional heterogeneity of cholinergic receptors in oligodendroglial development and function in disease.

Early developmental studies reported acetylcholinesterase activity including butyrylcholinesterase; a nonspecific cholinesterase enzyme shows a sharp increase in activity at a time coinciding with active myelination and synapse formation in chick spinal cord cultures (Kim et al., 1972). Another study revealed that acetylcholinesterase activity was found in the spinal cord white matter of the developing chick only at the time of glial cell aggregation in the white matter and after myelin formation (State et al., 1977). Turbow and Burkhalter also reported the presence of acetylcholinesterase activity in the white matter only during myelination in chick embryos and then replaced by butyrylcholinesterase activity (Turbow and Burkhalter, 1968). These studies together suggest that the enzyme plays a role in myelin development, and since the enzyme activity in these studies was detected pre-synaptically; this suggests that the process of myelination in chick development is not

cholinergic receptor mediated. It is yet to be determined whether this event is conserved among species.

Other more recent studies of remyelination post injury however do suggest the involvement of cholinergic receptors in promoting remyelination. It is not known whether oligodendrocytes synthesise acetylcholine to regulate their own behaviour, however, they do express the synthesizing enzyme choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAhT) (MacDonald et al., 2002). This study cultured neurospheres from spinal cords of E15-E18 mouse embryos and identified oligodendrocytes immunohistochemically with antibodies against galactocerebroside (GalC); the major glycolipid in myelin and a cell surface marker for rat oligodendrocytes in culture (Raff et al., 1978), and reported that almost all GalC positive cells displayed extensive process arborization and round cell bodies characteristic of oligodendrocyte morphology in culture were also immunopositive for ChAT (MacDonald et al., 2002). Some of the $ChAT^+$ $GalC^+$ positive oligodendrocytes were also positive for VAhT; indicating that some oligodendrocytes are capable of packaging ACh into vesicles. This study also reported that the number of galactocerebroside positive oligodendrocytes cultured in the presence of the muscarinic antagonist atropine was reduced indicating the importance of these receptors in oligodendrocyte development (MacDonald et al., 2002). In the context of remyelination post disease or injury however, more recent research into the role of muscarinic acetylcholine receptors reveal potential effective adjunct therapies by inhibiting muscarinic receptors to enhance remyelination in combination with immunomodulation in multiple sclerosis. For instance, the direct antagonism of M1 and or M3 receptors *in vivo* and *in vitro* by benztropine; a muscarinic antagonist resulted in the enhancement of remyelination in a chemically-induced demyelination model using cuprizone and the experimental autoimmune encephalomyelitis model (Deshmukh et al., 2013). Furthermore, (Abiraman et al., 2015) also employed M3 muscarinic antagonism *in vitro* which resulted in oligodendrocyte differentiation. This study employed human fetal forebrain (17-22 weeks of gestation) of consenting patients which were minced, serially stained with a variety of oligodendrocyte and oligodendrocyte precursor cell markers (PDGFRa, CD140a, O4, Olig2, Sox10) and Ki67 and FACs sorted. They employed qPCR analysis to detect muscarinic receptor gene expression and determined that M3 is expressed by

human fetal oligodendrocyte precursor cells. When CD140a^{+} / PDGFRa^{+} human OPC were cultured in the presence of a potent non-selective muscarinic agonist (oxotremorine), there was a dose-dependent decrease in cell differentiation, interestingly however, when the human OPCs were cultured in the presence of ChAT positive neurons and an M3 receptor antagonist, a significant increase in OPC differentiation occurred (Abiraman et al., 2015). To test if M3 receptor antagonism would cause enhanced OPC differentiation and myelination *in vivo*, they subcutaneously injected the antagonist into the corpus callosum of mouse pups (from P4 to P9). They reported a significant increase in MBP staining in the injected area (Abiraman et al., 2015). Indeed, in an engineered model of conical micropillar arrays which allows two-dimentional imaging of cultured oligodendrocyte precursor cells (obtained from postnatal mouse and rat brain cortices) and their concentric myelin wrapping around the pillars, eight FDA-approved anti-muscarinic and anti-histaminic drugs out of a thousand screened have resulted in enhanced oligodendrogenesis and enhanced myelination (Mei et al., 2014b). Out of the eight compounds, clemastine; an anti-histaminic and anti-muscarinic drug was the most effective in remyelination after LPC induced injury in the dorsal funiculus and ventrolateral white matter of adult mice (Mei et al., 2014a). Moreover, D'Intino reported that chronic acetylcholinesterase inhibition with donepezil or rivastigmine restored acetylcholine levels, choline acetyltransferase activity and nerve growth factor mRNA expression which in turn restored cognitive function in an experimental allergic encephalomyelitis rat model (D'Intino et al., 2005). Together these studies highlight the involvement of cholinergic signalling in white matter disorders associated with cognitive decline and demyelination, and emphasize potential therapeutic targets to ameliorate these disorders through modifying cholinergic signalling.

Interestingly, Bartzokis suggested acetylcholinesterase inhibition in people with Alzheimer's disease can enhance myelin integrity through protective trophic non-synaptic mechanism of action on oligodendrocytes or other non excitable cells (Bartzokis, 2007). This hypothesis is based on the link between the involvement of cholinergic signalling in central nervous system development which is mediated partly through increasing neurotrophin production that can in turn affect or enhance myelination. Since this is yet to be proven, we can only postulate that the mechanism

by which donepezil enhances oligodendrogenesis when administered along with α7nAChR potentiation in our experiments is solely via elevating acetylcholine levels and enhancing cholinergic transmission. The significant difference seen in animals treated with PNU-120596 alone and baseline oligodendrogenesis is clearly mediated through enhanced synaptic cholinergic transmission after α7nAChR potentiation.

4.3.2 The LPC animal model of demyelination

Lysolecithin injections cause an acute (1-3 days) injury localised in a focal area inducing demyelination which is followed by prompt oligodendrocyte progenitor cell infiltration and remyelination (Keough et al., 2015). The simplicity of this model allows for remyelination to be examined in the absence of chronic damage. For our therapeutically promising results seen using this model to be translational, it is crucial to test our drugs in a model which incorporates the chronic involvement of lymphocyte infiltration that is characteristic of multiple sclerosis. LPC focal injections are only associated with myeloid inflammation (Plemel et al., 2017). Demeyelinating lesions in multiple sclerosis or even spinal cord injuries are associated with a variety of micromolecules that may hinder oligodendrocyte progenitor differentiation or remyelination (Plemel et al., 2017) therefore understanding the pathophysiology of multiple sclerosis requires understanding the changes that occur in the extracellular matrix within the lesions.

LINGO-1 for example is a membrane bound protein reportedly present in chronic active multiple sclerosis lesions identified by immunohistochemistry on astrocytes and microglia (Satoh et al., 2007), and is a component of the myelin associated axonal regrowth inhibitor NOGO signalling complex. LINGO-1 is also present on oligodendrocytes and negatively regulates oligodendrocyte differentiation and myelination (Mi et al., 2005); antagonism of LINGO-1 or expression reduction by LINGO-1 RNAi lentivirus resulted in oligodendrocyte differentiation and abundant myelination in culture, whereas overexpression of LINGO-1 had the opposite effect. These results were also consistent with their *in vivo* studies in LINGO-1 knockout mice. Chondroitin sulfate proteoglycans (CSPGs) a family of extracellular matrix

proteoglycans and hyaluronan are also remyelination inhibitors (Back et al., 2005, Ahmed and Sobel, 2001). The accumulation of the glycosaminoglycan hyaluronan was detected in very small amounts in LPC induced lesions which is characterized by a limited inflammatory response (Back et al., 2005), however in EAE mouse models and individuals with multiple sclerosis, hyaluronan accumulated in inflammatory lesions inhibiting oligodendrocyte maturation in chronic lesions and prevented oligodendrocyte differentiation in culture (Back et al., 2005). It is thought to be produced by activated T cells and microglia that potentially promote T cell activation in early lesions while a higher molecular weight form of hyaluronan is produced by astrocytes in chronic lesions (Back et al., 2005). Interestingly, the high molecular weight form of hyaluronan inhibits remyelination by inhibiting oligodendrocyte precursor cells (O2A- lineage cells) in culture from proliferating, maturing and differentiating (Marret et al., 1994). Immunohistochemistry revealed that the amount and distribution of chondroitin sulfate proteoglycans in white matter extracellular matrix or multiple sclerosis lesions coincide with the inflammatory phase and are likely playing a role in plaque expansion, inhibition of axonal regrowth and remyelination (Ahmed and Sobel, 2001).

Another component of the extracellular matrix, matrix metalloproteinases (MMPs) which are endopeptidases that mediate the degradation of proteins in the extracellular matrix (Mott and Werb, 2004), and are produced by neurons and glial cells in the central nervous system (Gray et al., 2008). These are variably elevated in the white matter in multiple sclerosis (Anthony et al., 1997). Active levels of matrix metalloproteinases -9 (MMP-9) in particular are found elevated in demyelinated cortical areas where neurons appear to be affected by the accumulation of phosphorylated neurofilament proteins in their cell bodies in combination with the loss of perineural nets (Gray et al., 2008). It is unknown however if this occurs at all in the LPC model of demyelination and whether alterations in the perineural nets affect synaptic plasticity and function.

The experiments in this chapter were conducted on both male and female mice. There were no noticeable differences in cell proliferation and differentiation between female and male mice in each experiment. This is relevant since, multiple sclerosis is a

disease known to have a higher incidence and progression in females than males, and this is hypothesized to be due to sex-specific hormones underlying the pathophysiology and disease progression (Tomassini and Pozzilli, 2006). Gender- dependent studies in rodents have been conducted to elucidate cellular and / or molecular differences in remyelination after injury. A study on EAE rat models concluded a strong gender-dependent difference in the clinical severity of the disease (a higher clinical score in female rats than male rats) and male rats showing a more pronounced recovery than the females. Interestingly, the inflammatory cellular infiltration score in the spinal cord of female rats is lower yet associated with a more severe case of astrogliosis than in male rats (Massella et al., 2012). Demyelination of the gracile fasciculus of lumbar spinal cord was not different among genders, while the levels of mRNA expression for MBP and PDGFRa in the spinal cord and cerebellum was different among genders; PDGFRa was 2.5 times higher in healthy males than in healthy females and MBP was 5 times higher in healthy males than in healthy females. Both genes were downregulated during all phases of EAE induction, although, there was a significantly lower MBP gene expression in female rats compared to male rats at 40 days post EAE induction (Massella et al., 2012).

Finally, a proper interpretation of our results lies in understanding the exact mechanism of action by which the injury occurred. Since LPC is an endogenous lisophospholipid (Aoki, 2004, Plemel et al., 2018), understanding how it is maintained in health and the mechanism by which it produces demyelination is essential. Elevated levels of endogenous LPC have been reported following spinal cord injuries (Hanada et al., 2012) in the spinal cord grey matter of patients with amyotrophic lateral sclerosis (Hanrieder and Ewing, 2014) and in the cerebrospinal fluid of patients with multiple sclerosis (Pieragostino et al., 2015) to name a few. In a study conducted on C57/BL6 mice aged (6-8 weeks), similar to our experiments in this chapter, (Plemel et al., 2018) investigated the mechanism of action of LPC. They found that the initial consequences of injecting LPC into the white matter of the ventral column of the spinal cord (4 hours post injection) resulted in a lesion intensely immunoreactive for MBP depicting the occurrence of demyelination which plateaued 4-24 hours post injection. They hypothesized that the intense labelling may be due to the fact that the compact myelin

sheath had been permeabilised by LPC which facilitated the access of the antibody. More importantly, LPC exerted its permeabilising effects on all cells present in the injury site; astrocytes, oligodendrocytes and oligodendrocyte precursor cells were lost based on reduced reactive cells for glial fibrillary acidic protein (GFAP), Olig2 and PDGFRα respectively. Complete oligodendrocyte loss occurred at 4 hours post injection, and oligodendrocyte precursor cell recruitment near the site of injury began 72 hours post injury. Ex-vivo two-photon imaging of live spinal cord preparations labelled with a lipophilic dye to observe axons and myelin showed that they were not injured by perfusion methods, and that bathing sections in 0.5mg/ml of LPC was sufficient to induce robust injury to both axons and myelin indicated by the presence of axonal and myeloid spheroids. They assessed the possibility of LPC inducing toxicity through targeting known downstream receptors in culture, however astrocytes and oligodendrocytes subjected to LPC toxicity *in vitro* were not saved by the application of receptor inhibitors. Furthermore, they investigated whether LPC induces inflammation as a mechanism to myelin injury, although, they found that inflammation occurred after LPC had cleared and caused a substantial lesion (within 24 hours) *in vivo*. After LPC had cleared, an increase in immune activity was present suggesting that demyelination is not caused by inflammation in this model. Moreover, they concluded that LPC exerts its action in a dose dependent manner, integrating into cell membranes once it had reached a specific concentration. This effect was recapitulated using other lipid disrupting agents (Tween 20 and Brij 35) in culture. LPC, Tween 20 and Brij 35 all had different degrees of toxicity *in vitro*, and their respective lesion volume *in vivo* depended on the concentration injected. What's more, they revealed that since injected LPC is cleared by 24 hours post injection, endogenous LPC levels measured by mass spectrometry imaging increased significantly five days post injection. This was localised along the needle injection site accompanied by an increase in immunoregulatory lipid (phosphatidylinositol) reportedly capable of mitigating proinflammatory cytokine release. Surprisingly, they also report that LPC toxicity in culture can be buffered using bovine serum albumin which reduced toxicity by ten-fold suggesting one way in which endogenous LPC levels can be naturally neutralised *in vivo*. In contrast to the lack of phenotype presentation when LPC is injected dorsally, ventral LPC injections into the spinal cord can lead to mild paralysis in the hind limbs and uncoordinated gait control (Plemel et al., 2018).

4.3.3 Semliki forest virus: how can this model be optimized?

The semliki forest virus has been successfully used to generate a model of demyelinating encephalomyelitis in the central nervous system of mice (Mokhtarian et al., 2003). This study used the same strain of virus (The avirulent A774 strain of SFV) and the same strain of animals (C57/BL6 mice of either sex) with a similar age range (5-6 weeks old) compared to mice used in our experiment (9 week old male mice). Furthermore, the virus was inoculated intraperitoneally, thus is the same route of administration as used here. The dose of virus inoculated per mouse was lower (10^4 PFU) compared to (10^5 PFU) the dose used in our experiment. (Mokhtarian et al., 2003) reported initial inflammation characterized by the presence of microglia, the absence of symptoms indicative of infection along with the clearance of the virus within 6-8 days post injection, which was followed by the occurrence of initial demyelination in the cerebellum, brainstem and corpus callosum by days 15-21 post injection. Remyelination also occurred and was complete by day 35 post injection. Hence given the similarity of experimental design, excluding the minimal difference in doses given, the only explanation for the lack of infection seen in our experiment would be the loss of viral viability prior to its use since our mice did not exhibit any symptoms of pathology and the entirety of tissues analysed were devoid of the virus. It must be noted however, that this study did not use a fluorescently tagged virus; which presents a possible variable affecting the viability of the virus used in our experiment.

Acute encephalomyelitis mouse models have been generated for many years to investigate the pathogenesis of white matter diseases like multiple sclerosis through infecting the central nervous system with the A774 strain of the semliki forest virus (Fazakerley et al., 1983, Subak-Sharpe et al., 1993), although, various strains of mice were used other than C57Bl/6 mice. The semliki forest virus has also been shown to increase susceptibility to inducing experimental autoimmune encephalomyelitis in C57Bl/6 mice which are known to be less susceptible to the disease by inducing damage to the central nervous system tissue facilitating subsequent immunization (Mokhtarian and Swoveland, 1987). This study also employed similar methods of infection; A774 strain intraperitoneally injected (10^4 PFU) in 7-8 week old mice which resulted in a non-fatal infection of the central nervous system along with transient mild

paralysis and demyelination of the cerebellum. The induction of allergic experimental encephalalomyelitis in these mice was carried out by immunization with lymphocytes from myelin basic protein primed donors of SJL/J mouse strains. Interestingly, this method of EAE induction was not successful when administered to non-semliki virus infected wild type mice suggesting that virus induced damage facilitates the expansion of reactive lymphoid cells. Taken together, these studies justify the strain of mouse used, the strain of virus and route of administration. Thus in order to reproduce the inflammatory demyelinating disease to test our drugs, we must adjust the dose of inoculate administered *in vivo*, test for virus viability and infectivity *in vitro* prior to *in vivo* studies, and compare the viability of the virus with and without fluorescent tags.

The pathogenesis of the semliki forest virus in intraperitoneally inoculated C57Bl/6 mice constitutes an acute encephalitis phase that clears the virus within 8 days post infection from blood and brain. The immune-mediated demyelination occurs between days 14 and 21 post infection in the absence of viral persistence. This is then followed by remyelination by day 35 post infection (Mokhtarian et al., 2003). Once the virus crosses the blood-brain barrier it mainly infects neurons and oligodendrocytes (Atkins et al., 1999). Oligodendrocytes can either die as a result of virus infection or by being targeted by cytotoxic T cells following infection and expression of viral proteins (Atkins et al., 1999). The immune response in semliki forest virus infected mice include $CD8^+$ T cells and monocytes during the first 8 days of infection which decrease in expression before the start of demyelination. After the first week of infection, B cells begin to secrete immunoglobulins against the viral proteins (Mokhtarian et al., 2003). Remyelination is usually complete by day 35 post infection (Mokhtarian et al., 2003).

Chapter 5

The role of calcineurin-nuclear factor of activated T-lymphocytes pathway in spinal cord cell proliferation

5.1 Introduction

The calcineurin-nuclear factor of activated T lymphocytes (NFATs) pathway is involved in various roles including Schwann cell myelination, synaptic plasticity, neurotransmission, central and peripheral nervous system migration, proliferation and differentiation in the adult nervous system reviewed in (Kipanyula et al., 2016). The calcineurin / NFAT signalling pathway, although predominantly known as the calcium / calmodulin- dependent proinflammatory pathway is also implicated in the physiology of the developing and adult nervous system and neuropathologies (Gafter-Gvili et al., 2003, Graef et al., 2001, Kao et al., 2009, Heit et al., 2006). The α7nAChRs are known to have the greatest permeability to calcium compared to other nAChRs and have been shown to propagate cytoplasmic calcium signals by direct influx through activated receptors, indirect influx via voltage-dependent calcium channels that are activated by nAChR depolarization and through calcium-induced calcium release from the endoplasmic reticulum (ER) via the ryanodine receptors and inositol (1,4,5)-triphosphate receptors that can be initiated by either direct or indirect calcium influx (Shen and Yakel, 2009). Activation of the ligand gated, calcium permeable α7nAChRs can lead to downstream events that activate cellular signalling cascades. For signalling pathways involved downstream of α7nAChR activation refer to chapter 1 (section 1.14). The focus of this study is to investigate whether one such signalling pathway is the calcineurin / NFAT signalling cascade and if it has a role in mediating cell proliferation in the spinal cord. Furthermore, to explore which nAChRs mediate the proliferative response and to what extent α7nAChRs mediate cell proliferation, organotypic spinal cord experiments in the presence of donepezil alone, donepezil and the specific muscarinic antagonist atropine, donepezil in the presence of both atropine and the specific non-α7nAChR antagonist (DHβE) and organotypic slices cultured with the partial nicotinic agonist cytisine and DHβE were carried out.

Calcineurin is known as protein phosphatase B; a calcium / calmodulin dependent serine / threonine phosphatase (Ho et al., 1976). Immunohistochemistry studies revealed that calcineurin isoforms are highly expressed by sensory neurons such as the corticohypothalamic pyramidal cells, by peripheral Schwann cells,

although, not by central oligodendrocytes (Bram et al., 1993, Usuda et al., 1996, Vihma et al., 2016). It is also present in SGZ and SVZ neurons (Graef et al., 1999, Quadrato et al., 2012, Serrano-Perez et al., 2015). Calcineurin is generally activated by the increase in intracellular calcium levels which in turn leads to the dephosphorylation of the normally phosphorylated NFAT proteins present in the cytoplasm and promotes nuclear translocation leading to NFAT-dependent gene transcription.

In contrast, the phosphorylation by protein kinases such as PKA, GSK3 or p38 retains NFAT proteins in the cytoplasm in an inactive state (Graef et al., 2001), figure 5.1. Although NFAT proteins (NFATc1-c4) are expressed by most immune cells and have an important role in the transcription of cytokine genes and other genes related to the immune response (Rao et al., 1997), they also have various roles within a variety of cells in the cardiovascular system, skeletal muscle, bone and nervous system (Graef et al., 2001).

NFAT proteins are generally activated by the stimulation of receptors coupled to calcium mobilization such as antigen receptors on T and B cells and receptors coupled to G proteins (Rao et al., 1997), ligand gated ion channels and calcium permeable tyrosine kinase receptors (Kipanyula et al., 2016). Since calcineurin is an upstream regulator for NFAT proteins, they are targets for the immunosuppressive drugs FK-506 and cyclosporine A (Rao et al., 1997), figure 5.1.

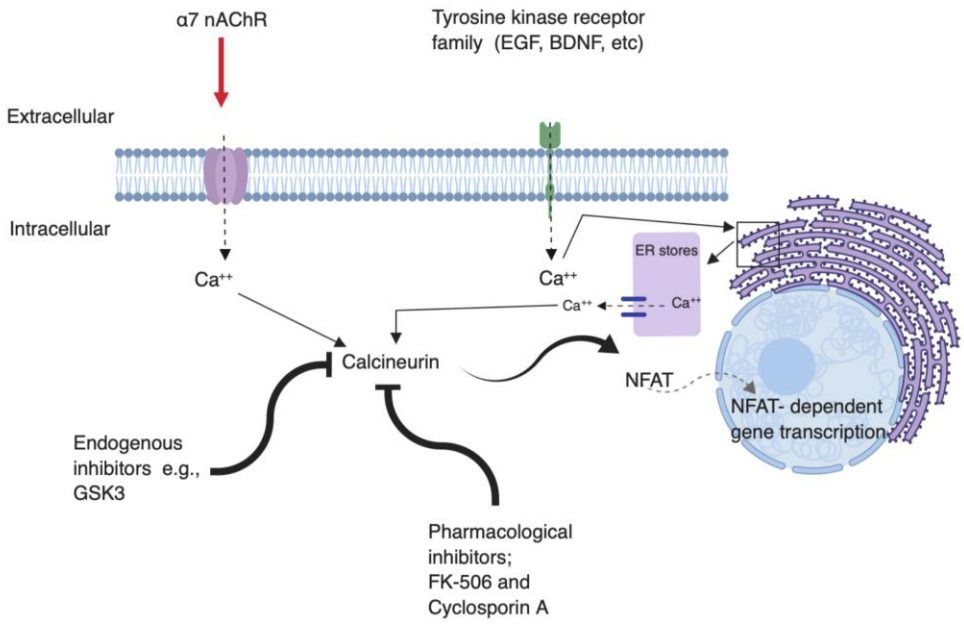

Figure 5.1 The calcineurin- NFAT calcium dependent signalling pathway. Increased intracellular calcium concentrations through ligand gated ion channels or induced via neurotrophins through their tyrosine kinase receptors leads to the activation of calcineurin and subsequent dephosphorylation of NFAT in order to translocate into the nucleus. The genes transcribed and overall effect of this signalling pathway are cell type and microenvironment dependent; reviewed in (Kipanyula et al., 2016). Figure created with BioRender.

In the adult mouse brain, the NFAT isoform NFATc4 was immunolocalised in various areas, including the hippocampus (Bradley et al., 2005). NFAT proteins (specifically NFAT c4 / NFAT3) translocate into the nucleus of hippocampal neurons and activate gene transcription which may affect synaptic plasticity and memory formation (Graef et al., 1999). Using in situ hybridization, endogenous NFATc4 RNA was located in both mouse and rat hippocampal cultures. Culture transfection with a reporter plasmid in which NFAT binding sites control the expression of a green fluorescent protein (GFP) revealed that activation of L-type voltage-gated calcium channels through NMDA receptors or after depolarization led to the cytoplasmic translocation of NFATc4 / NFAT3 to the nucleus in CA3 / CA1 cultured hippocampal

neurons and subsequent activation of NFAT-dependent gene transcription (Graef et al., 1999). Furthermore, GSK-3 can phosphorylate NFATc4 and export it from the nucleus of these hippocampal cells, thus reducing the NFATc4 dependent transcription (Graef et al., 1999).

5.1.1 The role of calcineurin-NFAT signalling in SGZ neurogenesis

The calcineurin-NFAT signalling pathway has been shown to regulate the expression of a transcription factor known as (Tox) during mammalian corticogenesis (Artegiani et al., 2015). Tox was transiently downregulated in neural stem cells during division and reinduced in newborn progenitor neurons. The role of Tox in the nervous system is still not well characterized, however, Artegiani and colleagues employed immunohistochemistry against Tox using antibodies on E 9.5 mice and found that the expression of the transcription factor coincides with the expression of the progenitor and proliferative marker Sox2 and is confined to the germinal layer of the SVZ but is absent in the SGZ (Artegiani et al., 2015). Furthermore, they injected the highly specific calcineurin inhibitor cyclosporin A into pregnant mice and collected embryos (E.13.5) 48 hours later. They showed by means of western blot analysis that a 30% decrease in Tox expression occurred in cyclosporin A treated mice; indicating that it may be indirectly regulated by calcineurin. Moreover, Tox overexpression (by means of electroporation in E 13.5 gestational mice together with BrdU administration to label proliferated cells) resulted in a doubling in the number of dendritic processes of newly born neurons (Artegiani et al., 2015). Thus, calcineurin can indirectly regulate cortical proliferation and neurite outgrowth.

Intraperitoneal injections of cyclosporine A in C57/Bl6 adult mice did not affect mature neurons or glial cells in the dentate gyrus in the context of damage or apoptosis, however, it significantly reduced neural stem cell proliferation. There were also fewer neuroblasts in the cyclosporine A treated animals indicating reduced neuronal differentiation. Furthermore, they report that the few cyclosporine A treated animal-derived neuroblasts exhibited significantly reduced dendritic outgrowth and complexity compared to vehicle treated animals (Hwang et al., 2010).

NFATc4 may also be required for BDNF-dependent survival of hippocampal adult- born neurons and spatial memory formation (Quadrato et al., 2012). These researchers compared the number of DCX positive adult born neurons throughout the entire dentate gyrus of adult C57/Bl6 and NFATc4 null mice which revealed a significant reduction in DCX positive cells in NFATc4 null mice compared to wild type controls. This was also accompanied by a significant reduction in PSA-NCAM positive cells (an antigen expressed by migrating neuroblasts) in NFATc4 null mice. Additionally, intraperitoneal delivery of cyclosporin A caused a significant reduction in the number of DCX positive adult born neurons in wild type mice treated with this highly specific calcineurin inhibitor which phenocopied what was seen in NFATc4 null mice (Quadrato et al., 2012). Interestingly however, intraperitoneal administration of cyclosporin A to both wild type and NFATc4 null mice revealed no difference in the number of BrdU positive cells; indicating that NFATc4 is not required for hippocampal neural precursor cell proliferation. A similar observation was concluded after culturing hippocampal neurospheres obtained from wild type and NFATc4 null mice or after treatment with cyclosporine A (Quadrato et al., 2012). However, tracing the survival of BrdU positive cells in wild type and NFATc4 null mice 21 days after BrdU administration revealed that the number of BrdU positive cells in the dentate gyrus of NFATc4 null mice was significantly reduced indicating that NFATc4 is required for hippocampal neuronal survival. To test whether NFATc4 affects fate commitment, they traced and measured the percentage of BrdU positive cells that were immunoreactive for the neuronal marker NeuN, 21 days after BrdU administration. They showed that the majority of progenitor cells in both wild type and NFATc4 null mice had committed to the neuronal lineage; thus NFATc4 does not influence neuronal verses glial differentiation. These results were reproduced by culturing neurospheres from wild type mice, wild type mice treated with cyclosporine A and NFATc4 null mice. They also cultured hippocampal neural precursor cells and found by means of immunohistochemistry that NFATc4 is expressed by differentiating neural precursor cells at 1 day *in vitro*. They then showed that the addition of BDNF to cultures significantly enhanced NFATc4 gene expression via both qPCR analysis and immunohistochemistry. Consistently, the addition of cyclosporine A to cultures blocked the BDNF-dependent events (Quadrato et al., 2012). To verify if the pro-survival effects were mediated through a BDNF-dependent NFATc4 mechanism, they measured and

compared the percentage of cell apoptosis between cultures derived from wild type and NFATc4 null mice in the presence of BDNF. This confirmed that BDNF rescues cells from apoptosis in wild type cells as opposed to NFATc4 null mice derived cells. Furthermore, in a series of learning and memory behavioural studies, NFATc4 was concluded to be important in spatial long-term memory but not contextual long-term memory in fear conditioning tasks (Quadrato et al., 2012).

5.1.2 The role of calcineurin-NFAT signalling in SVZ neurogenesis

The NFAT proteins also play major roles in survival, proliferation and differentiation of subventricular- derived neural precursor cells in culture (Serrano-Perez et al., 2015). This study used newborn mice with highly active neural precursor cells and two month old NFAT-luciferase mice as a reporter for NFAT activation. In all brain regions evaluated (cortex, hippocampus, subventricular zone and olfactory bulb), there was luciferase activity in all animals, although it was higher in young animals compared to older ones; indicating that NFAT signalling is active in the immature and adult brain with a potential developmental role in neural precursor cell maturation (Serrano-Perez et al., 2015) which was not investigated. NFATc1, c2, c3 and c4 mRNA was detected in neuroblasts derived from neural precursor cells isolated from the SVZ of 1 day old C57/Bl6 mice by means of qPCR analysis. These neuroblasts expressed the NFAT isoforms in a heterogeneous manner. The functionality of these NFAT proteins was tested through by evaluating the expression of RCAN1-4 (a specific NFAT target after NFAT activation) in neurosphere cultures after the addition of inomycin to increase intracellular calcium concentrations. This resulted in activated NFAT transcription and upregulation of RCAN1-4 expression which provided evidence of NFAT functionality in cultured neuroblasts. They then assessed the effect of NFAT on neurosphere density and proliferation. Neurospheres were cultured for 20 days prior to the addition of the highly specific NFAT activity inhibitor (VIVIT), which competes with calcineurin for binding NFAT. It was added to cultures at three different concentrations (1,2 and 4 µM) which resulted in a dose dependent reduction in the size of neurospheres 4 days after treatment (Serrano-Perez et al., 2015). The reduction in neurosphere size was due to a reduction in cell density; this was confirmed by neurosphere dissociation and subsequent treatment with VIVIT at (1,2 and 4 µM) that

recapitulated the dose dependent reduction in neural precursor cells. This was also mimicked by the use of other NFAT inhibitors (FK-506 and cyclosporine A). To identify if the decrease in density was due to a decrease in proliferation, they cultured neurospheres in proliferation medium in the presence of VIVIT (1 and 4 μM) and after the administration of Ki67 and BrdU. This resulted in a significant decrease in the BrdU positive population in a dose dependent manner along with an increase in the percentage of cells in the G0/1 phase and a reduction in cells at the S phase. This suggests that NFAT inhibition affects cell cycle progression and reduces proliferation (Serrano-Perez et al., 2015). The reduction in cell density after NFAT inhibition by VIVIT was also due to an increase in cell death and reduced survival shown in cultures in the presence of VIVIT by subsequently staining with propidium iodide (PI) - a small fluorescent stain that binds DNA of damaged plasma membranes of dead cells. Furthermore, the inhibition of NFAT by VIVIT (4 μM) in cultured neurospheres under differentiating conditions revealed that the inactivation of NFAT proteins caused a decrease in the distance travelled by cells from the neurospheres prior to undergoing differentiation. The inhibition of NFAT by VIVIT under neuronal differentiation conditions (in the presence of BDNF and the absence of EGF and bFGF to induce adhesion and differentiation) significantly decreased the percentage of immature neurons. Moreover, under these differentiation conditions; there was a significant reduction in the percentage of GFAP positive astrocytes derived from these neurospheres. In a third set of experiments to promote oligodendrocyte differentiation, there was no effect on the percentage of O4 positive cells (Serrano-Perez et al., 2015). Interestingly, the adenoviral transduction of NFATc3 expression in cultured neurospheres decreased proliferation, promoted cell adhesion, migration and differentiation (Serrano-Perez et al., 2015). Therefore, this study elucidates the regulation of NFAT transcription on SVZ neural precursor cell proliferation, migration and differentiation. Taken together, these results suggest the involvement of NFAT-mediated gene expression in hippocampal and subventricular cell survival, proliferation and differentiation.

5.1.3 The role of different calcium sources in cell proliferation

Somasundaram and colleagues sought to identify the role of calcium release activated calcium channels (CRAC) in neural precursor cell proliferation by means of pharmacology and transgenic mouse models lacking the CRAC channel proteins or expressing non-functional channels (Somasundaram et al., 2014). CRAC channels are generally activated by stimulation of cell surface receptors coupled to the phospholipase C signaling pathway, and EGF acts through activating tyrosine kinase receptors which stimulates phospholipase C and inositol triphosphate (IP3) signaling leading to the depletion of endoplasmic reticular calcium stores. They showed that both embryonic and adult mouse neural precursor cells exhibit store operated calcium entry (SOCE) mediated by CRAC channels. This was concluded by culturing neural precursor cells of C57Bl/6 mouse SVZ at three different stages of development; embryonic (E13 - E18), postnatal (P0 – P2, P5) and adult mice. All cultures were treated with (Thapsigargin) – an inhibitor of the endoplasmic reticulum calcium ATPase in calcium free Ringer's solution to evaluate the calcium influx after the addition of extracellular calcium. This identified the prominent presence of SOCE in neural precursor cells derived from all stages of development tested; and thus conserved throughout development. The inhibition of CRAC channels with specific channel inhibitors in culture reduced the release of SOCE indicating that calcium entry is mediated through the CRAC channels. Western blot analysis showed the presence of canonical CRAC channel proteins STIM1 and Orail; the absence or suppression of either canonical protein (use of transgenic mice) results in the loss of SOCE in neural precursors, and more importantly, caused a decrease in neural precursor cell proliferation in culture and in the subventricular zone of adult mice *in vivo*. Moreover, both acetylcholine (via muscarinic receptors) and epidermal growth factor (EGF) activated SOCE which stimulated gene transcription through calcineurin / NFAT signaling (Somasundaram et al., 2014). These results present an additional channel mediating calcium entry in neural precursors with a role in cell proliferation and a potential cholinergic target to manipulate in the context of neurogenesis.

5.1.4 Hypothesis

This chapter focuses on identifying whether the proliferative response of spinal cord stem / progenitor cells (ependymal and oligodendrocyte precursor cells respectively) is mediated via α7nAChRs, other nicotinic receptor subtypes or a mixture of both. Furthermore, we assess if the calcineurin / NFAT calcium signaling pathway downstream of activation of α7nAChRs play a role in spinal cord cell proliferation. This is of potential interest since we know that the adult spinal cord contains stem and progenitor cells capable of responding to injury and demyelination, however, there is still a lack of understanding in regards to what cellular signalling pathways influence their contribution to proliferation. Since both the ependymal cells lining the central canal and oligodendrocyte precursor cells express the α7nAChRs (Corns et al., 2015, Vélez-Fort et al., 2009, Rogers et al., 2001), it is possible for the calcineurin / NFAT calcium signaling pathway to play a role in both instances.

5.1.5 Aims

Thoracic and lumbar spinal cord organotypic slice culture experiments were established to evaluate the extent of cell proliferation under four different conditions; elevated levels of endogenous acetylcholine by adding donepezil in culture, elevated acetylcholine levels with donepezil and inhibiting muscarinic receptors with atropine, the addition of both donepezil and atropine in the presence of the non-α7nAChR inhibitor DHβE and a culture condition focusing on the role of α7nAChRs by adding the nicotinic acetylcholine receptor agonist cytisine in the presence of the non-α7nAChR inhibitor DHβE, figure 5.2.

Organotypic thoraco-lumbar spinal cord sections were also used to evaluate the effect of inhibiting calcineurin with two different calcineurin specific inhibitors; FK-506 and cyclosporine A in the presence of the α7nAChR positive allosteric modulator PNU-120596. The thymidine analogue EdU was administered in culture to label newly proliferated cells.

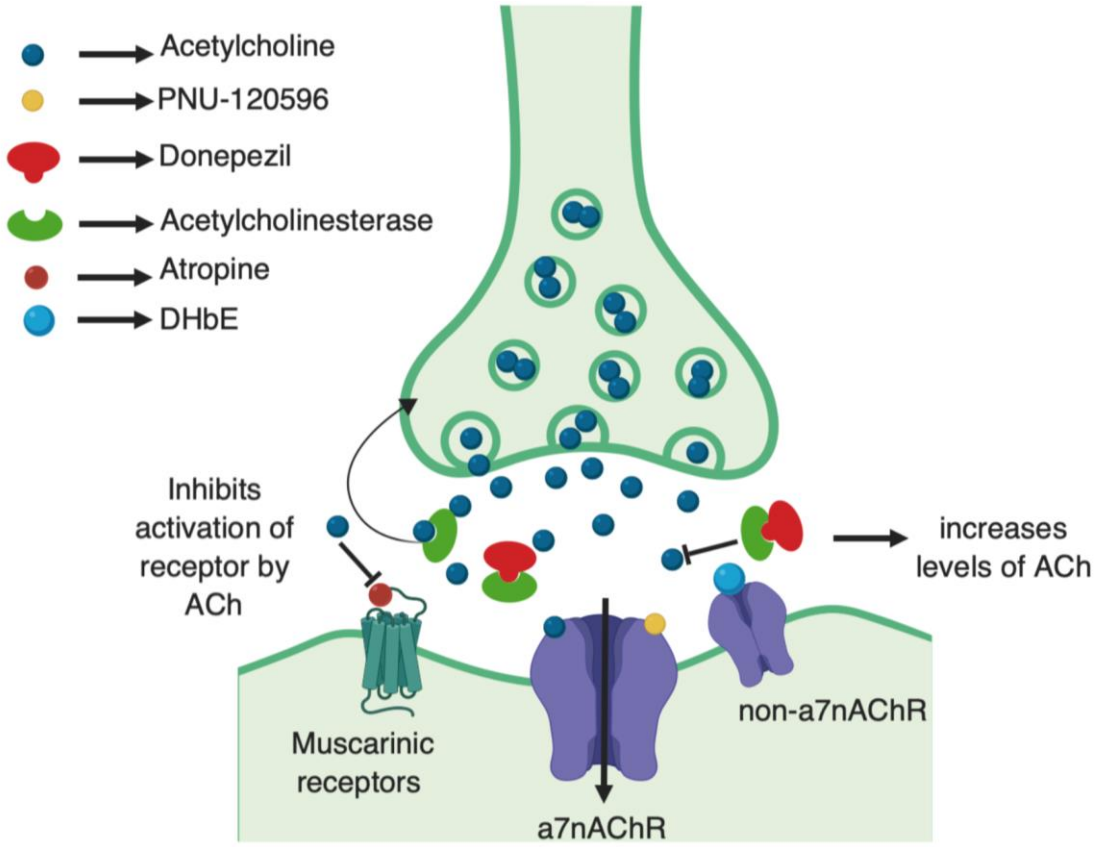

Figure 5.2 The effects of donepezil, PNU-120596, atropine and DHβE on cholinergic signalling.

ACh acts on both nicotinic and muscarinic receptors including the prevalent α7nAChR which mediates ion influx and action potential propagation. ACh is recycled back to the presynaptic terminal via the acetylcholinesterase enzyme which can be inhibited by donepezil; increasing endogenous levels of synaptic ACh. PNU-120596 increases the affinity of ACh for the active state receptor allowing for increased channel opening and ion influx. Atropine is a non-selective muscarinic inhibitor and DHβE is a selective non-α7nAChR antagonist. Figure created with Biorender.

5.2 Results

5.2.1 The α7nAChRs mediate a high proportion of the proliferative response to donepezil in adult spinal cord cultures

The numbers of EdU positive cells in the central canal of slices treated with donepezil + atropine, donepezil + atropine + DHβE and cytisine + DHβE were all significantly ($P < 0.0001$) higher than the extent of proliferation observed in slices treated with donepezil alone, figures 5.3 and 5.4 A and table 5.1. Furthermore, the numbers of EdU positive cells in slices cultured in the presence of donepezil + atropine + DHβE were significantly higher than the number of EdU positive cells in either donepezil + atropine or cytisine (the partial nicotinic receptor agonist) + DHβE ($P < 0.0001$), figures 5.3 and 5.4 A. Exact values are listed in table 5.1.

In the grey and white matter of spinal cord slices, donepezil + atropine treated slices had significantly ($P = 0.038$) higher numbers of EdU positive cells than donepezil alone treated slices. However, there were no significant differences between donepezil + atropine + DHβE and cytisine + DHβE treated slices compared to the donepezil alone condition, figures 5.3 and 5.4 B. The exact values are listed in table 5.2.

Condition	Donepezil	Don + atropine *	Don + atropine + DHβE *	Cytisine + DHβE *
Mean ± SE of difference	58 ± 8.7	126.2 ± 6.3	213.6 ± 13.1	123.6 ± 7.9
Animal and slice number	N = 3, n = 11	N = 3, n = 12	N = 3, n = 12	N = 3, n = 12

Table 5.1 The average number of EdU positive cells in the central canal of 300 µm thick cultured slices.

Here a Tukey's multiple comparison test following one-way Anova was conducted; comparing the mean of each condition with the other. All P values were < 0.0001.

Condition	Donepezil	Don + atropine *	Don + atropine + DHβE *	Cytisine + DHβE
Mean ± SE of difference	2411 ± 744.7	3958 ± 301.2	3843 ± 209.2	3406 ± 185.0
Animal and slice number	N = 3, n = 11	N = 3, n = 12	N = 3, n = 12	N = 3, n = 12
P value	------	0.0386	0.0598	0.2511

Table 5.2 The average number of EdU positive cells in the grey and white matter of 300 µm thick cultured slices.

The P value of each condition was generated in a Dunnett's multiple comparison test following one-way Anova. Here each condition was compared to donepezil alone.

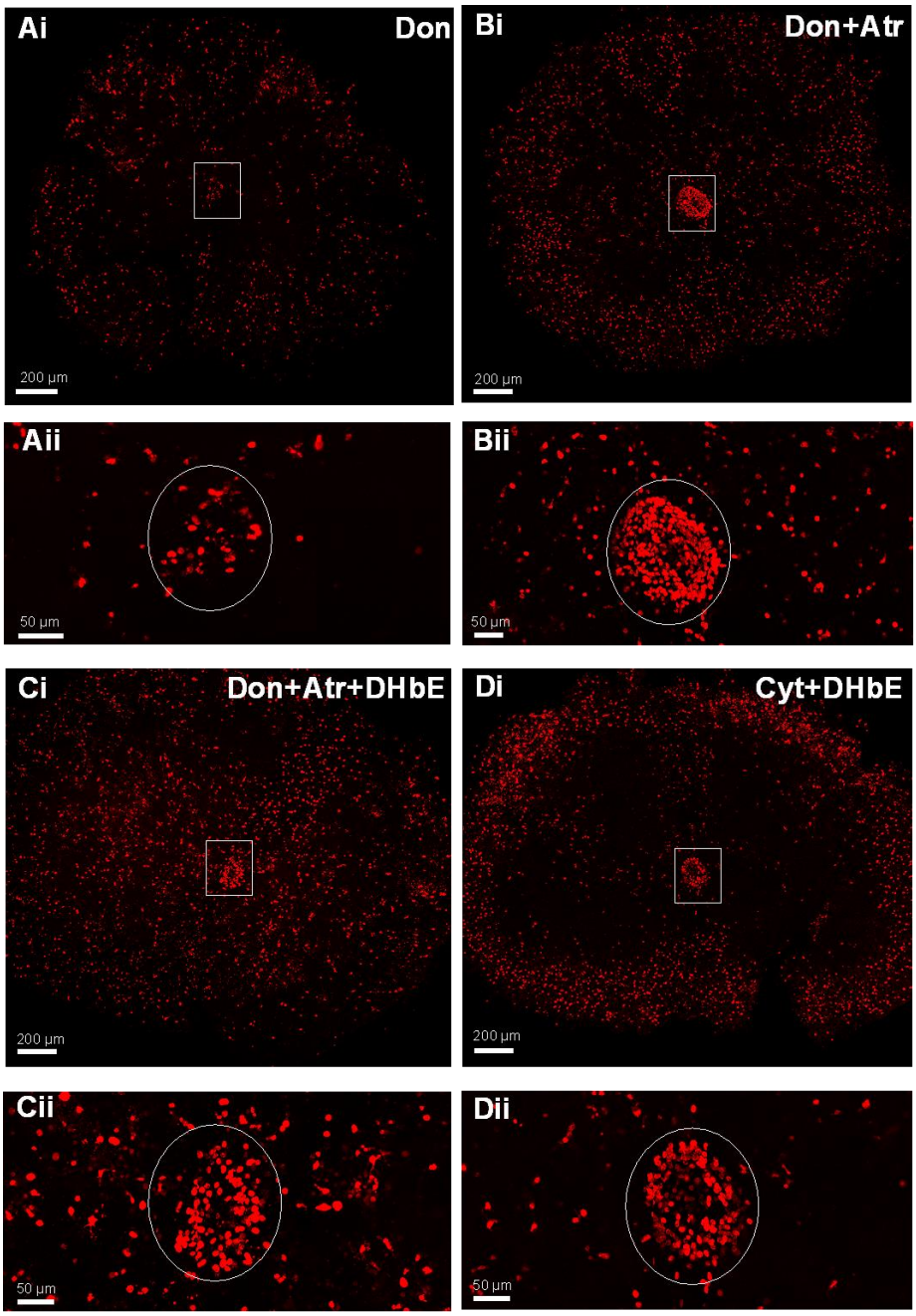

Figure 5.3 Organotypic spinal cord slices cultured in the presence of specific exogenous compounds.

Ai-Dii) Representative images of EdU positive cells in the central canal, grey and white matter of cultured spinal cord slices. **Ai)** Represents EdU^+ cells in a donepezil treated slice. **Bi)** Represents EdU^+ cells in a donepezil+ atrtopine treated slice. **Ci)** Represents EdU^+ cells in a donepezil+ atrtopine+DHβE treated slice. **Aii-Dii** Depict the central canal of each respective panel. Atrtopine = non-selective muscarinic antagonist. Donepezil= acetylcholinesterase inhibitor. DHβE= non-α7nAChR antagonist.

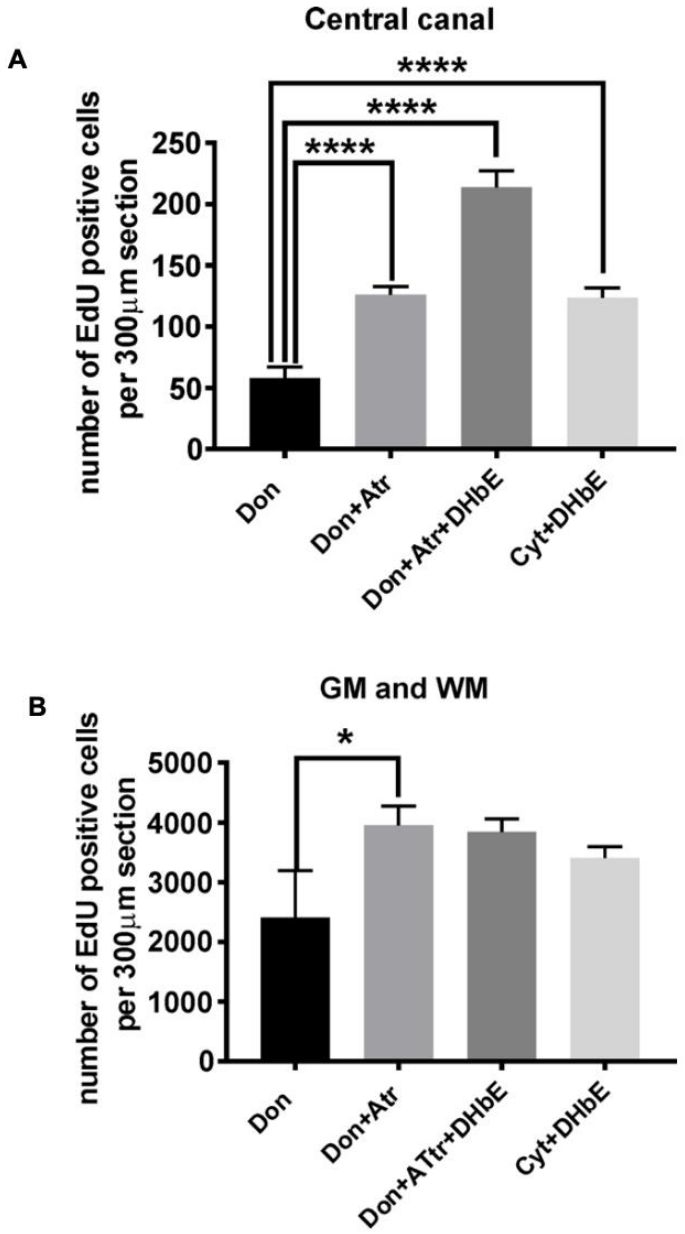

Figure 5.4 The α7nAChRs mediate the largest proportion of cell proliferation in the grey and white matter of the spinal cord.

A) Number of EdU labelled cells for each of the conditions. Data are shown as mean number of EdU labelled cells per 300 µm section ± SEM in the central canal of thoraco-lumbar spinal cord cultured slices. **B)** Number of EdU labelled cells for each of the conditions in the grey and white matter of thoraco-lumbar cultured spinal cord slices. ****; P <0.0001, *; P =0.038.

5.2.2 α7nAChRs may activate the calcineurin-NFAT signalling pathway to increase cell proliferation in the spinal cord

The number of EdU positive cells in the central canal of PNU-120596 and the calcineurin inhibitor cyclosporin A is significantly (P = 0.0190) lower than that observed in the PNU-120596 alone treated condition, figure 5.5. There was no significant change between PNU-120596 alone treated slices and those treated with PNU-120596 and FK-506 in the central canal. In the grey and white matter regions of cultured slices however, there were significantly (P < 0.0001) fewer EdU positive cells in the slices treated with either PNU-120596 and cylosporin A or PNU-120596 and FK-506 compared to PNU-120596 alone, figure 5.6.

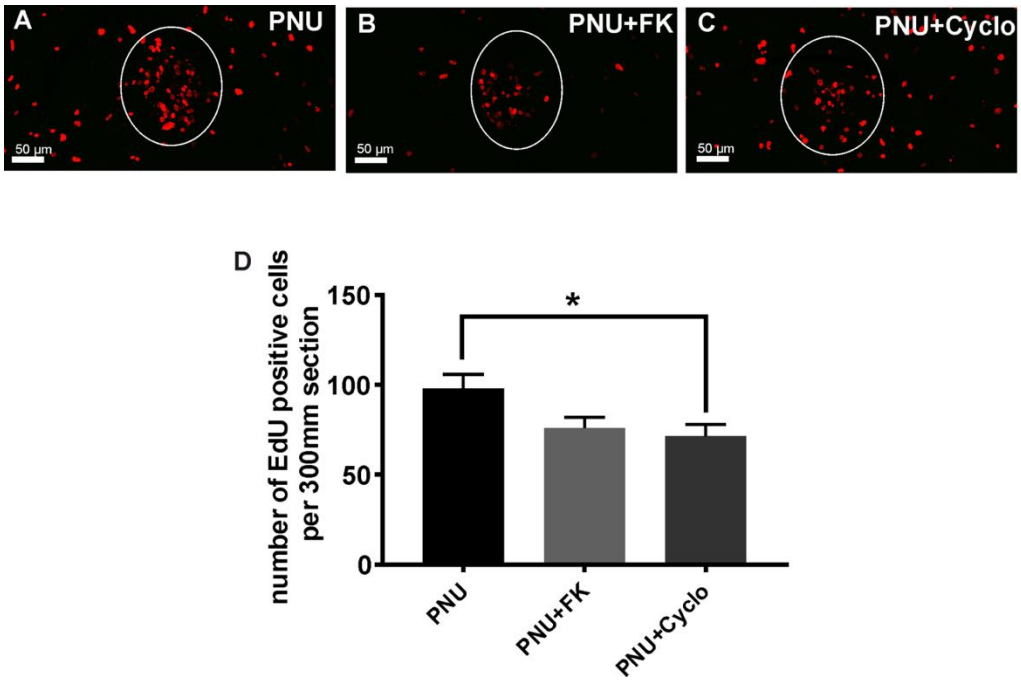

Figure 5.5 Slices cultured in the presence of PNU-120596 and cyclosporin A had significantly fewer proliferative cells in the central canal compared to PNU-120596 alone.

A) Representative image of EdU positive cells in the central canal of PNU-120596 alone treated slices. **B)** EdU positive cells in the central canal of PNU-120596 and FK-506 treated slices. **C)** EdU positive cells in the central canal of PNU-120596 and clyclosporin A treated slices. **D)** Number of EdU labelled cells for each of the conditions. Data are shown as mean number of EdU labelled cells per 300 µm section ± SEM in the central canal. *; P= 0.0190. PNU+FK had a P value of 0.0597.

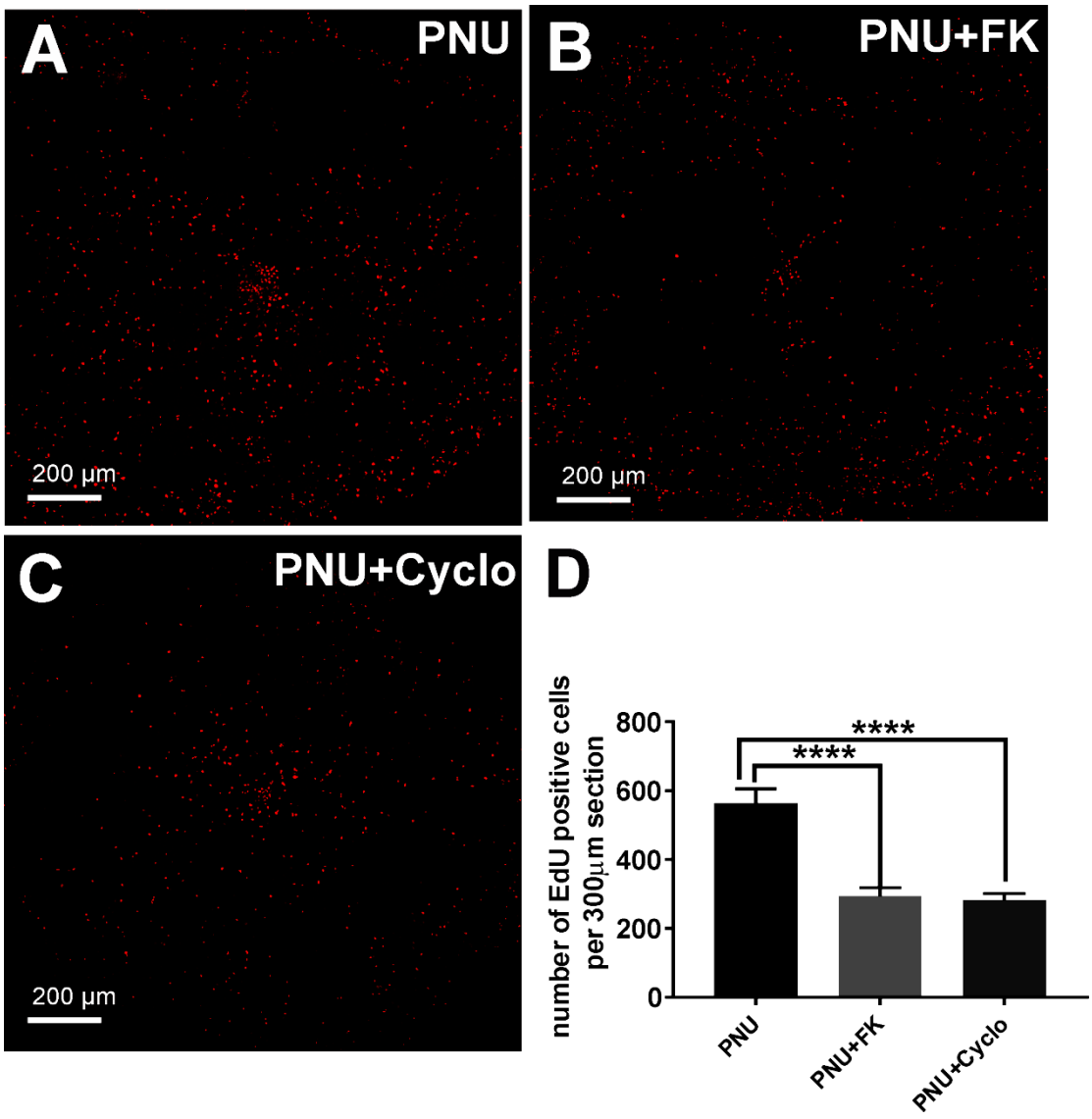

Figure 5.6 Slices cultured with PNU-120596 and either of the calcineurin inhibitors had significantly fewer EdU positive cells compared to those cultured with PNU-120596 alone.

A) Representative image of EdU positive cells in the grey and white matter of a PNU-120596 alone treated slice. **B)** Representative image of EdU positive cells in the PNU-120596 and Fk-506 treated slice. **C)** Representative image of EdU positive cells in the PNU-120596 and cyclosporin A treated slice. **D)** Number of EdU labelled cells for each of the conditions. Data are shown as mean number of EdU labelled cells per 300 µm section ± SEM in the grey and white matter of thoraco-lumbar spinal cord slice. ****; $P < 0.0001$.

5.3 Discussion

The *in vitro* experiments conducted in this chapter show that activating specific nicotinic acetylcholine receptors; especially α7nAChRs, enables the normally quiescent, self-renewing ependymal cells lining the central canal of the spinal cord to proliferate extensively and demonstrate potential plasticity. More importantly, we show for the first time that the calcineurin-NFAT calcium signalling pathway downstream α7nAChRs plays a role in spinal cord cell proliferation. Such findings are particularly critical not only to enable or enhance cell proliferation, but to also provide a means by which to suppress unwanted or detrimental cell proliferation responses.

5.3.1 Both α7nAChRs and non-α7nAChRs mediate cholinergic responses *in vitro*

Here, organotypic slice culture experiments of thoraco-lumbar spinal cord regions entailed the use of the nucleoside analogue EdU as a marker for cell proliferation, together with the application of donepezil to enhance endogenous levels of acetylcholine alone or in combination with the muscarinic receptor antagonist (atropine), nicotinic receptor activation with the partial nicotinic receptor agonist cytisine and the non-α7nAChRs antagonist (DHβE) or the addition of donepezil + atropine + DHβE.

In all regions of the spinal cord, cultured spinal cord slices in the presence of donepezil and the non-selective muscarinic receptor antagonist (atropine) had significantly more EdU positive cells compared to donepezil alone treated slices. This indicates that most of the proliferative response is mediated through nicotinic acetylcholine receptors, and potentially suggests an inhibitory role for muscarinic receptors in cell proliferation. This needs further elucidation; potentially by culturing spinal cord slices in the presence of nicotinic receptor antagonists and muscarinic receptor agonists. Furthermore, the central canal of slices treated with donepezil, atropine and the non-α7nAChR antagonist DHβE (with highest antagonism selectivity for α4 and β2 receptor subunits) had the highest number of EdU positive cells when

compared to donepezil alone, donepezil + atropine and cytisine + DHβE treated slices. This indicates that α7nAChRs are the receptors with the most prominent role in ependymal cell proliferation. These results are consistent with *in vivo* and *in vitro* α7nAChRs potentiation with PNU-120596 in C57/Bl6 mice and spinal cord cultures respectively (Corns et al., 2015) and my observations in chapter 3 on the extent of cell proliferation observed as a result of *in vivo* administration of donepezil and PNU-120596. However, since exogenous nicotinic acetylcholine receptor activation using cytisine had a smaller effect on cell proliferation compared to the increase in endogenous acetylcholine levels in slices treated with donepezil + atropine + DHβE; this may indicate an inhibitory role for non-α7nAChRs or may be attributed to the fact that cytisine is only a partial nicotinic agonist. Future experiments with a focused investigation on non-α7nAChRs and spinal cord cell proliferation is essential.

Taken together, these results revealed that the majority of the cholinergic proliferative response is mediated via α7nAChRs which indicates that the extent of cell proliferation in the central canal, grey and white matter can be modulated significantly through α7nAChRs. Given that oligodendrocyte precursor cells express this receptor subtype (Vélez-Fort et al., 2009, Rogers et al., 2001) in addition to ependymal cells of the central canal (Corns et al., 2015), either or both of these cell types may be undergoing cell proliferation.

5.3.2 The calcineurin-NFAT calcium signalling pathway plays a role in spinal cord cell proliferation

Given that spinal cord cell proliferation is largely mediated through the calcium permeable α7nAChRs, it is crucial to identify the downstream cellular pathways involved in this process. Here we describe one potential pathway with a role in spinal cord cell proliferation, however, whether this pathway affects cell survival or differentiation among other functions remains to be elucidated. Furthermore, the identity of NFAT isoforms mediating these responses in spinal cord cells, as well as, the full identity and role of gene transcription through calcineurin / NFAT signaling in this niche is still unknown.

Our experiments employed the use of cyclosporin A (a natural immunosuppressant) and FK-506, also known as tacrolimus (an antibiotic) that are structurally unrelated but have similar modes of action (Griffith et al., 1995). They bind their respective binding proteins (FK binding protein FKBP and the cytosolic protein cyclophilin) forming complexes that inhibit calcineurin which is normally activated via calmodulin upon increased intracellular calcium and are responsible for the dephosphorylation and translocation of NFAT into the nucleus for gene transcription (Kipanyula et al., 2016).

Here we show that the inhibition of calcineurin with a selective inhibitor (cyclosporine A) in the presence of the α7nAChR positive allosteric modulator PNU-120596 lead to a significantly lower number of EdU positive cells in all regions of the spinal cord compared to slices treated with PNU-120596 alone. Slices cultured in the presence of PNU-120596 and FK-506, had significantly fewer EdU positive cells in the grey and white matter of the spinal cord compared to slices treated with PNU-120596 alone. These results suggest that this particular pathway is involved in cell proliferation of cells expressing the α7nAChRs in the spinal cord niche.

Cell membrane signalling results in the formation of NFAT transcription complexes and gene activation in a cell-type dependent manner (Graef et al., 2001, Graef et al., 1999), and NFAT transcription complexes play various regulatory roles in neuronal maturation, survival, proliferation, neurotransmission, synaptic plasticity, outgrowth of embryonic axons, apoptosis and beneficial inflammatory responses reviewed in (Kipanyula et al., 2016, Graef et al., 2003). In order to better understand the full involvement of these transcription complexes in the spinal cord niche, experiments identifying the presence and functionality of each NFAT isoform could be carried out *in vivo* by employing NFAT isoform-specific mutant animals, or alternatively, by immunohistochemical identification of NFAT isoforms in culture given the availability of isoform-specific monoclonal antibodies. To assess whether the identified isoforms have functional roles; FoxJ1 reporter mice and ependymal cell derived neuroblasts could be cultured in the presence and absence of a calcineurin inhibitor to assess the potential role of NFAT transcription on cell survival. To assess the potential role in cell

differentiation; immunohistochemical markers to label the three neuronal cell types at different developmental stages could be used on ependymal cell derived neuroblasts in culture both in the presence and absence of a calcineurin inhibitor. This may help determine whether the calcineurin-NFAT signaling pathway is involved in promoting differentiation or if it favors cell turnover or proliferation. In addition, it may shed light on whether specific NFAT transcripts drive differentiation down a specific cell lineage.

Moreover, given that BDNF signalling through tyrosine kinase neurotrophic receptors is required to activate the calcineurin-NFAT signaling pathway upon increasing intracellular calcium for newborn neuronal cell survival in the dentate gyrus (Quadrato et al., 2012), it is plausible for extrinsic neurotrophins to contribute to spinal cord neurogenesis through calcineurin activation.

Since the lack of sufficient neurotrophic factors is one contributing factor to reduced endogenous spinal cord regeneration after injury (Widenfalk et al., 2001), and certain neurotrophins elicit beneficial effects when exogenously administered after spinal cord injury (Widenfalk et al., 2001, McTigue et al., 1998); identifying the mechanism of action of potential neurotrophic factors contributing to enhanced spinal cord recovery is essential.

5.3.3 The effects of neurotrophic factors on cell proliferation after injury and axonal outgrowth

Cultured primary neurons require the calcineurin-NFAT signalling pathway which is stimulated by neurotrophins and netrins for axonal outgrowth (Graef et al., 2003). This study demonstrated that netrin-1 (a laminin-related protein involved in axonal guidance and growth) can directly activate endogenous NFAT- dependent transcription in (E15.5) cultured cortical neurons which was successfully blocked by either FK-506 or clyclosporin A treatment resulting in impaired axonal outgrowth (Graef et al., 2003). Little axonal outgrowth were observed in neurons isolated from the trigeminal ganglion of (E10.5) NFATc2,3,4 triple mutant mice and cultured onto a collagen matrix or when wild type neurons were cultured in the presence of cyclosporin A compared to neurons

isolated from wild type mice. Additionally, when dissociated trigeminal neurons of triple mutant mice embryos were cultured on laminin in the presence of nerve growth factor (NGF) and fibroblast producing neurotrophin-3 (NT-3); fewer axons of shorter length were seen compared to wild type- derived neurons. The importance of calcineurin was further demonstrated in wild type-derived cultures in the presence of cyclosporin A; these exhibited similar outgrowth defects to cultures derived from triple mutant mice (Graef et al., 2003). Therefore, this study demonstrates the significance of NFAT gene transcription in neurotrophin-dependent axonal outgrowth independent of cell survival or differentiation.

In adult rat spinal cord dorsal contusion studies carried out at the eighth thoracic level (McTigue et al., 1998), NT-3, BDNF, CNTF, NGF and bFGF neurotrophin producing grafts or control beta-galactosidase grafts were transplanted subacutely into contused areas of the spinal cord and compared 10 days later. The grafts (implanted 2 days after injury) consisted of fibroblasts engineered to produce NT-3, BDNF, CNTF, NGF, bFGF or beta-galactosidase filling the cavity produced by the contusion. Significantly more axonal fibres were seen in NT-3 and BDNF grafts suggesting enhanced recovery demonstrated by more neurofilament labelled axons than other grafts. Moreover, they showed that the extent of myelination of axons was higher in NT-3 and BDNF grafts compared to all others (McTigue et al., 1998). They observed the effect of the various neurotrophins on the level of myelination in unmyelinated growing axons by double immunohistochemical labelling for MBP and neurofilament. The beta-galactosidase grafts had only a few axons ensheathed by myelin indicating low endogenous remyelination capacity, as opposed to NT-3 secreting grafts; almost all axons displayed MBP / neurofilament positive labelling. Interestingly, the identity of myelinating cells differed from one graft to another. Immunohistochemical labelling for oligodendrocytes and Schwann cells (RIP and P0 respectively) revealed that more Schwann cell-derived myelin was found in bFGF grafts despite the absence of P0 cells in the surrounding host tissue. In NT-3 or BDNF producing grafts however, oligodendrocytes were the primary source of myelin. This suggests that NT-3 and BDNF may have a role in recruiting oligodendrocytes to the graft / injury site (McTigue et al., 1998). Additionally, immunohistochemistry and BrdU administration revealed that oligodendrocyte proliferation was stimulated by both NT-3 and BDNF grafts. It would

be interesting to see whether this event is mediated by calcineurin / NFAT signalling given the expression of α7nAChRs by oligodendrocyte precursor cells (Vélez-Fort et al., 2009, Rogers et al., 2001) and the capacity of both NT-3 and BDNF to directly activate calcineurin.

Chapter 6

General discussion

6.1 Summary

Building upon the continuous progress in addressing fundamental questions and implications of adult mammalian neurogenesis, my study investigated the influence of the neurotransmitter acetylcholine on cell proliferation and differentiation in the adult murine spinal cord neurogenic niche. The ependymal layer surrounding the spinal cord central canal has attracted great interest due to the identification of a population of endogenous neural stem cells within this area. These cells exhibit neural stem cell properties *in vitro*; capable of forming neurospheres and differentiating into neurons, astrocytes and oligodendrocytes. Under normal conditions *in vivo*, these cells are under continuous cell turnover, and proliferate extensively following injury (Meletis et al., 2008, Barnabé-Heider et al., 2010, Johansson et al., 1999), where they differentiate into oligodendrocytes but rarely neurons.

This thesis focused on investigating the influence of enhanced acetylcholine transmission on spinal cord cell proliferation under both normal (intact) conditions and following focal white matter demyelination. These studies provided evidence that cell proliferation in the adult spinal cord is significantly enhanced both *in vivo* and *in vitro* through activating the α7nAChRs. They extended previous findings which demonstrated that ependymal cells proliferate in response to injury only when the injury occurs at or in close proximity to the central canal, and that very few ependymal cells proliferate in response to focal white matter injury (Lacroix et al., 2014, Ren et al., 2017b). We also show for the first time that spinal cord cell proliferation *in vitro* is mediated at least in part via the calcineurin-NFAT calcium signalling pathway downstream of α7nAChR activation.

The linage of these newly proliferated cells was also investigated. Under normal conditions *in vivo* following the exposure to donepezil and the α7nAChR positive allosteric modulator resulted in a significantly higher percentage of newly proliferated cells expressed the oligodendrocyte marker PanQKI in the grey and white matter of treated animals and the neuronal marker HUC/D in the grey matter of the spinal cord compared to control animals. There was no change in the proportion of cells

expressing the astrocytic marker S100β between control and donepezil + PNU-120596 treated animals. Despite significant ependymal cell proliferation, no proliferated cells still contained within the ependymal layer expressed markers for neurons, astrocytes or oligodendrocytes. In our *in vivo* dorsal focal white matter demyelination studies, the white matter of animals that had received the α7nAChR modulator PNU-120596 alone or in combination with donepezil following LPC injections had both a higher number of proliferated cells in response to demyelination, and a larger proportion of newly proliferated cells expressing the oligodendrocyte marker Olig2 compared to animals treated with LPC alone. More importantly, the extent of newly generated myelin 14 days following the induction of demyelinaton was significantly increased in PNU+120596 and donepezil treated animals compared to LPC and vehicle treated animals. Thus, targeting α7nAChRs proved to be successful in enhancing both the extent of cell proliferation and oligodendrogenic differentiation in the intact and demyelination murine model.

6.2 Can neurotransmitter modulation promote the proliferation and differentiation of ependymal cells in adult mice under normal conditions?

6.2.1 Ependymal cells do proliferate in the intact spinal cord and organotypic slice cultures; a process that can be enhanced by exogenous acetylcholine modulation.

The ability of the adult ependymal layer to exhibit plasticity after injury has been investigated and defined by many research groups including but not limited to (Meletis et al., 2008, Barnabé-Heider et al., 2010, Johansson et al., 1999). One of the aims of this study is activating specific acetylcholine receptors to transform the quiescent self-renewing ependymal cell population to a proliferative one. This will aid in a better understanding of adult neurogenesis in the spinal cord and provide an approach by which to naturally enable or disable cell proliferation and differentiation using exogenous compounds.

In our studies we identified the newly proliferated cells using the thymidine analogue EdU in postnatal mice *in vivo* and younger mice *in vitro*. *In vivo* experiments revealed a significantly higher number of proliferating cells in the central canal region after treatment with donepezil and PNU-120596 compared to control conditions. This suggests that the ependymal layer is responsive to cholinergic modulation. Vehicle treated control animals exhibited a few EdU positive cells in the ependymal layer suggestive of self-renewal. These observations are consistent with those of (Meletis et al., 2008, Barnabé-Heider et al., 2010) who defined this region as quiescent under normal conditions and a plastic neurogenic niche only following injury, as well as, results reported by Corns who compared ependymal cell proliferation under normal conditions to those treated with the α7nAChR positive allosteric modulator PNU-120596 (Corns et al., 2015). Electrophysiological studies showed that both the ependymal cells and CSFcCs lining the central canal are capable of responding to acetylcholine (Corns et al., 2015). They also showed via immunohistochemical studies the presence of cholinergic structures closely apposing ependymal cells and CSFcCs. The depolarising response of ependymal cells to acetylcholine in electrophysiological experiments was significantly enhanced in the presence of PNU-120596. These results suggest that the response is predominantly mediated by α7nAChRs expressed by these cells. Furthermore, α7nAChRs have been identified in the ependymal layer of mammalian spinal cord by using in situ hybridization techniques (Wada et al., 1989). Indeed, I also observed a significantly higher number of proliferating cells in the central canal, grey and white matter of slices treated with specific muscarinic and non-α7nAChR antagonists in culture compared to those treated with donepezil alone identifying the α7nAChRs as the main mediators of cell proliferation in all regions of spinal cord. Significant cell proliferation following cholinergic treatment *in vivo* was also observed in the grey and white matter compared to control conditions. This raises the possibility that PNU-120596 potentially activated α7nAChRs present on oligodendrocyte precursor cells (Vélez-Fort et al., 2009, Rogers et al., 2001) promoting these precursor cells to proliferate. Thus, the source of these EdU positive cells is not known from this study since fate mapping of the different cell types, such as ependymal cells, oligodendrocyte precursor cells or other stem / precursor cells was not possible.

PNU-120596 is a positive allosteric modulator of the α7nAChRs that acts by increasing the effect of endogenous acetylcholine; increasing the affinity of acetylcholine to the active state receptor and prolonging the agonist activated response (channel opening) in the continued presence of acetylcholine (Kalkman and Feuerbach, 2016) thus ultimately enhancing the effects of acetylcholine on α7nAChRs. This specific receptor subtype is a calcium-permeable ion channel, and activation of these channels raises intracellular calcium levels (Bertrand et al., 1993). Upon activating these receptors, a complex calcium response enables many downstream signalling effects, one of which is cell proliferation (Resende and Adhikari, 2009a). In slices cultured in the presence of PNU-120596 compared to those cultured in the presence of both PNU-120596 and the calcineurin inhibitor cyclosporine A, a significantly lower number of EdU positive cells was observed in the central canal of slices treated with PNU-120596 and cyclosporine A. This confirms the abundant presence of α7nAChRs in the central canal region, and identifies a pathway involved in regulating their cell proliferation. In addition to the central canal, cell proliferation was significantly inhibited in the grey and white matter of slices cultured in the presence of PNU-120596 and either of the calcineurin inhibitors (FK-506 or cyclosporine A) compared to PNU-120596 alone cultured slices. These findings are similar to what is known about the hippocampal postnatal neurogenic niche. The α7nAChR is one of the most prominent receptor subtypes in the hippocampal niche (Kaneko et al., 2006), and functional forms of this receptor subtype are found exclusively on the immature granule cells in the postnatal dentate gyrus of rats and mice (John et al., 2015). Furthermore, the downstream cellular signalling cascades are essential for the maturation and synaptic integration of adult born neurons in the hippocampus (Campbell et al., 2010).

6.2.2 Treatment with donepezil and α7nAChR potentiation did affect the proportion of cells differentiating into neurons and oligodendrocytes, but not astrocytes.

Our next step was to identify the fate of the newly proliferated cells by employing antibodies to label mature astrocytes (S100β), both mature and immature oligodendrocytes (PanQKI) and neurons (HUC/D) *in vivo* in the intact spinal cord under control conditions and following treatment with donepezil and the α7nAChR positive

allosteric modulator PNU-120596. These immunohistological studies did not include a specific label for ependymal cells, however, the proliferative marker EdU identified the occurrence of cell proliferation in the central canal area in addition to the grey and white matter of both vehicle treated and donepezil + PNU-120596 treated animals. This suggests that the newly proliferated cells in the neurogenic niche surrounding the central canal are the ependymal cells which are capable of proliferation under normal conditions and may provide a significantly greater number of new cells following donepezil and PNU-120596 administration.

There was no change in the proportion of cells differentiating down the astrocyte lineage between control and donepezil + PNU-120596 treated animals. This could be attributed to the lack of injury since both astrocytes and ependymal cells of postnatal mice are known to produce significant numbers of astrocytic progeny exclusively following injury (Barnabé-Heider et al., 2010, Meletis et al., 2008). In addition, unlike stem / progenitor-like astrocytes of brain neurogenic niches, astrocytes of the spinal cord lack *in vitro* neural stem cell potential and *in vivo* multilineage differentiation (Barnabé-Heider et al., 2010). This suggests that astrocytes of the spinal cord are different from those residing in other areas of the adult central nervous system, such as the SVZ and SGZ of the brain which have been described as the neural stem cells of those niches (Doetsch et al., 1999, Ma et al., 2004).

This result where the administration of donepezil and PNU-120596 did not affect the proportion of astrocytes generated is particularly valuable and beneficial since the glial scar and the persistence of large numbers of astrocytes can be detrimental to the process of remyelination and axonal regeneration (Frisen et al., 1995, Yiu and He, 2006, McKeon et al., 1991, Chen et al., 2000). These results complement the effect of donepezil and PNU-120596 on oligodendrocytic differentiation; which was significantly enhanced compared to vehicle treated controls. This effect is of significant importance since a couple of spinal cord injury studies have reported that ependymal cells give rise to fewer oligodendrocytes than astrocytes (Barnabé-Heider et al., 2010, Meletis et al., 2008). Moreover, *in vivo* and *in vitro* studies reported a greater proportion of EdU labelled cells that were immunoreactive for PanQKI after treatment with PNU-120596

alone compared to untreated controls (Corns et al., 2015). Taken together with results obtained from our experiments, these suggest that α7nAChR activation leads to an increase in the number of cells differentiating along the oligodendrocyte lineage providing a potential novel therapeutic strategy for generating one desired progeny after spinal cord injury where axonal regeneration is crucial and following demyelinating diseases. In support of these results, Gao and colleagues reported an increase in the number of oligodendrocytes in a murine model of multiple sclerosis following treatment with nicotine and a reduction in inflammation (Gao et al., 2015). As supplement to these results, donepezil has been shown to increase the levels of transcription factors necessary for the regulation of oligodendrocyte differentiation and neuronal maturation (Imamura et al., 2015b). This effect was inhibited by the nicotinic acetylcholine receptor antagonist mecamylamine suggesting that the effects are mediated via nicotinic acetylcholine receptors. These results in combination suggest the potential importance of α7nAChR activation as a target to increase the proportion of oligodendrocytes to ultimately enhance the process of remyelination of denuded axons following injury or disease. Therefore, treatment with PNU-120596 alone or in combination with donepezil following focal white matter demyelination was evaluated and discussed in section 6.3.

The largely dormant ependymal cells do proliferate extensively in response to injury *in vivo* and are capable of multilineage differentiation providing astrocytes and oligodendrocytes (Barnabé-Heider et al., 2010, Meletis et al., 2008). Resident oligodendrocyte precursor cells in postnatal mouse spinal cords have been shown to give rise to mature and immature oligodendrocytes under normal conditions (Barnabé-Heider et al., 2010). Therefore, both cell types are likely to be oligodendrocyte contributing candidates. Since the oligodendrocyte marker used in these experiments was a pan-oligodendrocytic marker (labels both mature and immature oligodendrocytes), the stage of maturity of this newly generated progeny is still unknown.

Treatment with donepezil and PNU-120596 also resulted in a significantly higher number of EdU positive cells immunoreactive for the neuronal marker HUC/D in the

grey matter of treated animals compared to control conditions. This marker is a pan-neuronal marker, thus the specific developmental stage of these newly generated neurons is still unidentified. This result, although preliminary, provides us with a potential alternative strategy to neuronal cell transplantation post spinal cord injury. An example of such is the study where the neural stem / progenitor cells from the ependymal layer of human spinal cord were isolated and passaged *in vitro* in the presence of EGF and FGF enriched medium that led to the generation of nestin and Sox2 positive neurospheres which differentiated into glial and neural cells under their respective specific differentiating conditions (Mothe et al., 2011). The transplantation of these human neural stem / progenitor cells into injured rat spinal cords resulted in the survival of the xenograft and cell differentiation into glial and neural cells. However, it is important to endogenously enhance neuronal production to avoid ethical and immunological limitations associated with stem cell transplants. Therefore, I aim to expand on promoting the generation of new neurons by administering exogenous compounds along with PNU-120596 in the future.

Many spinal cord injury studies and studies of the adult intact spinal cord identify the resident population of neural stem / progenitor cells but only describe their proliferation into glial cells *in vivo* (Meletis et al., 2008, Barnabé-Heider et al., 2010, Horner and Gage, 2000, Horky et al., 2006, Johansson et al., 1999, Corns et al., 2015). Therefore, it is imperative to identify the mechanism by which the combination of donepezil and PNU-120596 affect neuronal differentiation. Although the average number of neurons generated using our treatment combination in the intact spinal cord was low, approximately 3-4 cells per section in treated animals compared to ~1 cell in control animals, it provides a promising strategy with room for optimization. For instance, since potentiating α7nAChRs alone (PNU-120596 alone) is sufficient for the induction of significant numbers of oligodendrocytes after the promotion of proliferation, it can be administered along side an exogenous compound known to induce neural stem cell differentiation such as the small molecule isoxazole 9 (ISX9); an inducer of adult neural stem cell differentiation (Stem Cell Technologies, 2019). ISX9 has been shown to cross the blood / brain barrier without causing adverse effects and induce neurogenesis in the mouse adult hippocampal niche (Petrik et al., 2012). Long term intraperitoneal administration of ISX9 (a daily injection for 7 days followed by an

injection every other day) for up to 60 days resulted in the significant increase in the proportion of SGZ neuroblast proliferation ($Ki67^+$ DCX^+ cells) 12 days after the start of injections compared to control animals. They further analysed the effect of ISX9 on neuroblast proliferation and found a significant increase in the number of $BrdU^+$ DCX^+ at 30 days post ISX9 treatment when BrdU was administered after administrating ISX9 for seven consecutive days. Furthermore, there was a significant increase in the number of $BrdU^+$ $NeuN^+$ and $BrdU^+$ DCX^+ cells at 30 days post treatment compared to vehicle treated animals suggesting an increase in neuronally committed cell proliferation. In contrast, there was no change in the number of $BrdU^+$ $GFAP^+$ cells between treated and control animals. The number of SGZ DCX^+ newborn neurons 1, 12 and 30 days following treatment was significantly higher than that observed in control animals and these neurons exhibited increased dendritic complexity (almost double the number of branching nodes) compared to vehicle treated animals. Moreover, the hippocampal- dependent learning and memory test (Morris water maze) 3 weeks after 12 days of ISX9 treatment showed that ISX9 improved spatial memory by generating new neurons that incorporated into the hippocampal circuitry (Petrik et al., 2012).

Moreover, a recent publication reported that one of the effects valproic acid (a histone deacetylase inhibitor) exerts on adult spinal cord cultured NSCs is the induction of neuronal differentiation (Chu et al., 2015). The primary NSCs were derived from the compressed spinal cord region (20 mm centred injury site) of adult Sprague Dawley rats 15 days after the compression injury. The compression injury was done using transient (1 min) extradural compression of the spinal cord at T10 using aneurysm clips at a closing force of 30 g. They show by means of immunohistochemistry that NSCs cultured in the presence of valproic acid (1 mmol/L) exhibited 5.2-fold decrease in number of neurospheres compared to those treated with vehicle. By employing flow cytometry techniques, they showed that valproic acid arrests the NSC cell cycle in the G0/G1 phase compared to vehicle treated controls and significantly decreased proliferation. Furthermore, when neurospheres were induced to differentiate (in 5% FBS without growth factors for 10 days), valproic acid treated NSCs had 2.3-fold increase and 5.9-fold increase at (0.01 mmol/L) and (1 mmol/L) respectively in $tuj1^+$ neuronal populations and a 1.4-fold decrease in $GFAP^+$ astrocytic cells compared

vehicle treated cells. In order to mitigate the proliferative inhibitory effect of valproic acid *in vivo*, a delayed treatment (150mg/kg I.P.) starting at day 15 through 22 post compression injury was investigated. qPCR analysis of tissue from the injury epicentre revealed a 2.6-fold increase in mRNA expression of DCX at day 22 post injury and a 1.8-fold increase in NeuN (a nuclear protein expressed by mature neurons) protein expression by day 29 compared to vehicle treated animals. There was an even higher expression of these two proteins in adjacent segments to the injury epicentre. Interestingly, an 8 week long behavioural assessment using the Basso, Beattie, Bresnahan functional scale where rats were evaluated for their plantar stepping with full weight support and forelimb-hindlimb coordination revealed a significant enhancement in locomotion by week 4 compared to vehicle treated animals.

6.3 The potentiation of α7nAChRs alone or in combination with donepezil treatment following LPC-induced focal white matter demyelination enhances oligodendrogenesis significantly

Fourteen days following LPC injection into the dorsal lumbar white matter, PNU-120596 treated animals had significantly more EdU positive cells compared to animals administered LPC alone. There was a greater significance in the extent of proliferation between animals treated with both PNU-120596 and donepezil compared to those treated with LPC alone, as well as, a significantly higher number of proliferative cells in animals treated with PNU-120596 + donepezil than that observed in PNU-120596 alone treated animals. Similarly, in animals sacrificed 21 days post LPC injection, the outcome in cell proliferation after treatment with either PNU-120596 alone or PNU-120596 and donepezil compared to those administered LPC alone were consistent with the aforementioned experiment terminating at day 14. These results complement our results obtained following cholinergic enhancement of cell proliferation following PNU-120596 + donepezil administration in the intact spinal cord (chapter 3) and results reported by Corns following α7nAChR potentiation with PNU-120596 in the uninjured spinal cord in terms of enhanced cell proliferation (Corns et al., 2015) . However, since

the central canal was predominantly devoid of proliferation, are OPCs the main contributors? Did we miss ependymal cell proliferation due to their migration to the site of demyelination by days 14 and 21? The only way in which these questions can be definitively answered would be by employing transgenic animals for lineage tracing. To assess the contribution of ependymal cells to cell proliferation and / or differentiation, using an inducible knock-in mouse model of the transcription factor forkhead box j1 (Foxj1) can be employed. Foxj1 is expressed by cells committed to differentiate into epithelial cells with motile cilia contacting fluid or air-filled cavities during embryogenesis and postnatal development, it is also reliably induced via Cre-mediated recombination for genetic ependymal cell lineage tracing studies in mice (Muthusamy et al., 2014, Meletis et al., 2008). Given that OPCs arise from progenitor domains expressing platelet-derived growth factor receptor alpha (PGDFR α) (Pringle and Richardson, 1993) and PGDFR α knock-in mouse lines are reliably produced and used for OPC cell lineage tracing studies (Marques et al., 2018), it is feasible to determine the contribution of cell type to cell proliferation, migration and differentiation *in vivo*.

More importantly, potentiating α7nAChRs alone or in combination with donepezil significantly enhances oligodendrogenesis at both 14 and 21 days post LPC injections compared baseline oligodendrogenesis (LPC alone). The insignificant (~4%) difference in the percentage of $EdU^{+}olig2^{+}$ cells seen in PNU-120596 alone treated animals at 14 days compared to those treated with PNU-120596 + donepezil may be a result of variable degrees of demyelination induced among animals. Although we aimed to administer the same amount of LPC (1 µl) to each animal with the utmost precision and care, it is possible for minimal variabilities in the amount of LPC injected to occur between animals. Alternatively, natural biological differences in response to treatments among animals may present individual variabilities. The overall higher percentage observed in the PNU-120596 alone group can be attributed to a relatively higher number of colocalised cells seen in one of the 4 animals. Since this may be a result of technical variation, it can be hypothesized that this individual animal had a larger demyelinating lesion or more sensitive to the demyelinating agent which resulted in the requirement of more oligodendrocytes for repair.

6.3.1 Cell proliferation was confined to the focal demyelinating lesion

It must be noted that in all our focal demyelination experiments, cell proliferation was absent in spinal cord regions outside the focal LPC-treated lumbar region. The lack of proliferation was observed in thoracic and sacral regions taken from animals that received dorsal lumbar focal LPC injections followed by treatment with PNU-120596 alone or in combination with donepezil. This observation differs from our *in vivo* and *in vitro* cell proliferation studies involving cholinergic modulation with donepezil and PNU-120596 in the intact situation, as well as those reported by Corns, that show the generation of significant numbers of proliferating cells in all analyzed regions of the spinal cord; thoracic and lumbar (Corns et al., 2015). Since most *in vivo* LPC induced demyelination studies focus on the demyelinated lesions, none of the studies I came across looked into regions rostral and caudal to the demyelinated region for signs of cell proliferation and / or differentiation. In contrast, spinal cord injury studies do investigate these occurrences; reporting variable results based on type of injury, injury severity and the animal model used. Spinal cord transection injuries cause a rapid increase in mainly ependymal cell proliferation which is restricted to the site of injury, however, in contusion and compression injuries, the extent of the initial cell proliferation (encompasses ependymal and lateral parenchymal progenitor cells) is relatively lower but involves the entire rostrocaudal extent of the spinal cord (McDonough et al., 2012). The restriction of stem / progenitor endogenous proliferation to the occurrence of injury, and potentially local sites of injury, relies on intrinsic cellular and extrinsic microenvironmental factors that regulate their gene transcription according to the stimulus they receive (Navarro Quiroz et al., 2018). Additionally, stem / progenitor cell quiescence has been linked to the manifestation of inflammation (Kalamakis et al., 2019). In a study that explores the potential effects of aging on neural stem cell pools of the SGZ and SVZ, young and old mice were used to profile single transcripts using FACS and immunostaining along with sequencing and mathematical modelling techniques (Kalamakis et al., 2019). They report an age-dependent increase in neural stem cell quiescence in order to maintain a reservoir of stem cells in aged animals; a quiescence imposed by inflammatory signals as a result of an increase in the levels of interferon. The inhibition of interferon signals through deletion resulted in a similar number of neural stem cells in young and old animals. Furthermore, they discovered

that niche signals help to maintain these stem cells in a quiescent state. They show that by neutralizing (with use of a neutralising antibody to the antagonist *in vivo*) an endogenous non-canonical Wnt signalling antagonist found strongly expressed in the niche of old mice led to the generation of significantly more active neural stem cells. They also indicate that the neural stem cells of old animals are more resistant to exiting the quiescent state even after the occurrence of injury, however once activated they are just as functional as their younger counterparts (Kalamakis et al., 2019). Although the LPC model is independent of inflammation (i.e. demyelination is induced by the permeabilizing action of lysolecithin and not the infiltration of inflammatory cells), the induction of demyelination using LPC has been shown to cause mild myeloid / microglial inflammation following treatment (Plemel et al., 2018). Therefore it may be hypothesized that the lack of proliferation is potentially attributed to the presence of local endogenous inhibitory factors that are activated by inflammatory cytokines in neighboring regions inducing quiescence or the effect of signalling pathways such as Wnt signalling potentially maintaining ependymal cells, OPCs and / or other stem / precursor cells in a quiescent state (Navarro Quiroz et al., 2018, Kalamakis et al., 2019). Another instance demonstrating the importance of niche signalling is a study employing adult rat models of spinal cord transection injury revealing that Notch signaling is enhanced after the occurrence of the injury and its activation *in vitro* inhibited the differentiation of adult progenitor cells (Yamamoto et al., 2001). Conversely, the inhibition of Notch signalling in neurospheres obtained from injured spinal cords *in vitro* or induced expression of neurogenin 2 (Ngn2), a neurogenic transcription factor, substantially enhanced neurogenesis (Yamamoto et al., 2001). This suggests that the intrinsic factors of adult progenitor cells including the extracellular environment could explain the inadequate regenerative potential in adult mammalian species and identifies a target known to attenuate adult neurogenic potential (Yamamoto et al., 2001).

Alternatively, the cerebrospinal fluid contacting cells which reside in the subependymal and ependymal regions of the spinal cord may be transmitting signals from the CSF to ventral or ventrolateral regions of the spinal cord where their axons have been shown to terminate alerting them to the occurrence of demyelination in neighboring regions (Vigh et al., 1977, Shimosegawa et al., 1986). These cells contact

the cerebrospinal fluid with processes that contain steriocilia and are hypothesized to have roles in maintaining the homeostasis of the CSF (Shimosegawa et al., 1986, Vígh et al., 2004, Vigh et al., 1977, Vigh et al., 1983). Therefore, they represent a potential relay for messages that are important in cellular signalling and can potentially influence ependymal cell proliferation.

6.4 How can our findings be employed as potential therapeutic strategies in the treatment of either spinal cord injuries or multiple sclerosis?

There are two main potential methods for the employment of neural stem cells as a treatment for spinal cord injuries; one would be by transplanting isolated endogenous neural stem cells that are conditioned to differentiate into the desired cell type(s) (Mothe et al., 2011), another would focus on the recruitment of identified endogenous neural stem cells to the site of injury using exogenous compounds that also trigger their differentiation into the desired neuronal and glial cell types. Although many stem cell transplant studies conducted on animals show improved functional recovery (Karimi-Abdolrezaee et al., 2006, Keirstead et al., 2005, Koda et al., 2005, Wright et al., 2011, Mothe and Tator, 2013) and succeed in reaching the clinical trial phase, there are many immunological and ethical implications associated with this method of treatment. In addition, a study by Hofstetter revealed that transplanting primary neuronal stem cells derived from intact rat thoracic spinal cords into weight-drop injured rats caused hind paw allodynia-like responses compared to vehicle treated animals (Hofstetter et al., 2005a). However, animals which received transplants of virally transduced neural stem cells with neurogenin-2 (in order to promote oligodendrocyte production) had a significantly lower extent of the allodynia-like responses compared to naïve neural stem cell recipients.

In relapse-remitting forms of multiple sclerosis which constitute the majority of multiple sclerosis cases, immunomodulatory treatments are essential in slowing down the progression of the disease to reduce inflammation, however, there is still a need for

treatments that aid in the remyelination of demyelinated axons to prevent further axonal damage. Such treatments would ideally substantially enhance the level of endogenously occurring oligodendrogenesis that ultimately provides sufficient numbers of mature myelinating oligodendrocytes. Once a successful remyelinating treatment is identified and employed as an adjunct therapy to immunomodulation, there would be a great chance of proper disease intervention at an early stage to prevent neurological dysfunction and debilitation correlated with disease severity.

Our results obtained by the systemic administration (intraperitoneal injection) of the Alzheimer's disease approved treatment (donepezil) and the positive allosteric modulator for α7nAChRs (PNU-120596) are highly encouraging. Both drugs effectively cross the blood / brain barrier promoting the production of significant numbers of desired phenotypes (neurons and oligodendrocytes) that can potentially enhance functional recovery post injury. This combined treatment resulted in triggering the normally dormant, endogenously residing neural stem cells or OPCs to proliferate. It significantly enhanced ependymal cell proliferation in the central canal neurogenic niche, and also enhances overall spinal cord cell proliferation in the grey and white matter regions in the absence of disease or injury. It also led to a significant number of neuronal committed cells in the grey matter and oligodendrocyte committed cells in both grey and white matter regions under normal conditions, and most importantly, following focal white matter demyelination. Since treatment with PNU-120596 alone following focal white matter demyelination also significantly enhanced the level of oligodendrogenesis, similar to observations made by Corns where significant numbers of proliferated cells differentiated down the oligodendrocyte lineage following treatment with PNU-120596 alone *in vivo* or in culture which suggests that α7nAChRs activation mediates oligodendrocyte commitment, bringing to light the importance of identifying the precise mechanism(s) governing this process (Corns et al., 2015).

6.4.1 Enhancing α7nAChR activity is a widely targeted potential therapeutic strategy

Given the implication of α7nAChRs in various cognitive and neuropsychiatric disorders, the enhancement of their function is widely investigated as a potential therapeutic strategy and several agonists and positive allosteric modulators progressed to clinical trials (Yang et al., 2017). PNU-120596 is a class II positive allosteric modulator, and thus potentiates α7 currents in the presence of the agonist as well as delaying desensitization and reactivating desensitized receptors (Yang et al., 2017). This may explain the reduced extent of cell proliferation observed in animals treated with donepezil alone compared to control animals. Although PNU-120596 proved to induce cytotoxicity in clinical trials due to excessively high calcium permeability (Yang et al., 2017), utilizing PNU-120596 in animal studies to investigate the mechanisms and cellular signalling pathways downstream of α7nAChR activation governing enhanced proliferation, neurogenesis and oligodendrogenesis is crucial for the future employment of safe and effective alternatives with a similar mechanistic action to PNU-120596 for the treatment of patients. Therefore, in future experiments, it is important for me to employ one of the clinically approved α7 agonists or positive allosteric modulators. Since many of the agonists in clinical trials are only partial agonists to α7nAChRs, the best option forward would be the use of a type I selective α7nAChR positive allosteric modulator (AVL-3288, also known as, XY4083 or CCMI) which has been shown to improve cognitive and sensory deficits in rodent models both in the presence or absence of agonist and has advanced to phase I clinical trials for the treatment of schizophrenia (Yang et al., 2017).

6.4.2 The importance of neurogenic microenvironmental signals

Many studies investigating adult neurogenesis and central nervous system remyelination have shown that the behaviour of endogenous stem cells is governed to a great extent by their microenvironment (Plemel et al., 2017, Mei et al., 2016). Therefore, identifying the relevant cues to manipulate can promote the endogenous populations to produce the desired numbers and lineages of cells and / or reinforce

and provide micro-environmental support to sustain the effects generated with exogenous modulation.

For instance, remyelination depends on the proper recruitment of resident oligodendrocyte precursor cells (OPCs) and neural precursor cells (NPCs) and their maturation into myelin-producing oligodendrocytes; a process highly regulated by intracellular and extracellular factors yet challenged by insufficient differentiation, recruitment or migration in central nervous system demyelinating lesions (Hesp et al., 2015, Zhao et al., 2015, Trapp et al., 1999, Wolswijk, 2002, Alizadeh and Karimi-Abdolrezaee, 2016). Several neurotrophic and growth factors have been shown to regulate and support oligodendrocyte precursor cell survival, migration, differentiation, maturation and myelination. These include BDNF, PDGF, neuregulin-1 (Nrg-1), insulin-like growth fator-1 and leukemia inhibitor factor (LIF) (Plemel et al., 2017, Van't Veer et al., 2009, Mason et al., 2000, Du et al., 2006, Butzkueven et al., 2002) while a host of other factors are inhibitory to oligodendrocyte remyelination including Lingo-1 and CSPGs (Fancy et al., 2009, Mi et al., 2005, Kotter et al., 2006, Syed et al., 2011, Lau et al., 2012, Lau et al., 2013).

A recent study exemplified the importance of targeting and adjusting dysregulated levels of the oligodendrocyte and myelin supportive factor neuregulin-1 in demyelinating lesions (Kataria et al., 2018). In LPC induced thoracic focal demyelinated rat models, the levels of Nrg-1 proteins in LPC demyelinated lesions were significantly reduced (29%) at 3 days post injection, and further declined (47%) at 28 days. This was assessed by means of western blot tests and confirmed with Elisa analysis when compared to sham injured animals. They utilized a release-controlled delivery system (poly lactic co-glicolic acid; PLGA microparticles) which were intraspinally injected to locally provide a sustained level of the recombinant human Nrg-1 with a functional EGF domain to LPC-treated spinal cord tissue. This allowed them to assess the level of contribution that Nrg-1 makes to oligodendrogenesis and remyelination. They showed that treatment with Nrg-1 significantly promoted oligodendrocyte maturation and myelin thickness in remyelinated regions. More importantly, enhanced levels of Nrg-1 attenuated the levels of CSPGs expression in

LPC demyelinated lesions which are known to hinder myelin production (Kataria et al., 2018). It would therefore be interesting to compare the extent of oligodendrogenesis and remyelination in LPC induced focal demyelination using PNU-120596 alone or in combination with donepezil in the presence and absence of Nrg-1 modulation.

Moreover, similar to the murine spinal cord niche, the human ependymal region displays immature characteristics such as the expression of nestin, vimentin and Sox2 (Becker et al., 2018). Although aging can cause the central canal to shrink and the ependymal layer to become disorganized (Garcia-Ovejero et al., 2015). A recent study reported the presence of a largely conserved ependymal cell expression of 120 transcription factors and 1,200 genes between human and mice (Ghazale et al., 2019). This finding provides great promise for the translation of treatments aimed at modulating these cells in murine animals to clinical trials. Ghazale and colleagues described a sustained embryonic-like dorsal-ventral pattern of expression of these spinal cord developmental transcription factors including Foxj1, Pax6 and MSX1 in human tissue (Ghazale et al., 2019). Moreover, ependymal cells expressed solute carrier genes (129 in mice, and 34 in humans), and more importantly, a conserved expression of genes involved in Shh signalling (which is important in ventral fate specification during spinal cord development) was found between mouse and human samples. Human ependymal cells were also found to be enriched with genes involved in cell division, transcription factors (MEIS2 and PBX1) known to have regulatory roles in adult SVZ neurogenesis and express high levels of transcription factors (Sox9 and NFIA) involved in gliogenesis. Taken together, these results provide evidence that central canal neurogenic niche is conserved in humans and is genetically equipped with the capacity to proliferate.

6.5 Technical advantages and considerations

All experiments conducted in this project were carefully designed and carried out under the best technical conditions possible, however, there are limitations and disadvantages that must be considered.

Although *in vitro* spinal cord studies (acute slices and organotypic cultures) are not perfect experimental models, they provide a less expensive and time-consuming method to test numerous potential drug treatments, as well as allow us to test a variety of drugs on tissue obtained from the same animal; minimizing any variability introduced when using a cohort of animals. They also substantially reduce the number of animals required for these screens; making it a more ethical choice. Another advantage to these experimental models is the relatively conserved cytoarchitecture of spinal cord neuronal and glia circuits compared to dissociated cell cultures. Given that axonal afferents are severed causing the loss of synaptic neurotransmitter input to the slice, testing our drugs in an *in vivo* setting was imperative. Since some degree of cell death is inevitable due to the severance of axonal connections in generating transverse spinal cord sections, our method involves the use of ice-cold sucrose aCSF during perfusion and tissue handling while maximizing the efficiency and speed by which the tissue is extracted and incorporated in culture or acute treatment to obtain the best tissue viability possible. This was originally shown by Jillian Daniel (Daniel et al., 2016).

Spinal cord transverse organotypic slices were prepared and cultured in a similar fashion to the study conducted by Corns who proposed that the experimental model introduces a form of injury to the tissue (Corns et al., 2015). This consideration could explain the higher number of proliferative cells in the spinal cord slice as a whole, including the higher number of EdU positive cells in the ependymal layer compared to those observed in *in vivo* experiments.

In acute slice experiments, the slices are not allowed sufficient time to respond to the injury generated by sectioning before drug treatment, and therefore some of the proliferative response may be due to tissue injury. In cultures however, the slices are allowed to recover for 48 before any treatment is initiated. After the first 24 hours of culture establishment, the medium is changed to a serum-free condition in order to generate an environment with fully identified constituents since serum may contain unknown components that may have an effect on cultured slices. Furthermore, the viability of the tissue largely depended on the age of the animal; which prompted the

use of younger animals (P9-P16 mice). This however corroborated the findings obtained from *in vivo* studies which constituted older adult animals (6-9 weeks old). Thus, these experimental techniques proved to be appropriate for the desired investigation; acute cell proliferation and long term proliferative response to cholinergic transmission modulation.

This study required a cellular label to safely and reliably identify proliferation. The thymidine analogue EdU was used in all of the experiments to detect newly generated DNA in the S-phase of cell division *in vivo* and *in vitro* as detailed in chapter 2. EdU was considered here as a technically faster and less potent alternative to BrdU which requires a denaturing step using heat or acid with potential damage to the tissue antigens and structure (Zeng et al., 2010, Tang et al., 2007). Although EdU is comparable and arguably more convenient than BrdU in detecting cell proliferation (Zeng et al., 2010), it requires a substantial amount of copper to catalyze the click reaction which proved to hinder subsequent antibody-antigen reactions in some of my experiments. I can only hypothesize that the relatively high concentration of copper required for the EdU reaction caused some damage to antigens or perhaps disrupted the structure of epitopes making it difficult to conduct immunohistochemical reactions on EdU treated sections. This hypothesis evolved after troubleshooting a number of widely used antibodies in the literature on EdU treated and untreated sections which resulted in successful immunolabelling on non-treated sections only. Furthermore, when a section was first labelled immunohistochemically then treated with the EdU-required reagents; the immunolabelling was compromised. An example of these limitations was my inability to compare the number of relatively immature oligodendrocytes identified with Olig2 used in my focal demyelination studies with the number of mature oligodendrocytes generated after demyelination. The antibody anti-APC CC1 (Millipore) was incompatible with EdU treated sections. In addition, the only DCX antibody I managed to optimize to label immature granule neurons in the dentate gyrus sections discussed in (chapter 3) was also incompatible with EdU treated sections. Unlike Ki67 for example which detects different groups of cells (during G1, S, G2 and M phases), EdU (similar to BrdU) reliably only detects dividing cells (S phase). Thus, one way around this issue would be to optimise the lowest working concentration

of copper that permits the successful employment of immunohistochemical antibodies post EdU reaction.

Another limitation in this study was the methods used for cell counts *in vivo* and *in vitro*. The quantification of newly proliferated cells and / or cells positive for EdU and colocalised with differentiation markers was performed manually in acute slices experiments and *in vivo* experiments done under normal condition (in the intact spinal cord) as described in chapter 2 which potentially introduces human error. Semi-automated cell counting using the Zeiss Software on *in vivo* focal demyelination studies allowed for a more accurate method of counting, although, it was not applied for organotypic slices experiments given the substantial numbers of newly proliferated cells which prompted the use of a fully automated method employing image J. The use of a fully automated method required setting a different threshold for each imaged slice which could result in some level of error and variability. It must be noted however that although there isn't one perfect way to count cells that can be applied to all types of experiments, the best available method to count cell proliferation and / or differentiation was applied to each type of experiment.

Finally, the use of animals in research is necessary given the lack of alternatives. Digital simulations and *in vitro* systems do not allow for the full analysis of effects on the integrated and complex biological system, especially when studying the intricate central nervous system. Therefore, reduction, refinement and replacement (generally known as the three R's) were thoroughly considered in each experiment. For instance, since most of my experiments focused on the spinal cord, the brain of animals used were shared with colleagues studying various regions of the brain and not the spinal cord. Furthermore, all of my *in vitro* studies consisted of thoracic and lumbar regions, therefore sharing cervical and sacral regions with colleagues was commonly practiced. Undergraduate trainee students also made use of cervical and sacral spinal cord tissue to practice click chemistry and immunohistochemistry techniques. There are also disadvantages to using mouse models for disease. For instance, when studying diseases of aging such as Alzheimer's disease, mouse models would prove inadequate since they have a life span of approximately 2 years. Furthermore, many

mouse models of neurodegenerative disorders such as Alzheimer's disease or multiple sclerosis do not incorporate all the hallmarks of the disease. For example, what is considered the gold standard mouse model for multiple sclerosis is the EAE model. However, the inflammatory response is mainly mediated via $CD4^+$ T cells (Leuenberger et al., 2013) while scientists suspect $CD8^+$ T cells to mediate the inflammatory component in multiple sclerosis. Furthermore, the persistent inflammation in the EAE model makes remyelinating drug screening difficult to evaluate (Plemel et al., 2017).

6.6 Future experiments

The fundamental aim of this project was to produce efficacious treatments for patients suffering from spinal cord injuries and multiple sclerosis. There is a number of *in vivo* experiments to further investigate both ependymal cell and oligodendrocyte precursor cell proliferation and differentiation. My findings suggest that enhancing cholinergic transmission is a promising therapeutic avenue to achieve this aim.

For future investigations, an important experiment is to use transgenic fate mapping in normal intact spinal cord, spinal cord contusion injury and demyelinating lesions. First, transgenic animals allowing for the tracing of individual cell lineages must be employed to determine the precise effect of these drugs separately and in combination. This together with EdU to label newly proliferated cells, allows the analysis of cell proliferation and differentiation of both ependymal cells and OPCs under normal conditions. This study will allow us to monitor the source of the cells that are proliferating and differentiating. A second cohort of transgenic animals promoting the expression of either ependymal cells or oligodendrocyte precursor cells should be used to investigate whether PNU-120596 alone or in combination with donepezil successfully generate neurons and oligodendrocytes following contusion injury to the spinal cord. This experiment will also include the administration of EdU to identify newly proliferated cells. This study will enable us to answer questions such as:

1) Is the source of the newly generated cells in response to a common form of injury different from that under normal conditions?

2) Will there be a difference in the number of proliferated cells and differentiated cells between contused and control conditions?
3) Can the newly proliferated and / or differentiated cells migrate to the site of injury?
4) Will this result in a substantial number of astrocytes to contribute to the glial scar formation?
5) More importantly, to what extent do the newly generated cells integrate into the spinal cord circuitry post injury? This can be answered through behavioural studies analysing their motor skills before and after treatment.

A third cohort of transgenic animals promoting the expression of either ependymal cells (Foxj1) or oligodendrocyte precursor cells (PDGFR α) should be used to determine the effect of the drugs separately and in combination on ependymal cells and oligodendrocyte precursor cells following a focal demyelination injury. These experiments should allow for the clarification of the precise role or response of the spinal cord neurogenic niche (ependymal cells) to each injury type, and identify the extent of effect that these drugs have on oligodendrocyte precursor cells in the context of demyelination, contusion injury and normal conditions.

It is also of crucial importance to test these exogenous compounds in the same transgenic mouse lines separately and in combination in a model incorporating both the inflammatory and demyelination aspect of multiple sclerosis. This is important since acetylcholine may affect different cell types to those triggered by injury or inflammation. Troubleshooting the semliki forest virus model or generating experimental autoimmune encephalitis mouse models of multiple sclerosis will provide a mean to evaluate the effect of our drugs on oligodendrogenesis and remyelination. Scoring the behaviour of these animals pre- and post-treatment will allow for the assessment of the newly-generated myelin. The beam walking test for instance has been used to assess locomotor coordination following cuprizone focal demyelination (Skripuletz et al., 2010, Zhu et al., 2017). This generally entails training the mice to walk along a narrow horizontal beam to reach what they associate as a safe area (such as a box filled with

refinement and padding) to evaluate their motor skills post demyelination and during / after recovery.

6.7 Conclusions

This study provided evidence that the cells surrounding the adult mouse spinal cord central canal are capable of significant cell turnover and proliferation under normal conditions in response to enhanced cholinergic transmission using exogenous drugs. The proliferative response included the grey and white matter of the spinal cord which may partly be attributed to other types of resident progenitor cells such as OPCs. A significant number of these newly proliferated cells differentiated down the oligodendrocyte and neuronal lineage. Our focal white matter demyelination studies revealed that these exogenous compounds (PNU-120596 and donepezil) also enhanced oligodendrogenesis under demyelinating conditions. Enhanced oligodendrocyte production was achieved either by potentiating α7nAChRs alone or in combination with elevating endogenous acetylcholine levels using donepezil. More notably, enhancement of cholinergic transmission using these exogenous compounds significantly increased the extent of myelin production following focal demyelination. These results provide a promising base on which future studies utilize these beneficial outcomes in the development of clinically relevant treatments for spinal cord injuries or multiple sclerosis.

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