P2X7 Receptor Regulation of Hippocampal Neural Progenitor Cells

Hannah Leeson

BBioMedSc(Hons)

Griffith Institute for Drug Discovery

Griffith University

September 2017

Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

Abstract

Adult hippocampal neurogenesis plays an essential role in the formation and consolidation of new memories, spatial processing and some forms of learning. Identifying the molecular mechanisms that regulate hippocampal neural progenitor cells as they proliferate, differentiate, and are selected for either survival or cell death will provide a fundamental understanding of how this neurogenic niche coordinates these activities. Here, the roles of P2X7 receptors are examined for their influence over neural progenitor cell biology, particularly cell death, proliferation, and phagocytosis of apoptotic progenitors that have undergone programmed cell death. As a purinergic cation channel, P2X7 receptors are exceptionally versatile; their primary role is as ATP-gated calcium channels, and they have notable roles in the immune system, where they regulate cytokine release and form large transmembrane pores resulting in cell death. By acting as scavenger receptors, they can also mediate phagocytosis. These diverse roles were investigated in neural progenitor cells of the adult murine hippocampal neurogenic niche.

Primary cultures of hippocampal neural progenitor cells were derived from adult female C57BL/6 mice and characterised using multimarker immunocytochemistry as P2X7 receptor positive type 2 neural progenitor cells, as defined by $Sox2^{pos}$, $nestin^{pos}$, $BLBP^{pos}$, $Mash1^{pos/neg}$, $vimentin^{pos}$, $Pax6^{pos}$, $Prox1^{pos}$, DCX^{neg}, $GFAP^{neg}$ staining patterns. For some experiments, cultures derived from P2X7 knock out mice (Pfizer) were also used. Calcium influx assays using the indicator dye Fluo-8-AM demonstrated functional activity of P2X7 receptors with the general agonist ATP (1 mM) and the more specific agonist BzATP (100 µM). Ethidium bromide uptake demonstrated that P2X7 receptors were able to form large transmembrane pores, a

canonical function unique to this receptor, and confirmed the presence of a full length protein, as opposed to various splice variants. Live cell confocal microscopy revealed hippocampal neural progenitors are capable of phagocytosing fluorescent latex beads, and flow cytometry in conjunction with specific inhibitors demonstrated that P2X7 receptors are capable of facilitating this phagocytosis.

The effects of purinergic signalling on neural progenitor proliferation were assessed using the thymidine analogue EdU. P2X7 receptors activated with either extracellular ATP or BzATP showed a significant dose-dependent decrease in proliferation. Cell death was not observed under these conditions and proliferation could be rescued upon exchange of medium. P2X7 receptor inhibition reduced the effects of extracellular ATP on proliferation, and use of neural progenitor cultures derived from genetically null mice corroborated this observation. Convergence with growth factor signalling pathways was also explored.

The data presented here provides good evidence that P2X7 receptors function as scavenger receptors in the absence of ATP, allowing neural progenitor cells to phagocytose their apoptotic peers during target-independent programmed cell death, as well as governing rates of proliferation in the presence of ATP, possibly by regulating calcium dependent downstream signalling. Effector molecules of calcium signalling pathways were investigated following P2X7 receptor activation to determine some of the downstream mechanisms involved in P2X7 receptor mediated decreases in proliferation. Live cell calcium imaging identified the instigation of secondary calcium oscillations following extracellular ATP application; it was hypothesised that the decrease in proliferation was due to calcium dependent signalling cascades, involving calcium release from internal stores. Using confocal microscopy, calcium dependent

transcription factors NFκB and NFAT1 were evaluated for their potential to translocate to the nucleus following purinergic stimulation. Extracellular ATP did not cause translocation of NFκB or NFAT1. A possible convergence with growth factor signalling pathways was investigated as the growth factors present in culture conditions exert powerful regulation over the cells and also utilise calcium and endoplasmic reticulum signalling to exert their effects. Inhibition of proteins involved in endoplasmic reticulum signalling caused a decrease in proliferation, as did growth factor withdrawal. Transcription factor analysis revealed that withdrawal of both EGF and bFGF caused NFAT1, but not NFκB, to translocate to the nucleus, a novel finding in these cells.

The data presented here is among the first to examine the dichotomous signalling roles of P2X7 receptors in adult hippocampal neural progenitor cells. In mature neurons, P2X7 receptors have been implicated in various pathologies, and may present a therapeutic target for a number of neurological disorders. Understanding how these receptors regulate the physiology of stem and progenitor cells is an important first step in developing any regenerative therapies. Given the crucial role neurogenesis plays in both memory formation and hippocampal function, understanding these biological mechanisms is essential to addressing significant questions regarding neurogenesis and regeneration.

Statement of Originality

This work has not previously been submitted for a degree or diploma at any university. To the best of my knowledge and belief, this dissertation contains no material previously published or written by another person except where due reference is made in the text itself.

Hannah Leeson

September 2017

Acknowledgements

Thank you to my supervisors Michael and Jeremy for your advice and guidance over the years, and for not panicking too much when I disappeared to Europe for six or seven weeks. Thank you to Ben Gu for graciously hosting us at the Florey, and for providing resources, training, and advice. Kash, thank you for all the laughs, friendship, coffee (mental health) breaks, brunch dates (when we should have been working), and for late night lab 'ooga chaka' and 'Rasputin' sessions conducted at the top of our lungs (when we were working at one am instead of being home like normal people).

Thanks are also extended to Kelly (my forerunner and teacher of all the easier ways), Maria (for always fixing the microscope), Megan (for the rationale and sarcasm when good data was MIA), David (for chemistry assistance), Marie-Laure (for image analysis advice and support), Johana (for the Bowie shirt), Heidi (for coffees), Allie (for chicken nuggets), Ryo and Marco (for ramen), the Drug Runners and GRIDD FC (for the endorphins) and all the other staff and students (there are far too many to mention all of you wonderful people by name) for the friendship and support over the years. If it takes a village to raise a child, then it definitely takes an entire institute to produce a thesis.

To Janet, Andi, Stacie, Danielle and Paul, I've relied so much on your encouragement and support over the years, thank you for your friendship and I look forward to seeing more of you now that I've finished this degree.

Most of all, thank you to Jessica, the most amazing sister anyone could ask for, and to my parents, without whom I would not have made it this far.

Table of Contents

List of Figures and Tables

List of Abbreviations

A438079	P2X7 receptor inhibitor; 3-**[**[5-(2,3-Dichlorophenyl)-1H-tetrazol-1-yl]methyl**]** pyridine hydrochloride
ADP	adenosine diphosphate
AnCoA4	Orai1 inhibitor
ANOVA	one way analysis of variance
ATP	adenosine triphosphate
AZ16060120	P2X7 receptor inhibitor; *N*-[2-[[2-[(2-Hydroxyethyl)amino]ethyl] amino]-5-quinolinyl]-2-tricyclo[3.3.1.13,7]dec-1-ylacetamide dihydrochloride
BAY 11-7082	NFκB inhibitor
BDNF	brain derived neurotrophic factor
BLBP	brain lipid binding protein
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
BzATP	2', 3'-O-(4-benzoyl-benzoyl) adenosine 5' -triphosphate
CA1, 3	cornu ammonis (Ammon's horn, regions 1 and 3)
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
ChIP	chromatin immunoprecipitation
CNS	central nervous system
CO_2	carbon dioxide
Cpd5J-4	Orai1 inhibitor,
Cu^{2+}	copper
$CuSO_4$	copper sulphate
Cy3,5	cyanine 3, cyanine 5

DAG	diacylglycerol
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
DCX	doublecortin
DIV	days in vitro
Dlx2	distal-less homeobox2
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	embryonic day
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2’-deoxyuridine
EGF(R)	epidermal growth factor (receptor)
ELISA	enzyme-linked immunosorbency assay
ELMO1	intracellular engulfment and cell motility protein 1
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
F	fluorescence
FACS	fluorescence activated cell sorting
FGF(R)2	fibroblast growth factor (receptor) 2
FOV	fields of view
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
GPCR	G protein-coupled (metabotropic) receptors
GTPase	guanosine triphosphate (GTP) hydrolase enzyme
HBSS	Hank’s balanced salt solution
HEK293	human embryonic kidney cells

HEPES	hydroxyethyl piperazineethanesulfonic acid
IL	interleukin (-1β, -6)
IP_3(R)	inositol-1,4,5-triphosphate (receptor)
IPC	intermediate progenitor cell
K^+	potassium
LPC	lysophospatidylcholine
LPS	lipopolysaccharide
MAP2a/b	microtubule associated protein 2-ab
MAPK	mitogen-activated protein kinases
Mash1	mammalian achaete scute homolog-1
Mg^{2+}	magnesium
ML 9	STIM1 inhibitor
mRNA	messenger RNA
Na^+	sodium
NaN_3	sodium azide
NaOH	sodium hydroxide
NeuN	neuronal nuclear antigen
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor κB (kappa-light-chain-enhancer of activated B cells)
NGF	nerve growth factor
NGS	normal goat serum
NMM IIA	non-muscle myosin heavy chain IIA
NMR	nuclear magnetic resonance
NPC	neural progenitor cell
NSC	neural stem cell
NTDPase	nucleoside triphosphate diphosphohydrolase

OCT	optimal cutting temperature
Orai1	calcium-release activated calcium channel protein 1
OxATP	oxidized ATP
P	postnatal day (mouse age) *or* passage number (*in vitro* cultures)
P/S	Pen-Strep
P2X	ionotropic purinergic receptor
P2X7	P2X receptor 7
P2Y	G-protein coupled purinergic receptor
Pax6	paired box 6
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PD 158780	EGFR inhibitor; N^4-(3-bromophenyl)-N^6-methyl-pyrido[3,4-d]pyrimidine-4,6-diamine
PFA	paraformaldehyde
PIP_2	phosphatidyl-inositol-4,5-bisphosphate
PKC	protein kinase C
PLC (β,γ)	phospholipase C (β, γ)
PLO	poly-L-ornithine
PMCA	plasma membrane calcium ATPase
ppm	parts per million
Prox1	prospero-related homeobox 1
PS	phosphatidylserine
PS-1145	NFκB inhibitor
PVDF	polyvinylidene difluoride
ReNcell	immortalized human neural progenitor cell line
RIPA buffer	radioimmunoprecipitation assay buffer

RTK	receptor tyrosine kinases
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
SERCA	sarco-endoplasmic reticulum calcium ATPase pump
SGZ	subgranular zone (of the hippocampal dentate gyrus)
shRNA	small/short hairpin RNA
siRNA	small interfering RNA
SKF 96365	STIM1 inhibitor
SOC	store operated calcium (channel/entry)
Sox2	SRY (sex determining region Y)-box 2
SSEA-1	Lewis X; LeX
STIM1	stromal interaction molecule 1
SVZ	subventricular zone (of the lateral ventricles)
TAE	Tris acetate EDTA (buffer)
TBS	Tris buffered saline
TE	Tris EDTA (buffer)
TMA-DPH	trimethylammonium diphenylhexatriene
TMA-OH	tetramethylammonium hydroxide
TNF-α	tumour necrosis factor-α
TRP	transient receptor potential
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UDP	uridine diphosphate
UTP	uridine triphosphate
VGCC	voltage gated/dependent calcium channel
YG	yellow green latex beads
Zn^{2+}	zinc

1.0 INTRODUCTION

1.1 GENERAL INTRODUCTION

Since the discovery of neural stem cells in the adult mammalian brain, investigations into the role neurogenesis may play in mental disease, disorders and degeneration have continued in the hopes of aiding those afflicted by these maladies. Hippocampal neurogenesis is heavily regulated by a vast array of stimuli, both physiological and pathological. Niche derived growth factors and other signalling molecules are important in supporting and maintaining ongoing neurogenesis in the adult niches, providing protection and the physiological conditions permissible to continued neurogenesis (Ming and Song, 2011). Increased neurogenesis improves hippocampal function, in particular learning and memory formation (Kempermann et al., 1998). Alternatively, a decrease in neurogenesis has been correlated with a number of neurological disorders, such as Alzheimer's disease and depression (Danzer, 2012).

Neurogenesis is a tightly regulated balance between proliferation, differentiation and programmed cell death, and neural progenitors are selected for integration into neuronal networks depending on the requirements of the brain. Overproduction of neurons is a hallmark of neurogenesis, and programmed cell death is active in regulation of cell number in proliferative stages and the elimination of immature neurons that fail to correctly integrate into the existing cytoarchitecture (Ryu et al., 2016). Recent studies have shown that although microglia act as the professional phagocyte of the central

nervous system (CNS), adult neural progenitor cells (NPCs) are also capable of phagocytosing other apoptotic progenitor cells, contributing to maintenance of the neurogenic niche (Lu et al., 2011).

Purinergic signalling has emerged as an essential regulatory mechanism during hippocampal neurogenesis, contributing to proliferation, differentiation, migration and programmed cell death in both embryonic and adult progenitor populations in the hippocampus, and indeed in the entire CNS (Abbracchio et al., 2009). P2X7 receptors were initially identified in the immune system and have now been identified in the nervous system. The purinergic P2X7 receptor is activated by extracellular adenosine triphosphate (ATP) and is an ionotropic cation channel allowing passage of Ca^{2+}, K^{+} and Na^{+} ions, which initiate downstream signalling cascades. Their function as a cation channel allows signal transduction and at high agonist concentrations can additionally regulate cytoskeletal rearrangement and membrane blebbing, potentially resulting in apoptosis and/or necrosis (Surprenant et al., 1996). Thus, a role for P2X7 receptors in cell death events is currently the focus of much interest, as extracellular ATP is neurotoxic and elevated levels strongly correlate with neurological conditions, such as acute spinal cord injury and ischemia, and degenerative diseases, such as Parkinson's and Alzheimer's disease. Conferral of neuroprotection by inhibiting P2X7 receptor activity suggests that high levels of ATP present during an inflammatory response can result in further cellular damage (Wang et al., 2004).

Alternatively to their role in inflammatory events, P2X7 receptors are demonstrated to regulate cell proliferation, differentiation and axon branching. In mouse embryonic stem cells, accelerated cell cycle entry was observed following P2X7 receptor activation, while inhibition caused increased differentiation and axon growth and

branching (Glaser et al., 2014). The ability of P2X7 receptors to promote cell cycle entry demonstrates the ambiguous role P2X7 receptors may be playing in adult hippocampal neurogenesis.

P2X7 receptors have an additional role as scavenger receptors, facilitating phagocytosis of latex beads, bacteria and apoptotic neuroblasts in the absence of ATP. We recently demonstrated that during human embryonic development, NPCs express P2X7 receptors and that these neural progenitors were able to phagocytose via a P2X7 mediated pathway (Lovelace et al., 2015). The presence of ATP, P2X7 antagonists, or P2X7 receptor knockdown using siRNAs, inhibited this phenomenon and suggested that P2X7 can act as a scavenger receptor on neural progenitors within the developing human central nervous system. This may allow P2X7 to regulate the progression of hippocampal neurogenesis and play a fundamental role in the maintenance of the adult brain.

This research project aims to determine if P2X7 receptors are present in adult hippocampal NPCs and explore what roles they may play in regulating neurogenesis. The ability of hippocampal NPCs to phagocytose is investigated, as is the possible involvement of P2X7 receptors in facilitating this process. Purinergic regulation of proliferation and differentiation is explored, and subsequently the potential signalling mechanisms involved, including calcium encoding and transcription factor activation, are investigated. This research intends to provide an increased understanding of the multiple roles of P2X7 receptors in NPCs, and in turn, the molecular mechanisms regulating the process of adult hippocampal neurogenesis.

1.2 DISSERTATION OVERVIEW

This dissertation opens with a review on relevant and related literature, discusses the current knowledge in the field and highlights the literature gap that this project endeavours to cover. P2X7 receptors have determined roles in inflammatory processes and recent research has found they may play a role in the recovery of the brain following various types of neural disturbances. The general aim of this dissertation is focussed on understanding how P2X7 receptors may regulate hippocampal NPCs in the adult mammalian brain. In the current project hippocampal NPCs were isolated, identified, and the role of P2X7 receptors were investigated. This resulted in further investigations into purinergic, calcium and growth factor regulation in adult hippocampal NPCs. The general aims outlined below formed the basis of the experimental rationale, and are described in three results chapters. This dissertation concludes with a discussion on the wider implications of this research, particularly in relation to regulation and maintenance in the adult hippocampus, and suggests future studies to expand on the work presented here.

1.3 GENERAL AIMS OF RESEARCH

1.3.1 Aim one: To isolate and characterize primary cultures of adult hippocampal neural progenitor cells from the murine dentate gyrus

Neurospheres derived from the dentate gyrus of adult mice were comprehensively characterised using immunochemical methods. Multiple markers for proliferative or precursor cells were used to determine essential characteristics of NPCs, and to ensure cells *in vitro* represented their *in vivo* counterparts as much as possible. Their potential for differentiation to both neuronal and glial lineages was assessed as was changes in

expression patterns during mitosis. Effects of using the basement membrane Matrigel for culturing were investigated to determine any possible changes in protein expression patterns. It is essential that the properties of a newly established culture be fully identified prior to commencement of experiments, and this work laid the foundations for the rest of the current project, as well as subsequent projects. This work is described in Section 4.0.

1.3.2 Aim two: To determine if adult hippocampal neural progenitor cells express functional P2X7 receptors

Antibodies against P2X7 receptors, sourced from two different companies and raised against two distinct epitopes (intra- and extra-cellular), were applied using western blot and immunochemical methods to determine if NPCs express this protein. P2X7 receptor functionality was explored by utilising its canonical functions as a calcium channel and as a 'cell death' receptor in conjunction with agonists and antagonists.

1.3.3 Aim three: To determine if adult hippocampal neural progenitor cells are capable of phagocytosis, and if P2X7 receptors facilitate this process

The ability of adult NPCs to phagocytose has been recently reported. This study aims to confirm this finding and determine if any of this phagocytosis could be facilitated by P2X7 receptors. A number of techniques were employed in this endeavour, including live cell microscopy and flow cytometry. The ability of adult NPCs to phagocytose represents an exciting new insight into the mechanisms of neurogenesis in the adult brain, and a role for P2X7 receptors in this process has implications in potential therapeutic targets, especially in light of recent reports into the roles P2X7 receptors

might have in brain recovery following homeostatic disruption or traumatic events. Aims two and three are explored in Section 5.0.

1.3.4 Aim four: To investigate purinergic and calcium signalling mechanisms present in hippocampal neural progenitor cells and to determine the effects on proliferation, differentiation and transcription factor activation.

Signalling via purines is a powerful regulatory mechanism and results in downstream signalling by effector molecules, such as calcium ions. Extracellular calcium ions are conducted via plasma membrane bound ionotropic channels, such as P2X receptors, while metabotropic P2Y receptors signal to the inositol-1,4,5-triphosphate receptors (IP_3R) and ryanodine receptors, releasing calcium from the endoplasmic reticulum (ER) into the cytoplasm. The effects of purinergic signalling on neural progenitor proliferation and differentiation are investigated, as well as activation patterns of a number of calcium dependent transcription factors to extrapolate regulatory pathways. This work is detailed in Section 6.0.

1.4 SIGNIFICANCE

In an era of pronounced scientific advancement the human brain remains one of the greatest mysteries faced by researchers. Much of our knowledge of how the brain functions has been gained from studies where specific regions of the brain are damaged, resulting in particular changes in brain function. The current challenge revolves around gaining a deeper understanding of the molecular mechanisms involved, in the hopes of one day having the capabilities to prevent or reverse brain damage by disease or trauma.

Overproduction of NPCs, neuroblasts and neurons is a hallmark of CNS development and this physiological strategy is continually exploited within specialized niches of the adult mammalian brain to ensure synaptic integrity. Regulation of rates of proliferation and differentiation, as well as programmed cell death and the ensuing clearance of dead cells by phagocytosis is essential to generate the optimum cell numbers and to preserve the neurogenic niche. Purinergic signalling is known to regulate many cellular processes, from proliferation to cell death, but to date no previous work has addressed the role of P2X7 receptors in adult mouse hippocampal NPCs.

Research conducted here investigates purinergic regulation of neurogenesis, and more specifically the physiological role P2X7 receptors play in proliferation and differentiation of NPCs. The potential of adult NPCs to act as non-professional phagocytes via P2X7 receptors is also explored. This dissertation will provide insight into purinergic regulation of neurogenesis and provide an alternate and previously underreported molecular mechanism of debris clearance within the neurogenic niches, and could have a profound impact on our understanding of the mechanisms involved in maintenance of the niche and regulation of adult hippocampal neurogenesis.

2.0 LITERATURE REVIEW

2.1 NEUROGENESIS

Neurogenesis refers to the generation of new nervous tissue and begins with neurulation during early embryonic development, continuing throughout the mammalian life span. The term neural stem cell (NSC) is loosely applied to a subset of primary precursor cells that are defined as self-renewing and multipotent, able to give rise to all three primary cell types of the CNS: neurons, astrocytes and oligodendrocytes. Since the discovery of NSCs in the adult mammalian brain, investigations have continued to uncover the roles adult NSCs play in a wide range of physiological events from memory and olfaction to disorders, such as age-related neurodegeneration (Merkle and Alvarez-Buylla, 2006, Gotz and Huttner, 2005). Regenerative therapies, such as autologous stem cell transplantation and pharmacological manipulation of resident precursor pools, have been an area of much interest, though the current understanding of molecular mechanisms involved is lacking. This is in part due to the sophisticated and intricate nature of regulatory pathways, many of which can elicit opposing outcomes depending on a variety of factors. Nevertheless, it is hoped that NSC therapies may soon provide a treatment for neurological disease and injury.

2.2 ADULT NEUROGENESIS

In 1965, Joseph Altman provided the first anatomical evidence of the presence of newly generated granule cells in the hippocampus of adult rats (Altman and Das, 1965). Unfortunately, the dogma that neurogenesis was restricted solely to the developing embryo remained unchanged until the late 1970's and early 1980's, when adult neurogenesis was observed in non-mammalian species (Johns and Easter, 1977, Goldman and Nottebohm, 1983). A ground breaking study in 1992 found that cells isolated from the brain of adult mice were both proliferative and multipotent, demonstrating definitively that neurogenesis occurs in the adult mammalian brain (Reynolds and Weiss, 1992). Eriksson et al. (1998) was the first to demonstrate the regenerative potential of hippocampal granule neurons in a human study. By using post-mortem tissue from patients who had received 5-bromo-2-deoxyuridine (BrdU) for diagnostic purposes, and in combination with neuronal markers calbindin, neuronal nuclear antigen (NeuN), and neuron specific enolase, Eriksson discovered that the granule cell layer of the hippocampus contained a population of dividing neuronal cells. These findings demonstrated for the first time that adult humans also have progenitor cells within the dentate gyrus that are able to generate new neurons throughout a lifetime.

Under normal physiological conditions, neurogenesis is restricted primarily to two discrete neurogenic niches: the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the anterior lateral ventricles (Ming and Song, 2005, Gotz and Huttner, 2005). The SVZ houses resident radial-glial-like NSC capable of producing transit amplifying cells, which differentiate into neuroblasts and migrate via the rostral migratory stream to the olfactory bulb (Ming and Song, 2011)

and the striatum (Ernst et al., 2014). The SGZ of the hippocampus generates new granule neurons that play a crucial role in synaptic plasticity and the formation and consolidation of short-term memories (von Allmen et al., 2013, Squire and Alvarez, 1995, Kempermann and Gage, 2002, McEown and Treit, 2013).

Both neurogenic zones follow a similar process of neurogenesis beginning with the asymmetrical division of an adult NSC to a daughter stem cell (self-renewal) and a highly proliferative progenitor referred to as a transit amplifying cell (or type C cell) in the SVZ or an intermediate progenitor cell (IPC, or type 2 cell) in the dentate gyrus (Song et al., 2002, Kempermann et al., 2004). These NPCs symmetrically divide in an expansion phase before differentiating into more fate-restricted neuroblasts. Neuroblasts continue to proliferate as they migrate out of the niche to their target zones, where they differentiate into postmitotic immature neurons. These immature neurons integrate into the existing neural network by extending axons and increasing their connectivity, gradually obtaining the physiological characteristics of the local mature neurons (Ming and Song, 2011, Gotz and Huttner, 2005). Amplification of relatively large numbers of new neural progenitors is matched to the physiological requirements of the CNS. Of the newly formed neuroblasts, only a subset will go on to form immature neurons that are integrated into the neural circuitry of the target structure, the remainder undergo programmed cell death (PCD; Southwell et al., 2012).

During adult neurogenesis cellular maturation is both continuous and heterogeneous, unlike the orchestrated waves of cell proliferation and maturation observed during embryonic development. Niche derived growth factors and other signalling molecules provide support and maintenance of ongoing neurogenesis, ensuring the physiological conditions in the niches remain permissible to continued neuron production. Intrinsic

regulation of transcription factors and cell cycle regulators has been demonstrated to play a major role in maintaining the homeostatic environment of the neurogenic niches (Goritz and Frisen, 2012, Ming and Song, 2005). External stimuli, such as physical exercise, environmental enrichment, natural aging, mental illness, chronic pain and pathological states induced by seizure or stroke are also strongly correlated with altered rates of neurogenesis, particularly in the hippocampus (Steiner et al., 2008, Kempermann et al., 2002, Zheng et al., 2017).

2.3 NEUROGENESIS IN THE ADULT HIPPOCAMPUS

In humans, neurogenesis within the dentate gyrus continues throughout adulthood, generating granule cells crucial for memory formation. Recently, Spalding and colleagues examined the generation rate of granule cells using ^{14}C concentrations in genomic DNA, and estimated that 700 new neurons are added to the hippocampus each day, with only a modest decline with age (Spalding et al., 2013). Adult-born neurons display enhanced synaptic plasticity for a limited period of time, and have a key role in pattern separation and cognitive adaptability, allowing a relatively small number of granule cells to have significant influence on circuitry and function in the hippocampus (Ge et al., 2007). These new neurons represent a minority of the progenitor cells produced in the adult hippocampus; a large number of the newly generated cells undergo PCD and overall there remains a net loss of neuron numbers within the hippocampus.

Neurogenesis is vital for hippocampal function, in particular learning and memory formation, and the role of newly formed granule neurons in pattern separation is thought to be a key mechanism in this process (Sahay et al., 2011, Clelland et al., 2009).

Pattern separation processes neural inputs into distinct outputs, allowing memories to be stored without overlap or interference. Neurogenesis also modulates fear learning and supports association between events and predictive cues (Seo et al., 2015). Newly generated granule neurons have differing characteristics from mature neurons; they possess increased intrinsic excitability with higher potentiation amplitude and a lower induction threshold (Ge et al., 2007, Schmidt-Hieber et al., 2004). This results in enhanced synaptic plasticity and preferential activation of new granule neurons, and suggests they are major mediators of hippocampal synaptic plasticity (Yau et al., 2015).

While basal neurogenesis rates are thought to be genetic, the process is heavily regulated by both physiological and pathological stimuli. Mental disorders, such as stress, depression, schizophrenia and substance addiction have all been correlated with a decrease in hippocampal size and structural integrity, possibly due to decreased neurogenesis (Videbech and Ravnkilde, 2004, Heckers, 2001, Eisch et al., 2000). On the other hand, exercise, environmental enrichment and use of antidepressants have shown to promote hippocampal neurogenesis (van Praag et al., 1999, Santarelli et al., 2003, Malberg et al., 2000, van Praag, 2008). Mice and rats that are provided with an enriched environment and/or voluntary exercise consistently show increased rates of neurogenesis and display improved performance in learning and short term memory when assessed by the water maze task (Nilsson et al., 1999, Bruel-Jungerman et al., 2005, Iso et al., 2007).

A study by Steiner et al. (2008) demonstrated neuronal precursors to differentiate between different stimuli (exercise, environmental enrichment, and induced seizures) and respond with distinct modifications in proliferation patterns. The ability of the hippocampus to modulate neurogenesis in response to stimuli may lead to potential

therapies to treat a range of disorders, from stress and depression to other complex psychiatric disorders (Jun et al., 2012, Manji et al., 2000).

2.4 STRUCTURE AND FUNCTION OF THE ADULT HIPPOCAMPUS

The hippocampus is part of the limbic system and has well established roles in episodic memory formation, consolidation and storage, as well as sensory and spatial processing and integration, and some types of learning; each function has its own distinct groupings of efferent inputs (Aggleton, 2012, Squire and Alvarez, 1995, von Allmen et al., 2013, McEown and Treit, 2013). Hippocampal subregions are thought to contribute differently to memory acquisition and retrieval in fear-conditioning, as demonstrated by Lee and Kesner (2004). Their study demonstrated the CA1, CA3 and dentate gyrus contributed to acquisition, while memory retrieval involved only the CA1 and dentate gyrus. Extensive studies in humans, primates, and rodents have revealed that damage to the hippocampus or its connections results in deficits to episodic memory, object-place memory tasks and spatial memory tasks (Rolls, 2015). This becomes particularly evident when studying patients with hippocampal and/or fornix lesions, as exemplified by the famous case of H.M., who in 1953 underwent a surgery removing parts of the hippocampus in an attempt to treat epilepsy, and subsequently suffered severe anterograde amnesia (Squire and Wixted, 2011). Retrograde amnesia has also been observed; historic cases dating from 1957 to 1996 are reviewed by Nadel and Moscovitch (1997).

These studies are supported by functional neuroimaging that shows activation of the hippocampus by episodic memory tasks, spatial navigation and processing (Burgess et al., 2002, Chadwick et al., 2010, Iaria et al., 2003). The hippocampus temporarily

processes and stores new memories by utilizing a special type of synaptic plasticity to prepare memories for long term storage (Yau et al., 2015). Acquisition of spatial memories in the water maze task has been correlated with increased levels of neurogenesis in the hippocampus of adult mice, demonstrating the crucial role the hippocampus plays in synaptic plasticity (Kempermann and Gage, 2002). It may be that plasticity in the hippocampus is regulated by fate specification; evidence for this comes from lineage analysis experiments that demonstrate the ratio of NSCs to neurons depends on the animals experiences. Social isolation resulted in the accumulation of NSCs but not neurons, and indicates the presence of mechanisms that allow adaptation to environmental changes (Dranovsky et al., 2011).

A study by Gu and colleagues combined retroviral birth dating with optogenetic stimulation to examine the roles of new neurons during their integration and development. They found functional synapses as early as two weeks after initial division, and at four weeks the neurons displayed stable connections and enhanced plasticity. Optogenetic silencing of these four week old neurons, but not neurons at two or eight weeks of age, inhibited retrieval of hippocampal dependent memories, indicating a very specific role for new neurons in synaptic plasticity and hippocampal memory function (Gu et al., 2012).

2.4.1 Synaptic transmission in the hippocampus

The hippocampus consists of four distinct substructures; the dentate gyrus, the subiculum, the presubiculum, and Ammon's horn (cornu ammonis), which is further divided to CA1 and CA3 (Li et al., 2009, Yau et al., 2015). The dentate gyrus itself has a number of regions: the hilus, the granular cell layer, the molecular layer and the hippocampal fissure, refer to Figure 2.1.

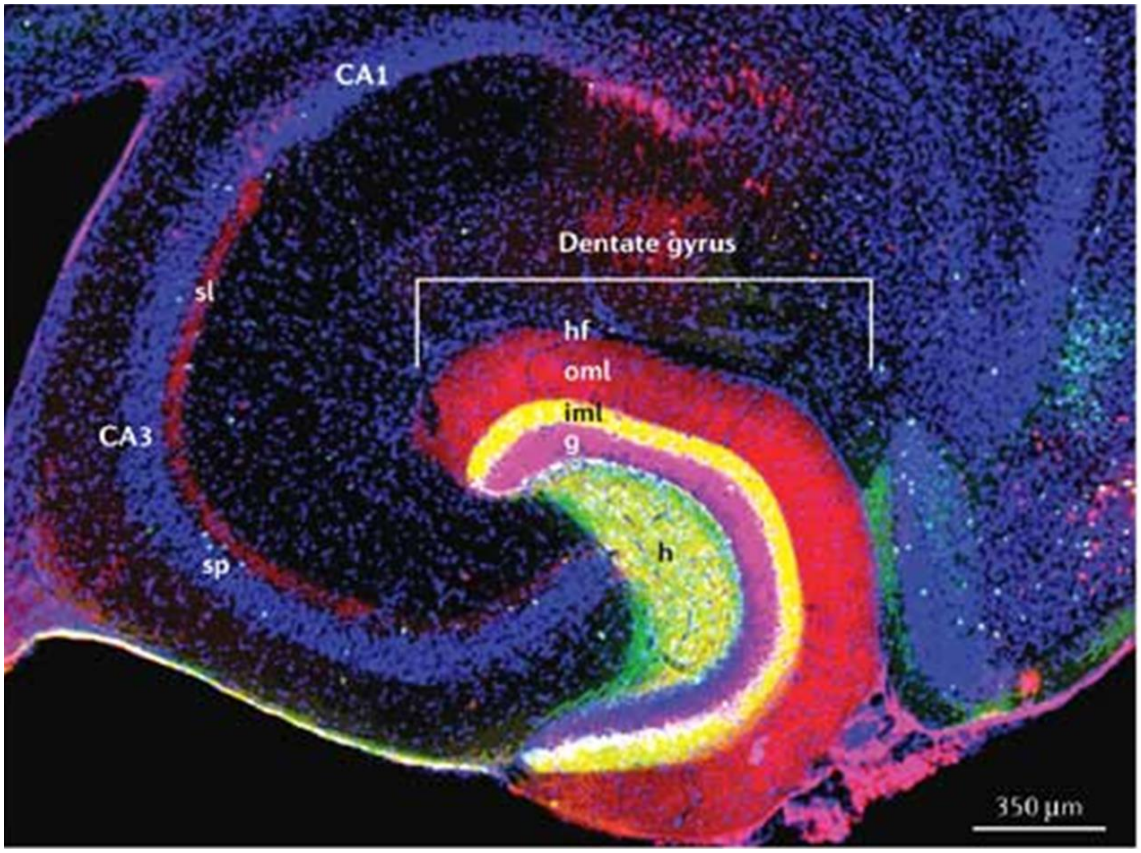

Figure 2.1 Section of the adult mouse hippocampal formation

Abbreviations: hf, hippocampal fissure; oml, outer molecular layer; iml, inner molecular layer; g, granule cell layer; h, hilar region; sp, pyramidal layer; sl, stratum lucidum. Cell nuclei have been stained with DAPI (blue) and calbindin (red) has been used to label granule neurons. The granule cell layer (thus depicted as purple) has dendrites extending through the molecular layer towards the hippocampal fissure, while their axons (mossy fibres) terminate in the stratum lucidum of CA3; these areas are strongly labelled red with calbindin. Fibres from the entorhinal cortex terminate in the outer molecular layer (red). NSCs are located in the subgranular layer, and migrate the short distance to the granule layer as they mature. Mossy cells in the hilus are stained for calretinin (green) and project their axons to the inner molecular layer (observed as yellow due to co-localisation with calbindin). Image reproduced with permission from Forster et al. (2006). Scale bar 350 µm.

Synaptic transmission is mostly unidirectional, whereby sensory information from the thalamus and other neocortical areas arrive at the hippocampal entorhinal cortex. Neocortical input includes information from the visual and auditory association areas of the temporal lobe, and the prefrontal, orbitofrontal and parietal cortices (Aggleton, 2012). The entorhinal cortex can also receive input from other structures, such as the amygdala and nucleus accumbens (Pitkanen et al., 2002).

Neurons in layer two of the entorhinal cortex project through the subiculum before arriving at either the molecular layer of the dentate gyrus or the CA3. In the dentate gyrus, fibres from the entorhinal cortex synapse with dendrites of granule neurons (Forster et al., 2006). Granule neuron cell bodies form the granule layer, and axons of these neurons (termed mossy fibres) synapse on the pyramidal cells of the CA3. Mossy fibres also synapse on mossy cells and interneurons located in the hilus. Axons of the CA3 pyramidal cells project mainly to the pyramidal cells of the CA1, which in turn synapse on the entorhinal cortex. The sensory signal then returns mostly to its neocortical region of origin. Output from the CA1 region of the hippocampus projecting to various cortical regions is likely responsible for memory recollection (Li et al., 2009, Rolls, 2015); see pathway illustration (Figure 2.2).

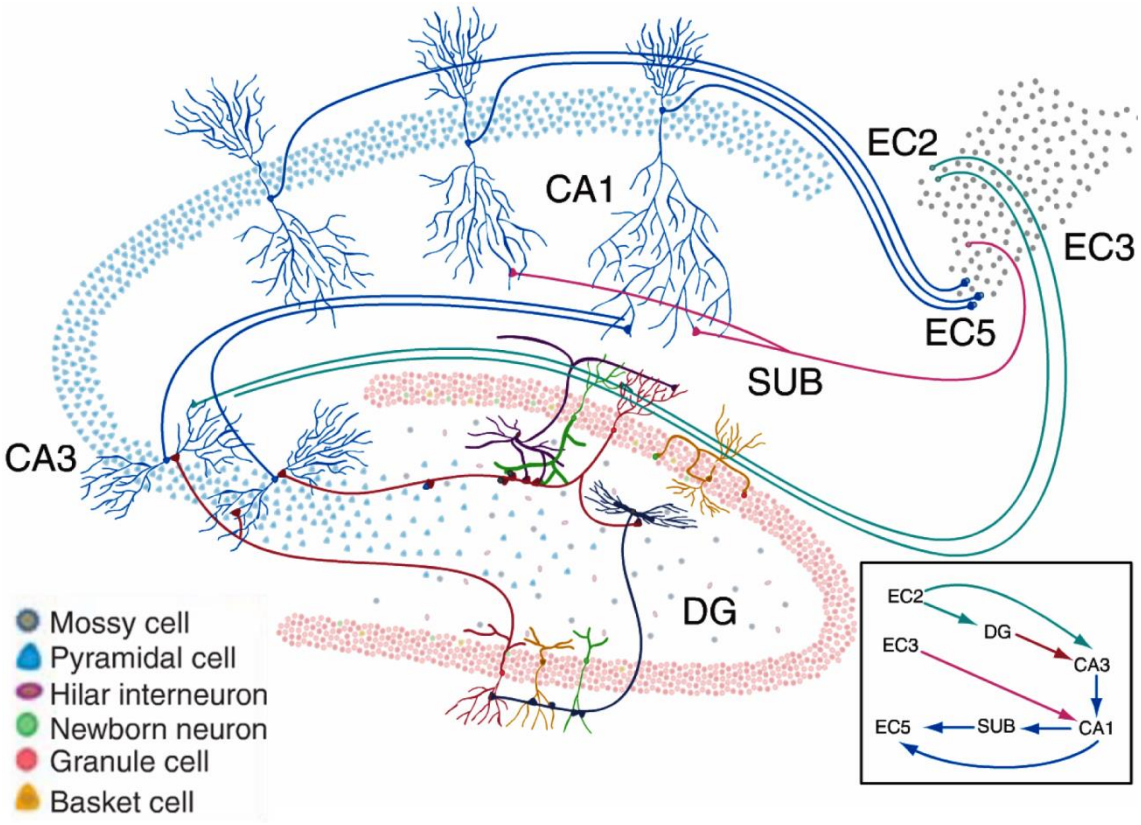

Figure 2.2 Synaptic transmission in the hippocampal formation

Abbreviations: EC, entorhinal cortex; SUB, subiculum; DG, dentate gyrus. Sensory information arrives at the entorhinal cortex, before travelling to the granule cells of the dentate gyrus and pyramidal cells of the CA3 region. The granule cells have axons known as mossy fibres, and these terminate on CA3 pyramidal cells, as well as mossy cells and interneurons in the hilus. CA3 pyramidal cells then project back to the CA1, which in turn synapse in the entorhinal cortex before returning the cortical region of origin. Reproduced with permission from Li et al. (2009).

2.5 PROGRESSION OF HIPPOCAMPAL NEUROGENESIS

2.5.1 Stages of neurogenesis

First described by Kempermann et al. (2004), contemporary neuroanatomical analysis of adult hippocampal neurogenesis characterizes the progression of NSCs to fully integrated mature granule neurons by separating cell types into five stages marked by specific developmental milestones. Each stage is differentiable both by the proliferative potential and the increasing neuronal phenotype of the cells. Neurogenesis begins with a NSC in the subgranular layer giving rise to intermediate progenitors of the neuronal lineage. These differentiate into neuroblasts and eventually mature into granule neurons. Granule neurons project their axons, called mossy fibres, to the CA3 region of hippocampus, while their dendritic arbours extend to the hippocampal fissure, and eventually they become fully integrated in the local neuronal network (Jessberger and Kempermann, 2003). The nomenclature used to define each cell type is relative as cells do not progress in discrete stages, but rather undergo gradual maturation with an ever increasing neuronal phenotype. Thus, there is overlap of some protein markers used to identify cell type and the use of these delineation stages is a convenience utilized primarily for analytical purposes (Ming and Song, 2005, Kempermann et al., 2004, Li et al., 2009, Ehninger and Kempermann, 2008). These stages are outlined below, and illustrated in Figure 2.3.

2.5.1.1 Stage 1: Asymmetric division

Hippocampal NSCs are referred to as type 1 cells and are located in the SGZ of the dentate gyrus. During the first proliferation stage, type 1 cells begin their development by dividing asymmetrically to give rise to an identical daughter cell and an intermediate progenitor (type 2) cell.

2.5.1.2 Stage 2: Progenitor proliferation

The second stage encompasses proliferation and fate specification as the intermediate type 2 progenitors gradually differentiate to type 3 neuroblasts. Type 2 cells can be divided into type 2a and type 2b cells, characterized by the expression of immature neuronal marker doublecortin (DCX) by type 2b cells.

2.5.1.3 Stage 3: Neuroblast differentiation and migration

Type 3 neuroblasts display an increasingly neuronal phenotype as they migrate to the granule cell layer, where their proliferation ceases and they differentiate into a postmitotic immature granule cell.

2.5.1.4 Stage 4: Targeting

Immature granule cells extend axonal projections to the hilus and the CA3 pyramidal cell region of the hippocampus, while their dendrites reach the molecular layer. Granule cells begin to compete for target-derived growth factors as maturation continues.

2.5.1.5 Stage 5: Integration and maturation

The final stage in hippocampal neurogenesis involves expansion of the dendritic arbour and full integration of the new granule cell into existing synaptic pathways. This completes the transformation from a neural progenitor in the SGZ to a mature neuron of the granule cell layer.

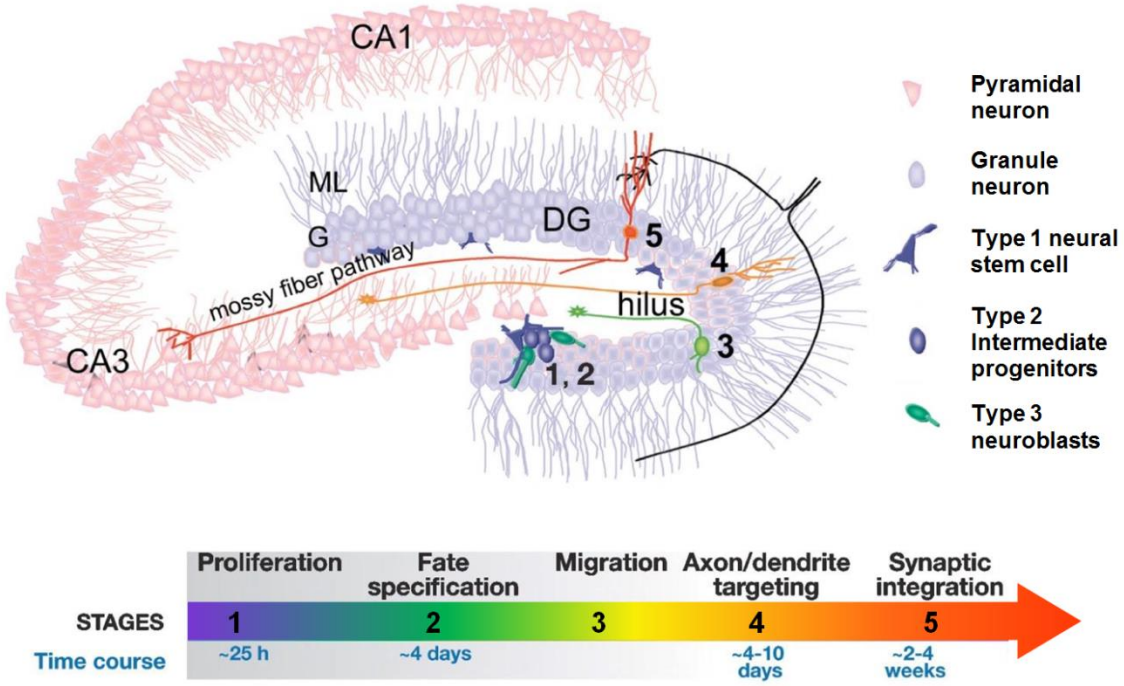

Figure 2.3 Developmental stages of hippocampal neural progenitors

Abbreviations: DG, dentate gyrus; ML, molecular cell layer; G, granule cell layer. In the first stage, type 1 NSCs asymmetrically divide in the subgranular zone, before differentiating to become proliferative intermediate progenitors (types 2a and 2b) in the second stage. In the third stage type 3 neuroblasts migrate to the granule cell zone. In the final stages of neurogenesis, immature neurons extend axons to the hilus and CA3 regions of the hippocampus, before being fully integrated in existing synaptic transmission pathways. Figure adapted from Ming and Song (2005).

2.5.2 Type 1 cells: The neural stem cell of the hippocampus

The putative NSC of the hippocampal dentate gyrus is often referred to as a radial glia cell and are similar to the type B stem cell of the SVZ (Merkle et al., 2004). Type 1 cells are generally quiescent, and primarily divide asymmetrically to a type 1 daughter cell and a type 2 intermediate progenitor cell with a more neuronal phenotype (Kronenberg et al., 2003). A distinguishing feature of type 1 cells is their expression of glial fibrillary acidic protein (GFAP; Seri et al., 2001). As GFAP is also expressed by

astrocytic cells, it must be used in combination with neural progenitor markers to identify type 1 cells (Kempermann et al., 2004). In addition, type 1 cells express the precursor marker nestin, a type VI intermediate filament protein expressed during development in the SGZ (Filippov et al., 2003), as well as radial glia marker brain lipid binding protein (BLBP) and SRY (sex determining region Y)-box 2 (Sox2), a transcription factor essential for maintaining the pluripotency of stem cells (Ehninger and Kempermann, 2008).

2.5.3 Type 2 cells: Intermediate progenitor cells

Type 2 intermediate progenitor cells have small soma with short processes and can be characterised by their expression of specific stem and progenitor markers in conjunction with increased proliferation (von Bohlen und Halbach, 2011). Type 2 cells continue to express nestin and Sox2 (Suh et al., 2007), and are concurrently negative for GFAP expression (Fukuda et al., 2003). Expression of mammalian achaete scute homolog-1 (Mash1, also known as ASCL1) defines entry of type 2 cells into the proliferative stage (Kim et al., 2008). Their most recognizable trait is their highly proliferative capabilities and the bulk expansion of new cells in the hippocampal neurogenic niche is driven by type 2 cell division. Type 2 cells are further subdivided into types 2a and 2b, depending on the expression of the immature neuronal marker DCX, which identifies type 2b cells only (Brown et al., 2003, Kempermann et al., 2004). DCX delineates the point at which type 2 cells begin to exhibit a neuronal phenotype (Lugert et al., 2010). Type 2b cells can also be identified by the transcription factor prospero-related homeobox 1 (Prox1), a granule cell specific transcription factor, which is expressed by type 2b cells fate restricted to the neuronal lineage (Steiner et al., 2006, Steiner et al., 2008).

2.5.4 Type 3 cells: Neuroblasts

Type 3 cells are proliferative neuroblasts; they migrate a short distance to the granule cell layer where proliferation decreases and they begin the process of differentiation (Kempermann et al., 2004). Type 3 cells do not express early precursor or glial markers and upregulate immature neuronal markers, such as DCX and Prox1 (Brown et al., 2003, Steiner et al., 2008). DCX is expressed by migrating neurons in both the central and peripheral nervous systems, and functions as a microtubule stabilising protein to facilitate migration and axon extension (Gleeson et al., 1999). When expressed in conjunction with proliferation markers, such as BrdU or Ki67, DCX is a valuable tool for studying adult neurogenesis in both rodents and humans (Knoth et al., 2010).

2.5.5 Postmitotic stages: Maturing granule neurons

Following the migration of type 3 neuroblasts to the granule cell layer, they cease proliferation and become postmitotic immature neurons, where they undergo a selection process and are either incorporated in the neural cytoarchitecture or undergo PCD. Post mitotic neuronal markers, such as NeuN, Map2a/b, βIII tubulin and granule cell markers calretinin (immature granule neuron) and calbindin (mature granule neuron) are expressed (Kempermann et al., 2004, von Bohlen und Halbach, 2011). It takes between four to ten days from the initial division for the new neurons to migrate to the granule cell layer and extend axonal projections to their targets in the CA3. By 24 days post division, the morphology of new granule neurons is mostly indistinguishable from the pre-existing mature neurons (Hastings and Gould, 1999, Ming and Song, 2005). A combination of morphological changes, proliferative potential and protein expression patterns are utilized to identify cells both in the hippocampus and in culture; these are summarised in Figure 2.4.

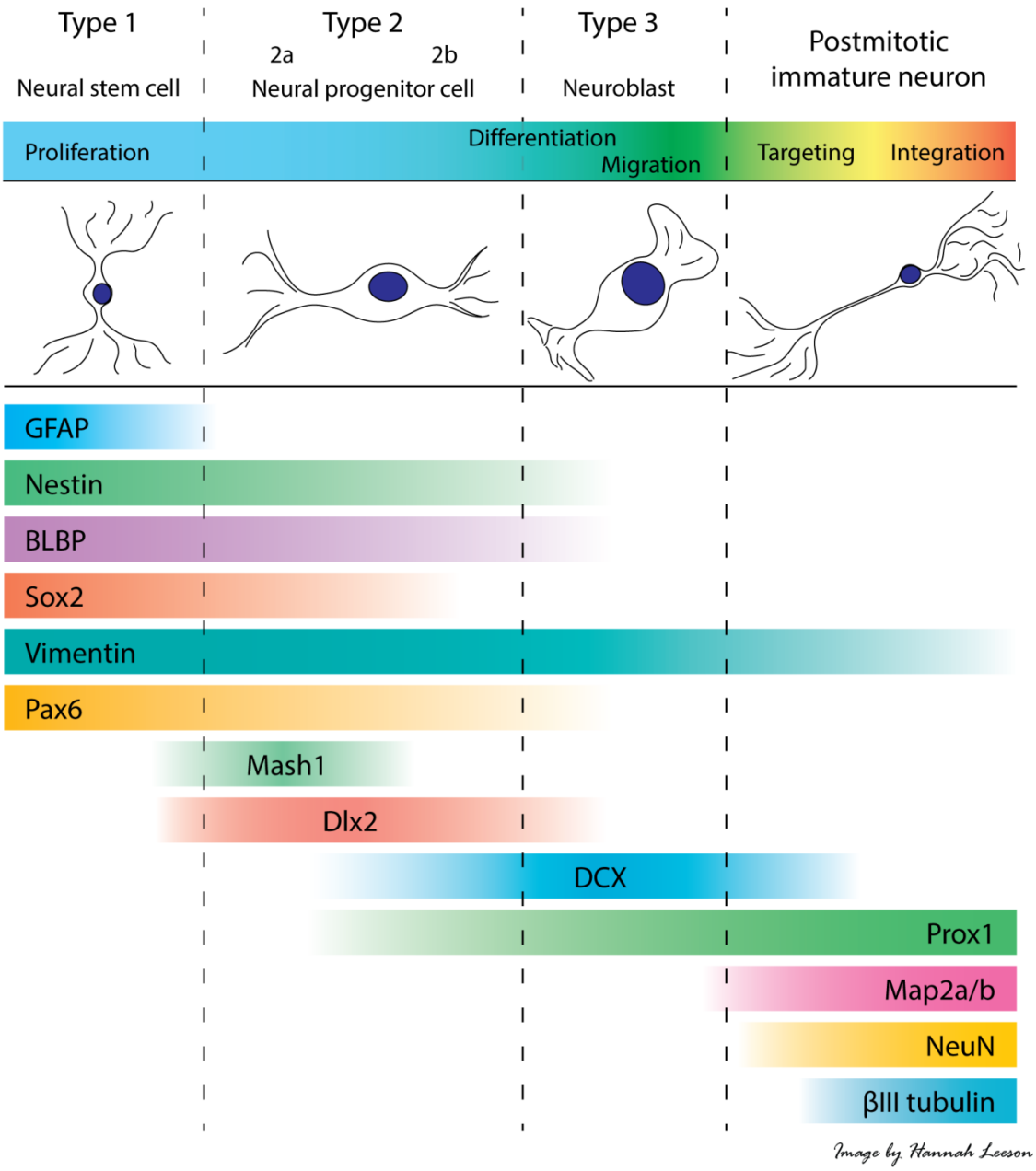

Figure 2.4 Lineage and markers of cultured hippocampal neural progenitor cells
Type 1 cells positively express GFAP and markers for early development and pluripotency including nestin, BLBP and Sox2. Type 2a intermediate progenitors lose GFAP expression and upregulate Mash1. Vimentin, Pax6 and Dlx2 are also expressed by these neural progenitors. Type 2b intermediate progenitors begin downregulating Mash1 and begin expressing DCX and Prox1, marking the beginning of maturation to a neuronal phenotype. Type 3 neuroblasts downregulate expression of the progenitor markers. Finally, postmitotic immature granule cell neurons continue to express DCX and Prox1, and upregulate Map2a/b, NeuN and βIII tubulin.

2.6 DYSREGULATION OF HIPPOCAMPAL NEUROGENESIS

2.6.1 Stress, depression and mental disorders

As an area of the adult brain that constantly remodels its synaptic connectivity in response to sense-data, it is not surprising that these experiences can also modulate neurogenesis. There is substantial evidence that psychosocial stress, anxiety and depression reduces neurogenesis in rodents via release of stress-related hormones (Dranovsky and Hen, 2006). Chronic corticosterone treatment has been used as a model for anxiety and depression in mice, and inhibits hippocampal neurogenesis, while treatment with antidepressants, such as fluoxetine, reverses this effect (David et al., 2009). In another study, deleting the pro-apoptotic gene Bax from NSC was sufficient to alleviate anxiety and depressive behaviours by increasing hippocampal neurogenesis (Hill et al., 2015). Environmental enrichment and exercise increased hippocampal neurogenesis and correlated strongly with decreased anxiety behaviours, faster habituation times and improved spatial learning (Meshi et al., 2006). Fate specification and synaptic plasticity has emerged as a possible mechanism for adapting to experiences and changes in the environment. Social isolation was correlated with an increase in the neural progenitor population (Dranovsky et al., 2011), and it may be that in times of stress NSCs accumulate until conditions favouring the progression of neurogenesis return. These data suggest that mechanisms regulating neurogenesis and fate determination can be modified in response to environmental factors.

2.6.2 Stroke and epilepsy

Adult hippocampal neurogenesis appears to play a role in the brain's ability to recover from some types of physiological trauma (Jin et al., 2010). For example, evidence

suggests that following stroke there is an increase in progenitor cell proliferation, and that these new cells become functionally integrated into the existing hippocampus (Geibig et al., 2012). Interestingly, another study observed that pharmacologically enhancing neurogenesis following stroke did not increase neuron survival or differentiation (Sun et al., 2015b). Epileptic seizures have been shown to cause abnormal hippocampal neurogenesis, with increased progenitor proliferation but aberrant integration, neuronal hypertrophy and altered excitability. This may prevent the hippocampus from properly regulating excitatory activity and may prompt further seizures (Danzer, 2012). Inhibition of hippocampal neurogenesis prior to inducing acute seizures with pilocarpine reduced the cognitive impairment associated with epilepsy, and this led to reduced seizure frequency and long term suppression of spontaneous recurrent seizures. The authors concluded that abnormal neurogenesis not only results from, but contributes to epileptic episodes (Cho et al., 2015). Whether or not epilepsy can be caused by altered or aberrant neurogenesis as the brain attempts repair following an initial injury remains a debated topic (Jessberger and Parent, 2015).

2.6.3 Other factors correlated to dysregulated neurogenesis

There is strong evidence suggesting that social interactions play an important role in helping prevent reduction in neurogenesis levels caused by stress events, and that social isolation can void the positive effects of exercise on rates of neurogenesis (Stranahan et al., 2006, Leasure and Decker, 2009). Social interaction can also assist in behavioural recovery by increasing rates of neurogenesis in mice suffering from stroke (Venna et al., 2014). Sex hormones also play a role in hippocampal neurogenesis, and appear to significantly modulate the formation of spatial and contextual memories (Duarte-Guterman et al., 2015). For example, sexual encounters with unfamiliar females

resulted in a significant reduction in neurogenesis rates in male rats when compared to encounters with familiar females (Spritzer et al., 2016). It is thought that this is due to changes in oxytocin and testosterone levels, both of which increase neurogenesis (Spritzer and Galea, 2007, Leuner et al., 2012). Interestingly, no significant differences were observed between familiar females and control, raising the possibility that unfamiliar females induced a stronger stress response.

Substance abuse can also lead to a reduction in hippocampal neurogenesis. Post mortem analysis of heroin addicts showed decreased numbers of NPCs accompanied by lowered rates of proliferation (Bayer et al., 2015). This study is supported by a number of animal studies reporting negative impacts of drug abuse on neurogenesis (Chambers, 2013, Arguello et al., 2009). Alcohol also has adverse effects on neurogenesis; it is capable of interfering by a number of different mechanisms, as reviewed by Geil et al. (2014).

2.7 REGULATION OF HIPPOCAMPAL NEUROGENESIS

Adult hippocampal neurogenesis is a complex and multi-faceted process, and many mechanisms are involved in regulating NPCs and the changes they undergo in the process of neurogenesis. Growth factors and neurotrophins are key regulators of adult neurogenesis, and facilitate proliferation, migration, transcription factor regulation and maturation processes (Oliveira et al., 2013). These pathways converge with cell death mechanisms to tightly regulate cell numbers. Purinergic signalling has also emerged as an important contributing factor during proliferation, differentiation, migration and PCD in adult progenitor populations in the hippocampus, and indeed in the entire CNS (Ulrich et al., 2012, Burnstock, 2016, Abbracchio et al., 2009).

2.8 ROLE OF GROWTH FACTORS IN THE NEUROGENIC NICHE

Survival and maintenance of adult NPC in the niche, as well as in culture, requires at least two growth factors: fibroblast growth factor-2 (FGF2) and epidermal growth factor (EGF). These two growth factors act as key regulators of many critical cellular functions including cell cycle progression, proliferation, differentiation, survival and metabolism (Lemmon and Schlessinger, 2010, Annenkov, 2014). Their receptors, FGFR2 and EGFR, belong to a family of receptor tyrosine kinases (RTKs), which are highly conserved and consist of an extracellular domain, a single transmembrane helix and a cytoplasmic region containing the tyrosine kinase domain, and are activated by the binding of a ligand that induces receptor dimerization (Ullrich and Schlessinger, 1990).

The proliferation of hippocampal NPCs is dependent on synergism between EGFR and FGFR signalling, requiring their use in standard protocols for the culturing of neural stem and progenitor cells *in vitro* (Gage et al., 1995). The neurosphere assay was developed in 1992 and has provided a reliable means of isolating and expanding NSC in culture. Reynolds and Weiss (1992) found that EGF in particular was essential for progenitor proliferation and that withdrawal of EGF from culture promoted differentiation of the NSC into neuronal or glial lineages. *In vivo*, EGFR signalling is vital for maintaining or recovering proliferation following trauma; in an ischemia model a combination of EGF and FGF2 increased NPC numbers in the neurogenic niches of the SVZ and SGZ, as well as the posterior periventricle and the hypothalamus (Oya et al., 2008). In the hippocampus, FGFR2 signalling is required for the generation of new NPCs and neurons, and inducible knock down significantly decreased spatial memory formation, a process heavily dependent on neurogenesis (Stevens et al., 2012).

2.9 PROGRAMMED CELL DEATH IN HIPPOCAMPAL NEUROGENESIS

Neurogenesis is marked by an overproduction of progenitor cells, which are selected for differentiation and integration into neuronal networks depending on the requirements of the brain. PCD is essential for controlling cell numbers in proliferative stages and for synaptic pruning and removal of immature neurons that fail to correctly integrate into the existing cytoarchitecture (Ryu et al., 2016). A number of hypotheses have emerged in trying to elucidate the molecular mechanisms driving PCD. Trophic dependence, where target-derived neurotrophic factors protect the developing neurons from entering apoptosis, has gained significant support (Fuchs and Steller, 2011). Genetic factors and local inhibitory signals in the microenvironment also contribute to determining if a cell should undergo PCD (Penaloza et al., 2006, de la Rosa and de Pablo, 2000). Another hypothesis involves the choice of cells to undergo PCD via phenotypic selection generated by an active gene rearrangement, similar to that seen in T cells of the thymus. Thus, cell death would be a natural outcome in the generation of large numbers of different phenotypes; this has interesting implications in the current dogma surrounding the process of neurogenesis, as it suggests a much greater level of genetic diversity than previously believed (Blaschke et al., 1996).

There are two general types of PCD, the first during early stage development is heavily influenced by internal regulators of apoptosis and is referred to as target independent cell death; this is followed by a second, later phase where cell survival is dependent on trophic support from the target tissue. This late stage development form of PCD is referred to as target dependent cell death (de la Rosa and de Pablo, 2000, and Dekkers and Barde, 2013).

2.9.1 Target independent programmed cell death

Target independent PCD occurs during early embryogenesis, as well as the beginning stages of adult neurogenesis. This form of PCD is independent of target interactions and depends more on internal regulation of cell death pathways and support from neurotrophins, such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), which are essential anti-apoptotic factors for promoting cell growth and survival (Frielingsdorf et al., 2007). In the adult dentate gyrus, only a small fraction of the proliferating cells migrate to the granule layer and mature into fully functional neurons, the majority undergo PCD one to four days after the first division (Sierra et al., 2010). In these situations, the apoptotic cells are progenitor cells still in the proliferation stages and have not yet matured into neurons capable of producing an axon to connect with a target, suggesting this form of cell death is regulated by cell autonomous signals or region specific signals.

2.9.2 Target dependent programmed cell death

Neuroblasts differentiate into postmitotic immature neurons and begin forming synaptic connections with their target cell or neuron. Though still influenced by trophic support, cell survival is now also regulated by synaptic activity and neurotransmitters present in the neurogenic niche, such as glutamate, GABA, serotonin, dopamine and acetylcholine, which all promote neuron survival in adult dentate gyrus (Yoshimizu and Chaki, 2004, Song et al., 2013, Diaz et al., 2013, Takamura et al., 2014, Campbell et al., 2010). Immature neurons compete with neighbouring neurons for growth factors and begin to propagate their synaptic connections. Neurotransmitters act as positive signals ensuring the correct scaffolding proteins are produced and maintained. Neurons that do

not form synaptic connects undergo target-dependent PCD and are removed by phagocytosis.

2.9.3 Role of programmed cell death in adult neurogenesis

In the SGZ of the adult rat dentate gyrus an estimated 9000 progenitor cells are produced each day (Cameron and McKay, 2001). Of these newly generated precursor cells, around 50% will undergo PCD at a steady rate during the first four weeks (Dayer et al., 2003). Young neurons surviving four weeks make up about 6% of the total population of granule neurons in the hippocampus (Cameron and McKay, 2001, Kempermann et al., 1998, West et al., 1991). By five months most of these neurons have matured and become incorporated into existing circuitry where they function together with the rest of the granule cells formed during development (Dayer et al., 2003).

Sierra and colleagues observed that the majority of neuroblasts underwent apoptosis one to four days post the initial division, and that microglia rapidly phagocytosed the dead cells (Sierra et al., 2010). Apoptotic bodies are cleared in just a few hours, meaning the total amount of cell death is difficult to estimate, and not necessarily reflected by the number of pyknotic or TUNEL positive nuclei observed (Chung and Yu, 2013). Apoptosis appears to be the major form of PCD initiated during hippocampal neurogenesis. Maturing neurons that fail to establish appropriate connections undergo PCD and this is almost completely blocked in Bax knockout mice. A study in rats observed a two fold increase in cell numbers in the dentate gyrus by twelve months, though not at two months, suggesting a greater role for Bax regulated apoptosis in the adult than in the embryonic brain, as well as a critical role for trophic support (Sun et al., 2004).

2.10 PHAGOCYTOSIS DURING ADULT NEUROGENESIS

Clearance of cell corpses following PCD is essential for maintaining homeostasis in the neurogenic niche, and dysregulation in phagocytosis results in build-up of cellular debris leading to brain dysfunction (Fuchs and Steller, 2011). The process of PCD and phagocytosis proceed together; initially, PCD begins and intracellular signalling cascades lead to morphological changes that include cell blebbing and shrinkage, chromatin condensation and DNA fragmentation (Ryu et al., 2016). Next, cells undergoing apoptosis release intercellular signals and these act as molecular attractants, signalling to and activating resident microglia, the professional phagocyte of the CNS, as well as non-professional phagocytes. These molecular signals include lysophosphatidylcholine (LPC) and phosphatidylserine (PS), the latter of which is expressed on the extracellular membrane of apoptotic cells and mediates interactions with the receptor on the phagocyte. Finally, engulfment occurs primarily via the Rho family of GTPases via their regulatory actions on intracellular actin dynamics (Fuchs and Steller, 2011).

2.10.1 Microglia: the professional phagocyte

Microglia appear in the cerebrum during the second trimester of human gestation and once present act as the principal phagocyte of the CNS, playing vital role in the maintenance of the adult hippocampus (Rezaie and Male, 1999, Sierra et al., 2010). During target dependent PCD, microglia play an important role in the removal of apoptotic neurons and also aid in the pruning of synapses during development (Kettenmann et al., 2011). The role of microglia goes beyond the phagocytosis of debris; they also have crucial roles in the reorganisation and repair of neural structures (Neumann et al., 2009).

Resting microglia monitor their environment by extending processes and initiating short term contact with neuronal synapses (Wake et al., 2009). A change in homeostasis caused by infection, injury or altered neuronal activity results in the release of microglial-activating and pro-inflammatory signals from the affected cells, including chemo-attractants, ATP, chemokines and growth factors. These 'find me' and 'eat me' signals are recognised by receptors on the surface of the microglia. In response, microglia are activated and migrate to the affected area, producing further pro-inflammatory mediators as they migrate (Wake et al., 2009, Kettenmann et al., 2011). Some evidence suggests that it is not necessary for microglia to become 'activated' in order to phagocytose apoptotic NPCs, suggesting microglia may be able to recognise and eliminate apoptotic cells using an alternative pathway to that activated by inflammation (Sierra et al., 2010).

Microglia undergo phenotypic changes that are dependent on the type of stimuli they encounter, and different stimuli are recognised by different receptors; microbes are recognised by Toll-like receptors, while apoptotic neurons activate phosphatidylserine mediated receptors, asialoglycoprotein-like lectins and vitronectin receptors (Witting et al., 2000). Scavenger receptors on the surface of microglia also play an important role in the initiation of phagocytosis. For example, P2X7 receptors can act as scavenger receptors in monocytes and macrophages in the absence of ATP (Wiley and Gu, 2012), and in primary microglial cultures phagocytosis was also facilitated by P2X7 receptors (Fang et al., 2009).

2.10.2 NPCs and astrocytes: the nonprofessional phagocytes

Until recently, microglia were assumed to be solely responsible for clearance of this apoptotic debris, though it has now been revealed that adult NPCs are capable of

phagocytosing other apoptotic progenitors (Lu et al., 2011). During early-stage embryonic neurogenesis when microglia have not yet arisen from the developing parenchyma, the principal phagocyte appears to be the neuroepithelial cells themselves (Gu et al., 2015). It is reasonable to suspect these mechanisms of cell removal are retained into adulthood. Observations in both the SVZ and SGZ demonstrate that NPCs are capable of phagocytosing other apoptotic neural progenitors, and this mechanism, seen in DCX^+ cells, was reliant on intracellular engulfment protein ELMO1, which promotes Rac1 activation and cytoskeletal rearrangements required for engulfment of apoptotic bodies (Lu et al., 2011). It was also recently demonstrated that human embryonic NPCs express P2X7 receptors and that these progenitors are able to phagocytose via a P2X7 mediated pathway (Lovelace et al., 2015). Resting astrocytes were likewise demonstrated to phagocytose via P2X7 receptors (Yamamoto et al., 2013). These data suggest microglial phagocytosis is not the only method used in the clearance of apoptotic NPCs, and that other mechanisms play important roles in maintenance of the neurogenic niche.

2.11 CALCIUM SIGNALLING IN NEURAL PROGENITOR CELLS

There are few signalling molecules as versatile as the calcium ion, and through specific modulation of internal stores and membrane channels, fluctuations in cytoplasmic calcium concentrations form complex signalling events. In this way a single ion can exert a wide influence over a large number of biological processes occurring in adult NSCs and NPCs, including excitability, synaptic transmission, gene expression, proliferation, differentiation and apoptosis (Tonelli et al., 2012). The regulation of internal concentrations is crucial and as cytosolic calcium concentrations are significantly lower than those in the extracellular fluid, cells expend a large amount of

energy to tightly control cytosolic concentrations through numerous ATP driven pumps, binding buffer proteins and channels. In this way the cell is able to control cytosolic calcium in a manner that generates distinct downstream signalling events that are eventually translated into biological changes through decoder proteins (Berridge et al., 2003, Tonelli et al., 2012).

2.11.1 Calcium encoding

Calcium holds influence over such a large number of mechanisms due to the diversity of its signalling events in terms of frequency, amplitude and spatiotemporal patterning. These are initiated by either global waves or localised spikes in cytosolic calcium concentration, and are controlled by finely-tuned co-ordination between channels and compartmentalisation mechanisms, such as the ER (Berridge, 1997). Receiver proteins decode the calcium signals and alter the biological activity accordingly, through activation of transcription factors or other secondary signalling proteins (Smedler and Uhlen, 2014).

2.11.2 Calcium channels

Calcium homeostasis is governed by a number of membrane-bound ion pumps that actively move calcium against its concentration gradient, thus preparing cell for subsequent calcium signalling events. Cytosolic calcium is pumped into internal stores in the ER, the nucleoplasmic reticulum, the mitochondria or the nucleus. In the ER, this process is often regulated by the sarco-endoplasmic reticulum ATPase (SERCA) pump (Periasamy and Kalyanasundaram, 2007). Calcium stored in the ER lumen is released into the cytosol by activation of IP_3Rs or ryanodine receptors. Together, the SERCA pumps work in conjunction with IP_3Rs, ryanodine receptors and ionotropic channels to

generate, regulate and maintain cytoplasmic calcium oscillations (Berridge, 2009, Kapur et al., 2007).

IP_3Rs are ligand-gated calcium channels located on the ER membrane and they play an essential role in converting extracellular stimuli, such as growth factors (RTK activity) and neurotransmitters to intracellular calcium signals (Fain and Berridge, 1979, Berridge, 2009). IP_3 concentration is regulated by activation of phospholipase C (PLC) linked pathways, which hydrolyse phosphatidyl-inositol-4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG) (Berridge, 2003). Depletion of ER calcium concentrations leads to the activation of store operated calcium (SOC) channels and subsequent refilling of the ER. Two proteins vital for this process to occur are stromal interaction molecule 1 (STIM1) and calcium-release activated calcium channel protein 1 (Orai1). STIM1 senses calcium concentrations inside the ER, and when concentrations drop it forms a complex with Orai1 channels on the plasma membrane to allow calcium influx and ER refilling (Hewavitharana et al., 2007, Frischauf et al., 2008).

Channels on the cell membrane also allow influx of calcium ions. Voltage-gated calcium channels (VGCC) are typically expressed on excitable cells and elicit short term spikes in internal calcium important for generation of calcium oscillations and cell-cell interactions (Atlas, 2010). VGCC also facilitates the docking of neurotransmitter containing vesicles, allowing the transmission of neural impulses (Striedinger et al., 2007). Purinergic channels are another class of receptor that can mediate signalling via calcium ion influx; these are discussed in Section 2.12. The channels and receptors described here are just a few of the mechanisms capable of modulating calcium

signalling in NPCs, and given the complexities of these signalling mechanisms, elucidating the pathways involved is an ongoing process (Tonelli et al., 2012).

2.12 PURINERGIC SIGNALLING

Purinergic signalling is mediated by purine and pyrimidine nucleotides, such as ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP) and uridine diphosphate (UDP), and is an important modulator of cellular activity, having roles in signal transduction and cell-cell communications. The capacity of ATP to act as a neurotransmitter was identified in the 1970s, and since then the role of purinergic receptors in neural tissue has attracted much interest (Burnstock, 1972). Purinergic receptors mediate the effects of purines via three distinct receptor classes; P1, P2Y and P2X receptors. P1 receptors are metabotropic G protein-coupled receptors (GPCR) activated by adenosine. P2 receptors respond to the binding of extracellular purinergic molecules, and are divided into P2X and P2Y subfamilies. P2X receptors 1 through 7 are ionotropic cation channels activated by extracellular ATP and allow for the passage of Ca^{2+}, K^{+} and Na^{+} ions (Ulrich et al., 2012). P2Y receptors on the other hand are GPCRs and may be activated by ATP, as well as its derivatives ADP, UTP and UDP. Most P2Y receptors appear to signal via PLC, leading to an increase in IP_3 concentrations and the release of calcium from the ER (Grimm et al., 2010). Thus both P2X and P2Y signalling can cause influx of calcium ions to the cytosol and initiate downstream signalling cascades to regulate transcription factor activation, cell cycle events, differentiation, migration and cell death (Abbracchio et al., 2009).

In neurotransmission, purinergic signalling is involved in neuron-glial interactions, and P2X receptors can regulate rapid synaptic signal transmission and synaptic plasticity.

Purines are also involved in control of learning, memory, feeding and sleep behaviour, as well as pathophysiologies, neurodegenerative and neuropsychiatric disorders (Burnstock, 2013). P2X receptors are known to play a critical role in pathological processes, such as thrombosis, inflammation and neuropathic pain (North, 2002, Kaczmarek-Hajek et al., 2012). P2X7 in particular has been heavily implicated in inflammatory responses, thereby providing possible therapeutic applications for endogenous tissue repair and stimulation of neurogenesis in cases of neurodegenerative diseases (Glaser et al., 2012, Ulrich et al., 2012).

2.12.1 Purinergic regulation of proliferation and differentiation

Purinergic signalling has been reported to drive proliferation in cancer cells (Dixon et al., 1997), smooth muscle cells (Wang et al., 1992), retinal cells (Sanches et al., 2002) and vascular cells (Burnstock, 2002). In immortalised human embryonic neural progenitor cultures, ATP induced proliferation via intracellular calcium and PI_3 kinase dependent pathways (Ryu et al., 2003), while in embryonic radial glia cells, proliferation was accompanied by P2Y1 receptor activation, and decreased following disruption of calcium waves (Weissman et al., 2004). Evidence suggests the mitogenic effect of P2Y receptor activation is mediated by the Ras/Raf/MEK/ERK pathway in glioma cells (Tu et al., 2000), demonstrating the possibility for interactions with growth factor signalling pathways. Confirming this concept, synergistic activity between nucleotides and EGF was observed in adult SVZ primary progenitor cells (Grimm et al., 2009).

Cell-cell interactions are an important contributing factor to the regulation of neuronal processes. ATP released from astrocytes has been demonstrated to positively regulate NPC proliferation in cultures obtained from the adult hippocampus, in a P2Y1 mediated

process (Cao et al., 2013). Real time bioluminescence imaging revealed that embryonic NPCs release ATP in episodic bursts, and that this ATP acts to drive proliferation via P2Y receptors. Interestingly, enzyme histochemistry of the SVZ and dentate gyrus of the adult brain revealed NTDPase activity, which degrades ATP. The authors suggest that as they observed P2Y receptor inhibition to suppress proliferation and permit differentiation *in vitro*, then this NTDPase activity in neurogenic niches may help drive differentiation of NPCs (Lin et al., 2007). However, the neurogenic niches are also sites of significant proliferation, which according to their findings, would require ATP; it is clear that multiple mechanisms are involved.

2.13 PURINERGIC SIGNALLING: P2X7 RECEPTORS

The P2X7 receptor was first described as the P2Z receptor, or the 'cell death' receptor, as its activation with high concentrations of ATP results in the opening of a pore that allows macromolecule exchange leading to cell death (Surprenant et al., 1996). They were first detected in the immune system on antigen presenting immune cells, where they are rapidly activated in response to inflammatory stimuli in immune cells, releasing pro-inflammatory mediators, including cytokines, such as IL-1β and tumour necrosis factor, from the cytosol as part of the host defence reactions (Tsukimoto et al., 2006, North, 2002). P2X7 receptors have since been shown to have a number of non-immune functions, with distinct responses depending on exposure time and concentration. Brief activation results in cation influx for the purposes of neurotransmitter and signal transduction (Papp et al., 2004), while prolonged activation results in the formation of a large transmembrane pore permeable to molecules up to 900 Da. This leads to cytoskeletal rearrangement, transmembrane pore formation, and potentially apoptosis and/or necrosis (Delarasse et al., 2009). The latter has significant

implications in the immune system, where P2X7 receptors are rapidly activated in response to inflammatory stimuli, releasing pro-inflammatory mediators, such as IL-1β and tumour necrosis factor (Sperlagh and Illes, 2014). In the absence of ATP, P2X7 receptors have been demonstrated to facilitate phagocytosis in both the immune system and the nervous system (Wiley and Gu, 2012, Lovelace et al., 2015).

Activation of transcription factors is thought to be one method utilised by P2X7 receptors to affect downstream responses. In macrophages, stimulation of P2X7 with ATP resulted in activation of nuclear factor of activated T cells (NFAT) 1 and 2 (Ferrari et al., 1999), as well as nuclear factor κB (NFκB) (Ferrari et al., 1997). In neurons of the hippocampus, purinergic activation of NFκB through P2X7 receptors have been of interest due its implications in the pathophysiology of neurological damage or trauma (Kim et al., 2013). Finally, a role for P2X7 receptors in cognitive dysfunction and traumatic or ischemic events in neurogenic niches currently the focus of much interest, and is discussed in greater detail in Section 2.13.2 (Yu et al., 2013, Liu et al., 2017, Engel et al., 2012).

P2X7 receptors share the least homology (35-40%) with other P2X receptors and have a number of important physiological functions that distinguish it from the others in its family (North, 2002, Sperlagh et al., 2006). Endogenous ATP has a relatively low potency against P2X7 receptors, generally requiring concentrations 100 μM and above, compared to other P2X receptors, which are activated by ATP in the low micromolar range (Virginio et al., 1999). As an agonist, 2', 3'-O-(4-benzoyl-benzoyl) adenosine 5' -triphosphate (BzATP) is approximately 10 to 30 times more potent than ATP, and although it is not specific to P2X7 receptors, its higher affinity to P2X7 makes BzATP a useful tool for examining the pharmacological activity of P2X7 receptors (Bianchi et

al., 1999). Antagonists for P2X7 receptors include Brilliant Blue G (Jiang et al., 2000) and oxidized ATP (oxATP, Murgia et al., 1993). Chemical inhibitors A438079 and AZ10606120 are also widely used (Diaz-Hernandez et al., 2008, Heinrich et al., 2012).

P2X receptors form ion-channel complexes in the membrane by forming subunit multimers, either homomeric or heteromeric; P2X7 appears to be the only subunit unable to form a heteromeric complex (North, 2002). The P2X7 receptor contains two hydrophobic regions that transverse the plasma membrane, with both the N- and C-terminus located intracellularly. The P2X7 receptor forms a homo-trimeric structure, with the bulk of the protein on the external membrane surface, see Figure 2.5. The three agonist binding sites can also be seen in B, located in the cleft between each subunit (Jiang et al., 2013).

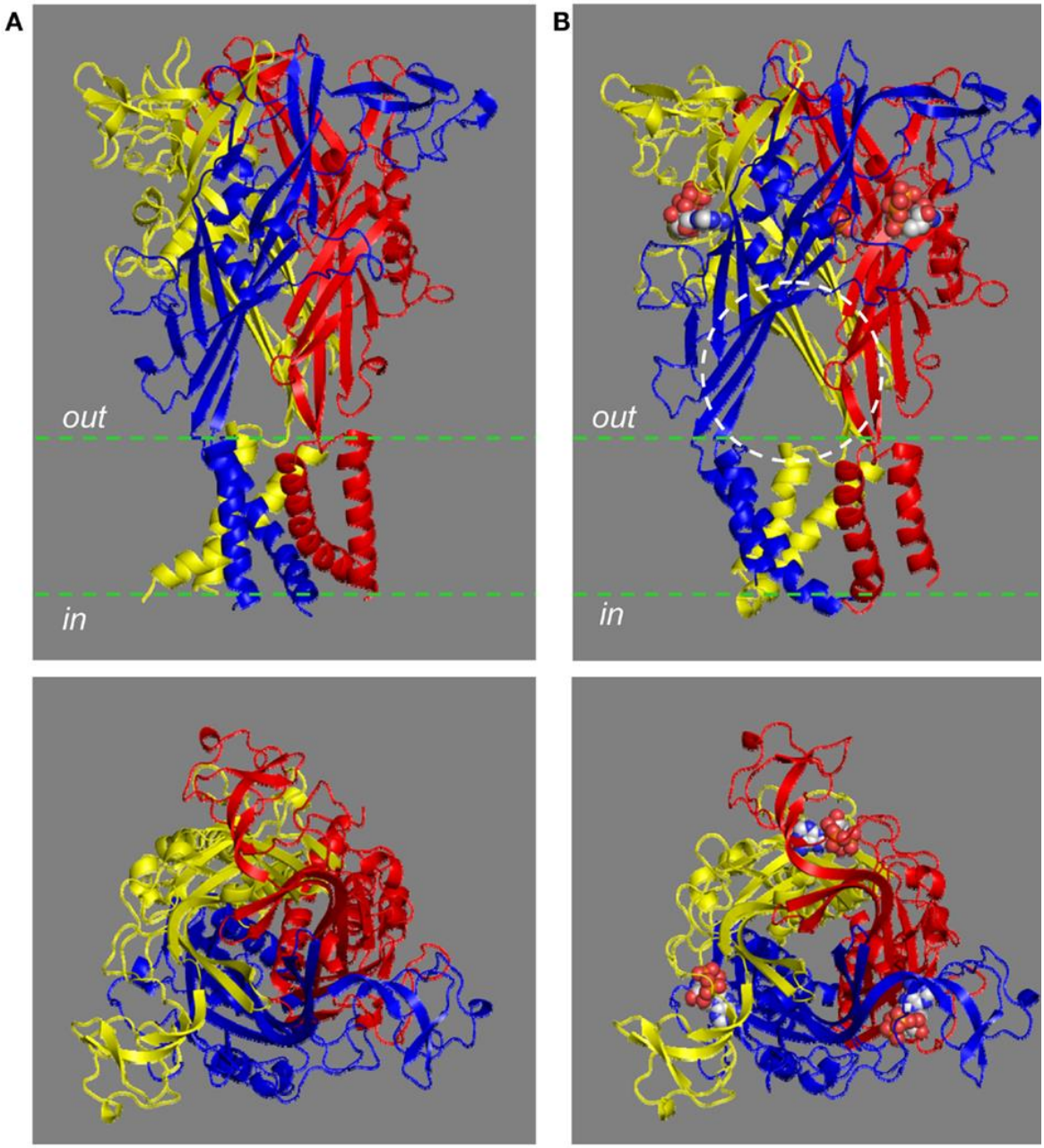

Figure 2.5 Homo-trimeric structure of the human P2X7 receptor in closed and open states.

The structure of the receptor in both the closed (A) and open (B) states when viewed parallel (top) or above (bottom) the plasma membrane. B shows the three ATP binding sites and the circle depicts one of the lateral fenestrations that allow ion transport. Reproduced with permission from Jiang et al. (2013).

2.13.1 P2X7 receptor signalling in inflammation

Inflammation is a complex immune response to defend an organism from a range of noxious stimuli and is regulated by a range of mediators including enzymes, chemokines, cytokines and nucleotides (Virgilio et al., 2009). Cytosolic ATP has a relatively high concentration compared to the extracellular milieu and is released in large quantities when membrane integrity is compromised. In high concentrations, extracellular ATP is neurotoxic and elevated levels strongly correlate with neurological conditions, such as acute spinal cord injury and ischemia, and degenerative diseases, such as Parkinson's and Alzheimer's disease (Virgilio et al., 2009, Wang et al., 2004, Parvathenani et al., 2003). Excessive inflammation caused by high concentrations of ATP may be counterproductive to attempts by the healthy nervous tissue to repair the acute damage, as the cytotoxic effects of these modulators impact on healthy tissue and exacerbate the initial injury (Fiebich et al., 2014).

P2X7 receptors have well demonstrated roles in inflammatory responses. High ATP concentrations can induce the release of plasminogen, tumour necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) via P2X7 receptor mediated pathways regulated by mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (ERK) and calcium dependent signalling (Fang et al., 2009, Inoue, 2008). These cytokines and chemokines can act to exacerbate the inflammatory response. P2X7 mediated pore formation also plays an important role in ATP induced cell death (Le Stunff et al., 2004) and microglial activation (Monif et al., 2009), further validating a role for P2X7 receptors in inflammation (Tsukimoto et al., 2006). More recently, P2X7 receptor pore formation has been implicated in inflammatory bowel disease (Gulbransen et al., 2012) and chronic pain sensitivity (Sorge et al., 2012), while P2X7

receptor inhibition abolished chronic inflammation, neuropathic pain and hyperalgesia (Chessell et al., 2005, Fulgenzi et al., 2008)

2.13.2 P2X7 receptor function in neural disease and disorder

P2X7 function in the CNS is generally associated with the induction of cell death (Ulrich et al., 2012). Higher than normal amounts of ATP released from necrotic cells following an ischemic event activates P2X7 receptors on the surface of both neurons and glial cells, allowing inward current and an overload of cytosolic calcium levels, leading to mitochondrial depolarisation, oxidative stress and cell death. In embryonic NPCs, prolonged ATP exposure resulted in membrane disruption and cell death via activation of the P2X7 receptor (Delarasse et al., 2009). A number of recent studies demonstrating conferral of neuroprotection by modulation of P2X7 receptor activity in ischemic brain injury, epilepsy and stroke have highlighted the therapeutic potential of targeting P2X7 receptors in cerebrovascular diseases (Sperlagh and Illes, 2014).

Pharmacological inhibitors of the P2X7 receptor demonstrated neuro-protective qualities in ischemia and spinal cord injury, by reducing cell death in the peritraumatic zone and improving functional recovery (Wang et al., 2004). Reduced release of pro-inflammatory mediators and microglial activity was also observed. Supporting this observation, Choi et al. (2007) showed inhibition of P2X7 receptors to decrease both pro-inflammatory mediators and NFκB activation, subsequently increasing neuronal survival rates in the striatum following lipopolysaccharide (LPS) injection.

In mice with pilocarpine and kainic acid induced seizures, an increase in P2X7 receptor immunoreactivity and sensitivity was observed in hippocampal NPCs (Rozmer et al., 2017). Blocking the P2X7 receptor in this study prevented the neuronal degradation of

CA3 pyramidal cells, though also caused an increase in the number and severity of subsequent spontaneous seizures. Status epilepticus (prolonged seizures) has also been found to increase levels of P2X7 in the granule neurons of the dentate gyrus, and that antagonising P2X7 receptors reduced both seizure duration and subsequent neuronal death (Engel et al., 2012). Similar observations were made in the CA1 area of the hippocampus, where P2X7 receptor inhibition reduced the amount of delayed neuronal death in ischemic injury (Yu et al., 2013). An increase in receptor expression levels was also observed.

Glial cells are also heavily impacted by P2X7 receptor signalling during inflammatory events (Verkhratsky et al., 2012). Following an acute event, such as ischemia or trauma, as well as chronic neuropathies, such as multiple sclerosis, Parkinson's and Alzheimer's disease, expression of P2X7 receptors on the surface of microglia are often upregulated (Franke et al., 2004). This upregulation is also observed in oligodendrocytes, where ischemic damage is partly caused by glutamate toxicity and compounded by increases in extracellular ATP concentrations. The irreversible increase in cytosolic calcium concentrations severely damaged oligodendrocytes and myelin, and the ATP degrading enzyme apyrase and P2X7 receptor antagonists alleviated the damage caused by ischemia, as well as improving action potential recovery (Domercq et al., 2010). Together, these studies heavily implicate P2X7 receptor activity in processes of inflammatory cell death.

Recently P2X7 receptors have also emerged as a new target for depression and cognitive dysfunction studies (Liu et al., 2017). Inflammation is a key pathophysiological mechanism contributing to neuropsychiatric disorders, and pro-inflammatory cytokines IL-6, IL-1β and TNF-α can mediate many of the psychological

changes associated with depression. Chrysophanol, a traditional Chinese medicine with anti-inflammatory properties, was demonstrated to impart anti-depressant effects in LPS depression models, and reduced the expression of P2X7 receptors, as well as serum levels of IL-6, IL-1β and TNF-α (Zhang et al., 2016). The authors speculated that the antidepressant effect of Chrysophanol was mediated by a P2X7/NFκB signalling pathway. This hypothesis is supported by P2X7 receptor knock out mice that display antidepressant-like profiles in forced swim and tail suspension tests (Basso et al., 2009).

2.13.3 P2X7 receptors in phagocytosis

In the absence of ATP, P2X7 receptors have been not only demonstrated to facilitate phagocytosis, but can confer the ability to phagocytose. P2X7 receptors expressed on the surface of macrophages are involved in the engulfment of latex beads, as well as both live and heat-killed bacteria in the absence of ATP and serum, while transfection of P2X7 receptors into HEK293 cells conferred the ability of HEK293 cells to phagocytose (Wiley and Gu, 2012). Within the CNS, the P2X7 membrane complex is thought to play an important role in innate immunity, as it can mediate the phagocytosis of non-opsonised particles including beads, bacteria and apoptotic neuronal cells (Wiley and Gu, 2012).

P2X7 receptors have been shown to tightly associate with heavy chain IIA of the non-muscle myosin complex, a major cytoskeletal component and essential for internalising particles during phagocytosis (Gu et al., 2009). It was later discovered that an intact P2X7-nonmuscle myosin complex was required for phagocytosis, and that extracellular ATP causes the dissociation of the P2X7 complex from myosin IIA, resulting in inhibition of particle uptake (Gu et al., 2010). This suggests that in the absence of ATP and serum proteins, P2X7 receptors may have a function distinct to the inflammatory

response, and can act as a scavenger receptor for bacteria, debris and apoptotic cells in the CNS.

It was recently demonstrated that during human embryonic development, NPCs express P2X7 receptors and that these neural precursors and neuroblasts are able to phagocytose apoptotic ReNcells and apoptotic neuroblasts, as well as latex beads via a P2X7 mediated pathway (Lovelace et al., 2015). Presence of ATP, P2X7 antagonists or siRNA knockdown inhibited this phenomenon, suggesting that P2X7 can act as a scavenger receptor on neural progenitors within the developing human central nervous system (Lovelace et al., 2015). This alternate function may allow P2X7 to act as a scavenger receptor in the nervous system, where the balance between proliferation and PCD plays a fundamental role in the maintenance of the adult brain.

2.13.4 P2X7 receptors in the adult hippocampus

An early report based on in situ hybridisation studies suggested that in the adult brain P2X7 receptor mRNA was restricted to the ependymal layer of the third ventricle and activated microglia (Collo et al., 1997). P2X7 receptors were thus believed to be absent from neurons until later studies demonstrated their presence. Using in situ hybridization and electron microscopy, P2X7 receptors were localised to the excitatory terminals in the CA1, CA3 and the dentate gyrus, and implicated in the regulation of GABA and glutamatergic signalling (Sperlagh et al., 2002). P2X7 receptor involvement in modulating GABA and glutamate release in the hippocampus has been further confirmed by immunohistochemistry and located mostly to pre-synaptic nerve terminals (Atkinson et al., 2004) and by glutamate and GABA release and uptake experiments in mice lacking the P2X7 receptor (Papp et al., 2004). Despite this

evidence, controversy over the presence of P2X7 receptors in neurons still ensues (Miras-Portugal et al., 2017, Illes et al., 2017).

So far, P2X7 receptors have been reported at embryonic stages in the SVZ (E15.5), and the SGZ (E18.5 and P4), with P2X7 receptor mRNA expressed in terminally differentiated neural cells (Tsao et al., 2013). The receptor has also been identified in embryonic progenitor cells derived from the striatum (Delarasse et al., 2009) and the human telencephalon (Lovelace et al., 2015) and in adult neural progenitors of the subventricular zone (Messemer et al., 2013). Despite this, and the abundance of literature dedicated to P2X7 receptor functions in the hippocampus, research involving P2X7 receptor function on hippocampal NPCs is limited. Hogg et al. (2004) reported application of ATP and BzATP evoked inward current and depolarisation, as well as transient cytosolic calcium increases in NPCs derived from the hippocampus of adult rats. Further, the authors demonstrate positive P2X7 immunochemistry in undifferentiated and differentiated NPCs (Hogg et al., 2004). In juvenile mouse hippocampal slice preparations, ATP and BzATP induced membrane currents in NPCs, granule cells, and astrocytes, but not GABAergic or glutamatergic interneurons of the hilus. These NPC currents were inhibited by presence of A438079, and were not observed in slice preparations from P2X7 deficient mice (Rozmer et al., 2017).

2.13.5 P2X7 receptor regulation of proliferation and differentiation

P2X7 receptor activity has been shown to regulate proliferation and differentiation pathways, with activation promoting cell survival and proliferation, with inhibition resulting in differentiation and axon growth. As P2X7 receptors are generally associated with cell death pathways, this presents an interesting and alternate role for the receptor (Tang and Illes, 2017). Contrary to other findings, activation of P2X7

receptors with BzATP was found to decrease neuron damage following status epilepticus, while inhibition with oxATP or A438079 resulted in increased neuronal death in the CA3 region of the hippocampus. This effect was mediated by release of TNF-α and subsequent NFκB phosphorylation (Kim et al., 2011). A possible explanation for this ambiguity is different expression patterns of splice variants, most of which are truncated at the C-terminus and no longer have the ability to form transmembrane pores (Cheewatrakoolpong et al., 2005). In human embryonic kidney (HEK293) cells transfected with P2X7B, a naturally occurring truncated splice variant, ATP or BzATP application induced increases in ER calcium concentration, activated NFAT1 and stimulated growth (Adinolfi et al., 2010).

Using mouse embryonic stem cells, Ulrich and colleagues recently observed accelerated cell cycle entry following BzATP application, and that inhibition of P2X7 by pharmacological antagonists increased differentiation, measured by the number of cells expressing markers of early neuronal phenotypes (Glaser et al., 2014). They also demonstrated that levels of P2X7 receptor mRNA and protein decrease with increasing differentiation, and suggest P2X7 receptors have a role in proliferation and differentiation. Contrary to this data, another study found P2X7 receptor activation caused a decrease in proliferation and enhanced the expression of neural markers in embryonic NPCs; this neuronal differentiation was regulated by the protein kinase C (PKC) ERK1/2 signalling pathway (Tsao et al., 2013).

One of the most important aspects of neurogenesis is the migration of the axon's growth cone towards its target. By responding to positive and negative stimuli, the growth cone is able to establish correct neuronal circuits. Exposure of cultured hippocampal neurons to ATP inhibited axonal growth via P2X7 receptor mediated calcium transients.

In this study, inhibition or silencing of P2X7 receptors generated growth cone extension and longer and more branched axons (Diaz-Hernandez et al., 2008). This effect was also observed when the neuron culture was treated with alkaline phosphatase (Diez-Zaera et al., 2011). Neurite outgrowth was also reported in neuroblastoma cells in response to P2X7 inhibition (Wu et al., 2009). Together, these studies demonstrate the ambiguous roles P2X7 receptors can play, by regulating cell cycle, proliferation and differentiation.

In summary, there are at least three distinct functions P2X7 receptors may play in the adult hippocampus, depending on the conditions present in the extracellular environment. The first is initiation of cell death in the presence of inflammation and extracellular ATP. The second is calcium mediated signal transduction in response to ATP signalling, which may in turn regulate biological functions, such as proliferation and differentiation. The third and non-canonical function of P2X7 receptors is to promote phagocytosis in the absence of extracellular ATP. These alternate facets of P2X7 signalling have somewhat juxtaposed outcomes in terms of function. This project endeavours to investigate the roles of P2X7 receptors in adult hippocampal NPCs, an area relatively untouched by current literature. Understanding these mechanisms is essential to addressing important questions that remain regarding neurogenesis and regeneration in the adult brain.

3.0 MATERIALS AND METHODS

All reagents were obtained from Sigma Aldrich unless otherwise stated.

3.1 CELL CULTURE

3.1.1 Dissection and isolation of primary hippocampal tissue

Primary cultures of hippocampal neural progenitor cells (NPCs) were derived from adult female C57BL/6 mice and P2X7 knock out mice (Pfizer; C57BL/6 genetic background; Solle et al., 2001) aged between 8 and 12 weeks. Mice were treated in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and used under the Griffith University Animal Ethics approval numbers ESK/09/12/AEC and ESK/02/16/AEC. Cultures generated from P2X7 knock out mice were handled under University Biosafety Committee number ED/005/16. Generally, two mice were used to generate a single culture. Briefly, mice were euthanized by CO_2 inhalation and the skull removed. Tissue and bone were removed to expose the brain, which was transferred to a sterile dish containing HBSS with Pen-Strep (P/S). The hippocampi were removed and processed by mechanical dicing followed by a 30 minute incubation with 0.25% trypsin. The cells were washed and triturated using a fire polished pipette to create a single cell suspension, then passed through a cell strainer to remove excess debris.

3.1.2 Culture and passage of hippocampal neural progenitor cells

Hippocampal NPCs were cultured in growth medium consisting of NeuroCult Basal Medium (Stemcell Technologies) supplemented with NeuroCult Neural Stem Cell Proliferation Supplement (Stemcell Technologies), glutamine (2 mM, Thermo Fisher Scientific), EGF (20 ng/mL, PeproTech), bFGF (10 ng/m, PeproTech) and heparin (2 µg/mL). Antibiotics and serums were not included. Primary cultures (P0) were maintained at 37°C, 5% CO_2 for approximately 21 days in vitro (DIV) following dissection. The resultant neurospheres were dissociated using TrypLE (Thermo Fisher Scientific), and subsequent passages were conducted every 7-10 days as necessary. For experiments described here, the passage number was limited to P8 in order to minimise the impact of long term culturing conditions on cell phenotype. This protocol has been adapted from Walker and Kempermann (2014) and Babu et al. (2011).

3.1.3 Cell plating

Hippocampal NPCs were plated either as spheres or a single cell suspension on glass coated with poly-L-ornithine (PLO; 5 µg/ml) and natural mouse laminin (2.5 µg/ml, Thermo Fisher Scientific). For some experiments Matrigel (1:10, Corning) was used. For immunocytochemistry, 24 well plates with 12 mm glass coverslips were used, while for calcium imaging and live cell microscopy, 35 mm glass bottomed dished were used. Cells cultured for western blot analysis required plastic 6 well dishes coated with PLO and laminin. Unless otherwise stated, single cell suspensions were plated at a density of 30 000 to 50 000 cells per mL and allowed to grow until approximately 70% confluent before used in experimentation.

3.1.4 Differentiation of precursor cells to a neuronal phenotype

Hippocampal NPCs were plated at low density (5000 cells per mL) and allowed to adhere overnight. The following day, 50% of the growth medium was exchanged for differentiation medium, consisting of NeuroCult Basal Medium supplemented with NeuroCult Differentiation Supplement (Stemcell Technologies). The medium was replaced daily with fresh differentiation medium for 6 DIV, by which time the cultured cells had achieved complex neurite arborisation, coinciding with a confluency of approximately 80%.

3.1.5 Cryopreservation of hippocampal neural progenitor cells

Hippocampal NPCs generated from dissections were preserved in liquid nitrogen for long term storage. Cells were dissociated using TrypLE and resuspended in medium before the addition of cryopreservation medium (HyCryo-STEM, 2x concentration, GE Healthcare Life Sciences) for a final concentration of 1 x 10^6 cells per mL. Aliquots of 500 µL were frozen to -80°C in a freezing container before being transferred to liquid nitrogen storage. Vials were thawed rapidly by immediate immersion in a 37°C water bath then washed twice with warmed HBSS to remove cryopreservation medium. The cells were placed in growth medium and allowed one passage cycle to recover before experimental use.

3.2 IMMUNOCHEMISTRY

Images were captured using an Olympus FV1000 confocal microscope with Olympus FluoView software, or a Zeiss AxioObserver Z1 microscope with Zeiss AxioVision and Zeiss Zen software. Images were analysed and prepared for presentation using ImageJ.

3.2.1 Immunohistochemistry

Mice were perfused with PBS followed by 4% paraformaldehyde (PFA) for approximately 10 minutes. The brains were removed and drop fixed in 4% PFA for a further 4 hours. Brains were equilibrated with increasing concentrations of sucrose (up to 30%) across 3 days, before finally being transferred to Tissue-Tek Optimal Cutting Temperature (OCT) Embedding Compound (Sakura). The brains were frozen and sectioned using a Leica cryostat between 15 and 20 µm in thickness and the sections were stored at -20°C until required. For immunohistochemical staining, tissue sections were treated with saponin and 10% normal goat serum for 1 hour then washed 3 times with 0.1% Tween-20. Sections were incubated with primary antibody at an appropriate dilution in normal goat serum (NGS) for 16 hours at 4°C. Refer to Table 3.1 for a list of antibodies used. Secondary antibodies (Molecular Probes) conjugated to cyanine 3 (Cy3), cyanine 5 (Cy5), or Alexa Fluor 488, 594 or 647 were incubated at room temperature for 2 hours. Nuclei were counterstained using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Secondary antibody controls and isotype controls were conducted accordingly, while positive and negative controls were completed upon purchase of the antibody to confirm specificity.

3.2.2 Immunocytochemistry

All immunocytochemistry was carried out following fixation of the cells with 4% PFA on ice for 10 minutes. For the detection of cytoplasmic and nuclear antigens, cells were permeabilized with 0.5% Triton X-100. Cells were blocked with 3% NGS for 1 hour to prevent non-specific staining. Primary and secondary antibodies were incubated as previously described, with concentrations adjusted accordingly.

Table 3.1 Primary antibodies for immunochemistry

Antibody	*Supplier*	*Isotype*	*Concentration*
βIII tubulin	Promega	Mouse IgG1	1:2000
BLBP	Merck-Millipore	Rabbit polyclonal	1:1000
Caspase 3	ProSci	Rabbit polyclonal	1:500
DCX	Cell Signalling	Rabbit polyclonal	1:500
Dlx2	Abcam	Mouse IgG2a	1:1000
DYRK1A	ProSci	Rabbit polyclonal	1:1000
EGFR	Abcam	Mouse IgG2b	1:1000
FGFR2	Abcam	Rabbit polyclonal	1:2000
GFAP-cy3 conjugate	Sigma-Aldrich	Mouse IgG1	1:1000
Iba1	Abcam	Goat IgG	1:300
IP_3R1	Sigma-Aldrich	Mouse IgG1	1:500
IP_3R2	Alomone	Rabbit polyclonal	1:50
IP_3R3	BD Biosciences	Mouse IgG2a	1:500
Map2a/b	Merck-Millipore	Mouse IgG1	1:1000
Mash1	BD Biosciences	Mouse IgG1	1:500
Nestin	Abcam	Rabbit polyclonal	1:200
NeuN	Merck-Millipore	Mouse IgG1	1:100
NFAT1	Abcam	Mouse IgG1	1:200
NFAT2	Abcam	Mouse IgG1	1:200
NFAT3	Abcam	Rabbit polyclonal	1:100
NFAT4	Santa Cruz	Rabbit polyclonal	1:500
NFκB	Abcam	Rabbit polyclonal	1:500
O4	Sigma-Aldrich	Mouse IgM	1:200
Orai1	ProSci	Mouse IgG2b	1:100
P2X7	Santa Cruz	Rabbit polyclonal	1:500
P2X7-004 (intracellular)	Alomone	Rabbit polyclonal	1:1000
P2X7-008 (extracellular)	Alomone	Rabbit polyclonal	1:2000
P2X7-008-488 conjugate	Alomone	Rabbit polyclonal	1:1000
Pax6	Thermo Fisher	Rabbit polyclonal	1:500
Prox1	Sigma-Aldrich	Mouse IgG2b	1:200
Sox2	Abcam	Rabbit polyclonal	1:2000
Sox2-647 conjugate	Abcam	Rabbit polyclonal	1:2000
Sox4	ProSci	Rabbit polyclonal	1:500
STIM1	ProSci	Rabbit polyclonal	1:2000
Vimentin	Sigma-Aldrich	Mouse IgM	1:1000

3.2.3 EdU click chemistry

Proliferating cells were labelled *in vivo* and *in vitro* using thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU). Mice were injected intraperitoneally with EdU (50 mg/kg) for 4 consecutive days. On the 5^{th} day mice were sacrificed and perfused with 4% PFA. Cells cultured *in vitro* were labelled by addition of 10 µM EdU for 4 hours prior to fixation. Click reaction was modified slightly from the manufacturers protocol (Click-iT^{TM} Molecular Probes). Briefly, 1 mL of reaction cocktail consisted of 860 µL PBS, 40 µL $CuSO_4$ (100 µM), fluorescent azide dye (0.8 µL for cells, 2 µL for tissue) and 100 µL ascorbic acid. For tissue sections, approximately 200 µL was applied to the whole slide for 1 hour; for coverslips 30 µL for half an hour was used. EdU click chemistry is compatible with other immunostaining, and once the sample was washed primary antibodies could be subsequently applied.

3.3 LIVE CELL ASSAYS

Experiments were conducted using a Zeiss AxioObserver fluorescent microscope fitted with a temperature and 5% CO_2 chamber. Hippocampal NPCs were cultured in proliferation medium as either a single cell suspension or whole as neurospheres in a 35 mm glass bottom petri dish coated with PLO and laminin for 1 to 3 days prior to being transferred to the microscope for analysis. Cells were maintained in culture on the live cell microscope for up to 4 days without observable adverse effects.

3.3.1 Live cell calcium assays

Intracellular calcium influx was detected using Fluo-8 AM dye. Cells were loaded with 2 ng/mL dye with 10 µL 5% pluronic acid diluted in 250 µL of medium for 20 minutes

before being washed, returned to the medium and allowed to de-esterify for 10 minutes. The medium was then exchanged for phenol red free Neurobasal medium for imaging. ATP was applied at various concentrations and images were taken at 100 ms intervals over a one minute period. Approximately 30-50 regions of interest were selected at random from monolayer areas and the change in fluorescence (F/F0) was recorded as calcium entered the cytosol in response to receptor activation.

3.3.2 Live cell phagocytosis assays

Yellow green (YG) latex beads were used to measure the occurrence of innate phagocytosis of non-opsonised particles. To facilitate visualisation of the lysosomes, NPCs were labelled with LysoTracker Red (1 µM, Molecular Probes) for 20 minutes in 37°C. CellTracker Red (1 µM, Molecular Probes) was also used in some experiments. Serum addition, as well as ATP and oxATP pre-treatment, were used to inhibit P2X7 receptor induced phagocytosis as previously described by Lovelace et al. (2015). YG beads were added and the culture incubated at 37°C, 5% CO_2 in the Zeiss AxioObserver live cell microscope. The culture was imaged overnight with multiple fields of view (FOV) selected from monolayer areas. Co-localisation of the LysoTracker Red and the YG beads fluoresces yellow and indicated a phagocytosed bead. Percent total cells engaged in engulfment, as well as average YG beads per positive cell were recorded.

3.3.3 Proliferation assays

Cells were cultured in a 24 well plate with coated coverslips until approximately 60% confluent. Purines (ATP, ADP, UTP, UDP, BzATP) were applied at various concentrations for 18 hours. Some experiments were conducted in the absence of growth factors, for other experiments inhibitors were applied. These include P2X7

receptor inhibitor A438079, IP_3R inhibitors 2-APB and Xestospongin C, EGFR inhibitor N^4-(3-bromophenyl)-N^6-methyl-pyrido[3,4-d]pyrimidine-4,6-diamine (PD 158780), Orai1 inhibitors AnCoA4 and Cpd5J-4, STIM1 inhibitors ML 9 and SKF 96365, NFAT inhibitors Cyclosporin A (indirect through calcineurin (Hunt et al., 2010)) and direct NFAT Inhibitor (VIVIT peptide), NFκB inhibitors BAY 11-7082 and PS-1145. EdU (10 μM) was applied 4 hours prior to fixation. Click chemistry was conducted as above and the nuclei stained with DAPI. Unless otherwise stated, 6 random FOV (normally containing between 400 and 800 cells) were selected using only the DAPI channel, with 2 technical repeats per treatment and at least 3 biological repeats. Imaging was conducted at 20X objective on a confocal microscope and ImageJ was used to analyse the images. Briefly, EdU chemistry was quantified using consistent fluorescence intensity thresholding followed by particle counting. For both EdU and DAPI channels, an intensity threshold was selected where intensities above a certain level were considered positive while any fluorescence below was considered negative. Pixels were converted to binary form in ImageJ and the nuclei were counted using the particle analysis function. Particles below 20 pixel units represented debris or background noise and were excluded from analysis. Percentage positive cells per FOV were then calculated.

3.3.4 Transcription factor translocation assay

Hippocampal NPC transcription factor activation in response to purines was measured by translocation of the transcription factors to the nucleus as observed by immunochemistry. This method has been previously validated as described by Noursadeghi et al. (2008), and Agley et al. (2012). Briefly, cells were cultured in a 24 well plate with coated coverslips. Purines ATP, ADP, UTP and UDP were applied at

500 µM concentration for 15, 30, 60, 120 and 180 minutes. Coverslips were fixed and stained for transcription factors, and the image analysis program Columbus was used to calculate nuclear fluorescent intensities.

3.3.5 Growth factor withdrawal

Hippocampal NPCs were cultured in 24 well plates until approximately 40 to 50% confluent. Using forceps the coverslips were gently lifted out and placed into new wells containing proliferation medium minus growth factors. Withdrawal assays were conducted without EGF and FGF, without EGF only, and without FGF only, to determine impact of the individual growth factors. Coverslips were fixed following respective incubation periods and analysed by immunocytochemistry.

3.4 FLOW CYTOMETRY

Assays were performed on a Beckman Coulter CyAn flow cytometer (Eskitis Institute, Griffith University), or a FACSCalibur flow cytometer (Becton Dickinson) fitted with a Time-Zero Module (Cytek Development) allowing temperature modulation and continuous magnetic stirring for real-time flow cytometry (Florey Institute, University of Melbourne). General media used in these assays included Na^+ medium (140 mM NaCl, 5 mM NaOH, 5 mM KCl, 10 mM HEPES, 5 mM glucose, 0.1% BSA, 0.1 mM $CaCl_2$), calcium-free Na^+ medium (with 0.1mM $CaCl_2$ withheld), and K^+ medium (145 mM KCl, 5 mM KOH, 10 mM HEPES, 5 mM glucose, 0.1% BSA, 0.1 mM $CaCl_2$). These methods were adapted from Gu et al. (2014) and Jursik et al. (2007). Unless otherwise stated, cytometry assays were conducted in quadruplicate.

3.4.1 Calcium assays by flow cytometry

Cells were suspended in 1 mL of calcium-free Na^+ medium and loaded with 2 ng/mL Fluo-8 AM with 10 µL of 5% pluronic acid for 30 minutes at 37°C. Following washes with calcium-free Na^+ medium, the cells were allowed to de-esterify on ice for 30 minutes. Cells were washed once more using K^+ medium and resuspended at a concentration of 1 x 10^6 cells in 500 µL of K^+ medium, with 3 mM $CaCl_2$ added a few minutes prior to running the assay. Samples were run for three minutes with treatments added at 40 seconds. Treatments included 1 mM ATP or 100 µM BzATP and the P2X7 receptor specific inhibitor AZ10606120 (1 µM, Tocris). Cells were incubated with the inhibitor for 10-15 minutes at 37°C prior to analysis. Stock ATP was dissolved in H_2O and adjusted to pH 7 with 1 M tetramethylammonium hydroxide (TMA-OH).

3.4.2 Ethidium bromide assays by flow cytometry

Cells were resuspended in conditioned medium at a concentration of 2 x 10^6 cells/100 µL/FACS tube and placed on ice until ready. K^+ medium was added for a final volume of 1 mL and the tubes were placed in a 37°C water bath for a few minutes to recover. Prior to the assays being run 25 µM ethidium bromide was added to the cells. To induce the formation of pores on the membrane, 1 mM ATP or 100 µM of BzATP was added 40 seconds after the start of acquisition. Samples were run at around 1000 events per second for around 6 minutes. Treatments included the specific inhibitor AZ10606120 (1 µM), which was incubated with the cells for 10-15 minutes at 37°C prior to running the assay.

3.4.3 Membrane fluidity by flow cytometry

The fluorescent probe trimethylammonium diphenylhexatriene (TMA-DPH) was used to measure fluidity of the cellular membrane. TMA-DPH (10 µM) was incubated with cells for 5 minutes at 37°C on a gentle rocker followed by 1 mM ATP for 10 minutes. The P2X7 receptor antagonist AZ10606120 (10 µM) was incubated for 15 minutes at 37°C. Following the incubation, cells were washed and resuspended in 1 mL of PBS for analysis by flow cytometry.

3.4.4 Phagocytosis assays by flow cytometry

YG latex beads were used as phagocytic targets for real time rapid phagocytosis assays. Cells were resuspended in conditioned medium and diluted to a concentration of 1 x 10^6 cells/mL with Na^+ medium. Samples were run for seven to eight minutes, and 5 µL of YG beads were added 15-20 seconds after the start of acquisition. Treatments were pre-incubated with the samples and included 1 mM ATP for 15 minutes, 300 µM oxATP for 40 minutes, 20 µM Cytochalasin D for 20 minutes, 4% PFA for 20 minutes or 5% human serum with no pre-incubation required .

3.5 WESTERN BLOT

3.5.1 Reagents

RIPA lysis buffer: 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris buffer pH 8.

2X SDS PAGE loading dye: 4% SDS, 20% glycerol, 100 mM Tris buffer pH 6.8, 80 µL bromophenol blue up to 20 mL dH_2O. DTT (100 mM final concentration) was added immediately prior to combination of loading dye with protein lysates.

Tris glycine buffer 10X stock: 144 g glycine, 30.5 g Tris up to 1 L dH_2O.

SDS running buffer 10X stock: 144 g glycine, 30.5 g Tris, 1% SDS up to 1 L dH_2O.

Transfer buffer: 14.4 g glycine, 3.05 g Tris 200 mL methanol up to 1 L dH_2O.

TBS 10X stock: 24.23 g Tris, 80.06 g NaCl up to 1 L dH_2O, pH adjusted to 7.4 to 7.6.

Blocking solution: 5% skim milk powder in 1X TBS with 0.05% Tween-20.

3.5.2 Protein preparation

Hippocampal NPCs were cultured in 6 well plates until 80% confluent and harvested using TrypLE. Once washed and resuspended, the pellet could be stored at -80°C until required. Protein was extracted with RIPA lysis buffer plus 20 µL/mL of protease inhibitor (10X stock), approximately 100 µL per sample. Following resuspension, DNA was sheared with a 27 gauge needle, the sample was spun and supernatant collected. Protein concentration was estimated using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

3.5.3 SDS PAGE

The required volume of lysate containing 20 µg of protein was calculated per sample and combined with equal volumes of 2X SDS PAGE loading dye with DTT. Samples were heated at 95°C for 5 minutes to ensure complete denaturing of disulphide bonds. At this point samples could be frozen or loaded directly onto precast gels (Bio-Rad), along with a protein ladder. Gels were run at 150 volts for an hour in 1X SDS running buffer then prepared for western blotting.

3.5.4 Western blot preparation

Resolved protein was transferred from gels to polyvinylidene difluoride (PVDF) membranes for analysis by immunoblotting. The PVDF membrane was prepared by activating it with methanol for 10 seconds then submerging in chilled transfer buffer for 20 minutes. The gel was placed against the membrane and secured in the transfer cassette with filter paper and sponges. The tank was filled with transfer buffer and run at 100 V for 3 hours.

3.5.5 Immunoblotting

Membranes were blocked in 5% skim milk for 30 minutes prior to overnight incubation at 4°C with the primary antibody, as detailed in Table 3.1. For P2X7 blotting, anti P2X7-004 at 1:2000 was used. Following washes with TBS Tween, a goat anti-rabbit horseradish peroxidase secondary antibody (1:10 000) was applied for 45 minutes at room temperature. The membrane was washed again and imaged with chemiluminescent substrate (BioRad) on a VersaDoc imaging station.

3.6 PCR

3.6.1 DNA extraction

Cells were dissociated with TrypLE, washed and resuspended in 1 mL HBSS. To extract DNA, 3 mL of nuclear lysis buffer (10 mM Tris, 10 mM KCl, 2 mM EDTA, 4 mM $MgCl_2$, 0.75% SDS in dH_2O, pH 7.5) was added and samples were vortexed. Cells were incubated in 40°C for 10 minutes. Optional addition of proteinase K (5 µL) for 1 hour at 55°C resulted in a cleaner sample. Protein was precipitated with 300 µL of 10 M ammonium acetate. Samples were spun and the supernatant was transferred to 5 mL

100% chilled ethanol. DNA precipitate was pelleted and washed with 70% ethanol, which was then removed and allowed to evaporate. Once dried the DNA was resuspended in 20 µL TE buffer (0.1 M Tris, 1 mM EDTA, pH 7.4) and left overnight to dissolve at room temperature before the yield was quantified using a nanodrop.

For DNA extracted directly from tissue, REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich) was utilised. Briefly, tail snips were placed in an Eppendorf tube with 20 µL of Tissue Preparation Solution and 80 µL of Extraction Solution. This was incubated at room temperature for 10-20 minutes followed by a 3 minute incubation at 98°C. Immediately, 80 µL of neutralisation solution was applied; the sample could then be stored in 4°C until required.

3.6.2 Primer design

Primers were purchased from Integrated DNA Technologies and sequences are shown in Table 3.2. This protocol was designed to identify presence or absence of both the P2X7 gene and the neomycin resistance gene, which in the knock out ($P2X7^{-/-}$) mouse strain was used to disrupt the P2X7 gene (Solle et al., 2001). Primers for the neomycin resistance gene (NeoCassette, Sigma) were used as a control, along with a donated sample positive for the neomycin resistance gene.

Table 3.2 Primer design

Primer	*Sequence 5' - 3'*
P2X7 Intron Forward	GCA GCC CAG CCC TGA TAC AGA CAT T
P2X7 Exon Forward	GAC AGC CCG AGT TGG TGC CAG TGT G
P2X7 Reverse	TCG GGA CAG CAC GAG CTT ATG GA
Neomycin Gene Reverse	GGT GGG GGT GGG GGT GGG ATT AGA T
NeoCassette Forward	TGC TCC TGC CGA GAA AGT ATC CAT CAT GGC
NeoCassette Reverse	CGC CAA GCT CTT CAG CAA TAT CAC GGG TAG

3.6.3 Reaction conditions

Reaction master mix (Table 3.3) was prepared with GoTaq Hot Start Polymerase (Promega) based on the manufacturers direction and incorporating collaborators' protocol. Sample tubes were moved to a PCR machine and conditions were defined: stage 1, 95°C for 2 minutes by 1 cycle; stage 2 94°C for 1 minute, 58°C for 72 seconds, 72°C for 75 seconds by 35 cycles; stage 3 72°C for 10 minutes, followed by a cooling stage to end the reactions. Samples could then be stored at 4°C until analysed by gel.

Table 3.3 Reaction master mix

Master Mix	*Volume*
dH_2O	11.6 µL
5X reaction buffer with loading dye	5 µL
DMSO	2.5 µL
$MgCl_2$ (25 mM)	1.2 µL
dNTPs (5 mM)	1.2 µL
GoTaq Hot Start Polymerase	0.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
DNA template (20 ng/µL)	2 µL
TOTAL	25 µL

3.6.4 Gel analysis

A 2% agarose gel was made in Tris acetate EDTA (TAE) buffer with 10 µL of 10 mg/mL ethidium bromide. Samples (8 µL) were loaded along with 5 µL of ladder (50 bp, Bioline) and were run at 100 volts for approximately 1.5 hours. Gels were imaged under UV light.

3.7 NMR

The stability of working concentrations (100 µM) of ATP in NeuroCult Basal medium was determined by Fourier Transform ^{31}P nuclear magnetic resonance (NMR) spectroscopy. The spectra were acquired on a 500 MHz spectrometer at 298 K and D_2O was used for the deuterium lock. The ^{31}P NMR spectra reported in parts per million (ppm) were aligned relative to the inorganic phosphate present in the medium solution as an internal standard. Due to the low concentration of the substrate (100 µM), 10 000 scans were performed and the spectral width was set to 650 ppm to observe possible by-products that may be produced by any hydrolysis of ATP. The spectra were processed using MestReNova Version 11.01.

3.8 STATISTICAL ANALYSIS

Data are presented as the mean ± one standard error (SE). For comparisons of multiple sample groups, statistical significance was determined using one way analysis of variance (ANOVA). Effect of variance was assessed using Levene's test for homogeneity of variance, and where found to be insignificant a Tukey's HSD (honest significance test) was used to determine significant difference between groups. Where homogeneity of variance was violated (Levene's test was significant) a Welch one way ANOVA and Games-Howell post-hoc analysis used to determine significance as it is a more stringent test and reduces the chance of type 1 errors. The significance level for the difference between treatments was set at an α-value of 0.05, and *p* values are reported as calculated to three decimal points, except where the significance returned as 0.000, which was reported as $p < 0.01$. Analysis of data was conducted using SPSS 20.0 statistical analysis software (SPSS, IL, USA).

4.0 CHARACTERISATION OF HIPPOCAMPAL NEURAL PROGENITOR CELLS

4.1 INTRODUCTION

NSCs in the hippocampal neurogenic niche progress through a number of phases beginning with proliferation, then differentiation and migration before finally integrating into the existing neuronal circuitry (van Praag et al., 2002, Li et al., 2009). Maturation of hippocampal NPCs is a gradual progression with subtle shifts in cell phenotype over time. Contemporary neuroanatomical analysis of the hippocampus characterizes the neuronal maturation in five discrete stages each marked by specific developmental milestones as first described by Kempermann et al. (2004). Each stage is differentiable both by the cells' proliferative potential, as well as their increasing neuronal phenotype. The nomenclature used to define cell types is relative as cells do not progress in discrete stages; rather they undergo a gradual maturation with an ever increasing neuronal phenotype. Throughout this progression there's an overlap of protein markers during both the differentiation and maturation processes and these are often used to delineate different stages as a convenience utilized primarily for analytical purposes (Ming and Song, 2005, Kempermann et al., 2004).

Type 1 cells are the putative neural stem cell of the hippocampal dentate gyrus, and can be identified by their expression of the astrocytic marker GFAP in conjunction with other progenitor markers, such as nestin or Sox2 (Seri et al., 2001, Filippov et al., 2003,

Suh et al., 2007). Type 1 cells are typically a quiescent population and divide asymmetrically to generate a type 1 daughter cell (self-renewal) and a type 2 daughter cell with a more neuronal phenotype and greater proliferation potential (Kronenberg et al., 2003). Type 2 (intermediate progenitor) cells divide in an expansion phase characterised by the expression of nestin and Sox2, while losing GFAP expression (Fukuda et al., 2003). The expression of Mash1 by type 2a cells defines entry of progenitor cells into the proliferative stage (Kim et al., 2008). Type 2b cells are defined by the expression of the immature neuronal marker DCX (Couillard-Despres et al., 2005), and Prox1 indicates that type 2b cells are fate restricted to the granule cell lineage (Steiner et al., 2008). Type 2b cells differentiate into more fate-restricted type 3 cells or neuroblasts. Type 3 cells migrate out of the niche to their target zone where they differentiate into a postmitotic immature neuron. They no longer express early progenitor or glial markers and have upregulated immature neuronal markers DCX and Prox1. As maturation continues, post mitotic neuronal markers, such as NeuN, Map2a/b and βIII tubulin also begin to be expressed.

This chapter focuses on the characterisation of the primary neural cell cultures generated from the hippocampus of adult mice. These experiments were conducted to determine if the culture conditions used here produced a cell with morphological and immunological features aligned with contemporary literature, as well as to investigate expression patterns of proteins relevant to this project. Lastly, the data gathered here was used to establish culture protocols for the research presented in subsequent chapters.

4.2 INVESTIGATION OF PROLIFERATING CELLS *IN VIVO*

The dentate gyrus of the hippocampus is one of two areas in the mammalian brain known to generate new neurons throughout adulthood. The extent of this proliferation, as well as the presence of progenitor cell markers, was investigated *in vivo* using EdU in combination with a variety of immunohistochemical stains including Mash1, Prox1 and nestin. Adult female mice were given EdU (50 mg/kg) via intraperitoneal injection for four consecutive days before sacrificing. EdU click chemistry and immunohistochemistry was performed as described in Section 3.2.3.

EdU positive cells were observed in the hippocampus at substantially less numbers than those seen in the SVZ of the lateral ventricles, Figure 4.1, A-F. Quantification of EdU positive cells in the hippocampus (n = 498) and the SVZ/RMS (n = 628) revealed the SVZ and RMS had approximately a 10 fold increase in the number of EdU positive cells (23.0 ± 3.9%) when compared to the hippocampus (2.5 ± 1.2%; [$t(5) = 4.34$, $p = 0.007$]). EdU positive cells were found to be positive for Mash1, which was expressed through the dentate gyrus and is indicative of neural progenitors being in the proliferative phase of differentiation, see Figure 4.1, G-I. Not all cells that expressed Mash1 were positive for EdU, as EdU only labels cells in the S phase of the cell cycle where DNA is replicated. Cells of the hippocampus were also observed to be positive for Prox1, a granule neuron marker, and nestin positive cells were also apparent, indicative of the presence of a population of neural stem/progenitor cells; see Figure 4.1, J-L. Secondary controls demonstrated no non-specific binding of the secondary antibodies, see Appendix 1.

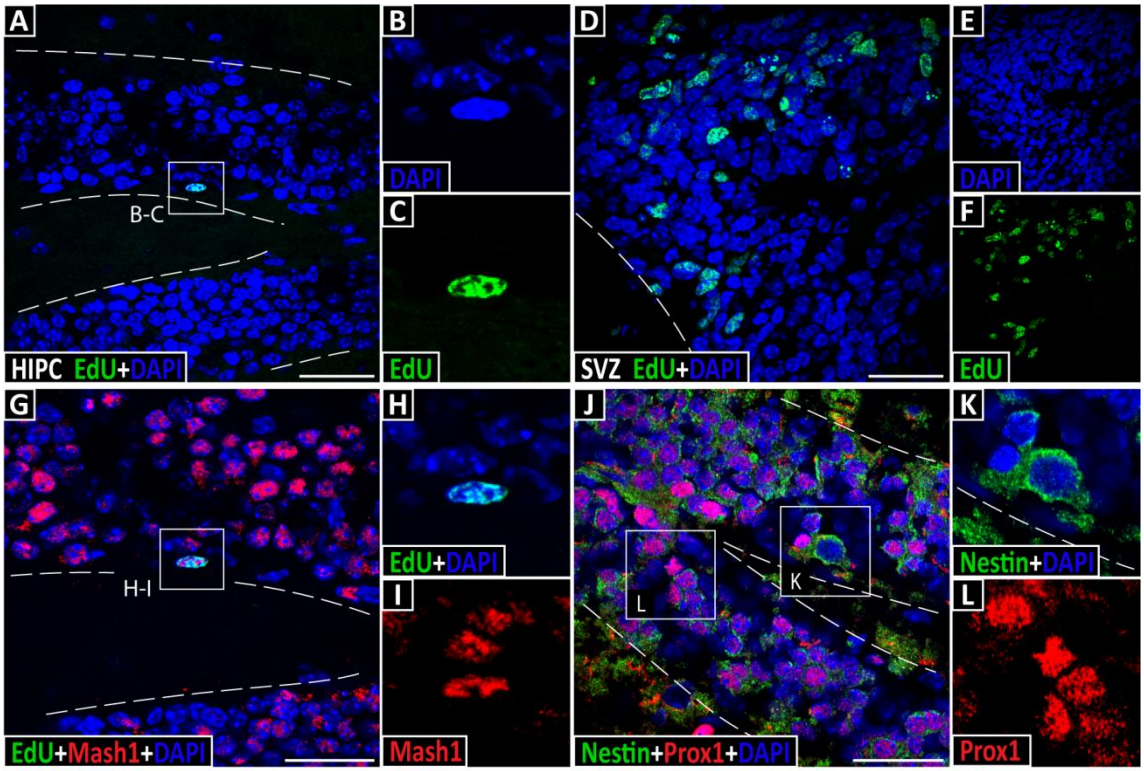

Figure 4.1 ***In vivo*** **analysis of adult mouse neurogenic niches**

Adult mice aged between 8 and 12 weeks were exposed to EdU, sacrificed and perfused with PFA. Brains were removed and cryosectioned before click- and immunochemistry was conducted. Click chemistry of the hippocampus (A-C) and SVZ (D-F) revealed EdU positive cells indicative of cell proliferation. Greater rates of proliferation were observed in the SVZ compared to the hippocampus. Hippocampal sections were probed with various neural progenitor markers. Mash1 was expressed though the hippocampus (G-I) and EdU positive cells were found to express Mash1. Nestin expression was detected, as well as granule cell marker Prox1 (J-L). Nuclei were stained with DAPI. Scale bar represents 20 µm.

4.3 ISOLATION OF HIPPOCAMPAL DERIVED NEUROSPHERES

Primary NPC cultures were derived from the proliferative cells in the hippocampus of adult mice, and characterised to determine if the cellular phenotype in culture was comparable to the EdU positive cells observed *in vivo*. Cell cultures were generated from dissociated tissue and seeded in proliferation medium as outlined in Section 3.1.1. Primary neurospheres routinely required 21 days to form in culture, and the number of spheres obtained from the hippocampal dissection was comparatively low, generally around 20 spheres, whereas upwards of 50 spheres could be obtained from the SVZ dissection of the same mouse under identical conditions. Hippocampal cultures were expanded through two or three passages prior to use in experiments, and cells were not used past passage eight. In culture neurospheres were observed to have a smooth spherical surface with a bright halo appearance and small microspikes, see Figure 4.2.

Once the neurospheres had reached approximately 100 to 150 µm in diameter they were passaged or used for experimentation. Cells were plated onto PLO and laminin coated glass coverslips as either whole spheres or a single cell suspension, and allowed to proliferate for typically between one and five days before experimentation. Experiments were ideally conducted on coverslips not exceeding 80% confluency. When plated as spheres cells migrate away from a densely packed centre, forming monolayers at the periphery. It was here that images were captured, to better observe individual cellular characteristics, see Figure 4.2. Coverslips were fixed with PFA for 10 minutes before proceeding to immunochemistry.

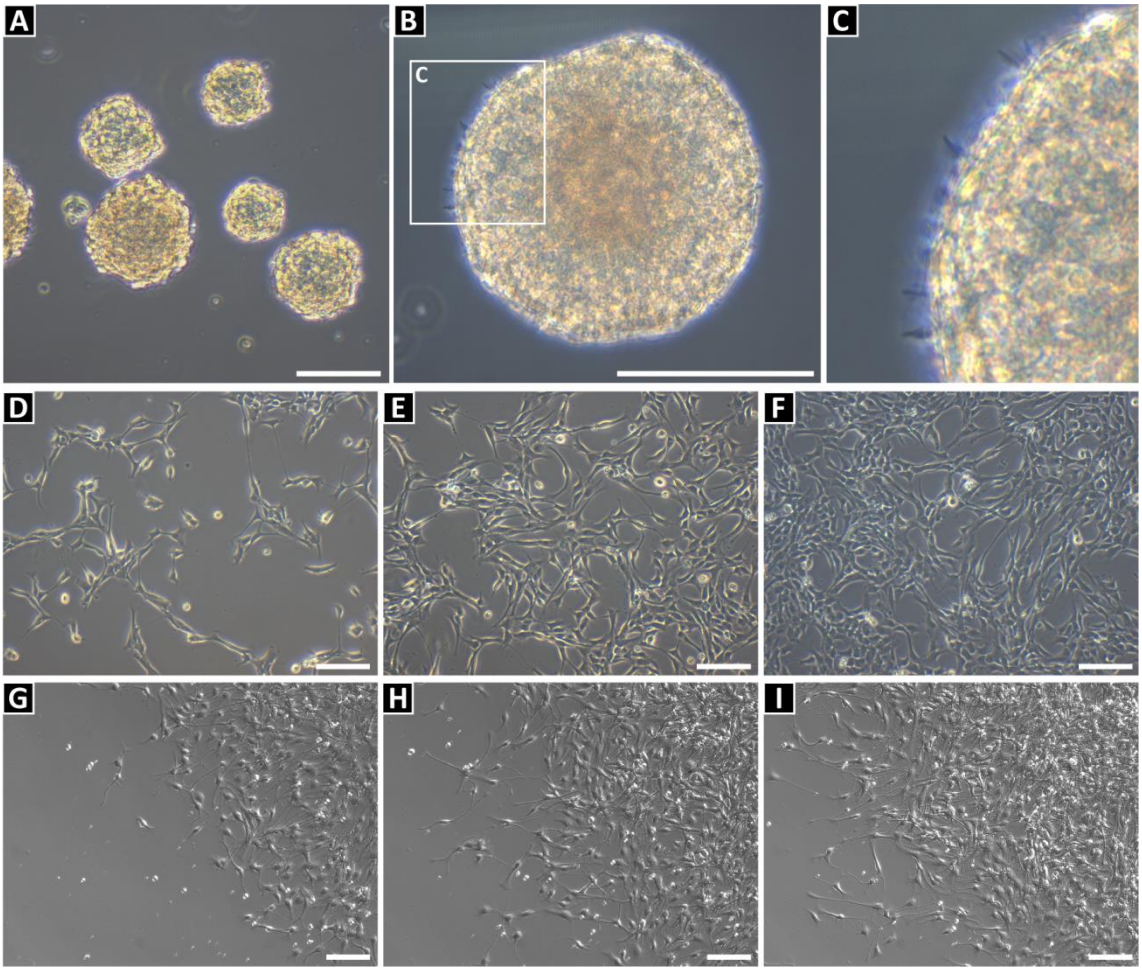

Figure 4.2 Hippocampal NPC cultures

Hippocampal NPCs were isolated from adult mice and cultured as neurospheres until approximately 100 to 150 µm in diameter (A). Neurospheres had a smooth periphery (B) and small microspikes could be observed on their surface (C). D-F demonstrates the typical NPC phenotypes achieved when cells were plated as a single cell suspension on PLO and laminin coated glass coverslips with increasing confluency. Experiments were ideally conducted on coverslips not exceeding 80% confluency (F). When plated as spheres (G-I), cells migrate away from the densely packed centre of the sphere, seen at the top right corner of the G-I panel. G indicates 3 hours of growth; H is 10 hours and I represents 18 hours since plating. Scale bars represent 100 µm.

4.4 CHARACTERISATION OF NEURAL PROGENITOR CELLS USING TRADITIONAL MARKERS

Immunochemistry was conducted on hippocampal NPCs cultured for two to four days to determine protein expression patterns with the aim of evaluating a more precise stage of neurogenesis. For all experiments, at least three biological replicates were conducted. No signal was detected using an isotype control or upon omission of primary antibody for all samples (data not shown).

The neuroepithelial stem cell marker nestin was used in conjunction with GFAP to identify type 1 cells, and the number of co-positive cells observed was relatively low (3.8 ± 0.8%, n = 1887) at 3 DIV, see Figure 4.3, A-C. When plated as neurospheres, it was noted that $GFAP^{pos}$/$nestin^{pos}$ cells tended to be located toward the original centre of the neurosphere. Almost all cells were observed to express nestin in culture (Figure 4.3, A-C). Immunocytochemistry conducted against Mash1 (Figure 4.3, D-F) revealed varied staining intensities ranging from high (12.5 ± 1.7%, indicated by an asterisk) to no expression (13.9 ± 2.3%, indicated by a hash symbol) with the majority of cells displaying medium expression levels (73.6 ± 3.1%, total n = 507). Sox2 immunochemistry revealed a positive stain in all observed cells (see Figure 4.3, G-I), similar to nestin observations.

Identification of the neuronal protein DCX required the use of a rabbit anti-DCX (Cell Signalling) as other antibodies were unable to differentiate between high and low immunoreactivity. This antibody was very useful in identifying DCX^{high} neuroblasts in culture and, together with morphological changes, was used to determine NPCs that had differentiated into type 3 neuroblasts and were beginning to express more neuronal like characteristics (Figure 4.3, J-L). These highly positive DCX neuroblasts only

comprised a few cells per coverslip (less than 0.1%, where the total number of observed cells was greater than 3000). The remainder of the NPCs showed faint DCX immunostaining localised to the cytoplasm and nucleus, possibly indicative of its function as a microtubule stabilising protein. Secondary controls were conducted and demonstrated no non-specific binding of the secondary antibodies upon omission of the primary antibody, see Appendix 1. Taken together, immunochemistry identified $GFAP^{neg}$/$nestin^{pos}$/$Sox2^{pos}$/$Mash1^{pos}$/DCX^{neg} cells as comprising the majority of the hippocampal NPC culture. These characteristics most closely match a type 2a neural progenitor; this identification is particularly supported by the high number of Mash1 positive cells and low number of DCX positive cells.

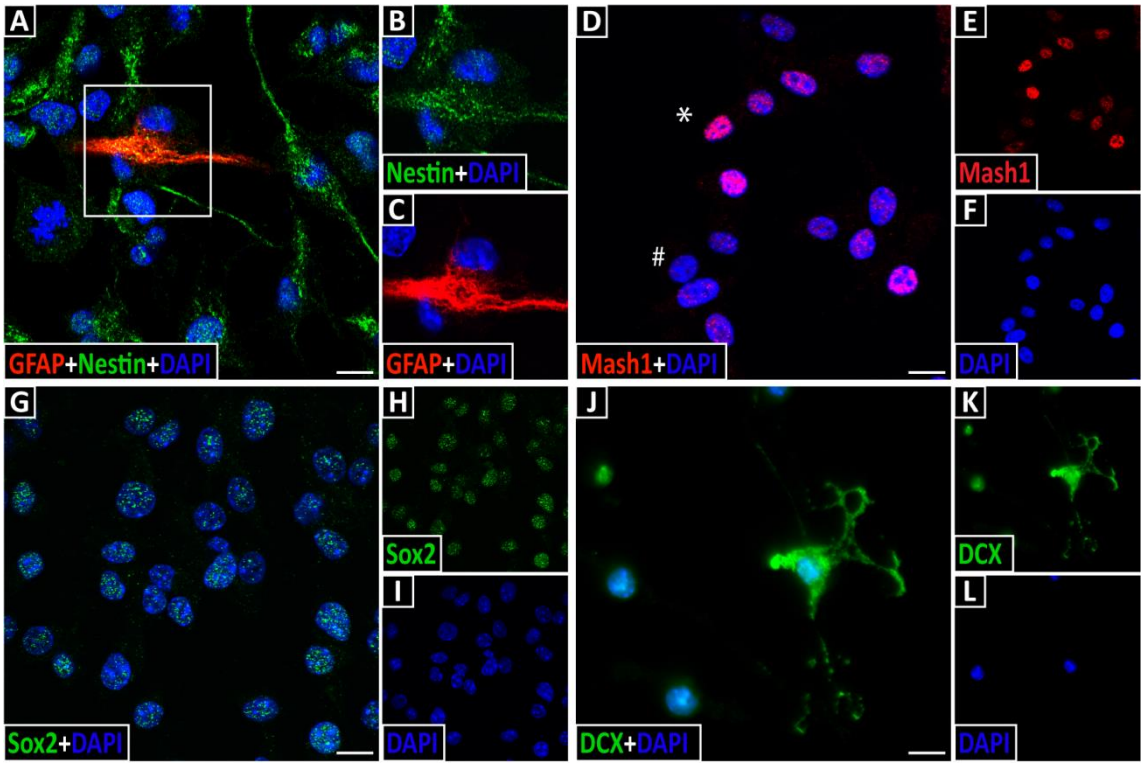

Figure 4.3 Identification of hippocampal NPCs using traditional markers

Hippocampal NPCs were plated on PLO and laminin coated glass coverslips for 3 days before fixation with PFA. Type 1 NPCs were identified by $GFAP^{pos}$/$nestin^{pos}$ staining, and comprised 3.8 ± 0.8% of the population, with nestin being positive in all observed cells (A-C). Mash1 (D-F) staining ranged from high expression (asterisk, 12.5 ± 1.7%), to no expression (hash, 13.9 ± 2.3%) with the majority of cells displaying medium to low expression levels (73.6 ± 3.1%). Sox2 was positive in all observed cells (G-I). DCX was highly expressed in less than 0.1% of cells, with other cells expressing low levels (J-L). Nuclei were stained with DAPI. Scale bar represents 10 µm.

4.5 EXTENDED NEURAL PROGENITOR CELL CHARACTERISATION

Additional immunocharacterisation was conducted using antibodies raised against BLBP and vimentin as both of these proteins are markers of neurogenesis expressed in radial-glia-like cells. BLBP was expressed in the cytoplasm and demonstrated a punctate staining pattern, while vimentin was filamentous and was observed to extend from the nuclear envelope out to the leading edge of processes, see Figure 4.4, A and B. Pax6, Dlx2 and SSEA1 (also known as LeX or CD15) are expressed by embryonic stem cells particularly during development. The transcription factors Pax6 and Dlx2 were observed to be positive; Pax6 was located in the nucleus while Dlx2 was observed to have even distribution between nucleus and cytoplasm, see Figure 4.4, C and D. SSEA1 was negative in the culture (Figure 4.4 E). Prox1 is a transcription factor required for the survival and cell fate determination of the granule cell lineage (Iwano et al., 2012). It was found to be positively expressed in the nucleus of the hippocampal NPC culture, see Figure 4.4 F.

To determine if neuronal lineage elaboration had occurred, immunochemistry was conducted against βIII tubulin, Map2a/b and the postmitotic marker NeuN, and no immunoreactivity was detected, see Figure 4.4, G-I. Refer to Table 4.1 below for an overview of the immunochemical markers used to identify and characterise the hippocampal NPCs. These further assays strengthened the conclusion of a mostly type 2a primary cell culture and that these cells have entered the highly proliferative phase of neurogenesis, though they had not yet fully begun to differentiate into immature neurons.

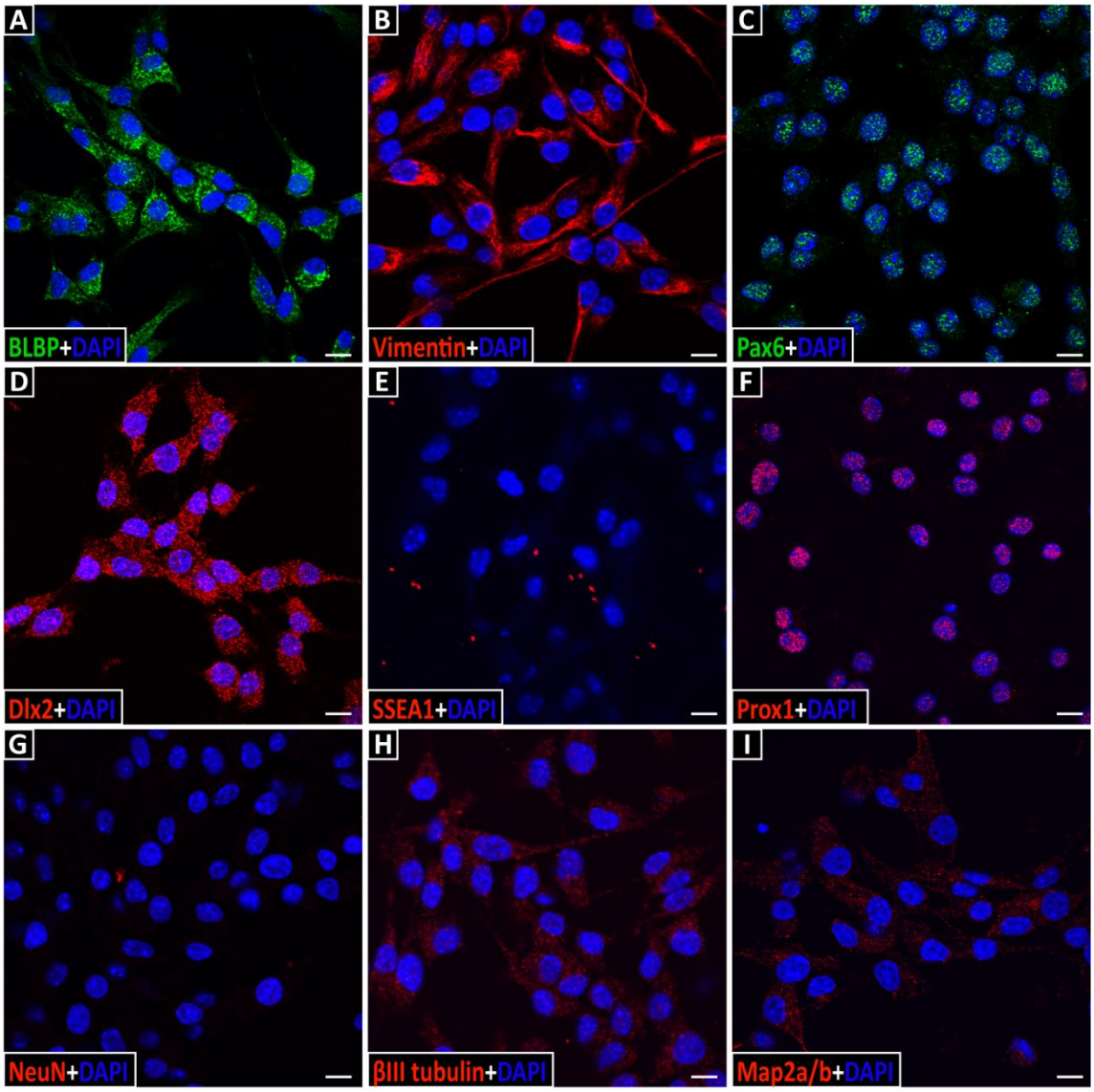

Figure 4.4 Hippocampal NPC extended characterisation

Hippocampal NPCs were plated on PLO and laminin coated glass coverslips and allowed to culture for 3 days. The cells were fixed with PFA and immunochemistry was conducted against a range of markers. BLBP (A) and vimentin (B) were observed to be positive, as were the embryonic markers Pax6 (C) and Dlx2 (D). SSEA1 (E) was negative. Granule cell marker Prox1 (F) was positive. Mature neuronal markers NeuN (G), βIII tubulin (H), and Map2a/b (I) were negative. Nuclei were stained with DAPI. Scale bar represents 10 μm.

Table 4.1 Overview of markers used to characterise hippocampal NPCs

Stain	*Description*	*NPC type*
GFAP *~4% positive*	Intermediate filament protein expressed in astrocytic cells and early neural progenitors	Type 1 cells
Sox2 *positive*	Transcription factor for maintaining stem cell pluripotency and self-renewal	Type 1, 2a, 2b cells
Nestin *positive*	Intermediate filament protein, required for self-renewal of stem cells	Type 1, 2a, 2b cells
BLBP *positive*	Developmental protein expressed in radial glia by activation of Notch receptors	Type 1, 2a, 2b cells
Mash1 *~85% positive*	A transcription factor transiently expressed in intermediate progenitors, marking entry into proliferative phase	Type 2a cells
Pax6 *positive*	Transcription factor present during development	Neurogenesis
Dlx2 *positive*	Transcription factor present during development	Neurogenesis
SSEA1/LeX/ CD15 *negative*	ECM associated carbohydrate expressed by embryonic stem cells and adult neural stem cells. Marker of pluripotency	Neurogenesis
DCX *negative*	Microtubule stabilising protein present in migrating cells; immature neuronal marker	Type 2b, 3 cells and postmitotic progenitors
Prox1 *positive*	A transcription factor expressed by the granule cell lineage	Type 2b, 3 cells and postmitotic progenitors
Vimentin *positive*	Intermediate filament protein expressed in radial glial-like cells	Neurogenesis
NeuN *negative*	Neuron specific nuclear protein	Postmitotic immature and mature neurons
βIII tubulin *negative*	Tubulin protein expressed in neurons	Neuronal phenotypes
Map2a/b *negative*	Microtubule associated protein, particularly associated with the dendritic arbour	Neuronal phenotypes

4.6 CHARACTERISATION OF PROTEIN EXPRESSION PATTERNS DURING MITOSIS

The expression patterns of a number of markers were assessed during stages of mitosis to provide a comprehensive characterisation of marker expression in hippocampal NPCs. DAPI was used to identify the characteristic chromatin patterns of NPCs undergoing prophase, prometaphase, metaphase, anaphase and telophase; these were compared to cells in interphase (Figure 4.3 and Figure 4.4). Prophase (Figure 4.5, A1) was identified by condensation of chromosomes into sister chromatids. Prometaphase (Figure 4.5, A2) follows the breakdown of the nuclear membrane and the chromatids begin to orient towards the midline. In metaphase (Figure 4.5, A3) the chromosomes align at the metaphase plate, ready to be separated toward opposite poles in anaphase (Figure 4.5, A4). Throughout telophase (Figure 4.5, A5) the cytoplasm separates to form distinct cells, the nuclear envelope reforms and the chromosomes decondense.

Sox2 was observed to be nuclear until the breakdown of the nuclear envelope at prometaphase and did not returned to the nucleus until after telophase was complete, see Figure 4.5, B1-B5. Nestin (Figure 4.5, C1-C5) maintained its cytoplasmic staining pattern throughout mitosis. Normal nuclear localisation of Mash1 was disrupted early during mitosis, with cytoplasmic staining being observed at prophase and persisting through to telophase (Figure 4.5, D1-D5). BLBP (Figure 4.5, E1-E5) and vimentin (Figure 4.5, F1-F5) were observed to remain cytoplasmic throughout mitosis, though vimentin expression was reduced from prophase to metaphase before returning to normal at anaphase. Nuclear Prox1 staining was detected at prophase, though was greatly reduced at prometaphase and metaphase before nuclear staining returned at telophase, see Figure 4.5, G1-G5.

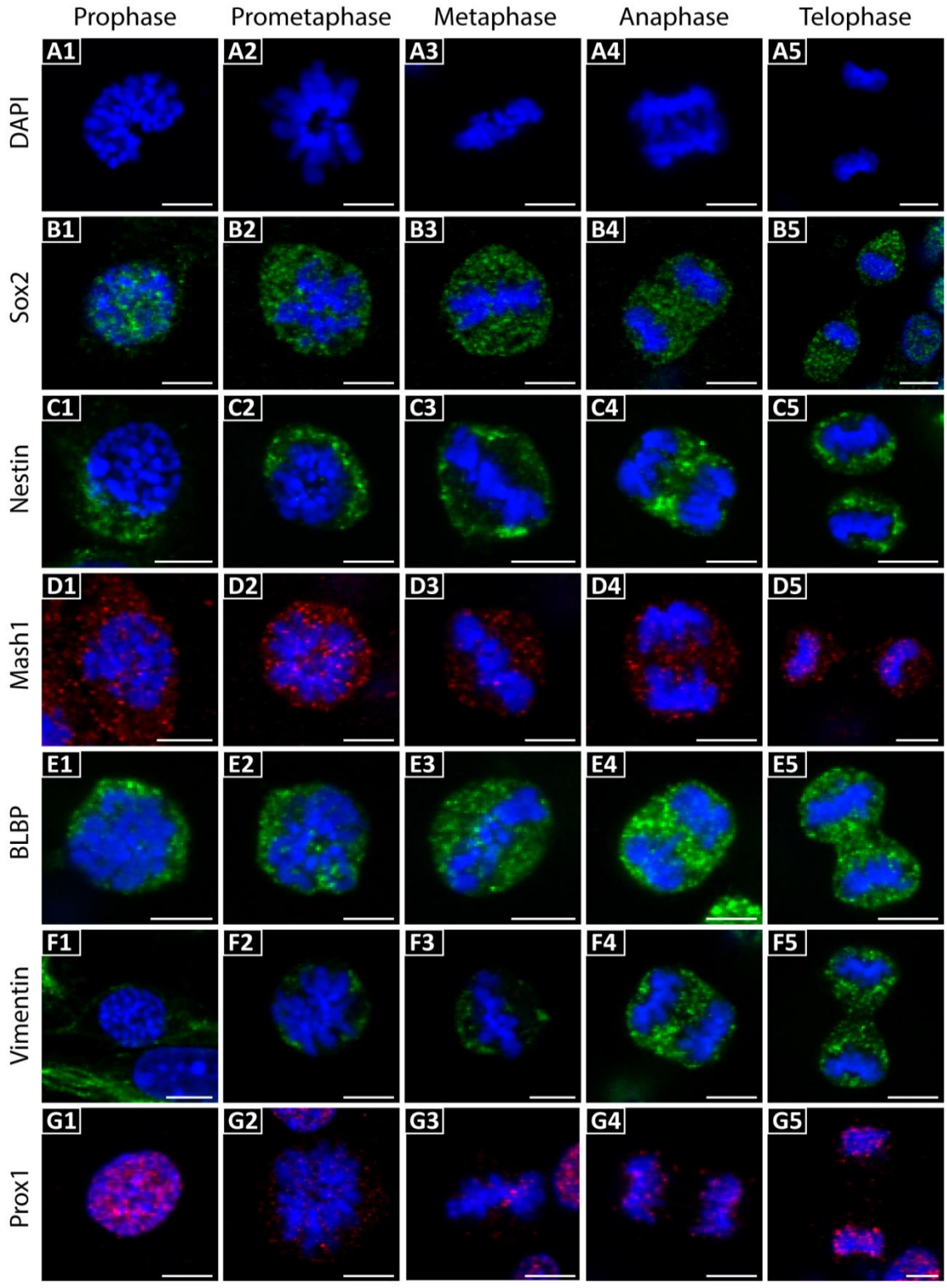

Figure 4.5 Expression patterns during mitosis

Hippocampal NPC protein expression patterns during mitosis were assessed. Cells were stained for DAPI (A1-A5) to identify nuclei in stages of mitosis. Sox2 (B1-B5), nestin (C1-C5), Mash1 (D1-D5), BLBP (E1-E5), vimentin (F1-F5), and Prox1 (G1-G5) expression was characterised. Scale bar represents 5 µm.

4.7 EFFECTS OF MATRIGEL ON PROTEIN EXPRESSION

Matrigel is a reconstituted basement membrane prepared from a mouse sarcoma rich in extracellular matrix proteins. It contains approximately 60% laminin, 30% collagen IV and 8% entactin. The remaining 2% consists of various growth factors, which are detailed in Appendix 2. By using a thin layer of 1:10 diluted Matrigel instead of laminin to prepare glass coverslips, it was found that both spheres and single cell suspensions adhered to the glass faster and grew rapidly in a more uniform monolayer. Morphological differences were noticed; cells grown on Matrigel had broader, more fibroblast-like processes compared to cells grown on laminin only, see Figure 4.6.

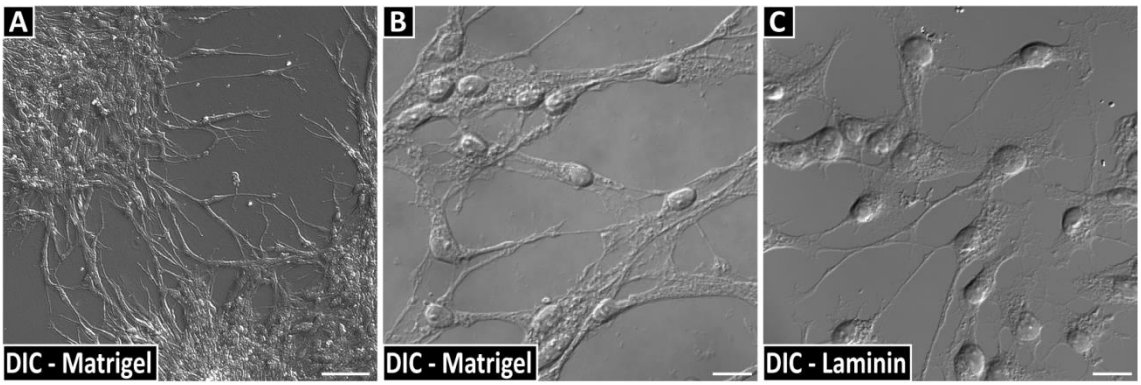

Figure 4.6 Morphological effects of Matrigel

Hippocampal NPCs were plated onto PLO and either Matrigel or laminin coated coverslips, to determine the suitability of Matrigel as a surface coat for cell culture, and to identify any morphological differences in hippocampal NPCs grown on the different surfaces. DIC microscopy (A) shows cells migrating away from a sphere centre. Matrigel can be seen in the textured surface of the glass. Cells grown on Matrigel (A-B) had broader, more fibroblast-like processes compared to cells grown on laminin (C), and tended to adhere faster and grow in a more uniform manner. Scale bars represent 100 µm (A) and 10 µm (B,C).

Immunocytochemistry was conducted to determine if the use of Matrigel as a surface coating had any significant effect on the immunological properties of hippocampal NPCs, see Figure 4.7. Nestin, vimentin (A-C), Mash1, Sox2 (D-F), Dlx2, Pax6 (G-I), Prox1 (J), GFAP (M) and BLBP (N) were all probed for and based on the staining profile used there was no difference observed between NPCs grown on Matrigel and those grown on laminin, with the exception of Dlx2, which localised to the nucleus only rather than expressing both cytoplasmic and nuclear staining, see Figure 4.7, G-I. No immunoreactivity was observed for the isotype control or the secondary antibody control (Figure 4.7, O).

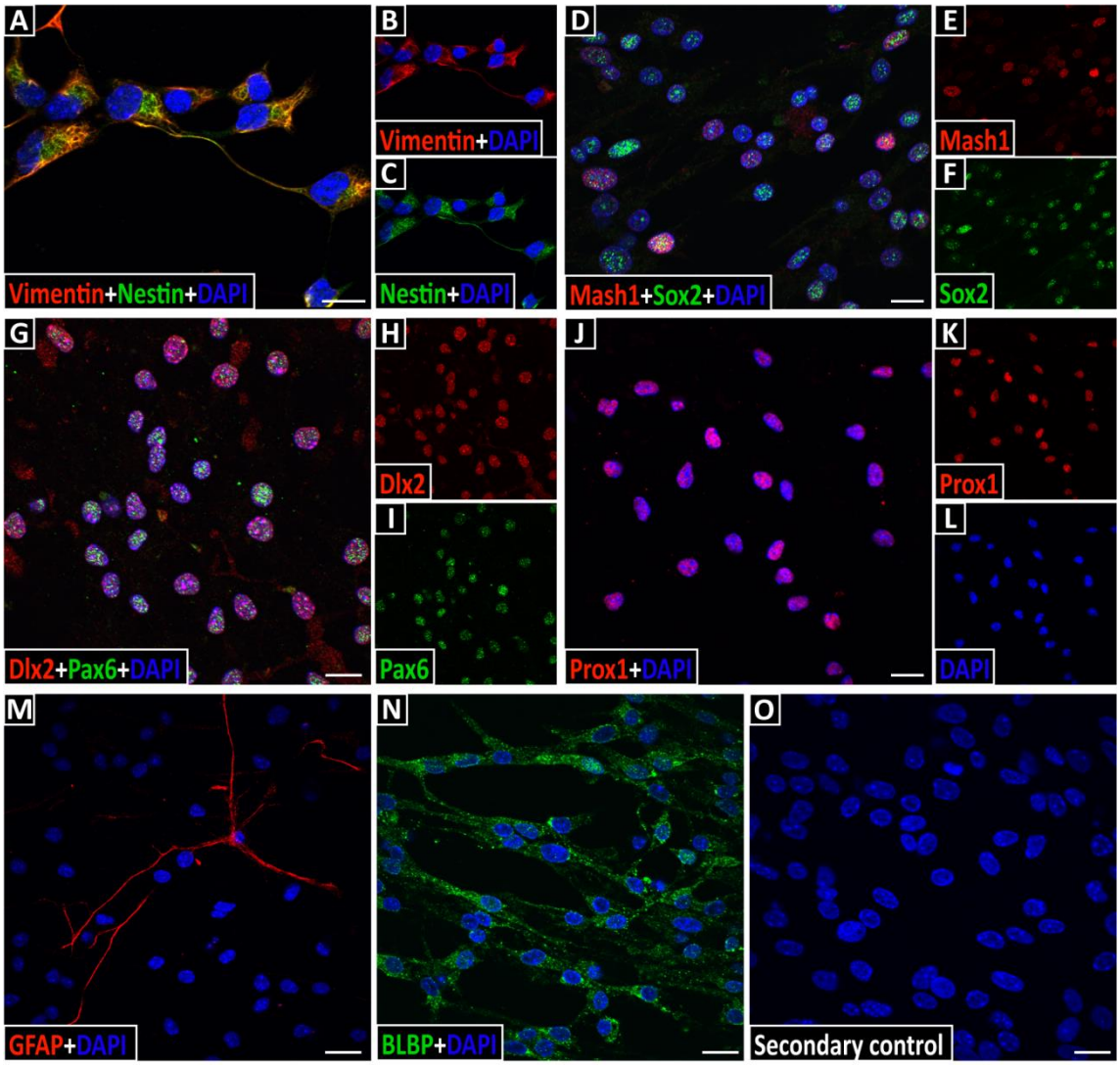

Figure 4.7 Immunochemical analysis of Matrigel cultured NPCs

Hippocampal NPCs were plated on PLO and Matrigel coated coverslips and stained for nestin, vimentin (A-C), Mash1, Sox2 (D-F), Dlx2, Pax6 (G-I), Prox1 (J), GFAP (M) and BLBP (N), to identify potential differences in marker expression patterns caused by culturing cells on Matrigel instead of laminin. No differences were observed between NPCs grown on the different surfaces, with the exception of Dlx2, which localised to the nucleus only when cultured on Matrigel, rather than expressing both a cytoplasmic and nuclear staining pattern when grown on laminin. No immunoreactivity was observed on the secondary control (O). Nuclei were stained with DAPI. Scale bars represent 10 µm.

4.8 CHARACTERISATION OF THE DIFFERENTIATED CULTURE

The potential of the hippocampal NPCs to differentiate into neurons, astrocytes and oligodendrocytes was assessed. Cells were plated at a low seeding density and cultured in NeuroCult Basal Medium supplemented with NeuroCult Differentiation Supplement for at least 7 days. The cells were observed to have smaller nuclei and soma, with long branching processes; see Figure 4.8.

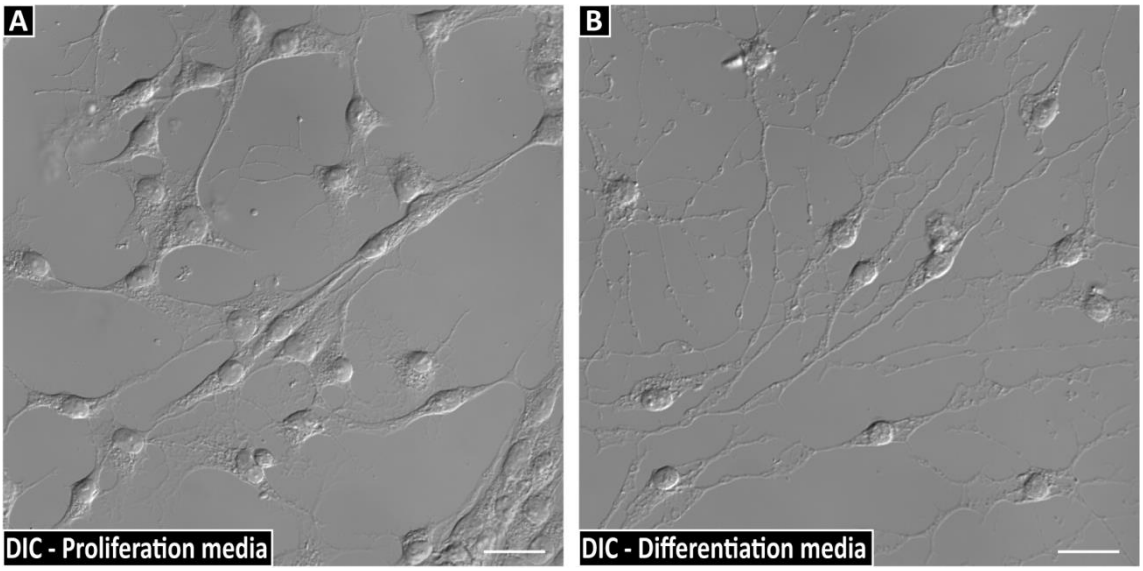

Figure 4.8 Morphology of differentiated hippocampal NPCs

Hippocampal NPCs were plated onto PLO and laminin coated glass coverslips and cultured in proliferation medium (A) or differentiation medium (B). Cells grown in differentiation medium were observed to have long, fine branching processes and in general had smaller nuclei and soma. Scale bar represents 20 µm.

Immunocytochemistry was conducted probing for neural progenitor markers BLBP, nestin, Sox2 and Mash1. Expression levels of BLBP (Figure 4.9, A-C) was reduced compared to when grown in proliferation medium, while nestin was negative in approximately half of the culture (Figure 4.9, D-F, example negative cells indicated by asterisk). Sox2 (Figure 4.9, G-I) was also reduced compared to proliferation medium, as was Mash1 expression (Figure 4.9, J-K), which was observed to have a higher amount of negative cells (67.4 ± 2.8%) than cultures grown in proliferation medium, while high expression was observed in just 1.4 ± 0.8% of cells, and medium expression in 31.1 ± 2.2% of cells. Statistical comparison of Mash1 expression between proliferation and differentiation media was conducted (Figure 4.9 M). Homogeneity of variance was violated as assessed by Levene's test [$F(5,99) = 3.11$, $p = 0.012$], thus a Welch ANOVA was used and a significant difference between medias was found [*Welch's* $F(5,99) = 197.17$, $p < 0.01$]. In the presence of differentiation medium, Games-Howell post hoc test identified an increase of 53.6% ± 3.6 SE in the number of cells negative for Mash1, while number of cells with low to medium expression decreased 42.4% ± 3.8 SE and number of cells with high expression decreased 11.1% ± 1.8 SE.

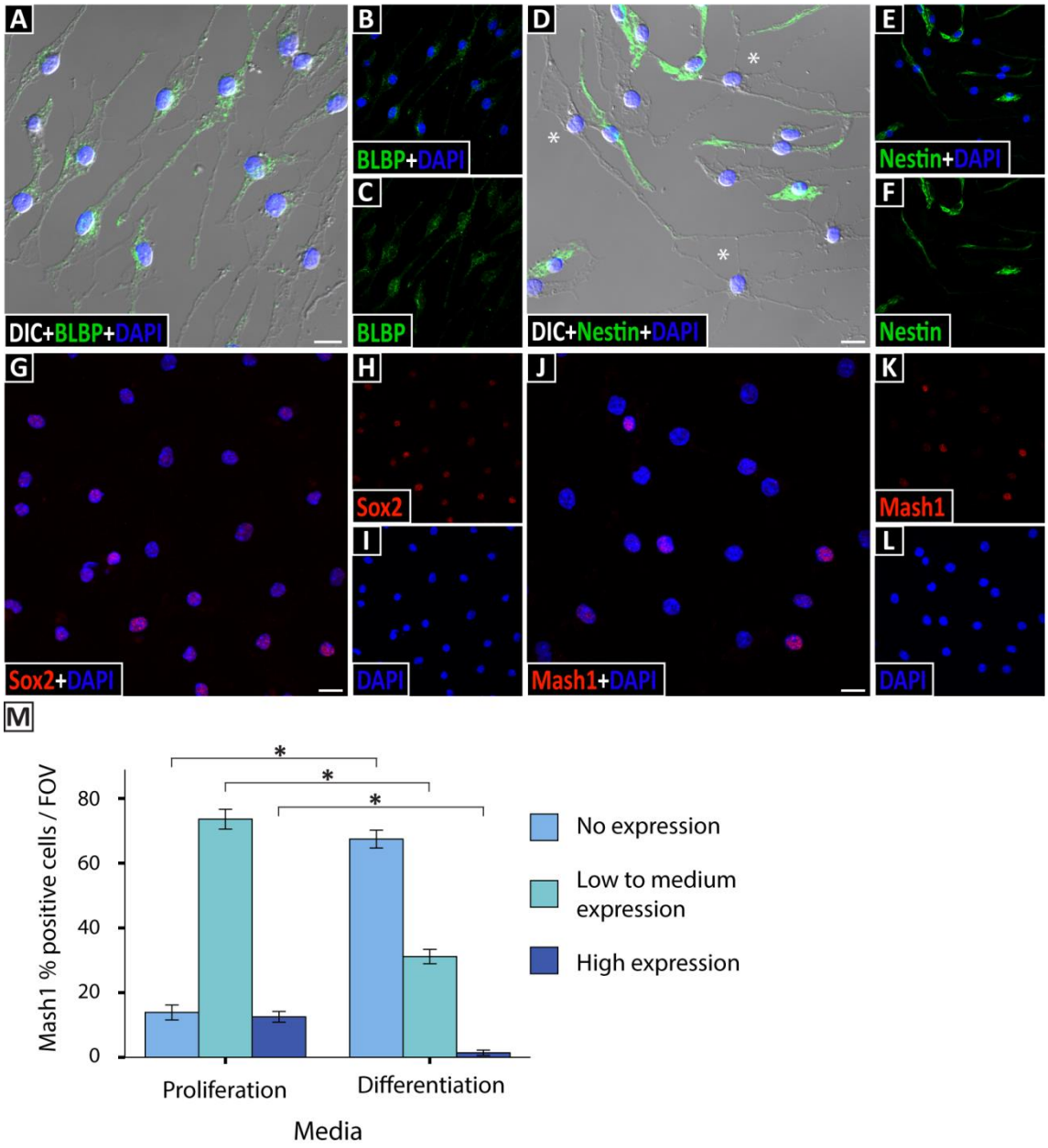

Figure 4.9 Neural progenitor characterisation of differentiated culture

Hippocampal NPCs were plated onto PLO and laminin coated glass coverslips and cultured in differentiation medium for at least 7 days prior to fixation. Immunocytochemistry was conducted against BLBP (A-C), nestin (D-F), Sox2 (G-I) and Mash1 (J-L), to determine changes in marker espression patterns in the differentiated culture. When compared to cultures grown in proliferation medium, expression levels of BLBP and Sox2 were reduced and nestin was negative in approximately half of the culture as indicated by asterisks. Nuclei were stained with DAPI. Scale bar represents 10 µm. A significant reduction in Mash1 expression between media was observed (M), asterisk indicates $p < 0.01$, error bars are ± 1 SE.

Potential for NPCs to differentiate into neurons was gauged by expression levels of neuronal markers; DCX (neuroblasts), Map2a/b and βIII tubulin (immature neurons), and NeuN (neurons). DCX positive staining was observed in less than 1% of the population (n > 1000, Figure 4.10, A-C), and the morphology appeared more consistent with an immature neuron rather than a neuroblast. Map2a/b and βIII tubulin stained positive in 16.7 ± 0.9% and 10.1 ± 0.8% of cells respectively, Figure 4.10, D-I and Figure 4.11 G. NeuN was positive in all observed cells, Figure 4.10, J-L.

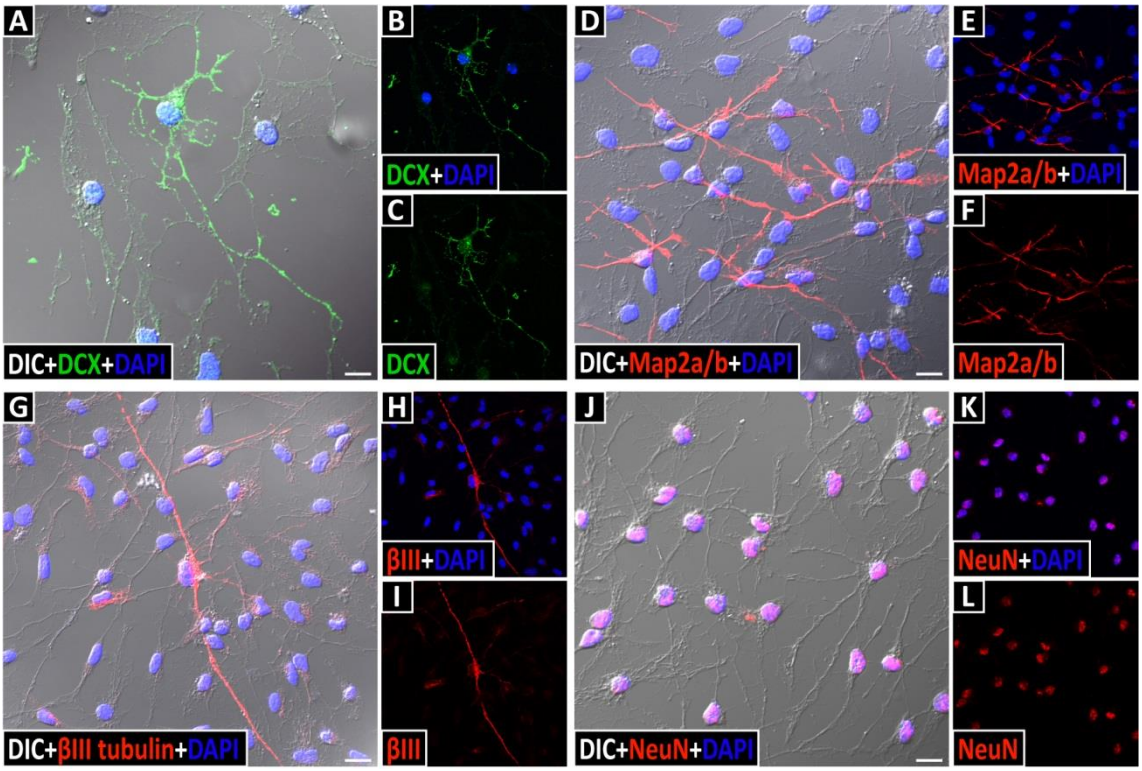

Figure 4.10 Neuronal characterisation of differentiated hippocampal NPCs
Hippocampal NPCs were plated onto PLO and laminin coated glass coverslips and cultured in differentiation medium for at least 7 days prior to fixation. Staining conducted against neuronal markers revealed less than 1% of cells positive for DCX (A-C), 16.7 ± 0.9% of cells were positive for Map2a/b (D-F), 10.1 ± 0.8% positive for BIII tubulin (G-I) and NeuN was positive in all observed cells (J-L). Nuclei were stained with DAPI. Scale bar represents 10 µm.

Differentiated NPCs were investigated for tripotentiality, using GFAP in combination with vimentin to reveal astrocytes and O4 to stain for oligodendrocyte precursor cells. Astrocyte precursors were identified by $GFAP^{pos}$/$vimentin^{neg}$ double stain and were found to comprise 4.9 ± 0.9% of the population (Figure 4.11, A-C). Occasionally more mature astrocytes were observed, as identified by their much larger morphology, see Figure 4.11 D. Staining for O4 revealed the NPC culture to be negative for cells of the oligodendrocyte lineage (n > 1000; Figure 4.11, E). NPCs derived from the SVZ served as a positive control for the O4 antibody, and are depicted in Figure 4.11, F. These data indicate the majority of hippocampal NPCs differentiate down the neuronal and astrocytic lineages, while the number of cells that mature into oligodendrocyte precursor cells is negligible.

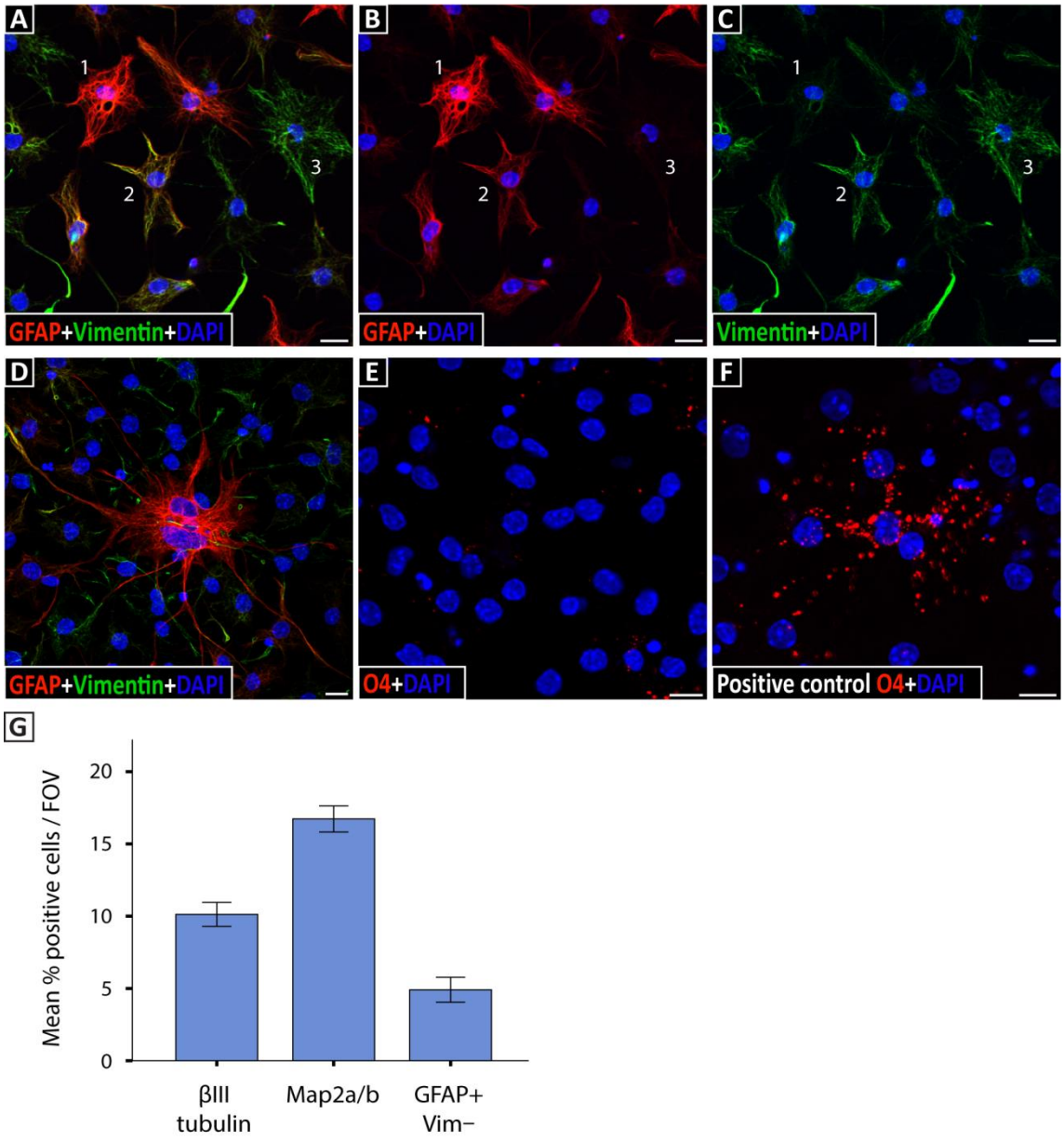

Figure 4.11 Tripotentiality of hippocampal NPCs

Hippocampal NPCs were plated onto PLO and laminin coated glass coverslips and cultured in differentiation medium for at least 7 days prior to fixation. Astrocyte precursors were identified by $GFAP^{pos}$/$vimentin^{neg}$ double stain and comprised 4.9 ± 0.9% of the population (A-C). More mature astrocytes were identified by their much larger morphology (D). Oligodendrocyte precursor cells were stained for using O4 antibody, and the culture was found to be negative (n > 1000; E). Differentiated SVZ NPCs served as a positive control for O4 (F). Nuclei were stained with DAPI. Scale bar represents 10 µm. Statistical analysis revealed the majority of hippocampal NPCs to differentiate down the neuronal and astrocytic pathways, rather than the oligodendrocyte pathway (G). Error bars ± 1 SE.

4.9 DISCUSSION

4.9.1 Isolation and identification of type 2 neural progenitor cells from the adult hippocampus

Neural progenitor cultures derived from the adult hippocampus are capable of self-renewal *in vitro* and can differentiate into neuronal and astrocytic lineages (Babu et al., 2007). Here the self-renewing properties of hippocampal progenitor cells were confirmed by careful dissection and the successful passaging and subsequent formation of neurospheres. *In vivo* analysis revealed the niche contained cells positive for stem cell marker nestin, while other cells negative for nestin expressed the granule cell marker Prox1. Dividing (EdU and Mash1 positive) cells indicated presence of a neural precursor cell. Once dissected the cells proliferated in culture in the presence of EGF and bFGF, consistent with previously reported protocols (Walker and Kempermann, 2014).

Monolayer cultures were assessed by immunochemistry. In order to accurately identify the cells multiple markers must be used, as proteins used as a marker for neurogenesis may also be expressed by other cell types (Kempermann et al., 2004, Mamber et al., 2013). Initial immunochemistry revealed cells to be $GFAP^{neg}$, $nestin^{pos}$, $Sox2^{pos}$, $Mash1^{pos}$, DCX^{neg}, and indicate the majority of the hippocampal NPC culture comprised of type 2a neural progenitors. $GFAP^{pos}$/$nestin^{pos}$ staining identified approximately 4% of the culture as type 1 NSCs. Nestin and Sox2 are heavily implicated in self-renewal and pluripotency, and are used as markers for type 1 and type 2 hippocampal NPCs. Type 2 cells could be further characterized by the expression of Mash1 by type 2a cells, indicating entry into a proliferative stage, and by DCX expression in type 2b cells. Absence of Mash1 in approximately 14% of cells may indicate a transitional phase

either into or away from type 2a cells, and could also reflect the loss of expression during mitosis. The presence of DCX positive cells in only a small number of cells indicates the culture did not yet contain a substantial number of cells differentiating into immature neurons when maintained in proliferation medium.

Further characterisation identified cells as BLBP^{pos}, vimentin^{pos}, Pax6^{pos}, and Dlx2^{pos} and further strengthened the conclusion of a type 2 cell characterisation. SSEA1 was observed to be negative in this culture, despite being used as a marker for pluripotency and neurogenesis (Ashwell and Mai, 1997). It's possible that despite SSEA1 expression having been previously reported in adult neural stem cells (Capela and Temple, 2006), these cultures have progressed through this stage. Expression of Prox1 also demonstrated the cells' potential to mature into neurons of the granule cell lineage.

The use of Matrigel was not observed to change expression patterns of the proteins stained for, with the exception of Dlx2, which localised to the nucleus only rather than expressing both cytoplasmic and nuclear staining. The vigour with which cells take to a Matrigel surface makes it a useful tool in neural cell culture. However, morphological changes were observed and cells had a more fibroblast-like appearance despite consistencies in marker expression. The faster growth rate and broad processes possibly indicated a culture shift to a cell type less neuronal than that obtained using laminin only. For this reason, as well as to remain as similar as possible to published protocols, Matrigel was not used for any further experiments in this project.

4.9.2 Multipotency of hippocampal neural progenitor cells

Multipotency is a key feature of progenitor cells and was established in this culture using NeuroCult Differentiation Supplement. Following differentiation treatment the

cells were observed to have smaller nuclei and soma, with long branching processes. As the progenitor cells matured they upregulated neuronal markers and downregulated progenitor markers. BLBP and Sox2 staining was faint, nestin was negative in approximately half of the cells and Mash1 expression levels were significantly decreased. Mature neuron markers βIII tubulin and Map2a/b, which were observed to be very low when grown in proliferation medium, were upregulated, and NeuN expression was observed in all cells. These stains indicate the NPC culture were capable of maturing down a neuronal lineage. Occasionally, overlapping expression of proteins that generally correspond with different cell type was observed, for example in cells that were $NeuN^{pos}$/$nestin^{pos}$/$Sox2^{low}$. The retained presence of early progenitor markers may indicate that the amount of time that the cells were cultured in differentiation medium was not long enough for cells to completely mature or to lose the expression of progenitor markers. For this reason, progression of the cells through their lineage is best thought of as a fluid and continuous process, rather than a discrete transition from one cell type to another at a single mitotic event.

Potential to differentiate down the astrocyte and oligodendrocyte lineages was also assessed. Astrocyte precursors were identified by $GFAP^{pos}$/$vimentin^{neg}$ double stain and O4 was used to stain for oligodendrocyte precursor cells. A dramatic increase in GFAP expression was observed, suggesting NPCs also had the potential to become astrocytes. Despite this, $GFAP^{pos}$/$vimentin^{neg}$ cells only comprised roughly 5% of the population. Expression levels of GFAP and vimentin are generally described as having an inverse relationship in maturing astrocytes, with GFAP expression levels increasing and vimentin decreasing with maturity (Gomes et al., 1999). Using GFAP and vimentin in combination to define astrocytes may be unreliable, as previous literature also suggests some astrocytes also express vimentin (Ridet et al., 1997, Sancho-Tello et al., 1995).

GFAP^{pos}/vimentin^{pos} cells comprised a substantial portion of the differentiated culture, and it is possible that requiring a negative vimentin stain results in an underestimate of the number of astrocyte precursor cells. Regardless, these stains clearly demonstrate the potential for the hippocampal NPC culture to mature down both neuronal and astrocytic lineages. O4 positive immunochemistry was not observed in the differentiated population, consistent with previous literature indicating that hippocampal NPCs do not routinely differentiate along the oligodendrocyte lineage unless induced (Braun et al., 2015, Sun et al., 2015a).

4.9.3 Conclusion

Taken together, these data demonstrate that the majority of NPCs derived from the hippocampus of adult mice and cultured in the presence of EGF and bFGF are type 2a neural progenitors. Proliferative capabilities were retained in culture, and the potential for differentiation into both neuronal and astrocytic lineages was established. Immunochemical analysis utilised stains widely accepted by the scientific community, and in conjunction with stem cell selective medium and careful dissection, potential contamination by other cell types posed a minimal risk. Subsequent experiments were undertaken with confidence in cell culture protocols and with minimal variation between culture replicates.

5.0 P2X7 RECEPTOR CHARACTERISATION AND FUNCTION IN HIPPOCAMPAL NEURAL PROGENITOR CELLS

5.1 INTRODUCTION

5.1.1 P2X7 receptors

Purinergic signalling is an important modulator of cellular activity, having roles in signal transduction and cell-cell communications. P2X7 receptors are activated by extracellular ATP and are ionotropic cation channels primarily allowing passage of Ca^{2+}, Na^{+} and K^{+}. Prolonged activation by high concentrations of ATP results in the formation of a large transmembrane pore, leading to cytoskeletal rearrangement, membrane blebbing and potentially apoptosis and/or necrosis (Surprenant et al., 1996). P2X7 receptors initiate the release of pro-inflammatory mediators; these features provide it an important role in the immune system. The P2X7 membrane complex also plays an important role in innate immunity, mediating the phagocytosis of non-opsonised particles including beads, bacteria and apoptotic cells in the absence of ATP (Wiley and Gu, 2012). This indicates a function distinct to the inflammatory response.

P2X7 function in the CNS is generally associated with cell death (Ulrich et al., 2012). Following an ischemic event, high amounts of ATP is released from necrotic cells, activating P2X7 receptors on neighbouring neurons and glia and leading to cell death. A number of recent studies have demonstrated that inhibition of P2X7 receptor activity confers neuroprotection in models of ischemic injury, and has highlighted the

therapeutic potential of targeting P2X7 receptors in cerebrovascular diseases (Sperlagh and Illes, 2014, Engel et al., 2012, Yu et al., 2013). P2X7 receptors have also emerged as a new target for depression and cognitive dysfunction studies as pro-inflammatory cytokines can mediate many of the psychological changes associated with depression (Liu et al., 2017)

5.1.2 P2X7 receptors in the adult hippocampus

In 2002, P2X7 receptors were identified in the CA1, CA3 and the dentate gyrus regions of the hippocampus and implicated in the regulation of GABA and glutamatergic signalling (Sperlagh et al., 2002, Atkinson et al., 2004). Despite these lines of evidence, controversy over the presence of P2X7 receptors in mature neurons still ensues (Illes et al., 2017, Miras-Portugal et al., 2017). P2X7 receptors in adult hippocampal NPCs have been reported with regard to membrane potential changes in response to ATP and BzATP application, and anti-P2X7 immunochemistry has also demonstrated positive staining (Hogg et al., 2004, Rozmer et al., 2017). Despite these studies, many aspects of P2X7 receptors in adult hippocampal NPCs remain unexplored and further research is required to fully comprehend the various roles that P2X7 receptors may have in the adult hippocampus.

During human embryonic development, NPCs express P2X7 receptors and these neural progenitors are able to phagocytose apoptotic neuroblasts via a P2X7 mediated pathway (Lovelace et al., 2015). These data suggest P2X7 acts as a scavenger receptor within the developing CNS, and this has implications with regards to the biological function and regulation of adult NPCs. Adult neurogenesis proceeds in the company of cell death, and while microglia were assumed to be solely responsible for the clearance of these apoptotic bodies (Sierra et al., 2010), recent observations in both the SVZ and

SGZ suggests that adult NPCs are also capable of phagocytosing other apoptotic neural progenitors (Lu et al., 2011).

In light of this evidence, and assuming presence of the receptor can be confirmed, there are at least three distinct functions P2X7 receptors may have in neural progenitor cells of the adult hippocampus depending on the conditions in the extracellular environment. The first of these functions is cellular signal transduction via cations, such as calcium; the second is transmembrane pore formation and cell death in the presence of inflammation and high concentrations extracellular ATP. A third additional role is the non-canonical function of P2X7 receptors in the absence of extracellular ATP, where they act to facilitate phagocytosis. These alternate roles can have quite distinct and varied outcomes in terms of function, and given the therapeutic potential P2X7 receptors have for assisting in the treatment of ischemic injury, the roles of this receptor in adult hippocampal NPCs must be understood.

5.2 IDENTIFICATION OF P2X7 RECEPTORS IN ADULT HIPPOCAMPAL NEURAL PROGENITOR CELLS

Immunohistochemistry was conducted on adult hippocampal sections to determine presence of P2X7 receptors *in vivo*. Immunochemistry was conducted using rabbit anti-P2X7 (intra- and extracellular epitopes; Alomone) and receptor presence was observed. Staining for the P2X7 receptor in conjunction with Mash1 demonstrated presence of the receptor in NPCs in the proliferative phase of neural differentiation, and was notably absent in cells also negative for Mash1, see Figure 5.1.

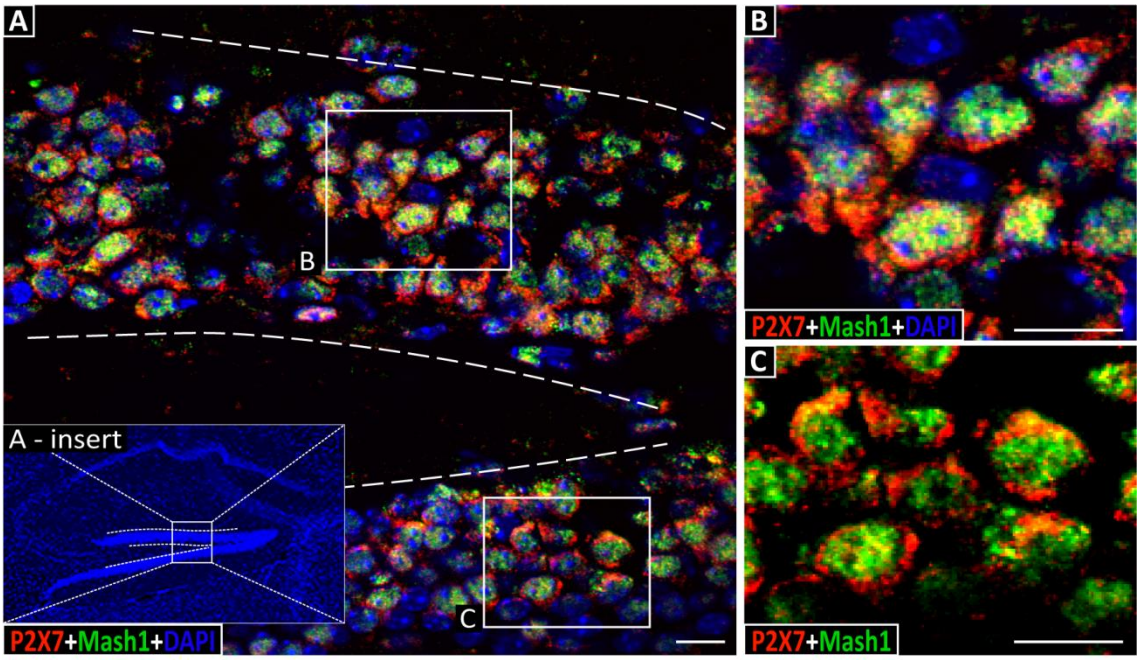

Figure 5.1 ***In vivo*** **identification of P2X7 receptors in the adult hippocampus**
Adult mice were perfused with PFA and the brains were sectioned. Immunohistochemistry was performed to identify P2X7 receptors in conjunction with Mash1 (A-C). A (insert) indicates the area of the hippocampus imaged in A. Zoomed areas are depicted in B and C, and show hippocampal cells positive for both P2X7 receptors and Mash1 in vivo. Mash1 staining was localised to the nucleus while P2X7 receptors were mostly cytoplasmic. Nuclei were stained with DAPI. Scale bar represents 10 µm.

Following the isolation and culture of NPCs from the hippocampus, immunocytochemistry was conducted to ensure continued presence of P2X7 receptors in the *in vitro* culture. Cells were cultured as previously described and stained for the P2X7 receptor using antibodies obtained from different companies, against different epitopes. Permeabilized conditions were used for these experiments. Antibodies from both Alomone (extracellular epitope, Figure 5.2, A-C) and Santa Cruz (internal epitope, Figure 5.2, D-F) were positive with similar staining patterns. An intracellular epitope antibody from Alomone was also used with similar results (data not shown). P2X7 receptor double labelling with GFAP and Prox1 was conducted to determine if the

receptor was expressed in both type 1 and type 2 NPCs respectively. The receptor was observed to co-express with both GFAP (Figure 5.2, G-I) and Prox1 (Figure 5.2, J-L) in all instances, indicating P2X7 receptors were present throughout the NPC maturation process. Expression patterns of P2X7 receptors were further investigated through the stages of division, and were found to remain consistently expressed in the cytoplasm, see Figure 5.3.

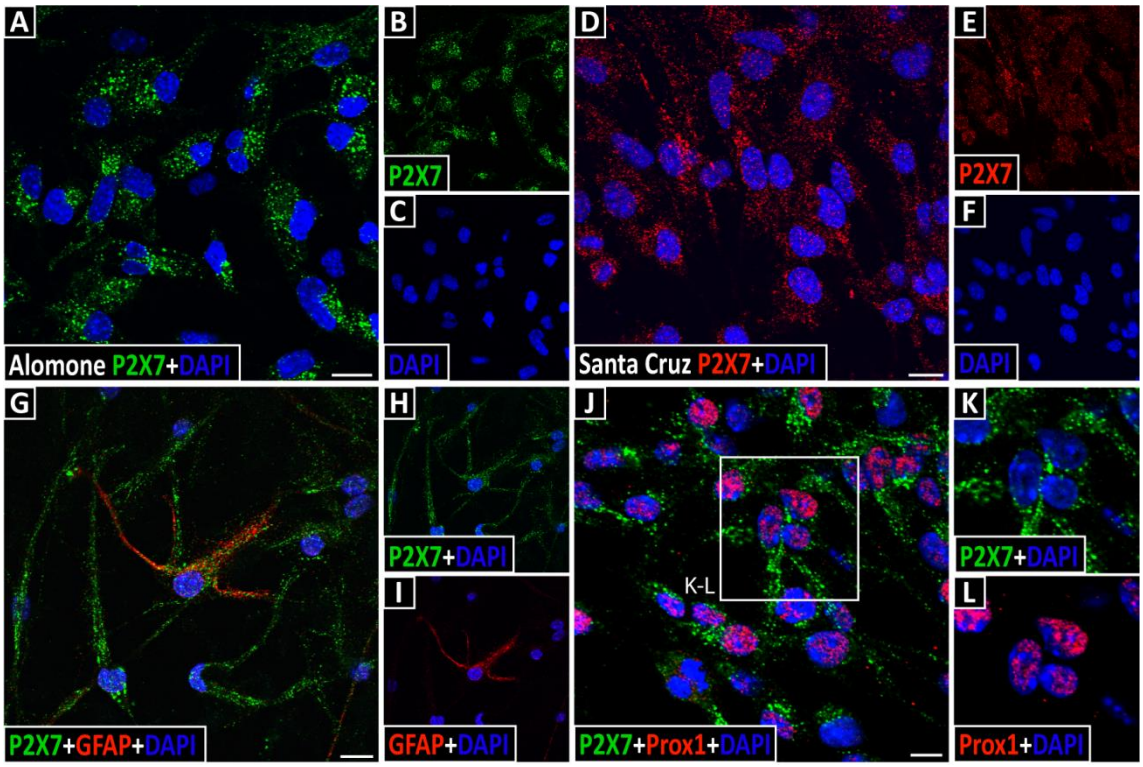

Figure 5.2 ***In vitro*** **identification of P2X7 receptors in cultured hippocampal NPCs.**

Hippocampal NPCs were plated on PLO and laminin coated glass coverslips and allowed to culture for 3 days. The cells were fixed with PFA and immunochemistry was conducted under permeabilized conditions. NPCs were observed to be positive for P2X7 receptors using antibodies from Alomone (extracellular epitope, A-C) and Santa Cruz (intracellular epitope, D-F). NPCs co-expressed P2X7 receptors with GFAP (G-I) and Prox1 (J-L), indicating P2X7 receptors were present in hippocampal type 1 NPCs, as well as type 2 NPCs, respectively. Nuclei were stained with DAPI. Scale bar represents 10 µm.

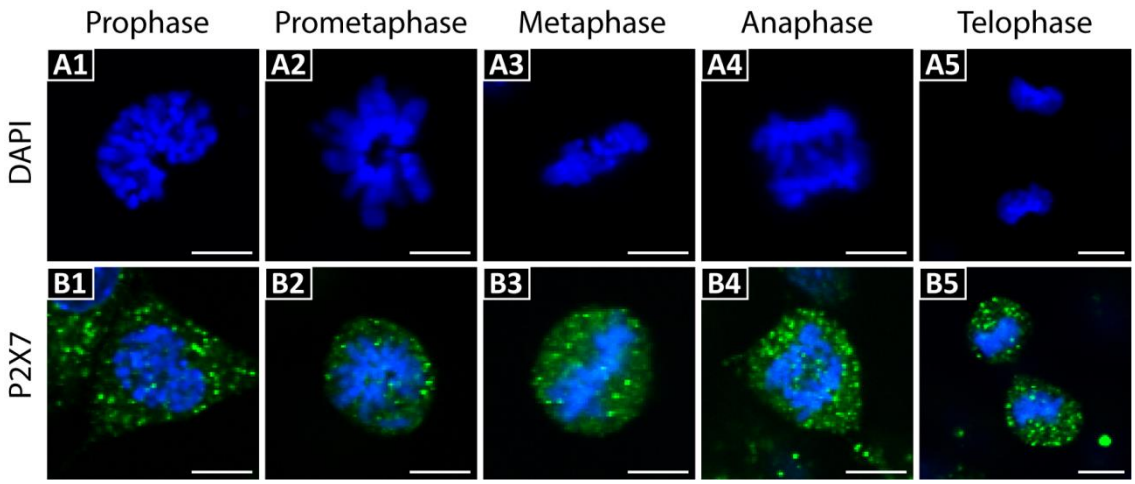

Figure 5.3 P2X7 receptor expression during mitosis

Hippocampal NPCs were plated on PLO and laminin coated glass coverslips and cultured for 3 days prior to immunochemical analysis being conducted for P2X7 receptors. DAPI was used to image the nuclei and determine the stage of mitosis (A1-A5) and staining against P2X7 receptors is depicted in B1-B5. Hippocampal NPCs expressed P2X7 receptors throughout the different stages of mitosis: prophase (A1, B1), prometaphase (A2, B2), metaphase (A3, B3), anaphase (A4, B4) and telophase (A5, B5) were imaged. Nuclei were stained with DAPI. Scale bar represents 5 µm.

5.3 SURFACE AND CYTOPLASMIC LOCALISATION OF P2X7 RECEPTORS

During characterisation experiments, the Alomone extracellular P2X7 receptor antibody was also trialled under non-permeabilized conditions. NPCs were negative for surface expression of the receptor in 9.9 ± 2.5% of cells (n = 1465 cells), see Figure 5.4, A-C. As this phenomenon was not observed under permeabilized conditions, a protocol was developed to investigate intracellular verse extracellular P2X7 receptor expression patterns. Briefly, NPCs were fixed, blocked and stained for the extracellular P2X7 epitope (Alomone APR-008), followed by goat anti rabbit secondary antibody conjugated to a fluorescent tag. The cells were fixed again with PFA, then

permeabilized and blocked. Anti P2X7 conjugated to a fluorescent tag was then applied (Alomone APR-008-488) to identify the same epitope located intracellularly. This method allowed distinction between receptors present on the surface and those sequestered in the cytosol, possibly in transport vesicles. Cells negative for surface expression of P2X7 were found to express the receptor internally, see Figure 5.4, D-F. This phenomenon was also frequently observed in dividing cells. Dividing cells were observed to divide either symmetrically (Figure 5.4, G-I) with both daughter cells expressing P2X7 receptors equally on the surface and internally, or asymmetrically (Figure 5.4, J-L) where one daughter cell was negative for surface expression. Dividing daughter cells both negative for surface expression of P2X7 receptors was not observed, nor was any cell observed negative for P2X7 receptors under permeabilized conditions. Given that the number of asymmetrically dividing cells was greater than the number of negative cells in culture, it is possible that negative surface expression is a transient phenomenon.

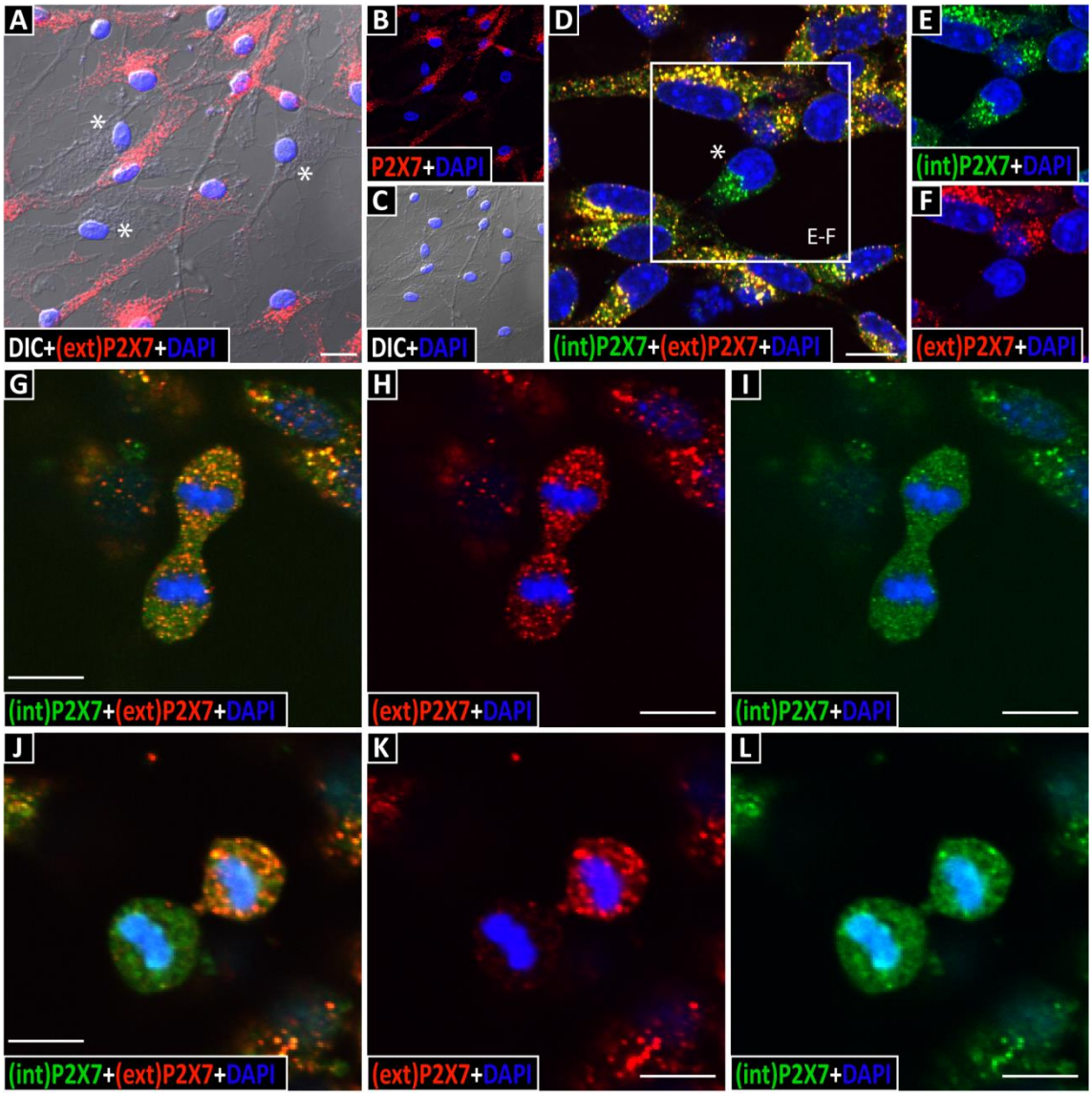

Figure 5.4 Extracellular and intracellular expression of P2X7 receptors

Hippocampal NPCs were cultured for 3 days prior to fixation and immunochemical analysis for both intracellular and extracellular P2X7 receptors. NPCs were stained for extracellular P2X7 receptors (red) under block only conditions. Cells were subsequently fixed again, permeabilized and re-blocked. Conjugated anti P2X7 was applied to locate receptors in the cytoplasm (green). Under block only conditions (A-C), 9.9 ± 2.5% of cells were negative for surface P2X7 receptors (asterisks). Co-staining for intracellular and extracellular P2X7 showed cells negative for surface expression had intracellular stores of the receptor (D-F). Dividing cells were observed to divide either symmetrically (G-I) with both daughter cells expressing P2X7 receptors equally, or asymmetrically (J-L) where one daughter cell was negative for surface expression. Nuclei were stained with DAPI. Scale bar represents 10 µm.

5.4 P2X7 RECEPTORS IN THE DIFFERENTIATED CULTURE

In the current study the presence of P2X7 receptors in cultured immature hippocampal neurons was confirmed. NPCs were cultured in differentiation medium for at least 7 days to allow immature neurons to develop, then were fixed and stained for surface expression of P2X7 receptors. As with NPCs, P2X7 receptors were identified around the soma of the immature neurons, though did not extend down the processes and were not observed at the growth cone terminals, see Figure 5.5, A-B. Similar to undifferentiated NPCs, approximately 10% of cells were negative for surface expression, as indicated by an asterisk.

Confirmation of antibody specificity was conducted by western blot in undifferentiated and differentiated NPC cultures, and a single band at 85 kDa was obtained, consistent with the glycosylated form of the receptor, see Figure 5.5 C. GAPDH was used as a loading control.

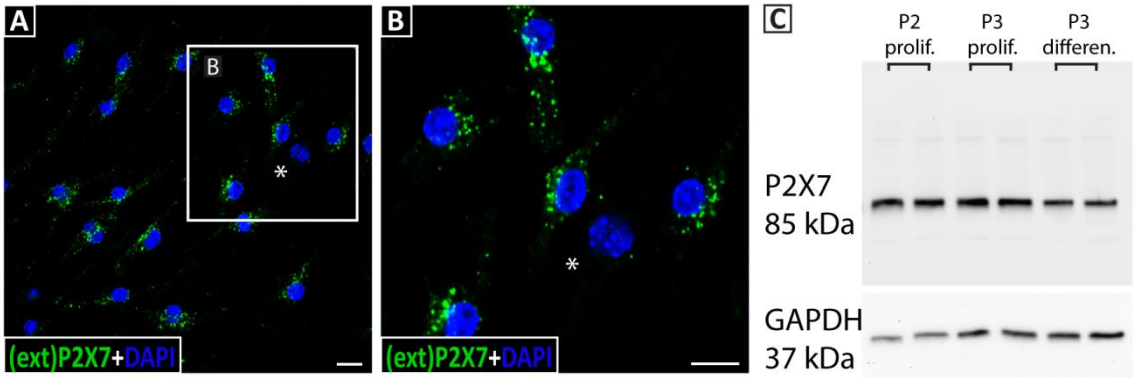

Figure 5.5 P2X7 receptors on differentiated hippocampal NPCs

Hippocampal NPCs were plated onto PLO and laminin coated glass coverslips and cultured in differentiation medium for at least 7 days prior to fixation. Cells were stained for P2X7 receptors (A-B) using the extracellular antibody under non-permeabilized conditions. Staining was localised to the soma, and did not extend down the length of the processes. Approximately 10% of cells were negative for surface expression (indicated by asterisk), consistent with P2X7 surface expression in NPCs cultured in proliferation medium. Nuclei were stained with DAPI. Scale bar represents 10 µm. Western blot confirmed specificity of the antibody to a single band protein (85 kDa) consistent with the molecular weight of the glycosylated form of the P2X7 receptor (C). Samples included NPCs cultured in proliferation medium at passages 2 and 3 (P2/3 prolif.) and differentiated NPCs (P3 differen.). GAPDH was used as a loading control.

5.5 FUNCTIONAL ACTIVITY OF P2X7 RECEPTORS IN HIPPOCAMPAL NEURAL PROGENITOR CELLS

5.5.1 P2X7 receptors function as calcium channels

P2X7 receptor function as a calcium channel was assessed using live cell microscopy and flow cytometry. NPCs were loaded with Fluo-8 AM calcium indicator dye, and fluorescence increases were observed upon purinergic stimulation, indicative of calcium influx into the cytosol. For live cell microscopy, application of the general P2X agonist

ATP (concentrations between 0.01 µM and 1000 µM) evoked influx of calcium in a dose dependent manner under live cell conditions (Figure 5.6, A-H). For each treatment, between 30 and 50 regions of interest were selected at random for quantification, and the fluorescence intensity ratios (F/F0) were calculated (total regions n = 1835, with minimum 3 biological repeats). The maximum F/F0 value for each region of interest was selected, as this represented the peak calcium response. Smaller secondary oscillations in cytosolic calcium concentration were observed, these values were not included in statistical analysis. The maximum F/F0 values for each region of interest were averaged and compared to control recordings. Fluorescence increase for each ATP concentration when compared to the control are as follows: 0.01 µM ATP increased fluoresce F/F0 value by 0.12 ± 0.04 SE, 0.1 µM by 0.62 ± 0.07 SE, 1 µM by 1.15 ± 0.08 SE, 10 µM by 1.37 ± 0.06 SE, 100 µM by 1.09 ± 0.03 SE, 500 µM by 1.05 ± 0.07 SE and 1000 µM by 1.08 ± 0.03 SE. A Welch ANOVA was used to determine significance [*Welch's* $F(8, 650) = 455.00$, $p < 0.01$], and concentrations 0.1 µM and above were deemed significantly different from the control, as indicated by the asterisk (Figure 5.6, I). Error bars are one standard error.

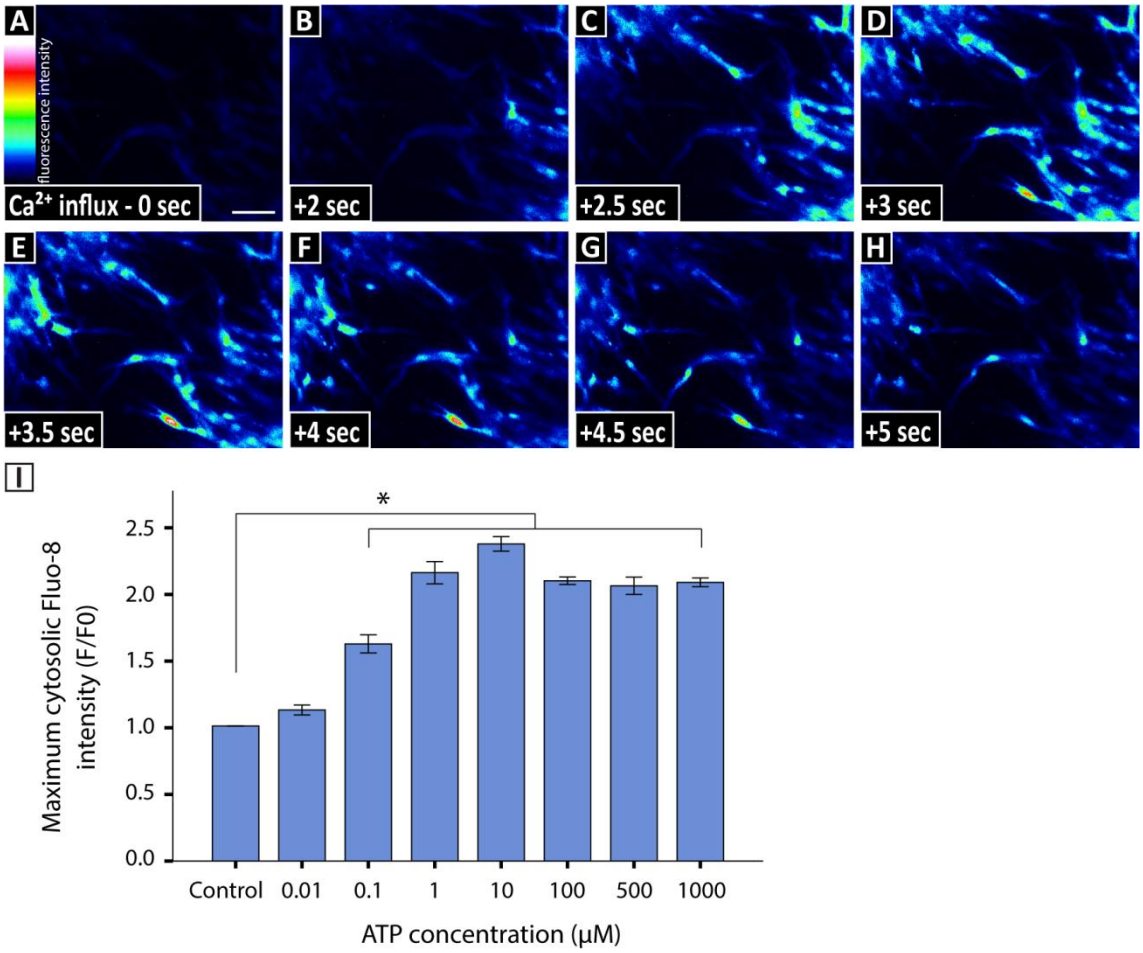

Figure 5.6 Calcium channel function of P2X7 receptors by live cell microscopy

NPCs were loaded with Fluo-8 AM calcium indicator dye and P2X7 receptor function as a calcium channel was assessed using live cell microscopy. Application of ATP evoked calcium influx (A-H, frame rate 0.5 seconds) in a dose dependent manner. Between 30 and 50 regions of interest were selected at random and the maximum cytosolic calcium concentrations (F/F0) from three biological repeats were quantified (I). Fluorescence increase for each ATP concentration when compared to the control are as follows: 0.01 µM ATP increased fluoresce F/F0 value by 0.12 ± 0.04 SE, 0.1 µM by 0.62 ± 0.07 SE, 1 µM by 1.15 ± 0.08 SE, 10 µM by 1.37 ± 0.06 SE, 100 µM by 1.09 ± 0.03 SE, 500 µM by 1.05 ± 0.07 SE and 1000 µM by 1.08 ± 0.03 SE. Scale bar represents 50 µm, error bars ± 1 SE, asterisk $p < 0.01$.

Time resolved live cell flow cytometry was used to more accurately gauge P2X7 receptor involvement in calcium influx (Figure 5.7, representative of four biological repeats). Events were captured at a rate of approximately 1000 events per second for three minutes. Application of ATP (1 mM) and the more specific P2X7 agonist BzATP (100 µM) at the 40 second mark (indicated by arrow) both evoked calcium influx similar to that recorded using live cell microscopy. Pre-incubation with the P2X7 specific inhibitor AZ10606120 (1 µM) reduced calcium influx in response to ATP, and abolished BzATP induced influx, indicative of P2X7 receptor functionality.

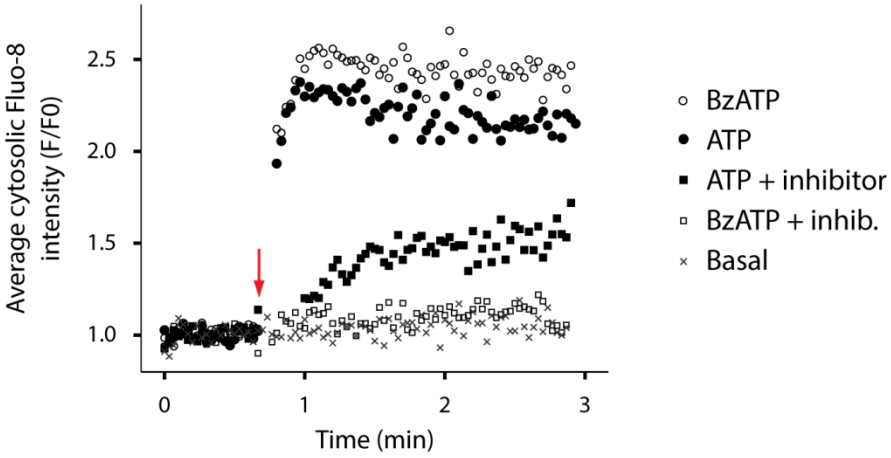

Figure 5.7 Calcium channel function of P2X7 receptors by live cell flow cytometry

NPCs were loaded with Fluo-8 AM calcium indicator dye and P2X7 receptor function as a calcium channel was assessed using time resolved live cell flow cytometry. Calcium influx was observed in response to ATP (1 mM) and BzATP (100 µM) by increases in Fluo-8 fluorescence. Pre-incubation with P2X7 specific inhibitor AZ10606120 (1 µM) decreased calcium influx in response to ATP and abolished BzATP induced influx. Agonists were applied at 40 seconds, indicated by the arrow. Figure is representative of four biological repeats.

5.5.2 Transmembrane pore formation as a function of P2X7 receptors

A canonical feature of P2X7 receptors is their ability to form a large transmembrane pore. This pore allows macromolecule exchange and can result in cell death. Ethidium bromide uptake was used to assess the transmembrane pore-forming ability of the P2X7 receptors in hippocampal NPCs, and was measured using time resolved flow cytometry (Figure 5.8 A, graph representative of four biological repeats). Application of the agonists ATP (1 mM ATP) and BzATP (100 µM) at the 40 second mark (indicated by arrow) resulted in the formation of a transmembrane pore, measurable by ethidium bromide fluorescence once intercalated with DNA. This effect was attenuated by the P2X7 specific inhibitor AZ10606120 (1 µM). The ethidium bromide uptake assay demonstrates a functional P2X7 receptor C-terminus (Cheewatrakoolpong et al., 2005) and is good evidence for full length P2X7 receptor expression. Live cell experiments confirmed membrane blebbing and cell death to occur after a one hour exposure to 1 mM ATP, see Appendix 3.

5.5.3 P2X7 receptor activation affects membrane fluidity: a preliminary study

Trimethylamine diphenylhexatriene (TMA-DPH) is a fluorescent probe used to label the outer cellular membrane and report on membrane dynamics and fluidity (Chazotte, 2011). Cells were incubated with ATP (1 mM) with and without P2X7 inhibitor AZ10606120 (10 µM) for 10 minutes prior to the application of 10 µm TMA-DPH and immediate analysis by flow cytometry (Figure 5.8 B). This preliminary study found ATP treated cells displayed a higher membrane fluidity compared to control. The effect was attenuated by pre-incubation with P2X7 inhibitor, indicating ATP affects plasma membrane structure via a P2X7 receptor mediated mechanism. Due to time constraints this assay was conducted on one biological repeat only.

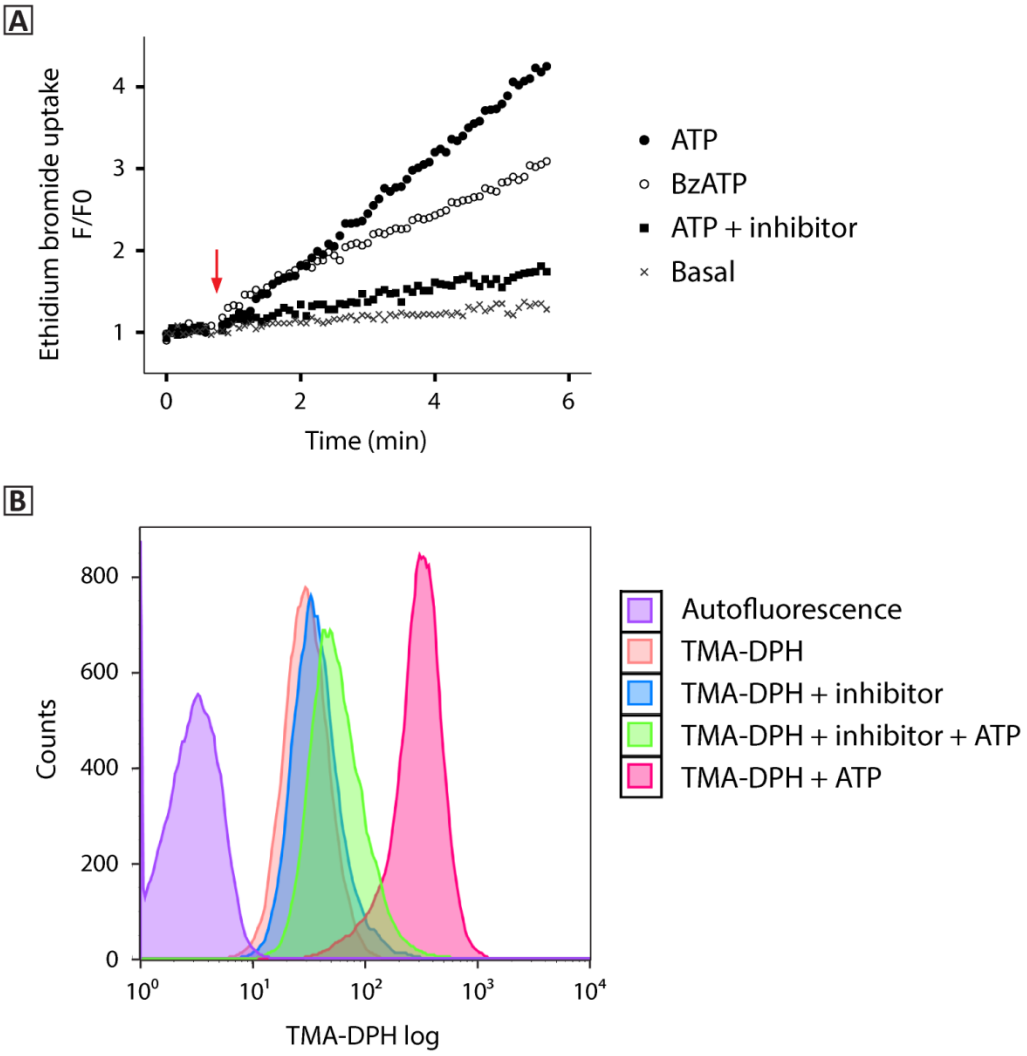

Figure 5.8 P2X7 receptor effects on NPC membrane integrity

Pore formation was assessed by ethidium bromide uptake (A). Agonists ATP (1 mM ATP) and BzATP (100 µM) applied at the 40 second mark (indicated by arrow) resulted in ethidium bromide uptake. This effect was attenuated by P2X7 inhibitor AZ10606120 (1 µM). Graph is representative of four biological repeats. In a preliminary study, TMA-DPH was used to assess the effects of P2X7 receptor activation on the fluidity of the cell membrane (B). ATP (1 mM) caused an increase in fluidity, which was negated by pre-incubation with P2X7 inhibitor AZ10606120 (10 µM).

5.6 P2X7 RECEPTOR FACILITATION OF PHAGOCYTOSIS BY HIPPOCAMPAL NEURAL PROGENITOR CELLS

Innate phagocytosis in adult NPCs was investigated by live-cell microscopy. Hippocampal NPC cultures were stained with LysoTracker Red to identify lysosomes and incubated overnight with 1 µm fluorescent yellow-green (YG) latex beads. Live-cell imaging captured NPCs phagocytosing YG beads in real-time, followed by their subsequent inclusion into lysosomes and rapid trafficking inside the cell. Engulfed beads could be identified as yellow (Figure 5.9, A-B, indicated by white arrow heads). Quantification revealed 62.4 ± 3.3% of NPCs contained beads with an average of 4.5 ± 0.3 beads per cell. Orthogonal reconstruction of confocal microscopy further demonstrated cellular localisation of YG beads within an NPC labelled with CellTracker (Figure 5.9 C, planes of orthogonal projection are indicated by yellow cross-hairs). NPC cultures were probed for microglial marker Iba1 to confirm absence of professional phagocytes (Figure 5.9, D-E). Iba1 control staining on a microglial culture is depicted in Figure 5.9 F.

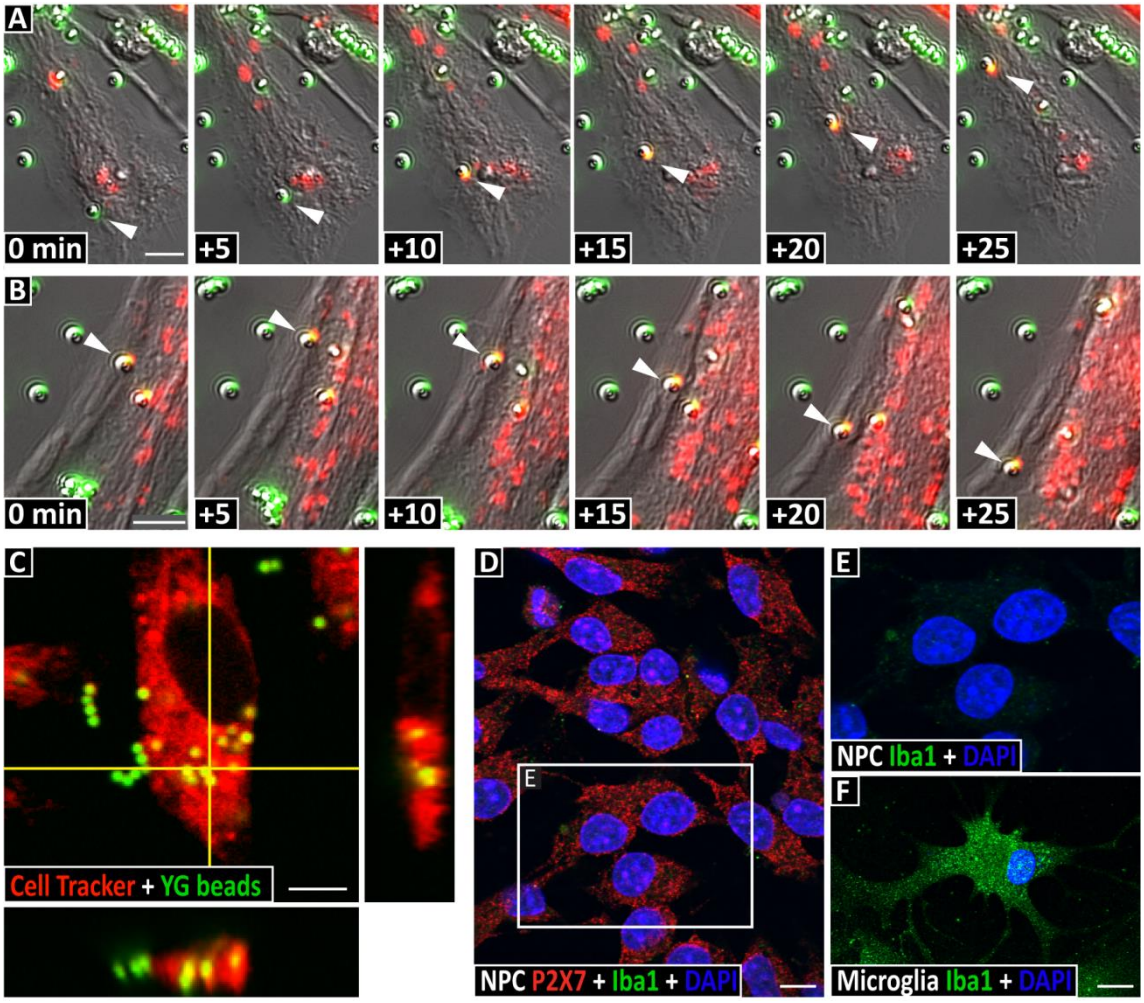

Figure 5.9 Hippocampal NPCs phagocytose YG beads

Overnight live cell microscopy was used to image hippocampal NPC cultures stained with LysoTracker Red and incubated with YG beads (A-B). YG beads were observed to be phagocytosed by the NPCs and rapidly incorporated into lysosomes and trafficked inside the cells. Engulfed beads and be identified as yellow, indicated by white arrow heads, scale bar represents 5 µm. In NPCs labelled with CellTracker, orthogonal reconstruction of confocal microscopy exemplified cellular localisation of YG beads (C). The planes of orthogonal projection are indicated by yellow cross-hairs, scale bar represents 5 µm. NPC cultures were stained for microglial marker Iba1 to confirm absence of professional phagocytes (D-E), Iba1 control staining on a microglial culture is depicted in F. Nuclei were stained with DAPI. Scale bar represents 10 µm.

P2X7 receptor involvement in hippocampal NPC phagocytosis was assessed using time resolved flow cytometry (Figure 5.10 A, representative of three biological repeats). Our group has previously demonstrated that extracellular ATP inhibits P2X7 mediated phagocytosis by dissociating the P2X7 C-terminus from the cytoskeleton, specifically the non-muscle myosin IIA complex (Gu et al., 2009, Gu et al., 2010), while inhibitors that interfere with the calcium or pore channel functions of P2X7, such as AZ10606120 and A438079, are ineffective in inhibiting the phagocytic function of P2X7 (Wiley et al., 2011). Uninhibited phagocytosis (basal) levels were established as the positive control. ATP inhibited phagocytosis of YG beads to the same extent as the non-specific inhibitors, PFA and actin polymerisation inhibitor cytochalasin D, while 5% serum completely abolished all innate phagocytosis. These data suggest P2X7 receptors can act to facilitate phagocytosis by adult hippocampal NPCs.

Population analysis was also conducted to confirm time-resolved flow analysis. Cells were pre-incubated with treatments before YG beads were added and the FACS tube was stirred gently for approximately eight minutes prior to analysis. Between 25 000 and 30 000 events were recorded per sample. This analysis confirmed the time-resolved flow data and revealed subpopulations within the NPC cultures that phagocytosed different numbers of beads per cell (Figure 5.10 B). This is most evident in the 5% serum treatment, which had two distinct populations, one of which had complete inhibition of phagocytosis and a smaller population that had reduced phagocytosis. ATP and PFA both reduced phagocytosis from basal levels; multiple peaks in the ATP treatment indicate sub-populations that are not as inhibited by ATP, indicating the presence of scavenger receptors additional to P2X7 that can facilitate phagocytosis in hippocampal NPCs.

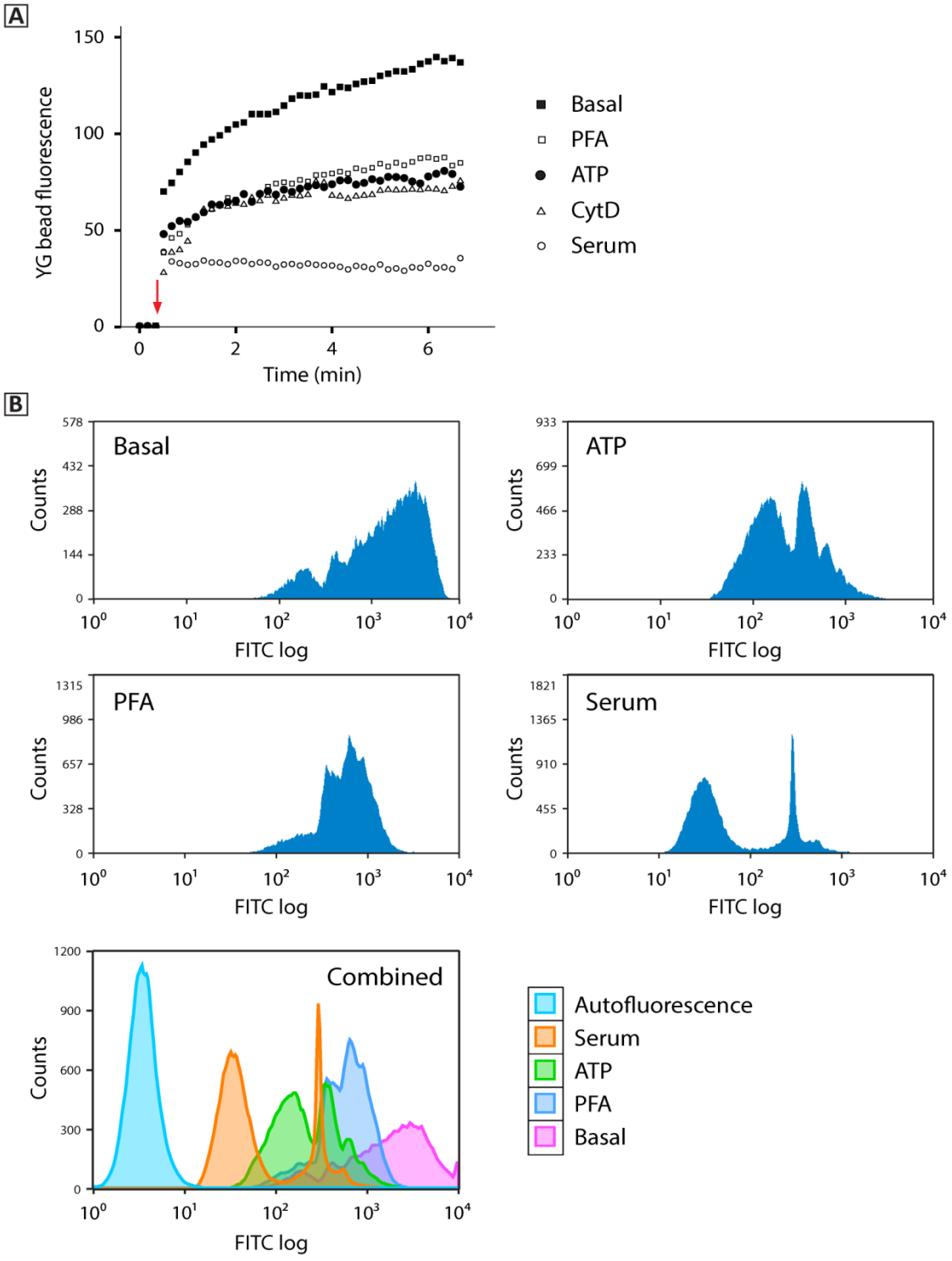

Figure 5.10 P2X7 receptors facilitate NPC phagocytosis of YG beads

Time resolved flow cytometry (A, representative of three biological repeats) showed YG bead uptake by NPCs. This was inhibited by ATP (P2X7 receptor specific inhibitor of phagocytosis), as well as PFA, cytochalasin D and serum (non-specific inhibitors of phagocytosis). Population analysis (B) confirmed the finding that ATP, as well as PFA and serum, inhibited phagocytosis in hippocampal NPCs.

5.7 DISCUSSION

5.7.1 P2X7 receptors in adult hippocampal neural progenitor cells

Immunostaining was conducted on hippocampal sections from adult mice; P2X7 receptor staining was observed in cells positive for Mash1, while being absent in cells negative for Mash1. This co-expression indicates P2X7 receptors are present in NPCs in the proliferative phase of neural differentiation, and supports arguments that while NPCs express P2X7 receptors, mature neurons may not (Illes et al., 2017). Cultures derived from the hippocampal neurogenic niche and defined as type 2 NPCs displayed positive immunoreactivity for P2X7 receptors using multiple antibodies. P2X7 receptors were also observed in type 1 progenitor cells, as identified by GFAP immunoreactivity, indicating P2X7 receptors were present throughout the NPC maturation process. A small percentage of NPCs had sequestered the receptor in the cytoplasm and displayed a negative surface stain; this phenomenon was observed at a much higher frequency in cells undergoing mitosis. Given that the number of asymmetrically dividing cells was greater than the number of negative cells in culture, it is possible that negative surface expression is a transient phenomenon, and may indicate a role for P2X7 receptors in the asymmetric division of NPCs as they progress through maturation processes. Alternatively, the asymmetry could be a by-product of the cytoskeletal rearrangement occurring as the cell undergoes telophase.

5.7.2 P2X7 receptors in hippocampal neurons

P2X7 receptor presence in mature hippocampal neurons is a contended subject, with initial reports suggesting the receptor was absent from adult neurons (Collo et al., 1997). Subsequently, receptor presence in excitatory terminals of hippocampal neurons

was described (Sperlagh et al., 2002, Atkinson et al., 2004) and recent literature has investigated the roles of P2X7 receptors in the hippocampal neurons of mouse epilepsy models (Engel et al., 2012, Kim et al., 2011, Rozmer et al., 2017). Despite evidence arguing the presence of P2X7 receptors in the mature neuron (Miras-Portugal et al., 2017) the poor selectivity of some agonists and antibodies, and the presence of multiple splice variants, some of which escape knock out in mouse lines, has led some researchers to propose the observations are due to cross talk between astrocytes and neurons, and it is actually the glial cells that are the targets of pathologically high extracellular ATP concentrations (Illes et al., 2017).

In the current study NPCs were differentiated down the neuronal pathway for 7 days prior to fixation. The cells possessed morphological and immunochemical characteristics of mature neurons, though still retained low expression of some progenitor markers. The P2X7 receptor immunofluorescence observed in these immature neuron cultures was concentrated around the cell body and was not observed in the distal processes, contrary to previous observations where the receptor was present in the excitatory terminals, and co-localised with glutamate transporters to facilitate the release of glutamate and GABA (Hogg et al., 2004, Sperlagh et al., 2002, Papp et al., 2007). The present culture conditions meant that the cells were not able to be cultured to full maturity, which by some estimates can require between 10 and 21 days (Kaech and Banker, 2006). It is possible that these cultures were not mature enough to provide representative expression patterns of P2X7 receptors in the mature neuron. Alternative methods may be required to confirm the presence or absence of P2X7 receptors in mature hippocampal neurons, and this is beyond the scope of the current project.

5.7.3 P2X7 receptor functionality by calcium channel and pore formation

Calcium influx in response to agonist binding was measured using Fluo-8 calcium indicator dye and confirmed the P2X7 receptors expressed on the surface of the NPCs were functional. The P2X7 inhibitor AZ10606120 was utilised in conjunction with agonists ATP and more specifically BzATP. No calcium influx was observed when using BzATP in the presence of AZ10606120, indicating specificity of both agonist and inhibitor. By using the broad P2 agonist ATP in conjunction with the inhibitor, an approximation of the effects other purinergic receptors were gained. These may include other P2X receptors, or P2Y GPCRs, which have also been identified in NPCs (Cao et al., 2013, Ulrich et al., 2012).

P2X7 receptor functionality was also confirmed by ethidium bromide uptake experiments. P2X7 receptors are normally tethered to the cytoskeleton by their C-terminus, which detach in the presence of agonists, allowing P2X7 to form a pore (Gu et al., 2010). The formation of a transmembrane pore large enough to allow ethidium bromide entry to the cell confirmed the presence of a full length functional receptor, as opposed to the Δ-C splice variant with a truncated C-terminus (Cheewatrakoolpong et al., 2005).

Prolonged ATP exposure of embryonic neural progenitors results in membrane disruption and necrotic cell death via activation of the P2X7 receptor (Delarasse et al., 2009). Functional P2X7 receptors in adult hippocampal NPCs thus have significant implications during an ischemic event, as high amounts of ATP released from necrotic cells can activate receptors, allowing inward current and an overload of cytosolic calcium levels, leading to mitochondrial depolarisation, oxidative stress and cell death. A number of recent studies demonstrating conferral of neuroprotection by modulation

of P2X7 receptor activity in ischemic brain injury, epilepsy and stroke has highlighted the therapeutic potential of targeting P2X7 receptors in cerebrovascular diseases (Sperlagh and Illes, 2014). Status epilepticus (prolonged seizures) has also been found to increase levels of P2X7 in the granule neurons of the dentate gyrus, and that antagonising P2X7 receptors reduced both seizure duration and subsequent neuronal death (Engel et al., 2012). Similar observations were made in the CA1 area of the hippocampus, where P2X7 receptor inhibition reduced the amount of delayed neuronal death in ischemic injury (Yu et al., 2013). An increase in receptor expression levels was also observed. Understanding the impacts that utilising P2X7 receptors as a target may have on adult NPC populations is a necessary step towards producing therapies for these disorders.

5.7.4 Phagocytosis in neurogenesis

Adult neurogenesis requires the progenitor cell population to be tightly controlled; proliferation is countered by large amounts of cell death regulated by target dependent and target independent PCD signalling processes. In the SGZ of the adult rat dentate gyrus approximately 9000 precursor cells are produced each day (Cameron and McKay, 2001); of these an estimated 50% undergo cell death at a steady rate during the first four weeks (Dayer et al., 2003). Clearance of cell debris following PCD is an important to prevent inflammation. Both professional phagocytes (microglia) and non-professional phagocytes contribute to this process; resident neural precursors and neuroblasts have been demonstrated to engulf the debris of neighbouring apoptotic cells via innate phagocytosis. It has been assumed that microglia are solely responsible for the clearance of these apoptotic bodies (Sierra et al., 2010), though recent observations in the adult SVZ and SGZ suggests that DCX positive NPCs are also capable of

phagocytosing other apoptotic neural progenitors (Lu et al., 2011). These data suggest microglial phagocytosis may not be the only method used in the clearance of apoptotic neural progenitors in the adult CNS and other mechanisms may play important roles in clearing debris from naturally occurring cell death without the involvement of an inflammatory response.

A defining feature of P2X7 receptors is their ability to mediate phagocytosis. Monocytes and macrophages can engulf latex beads, live and dead bacteria and apoptotic cells via P2X7 receptors and HEK293 cells transfected with P2X7 were also conferred the ability to phagocytose (Gu et al., 2011, Gu et al., 2010). This phagocytic function for the P2X7 receptor was later confirmed in resting astrocytes (Yamamoto et al., 2013) and, importantly, in human embryonic NPCs (Lovelace et al., 2015). Neuroblasts with high expression of both DCX and P2X7 receptors had the greatest phagocytic capability of developing CNS progenitors, and these neural progenitors were able to phagocytose apoptotic neuroblasts via a P2X7 mediated pathway (Lovelace et al., 2015).

In this study adult NPCs derived from the hippocampus were capable of phagocytosis via P2X7 receptors, despite low levels of DCX expression. This was inhibited by the presence of ATP. The carboxyl terminus of P2X7 receptors is associated with the heavy chain of non-muscle myosin IIA (NMM IIA); the activity of this motor protein enables the cytoskeletal rearrangements required for particle engulfment (Gu et al., 2015). Activation by extracellular ATP results in conformational changes that open the selective cation channel while dissociating P2X7 from the underlying cytoskeleton and abolishing its phagocytic function, a phenomenon also demonstrated by increased

membrane fluidity. This suggests P2X7 receptors may have a role in adult neurogenesis by facilitating phagocytosis of apoptotic NPCs.

5.7.5 Conclusion

These data demonstrate that functional P2X7 receptors are expressed by adult NPCs of the hippocampus. Given this evidence, there are at least three distinct functions P2X7 receptors may play depending on the conditions present in the extracellular environment: (i) as an ion channel P2X7 receptors are able to facilitate signal transduction via cations, such as calcium; (ii) as a transmembrane pore it may induce cell death in the presence of inflammation and high concentrations of extracellular ATP; and (iii) and as a scavenger receptor P2X7 receptors may be able to remove apoptotic bodies by facilitating phagocytosis in the absence of ATP. These alternate roles of P2X7 signalling have somewhat opposing outcomes in terms of function, and understanding these mechanisms is essential to resolving our incomplete knowledge of neurogenesis and regeneration in the adult brain.

6.0 PURINERGIC REGULATION OF HIPPOCAMPAL NEURAL PROGENITOR CELL PROLIFERATION

6.1 INTRODUCTION

P2X7 receptors have a number of physiological functions, the first being the formation of cation channels for the purposes of neurotransmitter and signal transduction (Papp et al., 2004, Ferrari et al., 1999). Calcium ions enter the cytosol where they initiate downstream signalling cascades. This signalling mechanism is routinely employed by the nervous system to regulate neurotransmitter release, and it can also be utilised in conjunction with RTK signalling as part of long term regulatory mechanisms to govern transcription factor activation, cell cycle progression, differentiation, migration and cell death. Though proliferation can be regulated by P2Y and subsequent IP_3 signalling, P2X7 receptors have also been implicated in the regulation of proliferation and differentiation (Tang and Illes, 2017). As P2X7 receptors are generally associated with cell death pathways through transmembrane pore formation, this presents an interesting and alternate role for the receptor in the adult neurogenic niche.

6.1.1 P2X7 receptor regulation of proliferation and differentiation

In murine embryonic stem cells, BzATP application was reported to increase cell cycle entry as indicated by the percentage of cells in S phase, while inhibition of P2X7 receptors using pharmacological antagonists decreased proliferation and simultaneously increased the expression of early neuronal markers (Glaser et al., 2014). The authors

also demonstrated that P2X7 receptor mRNA and protein levels decrease with differentiation, and suggested that P2X7 receptors may play a role in proliferation and differentiation. Contrary to these findings, another study found P2X7 receptor activation correlated with a significant decrease in proliferation and an enhanced expression of neural markers in embryonic NPCs; this neuronal differentiation was regulated by the PKC-ERK1/2 signalling pathway (Tsao et al., 2013). *In vivo*, increased proliferation of neural precursors has been observed in the dentate gyrus of P2X7 receptor deficient mice, in addition to altered neuronal morphology and a decrease in P2X7 mediated glutamate release (Sebastian-Serrano et al., 2016, Csolle et al., 2013). Taken together these findings suggest a role for P2X7 in neural stem cell proliferation.

P2X7 receptors have also been implicated in axonal elongation and migration. Exposure of cultured hippocampal neurons to ATP inhibited axonal growth via P2X7 receptor mediated calcium transients, and inhibition or silencing of P2X7 receptors generated longer and more branched axons (Diaz-Hernandez et al., 2008). These studies demonstrate the ambiguous roles P2X7 receptors can play at different stages of development, from regulating cell cycle, proliferation and differentiation of neural progenitors to axon guidance following neuronal maturation.

6.1.2 P2X7 receptor regulation of transcription factor activation

Activation of calcium dependent signalling cascades resulting in the nuclear translocation of transcription factors is one means by which P2X7 receptors are able to affect cellular responses. In macrophages, stimulation of P2X7 receptors with ATP resulted in activation of NFAT1 and NFAT2 (Ferrari et al., 1999), as well as NFκB (Ferrari et al., 1997). In HEK293 cells transfected with P2X7B, a naturally occurring

truncated splice variant, ATP or BzATP exposure caused increases in ER calcium concentration, activated NFAT1 and stimulated growth (Adinolfi et al., 2010). Likewise, purinergic activation of NFκB through P2X7 receptors has been of interest due its implications in the pathophysiology of neurological damage or trauma in hippocampal neurons. BzATP treatment prevented decreases in NFκB phosphorylation caused by status epilepticus and subsequently attenuated neuronal death in CA1-3 pyramidal neurons (Kim et al., 2013).

This chapter aims to elucidate possible signalling roles P2X7 receptors may play in the hippocampal neurogenic niche via intracellular regulation of calcium sensitive signalling pathways, and will focus on proliferation and possible downstream transcription factor activation. These data may be of particular relevance given recently published literature that has suggested the use of P2X7 receptor inhibitors to reduce neuronal death following various ischemic events. The findings presented here should be taken into consideration when analysing such studies.

6.2 EFFECTS OF PURINERGIC SIGNALLING ON NEURAL PROGENITOR CELL PROLIFERATION

The effect of ATP on proliferation rates of hippocampal NPCs was examined using EdU to identify the number of cells undergoing DNA replication, see Figure 6.1. A dose response was completed with ATP from 10 nM to 1 mM, to encompass previously reported concentrations used to effect proliferation changes in other cell models (Adinolfi et al., 2010). A dose dependent decrease in proliferation was observed for concentrations of ATP higher than 1 μM with significance detected at 50 μM, see Figure 6.1, Figure 6.2 A. Levene's test indicated a violation of homogeneity of

variance [$F(9,236) = 6.97$, $p < 0.01$], thus significance was determined by Welch ANOVA [*Welch's* $F(9,236) = 125.00$, $p < 0.01$]. Proliferation was lowest at 100 µM with 6.76 ± 0.53% of cells positive for EdU (a decrease of 23.89 ± 1.25% from control), and increased slightly again at 500 µM to 11.83 ± 1.64%. ATP at 1 mM concentration resulted in cell death after a few hours, consistent with data from the ethidium bromide assays (see Section 0) where high concentrations of ATP resulted in transmembrane pore formation. The apparent bimodal response at 500 µM may be attributed to recruitment of multiple purinergic signalling pathways.

A time course was also conducted to confirm the appropriate incubation time. The negative effect of purinergic signalling on proliferation was most evident after overnight incubation, 16 to 24 hours post ATP application, [*Welch's* $F(12,68) = 100.43$, $p < 0.01$], see Figure 6.2 B. This was an optimisation experiment and only one biological repeat was conducted. Recovery assays were conducted in combination with live cell observations to ensure the decrease in proliferation was not due to cells undergoing apoptosis or necrosis. Following removal of ATP and a one or two day recovery period with fresh medium, cultures treated overnight with ATP regained proliferation to control levels (Figure 6.2 C). Significance was confirmed by Welch ANOVA [*Welch's* $F(5,136) = 118.75$, $p < 0.01$]. Controls for each time point were conducted and proliferation remained constant until extreme confluency (approximately 150%) caused a decline in proliferation due to cell-cell inhibition.

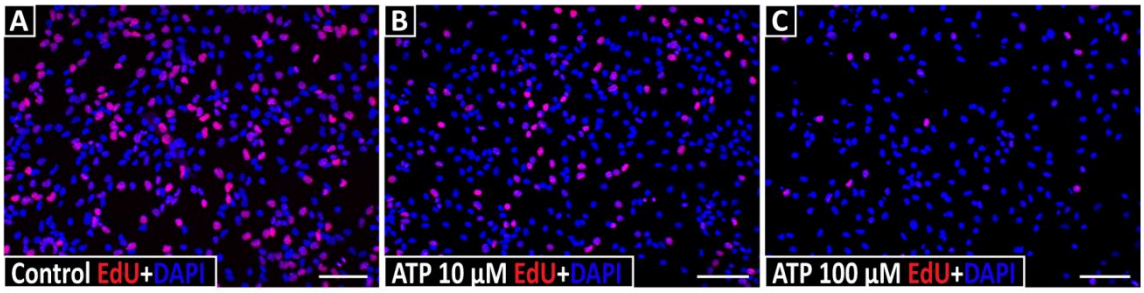

Figure 6.1 Proliferation decrease measured by EdU incorporation

Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips and treated with ATP, followed by a four hour incubation with EdU. Cells were fixed and permeabilized, and click chemistry was conducted to label EdU incorporated in dividing DNA. All nuclei were counterstained with DAPI. Images were captured with a confocal microscope. The percentage of EdU positive cells per FOV was calculated using ImageJ threshold intensities and particle count functions. A significant decrease in proliferation was detected at ATP concentrations exceeding 50 µM. Scale bar indicates 50 µm.

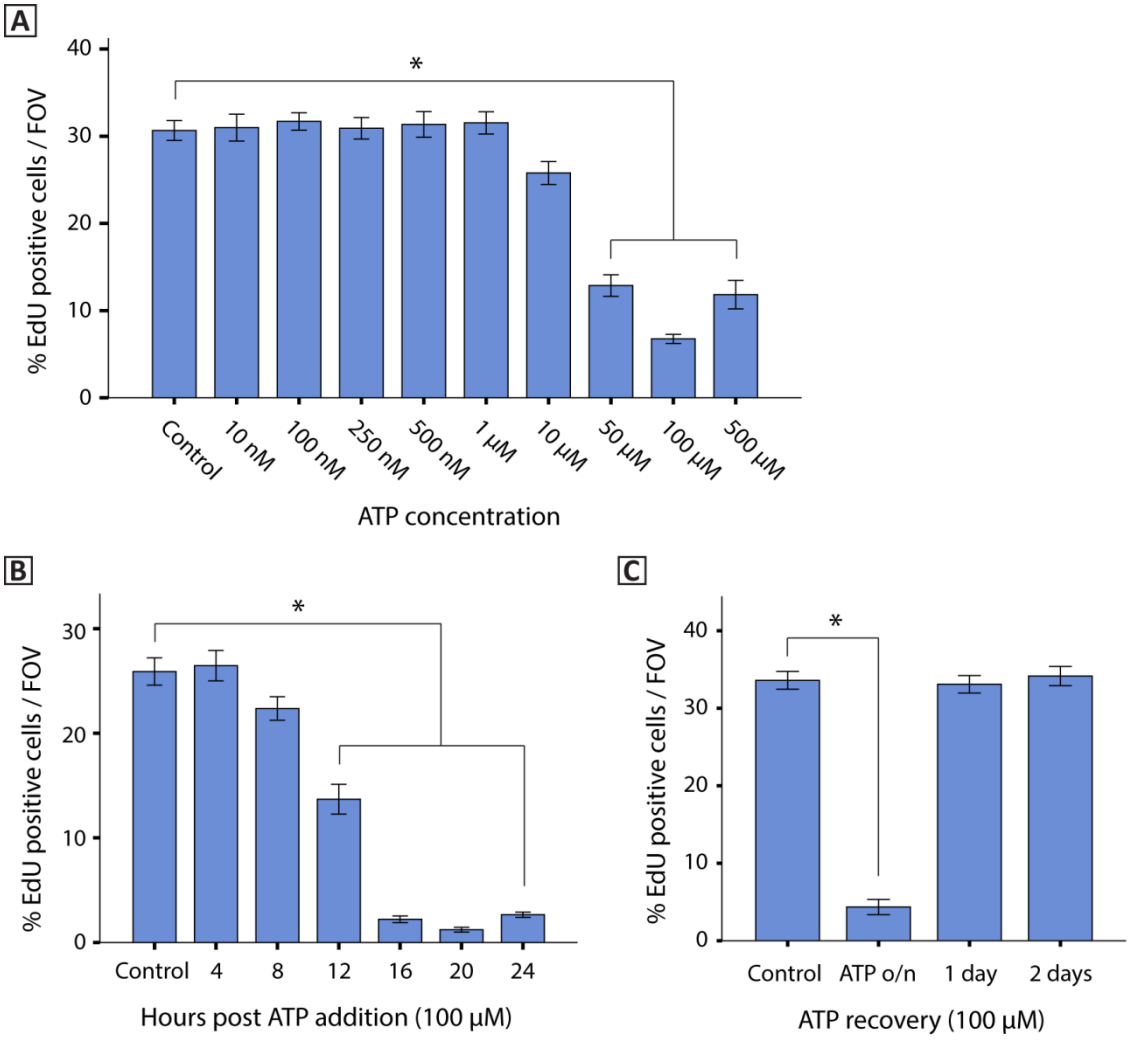

Figure 6.2 ATP signalling decreases NPC proliferation

Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips and treated with ATP, followed by incubation with EdU. The percentage of EdU positive cells per FOV was calculated. ATP was applied at concentrations from 10 nM to 500 µM overnight and a significant reduction was identified at 50, 100 and 500 µM (A). A time course confirmed overnight application of ATP was appropriate with 16, 20 and 24 hour time points showing the greatest decrease (B). Cultures given fresh medium recovered from ATP application after one and two days (C). Error bars ± 1 SE, asterisk indicates $p < 0.01$.

ATP may exert its effects by a number of purinergic receptors; it is probable that *in vivo* numerous receptors and signalling pathways are active in regulating proliferation. The activity of different purinergic receptors in hippocampal NPCs was assessed through the use of different purines. Effects of ATP were compared with 100 µM concentrations of ADP, UTP and UDP. ADP was found to decrease proliferation to a similar extent as ATP, while UTP and UDP had no effect on proliferation, [*Welch's* $F(4,106) = 44.20$, $p < 0.01$], see Figure 6.3. Failure of UTP and UDP to alter proliferation rates indicates the decrease in proliferation is mediated by P2X receptor activity. ADP is generally considered an agonist of some P2Y receptors and has a greater affinity than ATP. These data indicate multiple purinergic receptor pathways (both P2X and P2Y) may be active in the regulation of proliferation.

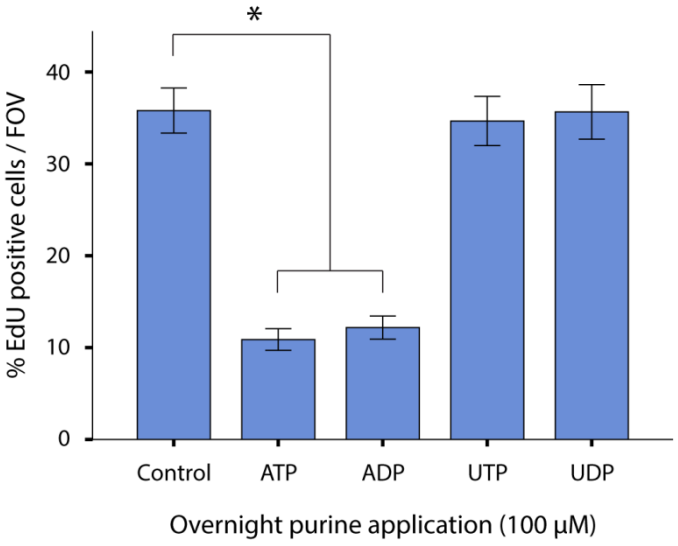

Figure 6.3 Effect of ATP derivatives on NPC proliferation

Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips and treated with 100 µM ATP, ADP, UTP and UDP, followed by incubation with EdU. The percentage of EdU positive cells per FOV was calculated. ATP and ADP decreased proliferation significantly, while UTP and UDP had no effect. Error bars ± 1 SE, asterisk indicates $p < 0.01$.

6.3 EFFECTS OF P2X7 RECEPTORS ON NEURAL PROGENITOR CELL PROLIFERATION

To determine if P2X7 receptors specifically have a role in regulating proliferation in hippocampal NPCs, assays using agonists and antagonists of P2X7 receptors were conducted. BzATP, which has a higher affinity for P2X7 than other receptors and is more potent an agonist than ATP, was applied at concentrations from 10 µM to 500 µM. A dose dependent decrease in proliferation was observed, [$F(7,143) = 130.12$, $p < 0.01$], see Figure 6.4 A. The P2X7 specific inhibitor A438079 was applied for four hours prior to ATP application, and competitively reduced ATP mediated decreases in proliferation, see Figure 6.4 B. Levene's test indicated an effect of variance [$F(5,108) = 3.90$, $p = 0.003$], and a Welch ANOVA [*Welch's* $F(5,108) = 85.50$, $p < 0.01$] indicated a significant effect of both 10 µM and 25 µM A438079 incubation. Games-Howell post hoc analysis indicated pre-treatment with 10 µM A438079 significantly reduced the inhibitory effects of ATP on proliferation by 7.14 ± 1.45% and by 16.15 ± 2.08% for 25 µM A438079.

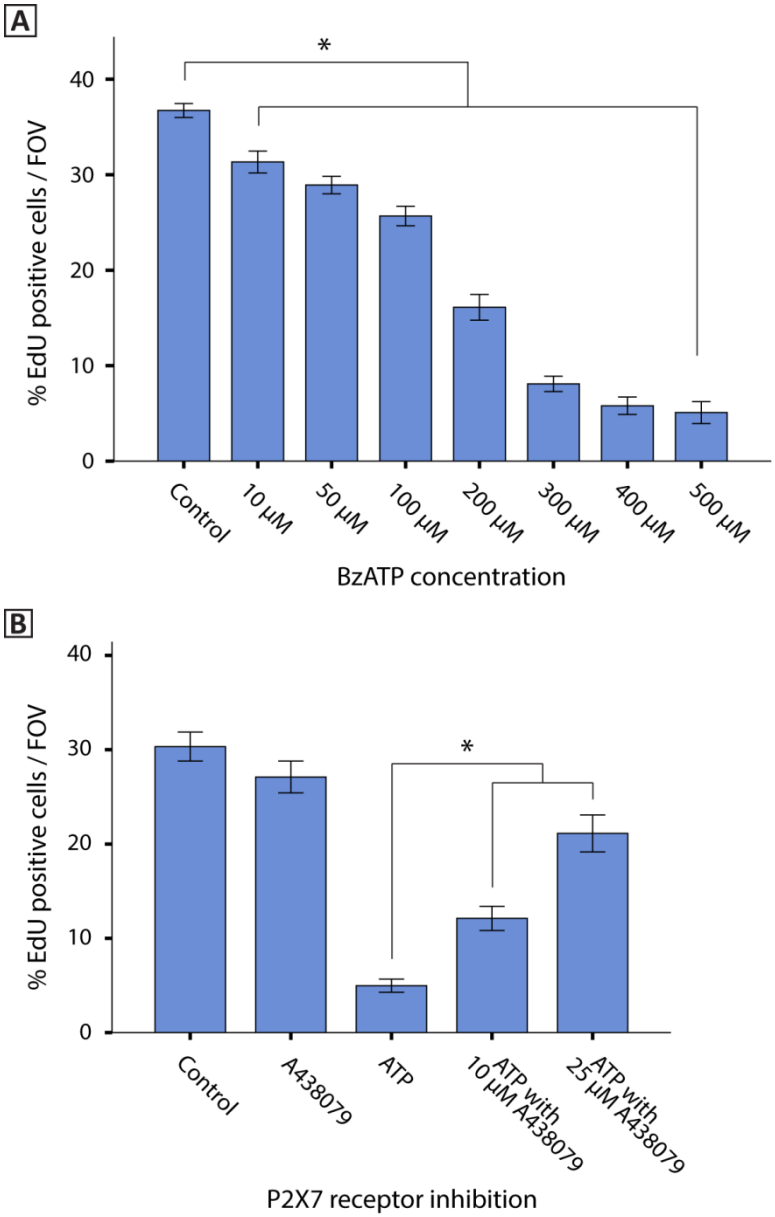

Figure 6.4 P2X7 receptor regulation of proliferation

Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips. BzATP was applied at concentrations from 10 µM to 500 µM overnight, followed by incubation with EdU. A dose dependent decrease in percentage of EdU positive cells per FOV was observed (A). Pre-incubation with P2X7 competitive inhibitor A438079 (10 µM and 25 µM) significantly reduced the inhibitory effects of 100 µM ATP (B). Error bars ± 1 SE, asterisk indicates $p < 0.01$.

6.3.1 Purinergic regulation of proliferation in cultures generated from P2X7 receptor knock out mice

To further confirm if P2X7 receptors were involved in regulating purinergic mediated decreases in proliferation, hippocampal NPC cultures were generated from Pfizer P2X7 knock out mice (Solle et al., 2001). Generally, cultures initiated from knock out mice required an extra two weeks before the first passage was completed. PCR confirmed the presence of the neomycin resistance gene used to induce the knock down, as well as the absence of P2X7 receptor gene, see Figure 6.5 A. Previous data obtained in our lab demonstrated that SVZ NPCs cultured from P2X7 knock out mice do not have functional P2X7 receptors as assessed by ethidium bromide uptake (Weible group, unpublished data, refer to Appendix 4).

Hippocampal NPC P2X7 knock out cultures were treated with increasing concentrations of ATP (from 10 µM to 500 µM) and compared to wild type cultures. The percentage of EdU positive cells per FOV was normalised to the control. Absence of P2X7 receptors in the knock out cultures significantly reduced the inhibitory effects of ATP on proliferation [*Welch's* $F(9,278) = 173.56$, $p < 0.01$], after Levene's test returned a significant effect of variance [$F(9,278) = 2.43$, $p = 0.011$]. This was evident at ATP concentrations 50 µM, 100 µM and 500 µM. A dose dependent decrease in proliferation was still observed, supporting the previous conclusion that other purinergic receptors can also have a role in regulating proliferation rates in hippocampal NPCs.

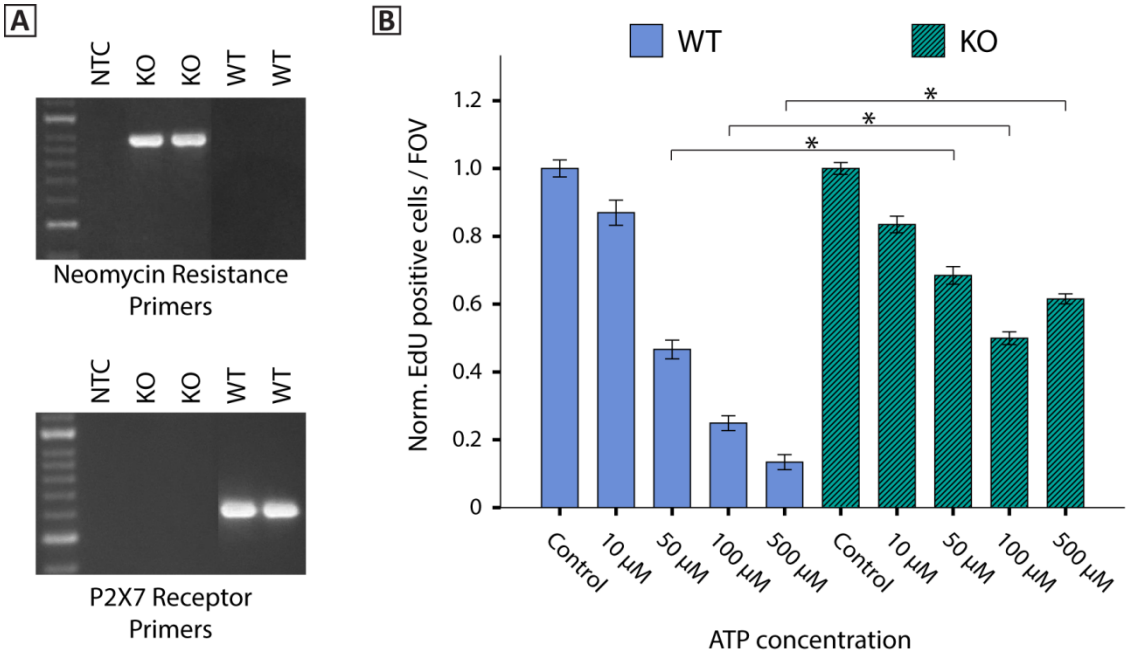

Figure 6.5 P2X7 knock out NPCs have a reduced response to ATP inhibition

Wild type and knock out NPC cultures were harvested and the DNA extracted for PCR. Primers specific for the neomycin resistance gene confirmed the presence of the neomycin insert in knock out cultures, while the P2X7 receptor gene was found to be absent, confirming knock down in hippocampal NPCs (A). Abbreviations: NTC, no template control; KO, knock out; WT, wild type. Both wild type and knock out NPCs were cultured on PLO and laminin coated glass coverslips and treated overnight with increasing concentrations of ATP. ATP had a significantly greater effect on the proliferation rates of wild type NPCs compared to cultures lacking P2X7 receptors (B, [*Welch's* $F(9,278) = 173.56$, $p < 0.01$]). A dose dependent decrease in proliferation was still observed in the knock out cultures, indicating activity of other purinergic receptors. Error bars ± 1 SE, asterisk indicates $p < 0.01$.

These data indicate that purinergic signalling decreases proliferation rates in hippocampal NPCs and that a portion of this occurs via P2X7 receptors. It is likely that other purinergic receptors also regulate proliferation rates, particularly in light of the observed decrease in proliferation in response to ADP, and the modest decrease that occurs in response to ATP in P2X7 knock out NPC cultures. There are a number of downstream cascades that may be active in translating the initial receptor activation to

cause a decrease in proliferation. P2X7 receptors primarily function as calcium channels, and P2X7 receptor signalling has been shown to activate both NFATs and NFκB (both calcium dependent transcription factors). Calcium is a powerful regulator of many cellular functions including proliferation and exerts its effects by subtle differences in concentration increases, which can occur as single spikes or repeating oscillations with varying frequency and amplitude. The following sections examine how calcium signalling and calcium dependent transcription factors may regulate the proliferation of hippocampal NPCs.

6.4 LINEAGE ELABORATION FOLLOWING PURINERGIC DECREASE IN PROLIFERATION

Lineage elaboration experiments were conducted to assess the possibility that hippocampal NPCs begin to differentiate following P2X7/purinergic receptor mediated decreases in proliferation. Cultures were treated with ATP for three days and immunocytochemistry was conducted to measure the change in DCX expression. The percentage of DCX positive cells per FOV was counted (n = 41 389 total cells counted). A small but significant increase in the number of DCX^+ neuroblasts generated following exposure to ATP was observed [$t(78) = 6.19$, $p < 0.01$], see Figure 6.6, suggesting an ATP induced decrease in proliferation may be accompanied by lineage elaboration in culture.

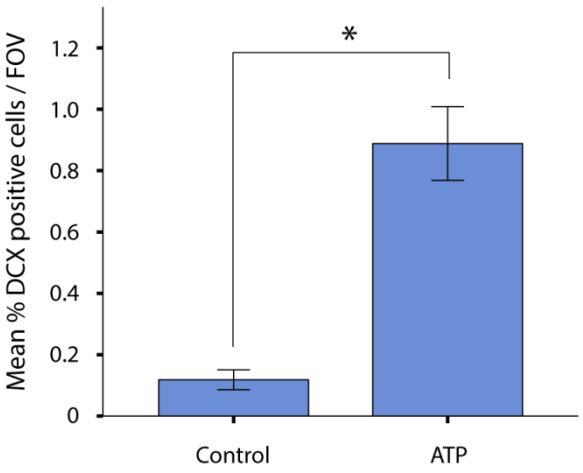

Figure 6.6 Increase in DCX expression following ATP application

Hippocampal NPC cultures were grown until approximately 40% confluent. ATP was applied at 100 µM for three consecutive days prior to fixation. The cells were stained for DCX and the percentage of DCX positive cells per FOV was counted (n = 41 389 total cells). A small but significant increase in the average number of DCX cells was observed. Error bars represent 1 SE, asterisk indicates significance at $p < 0.01$.

6.5 EVALUATION OF ATP STABILITY

ATP can hydrolyse to ADP rapidly given the correct conditions; for this reason ATP was made fresh for each experiment rather than frozen, and was applied to the cells within a few minutes of dissolving. The stability of 100 µM ATP in culture conditions was investigated using phosphorus NMR. Samples of ATP and ADP at 10 mM were used to establish reference peaks and then 100 µM samples were analysed. Three distinct peaks were observed for ATP, and two for ADP. A sample of ATP was spiked with ADP as a reference. No hydrolysis of ATP was observed after two hours, and the samples were stored at room temperature for three days before being re-analysed. After this time, still no hydrolysis was observed (see Figure 6.7), suggesting the ATP is stable in medium at the biologically relevant concentration for an appropriate amount of time.

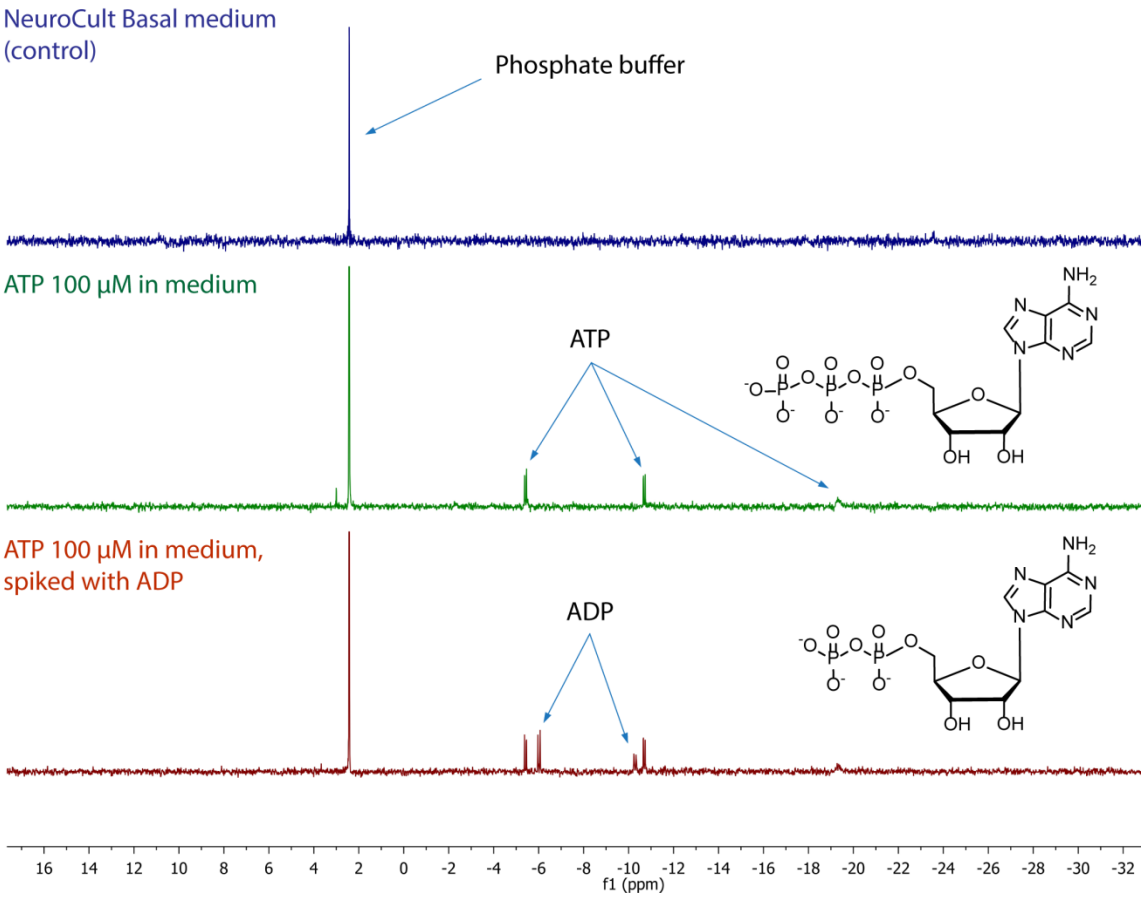

Figure 6.7 Phosphorus NMR spectra of ATP

Phosphorus NMR spectra for NeuroCult Basal medium identified a single peak corresponding to a phosphate buffer component. Spectra for ATP revealed three distinct peaks (labelled), while ATP spiked with ADP had two extra peaks and demonstrated the expected spectra if hydrolysis had been occurring. A minor unknown peak present in the ATP spectra was absent from both the 10 mM reference ATP sample (not shown) and the 100 µM ATP sample spiked with ADP. This is most likely a solvent contaminant and does not represent a breakdown of ATP.

6.6 PURINERGIC RECEPTORS SIGNAL VIA CALCIUM MODULATION

Intracellular calcium concentrations following purinergic receptor activation were profiled. Under resting conditions, hippocampal NPCs have infrequent spontaneous calcium spike or oscillation events, differing considerably to NPCs of the SVZ, which frequently oscillate spontaneously (Weible group, unpublished data). NPC cultures were loaded with Fluo-8 and imaged using a live cell microscope. Field of views were selected at random. Approximately 30-50 regions of interest were selected per field of view for analysis. Changes in fluorescence (F/F0) were plotted over time. Addition of ATP at the 10 second mark caused a large initial influx of calcium, see Figure 6.8, and is consistent with previous findings (refer to Figure 5.6). This cytosolic calcium is then rapidly transported either out of the cell or into internal stores, such as the ER (occurring around the 20 to 30 second mark). Following this, secondary calcium oscillations were observed (Figure 6.8, 35 second mark onwards). These apparent calcium-induced-calcium-release events are generally the result of calcium released from internal stores, most notably the endoplasmic reticulum.

Similar experiments were conducted by Weible group for ADP, UTP and UDP, see Figure 6.9, A, B and C respectively. Calcium influx was observed for all three, though not to the same extent as ATP and oscillating cells were rarely observed.

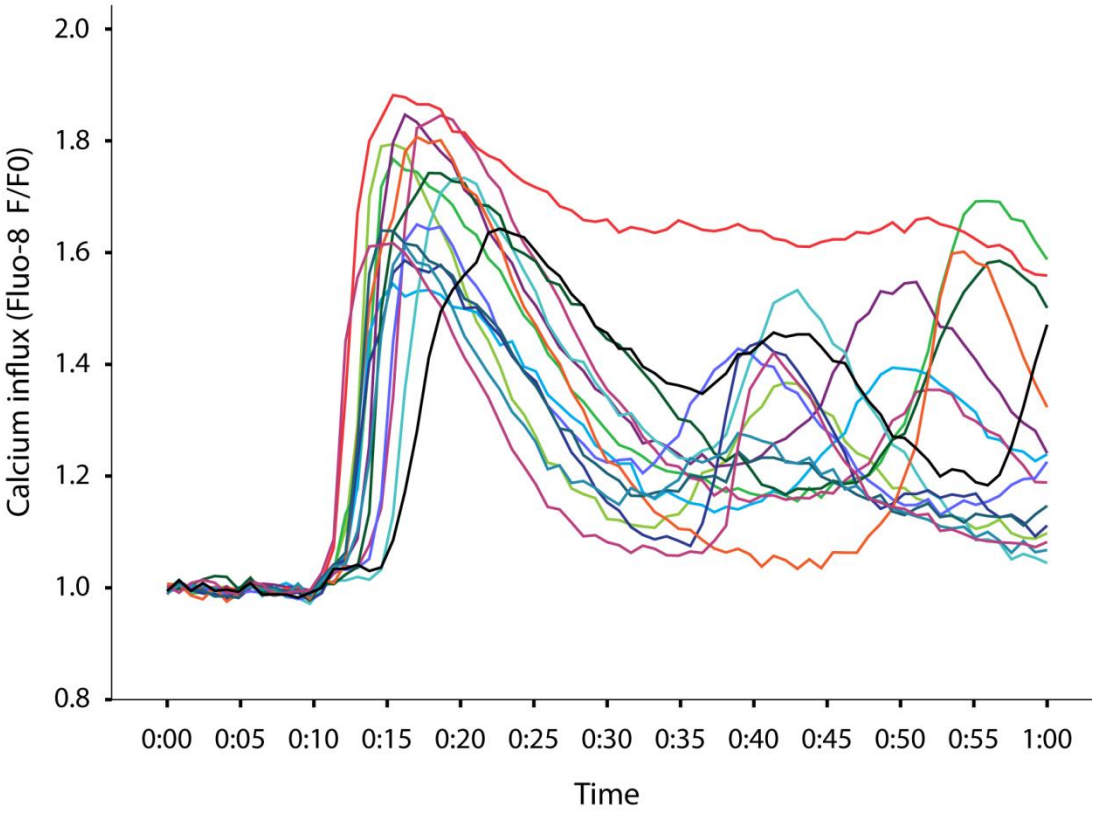

Figure 6.8 ATP causes calcium influx and oscillations in hippocampal NPCs

Hippocampal NPCs were cultured in a 35 mm glass bottomed dish coated with PLO and laminin. Cells were loaded with Fluo-8 and imaged in a live cell microscope over a one minute period. Random FOV were selected and the change in Fluo-8 fluorescence (F/F0) was recorded after application of 100 µM ATP at the 10 second mark. Initially, a large influx of calcium was observed (15 second mark), which was rapidly transported out of the cytoplasm (20 to 30 second mark). Following this initial influx event, cells began to oscillate as calcium was released from internal stores via calcium-induced-calcium-release activation of IP_3R on the ER.

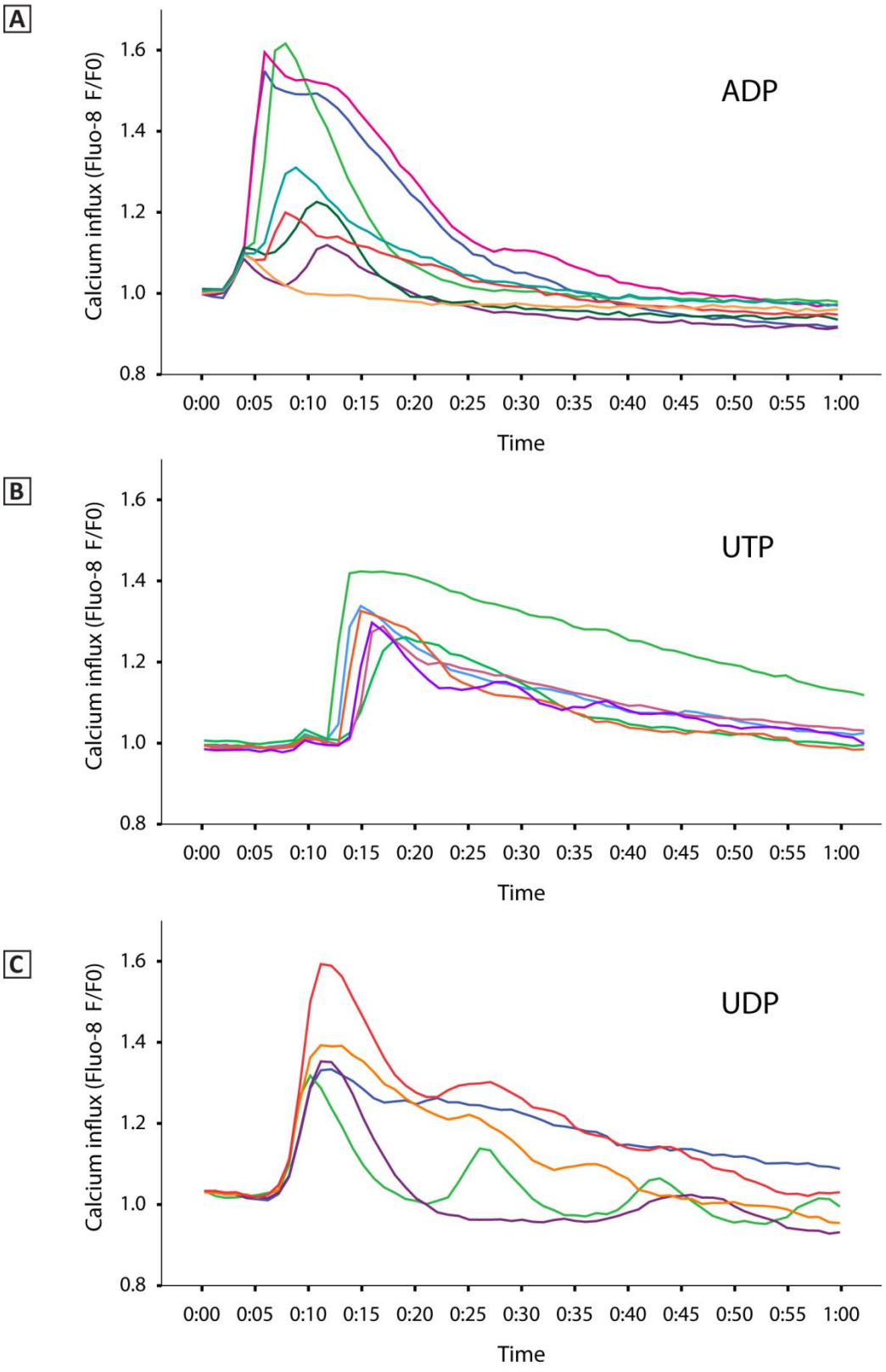

Figure 6.9 ADP, UTP and UDP causes calcium influx in hippocampal NPCs

Hippocampal NPCs were cultured in a 35 mm glass bottomed dish coated with PLO and laminin. Cells were loaded with Fluo-8 and imaged in a live cell microscope over

a one minute period. Random FOV were selected and the change in Fluo-8 fluorescence (F/F0) was recorded after 100 μM of ADP (A), UTP (B) and UDP (C). Initial calcium influx was observed, though at a lesser amplitude than the ATP induced calcium influx, and secondary oscillations were rarely observed.

6.7 CALCIUM DEPENDENT TRANSCRIPTION FACTOR SIGNALLING IN HIPPOCAMPAL NPCS

In an attempt to further elucidate the mechanisms downstream of P2X7 and purinergic receptor mediated proliferation decreases, calcium dependent transcription factors were investigated. P2X7 receptor activation has been demonstrated to result in nuclear localisation of NFATs and NFκB in microglial cultures (Ferrari et al., 1997, Ferrari et al., 1999). Translocation of NFAT to the nucleus is regulated by the frequency of calcium oscillations and is dependent on dephosphorylation by calcium-dependent phosphatase calcineurin (Tomida et al., 2003). Variations in calcium oscillation frequency and amplitude can also result in NFκB activation, via calcineurin activation (Smedler and Uhlen, 2014). It was hypothesised that purinergic signalling via P2X7 receptors could result in the precise calcium oscillation frequencies required to activate calcium dependent transcription factors.

Immunocytochemistry was initially conducted to determine if hippocampal NPCs expressed NFATs 1-4 and NFκB, and if so, to characterise the activation patterns under control conditions. A transcription factor activation assay utilising confocal microscopy was established and conducted to determine if purinergic signalling resulted in nuclear localisation of transcription factors. Inhibitors of the transcription factors were also assessed for possible roles in proliferation.

6.7.1 Purinergic regulation of NFκB in hippocampal NPCs

Immunocytochemistry against NFκB was conducted to determine expression patterns under control conditions. NFκB staining was punctate and present through the cytoplasm, while nuclear expression levels that varied between culture repeats. Expression of NFκB was generally low in the nucleus (Figure 6.10, A-C), though activation levels could approach equal distribution between nucleus and cytoplasm (as demonstrated in Figure 6.10, D-F). Some cultures were found to have no NFκB activation, with the nuclei being devoid of expression (Figure 6.10, G-I).

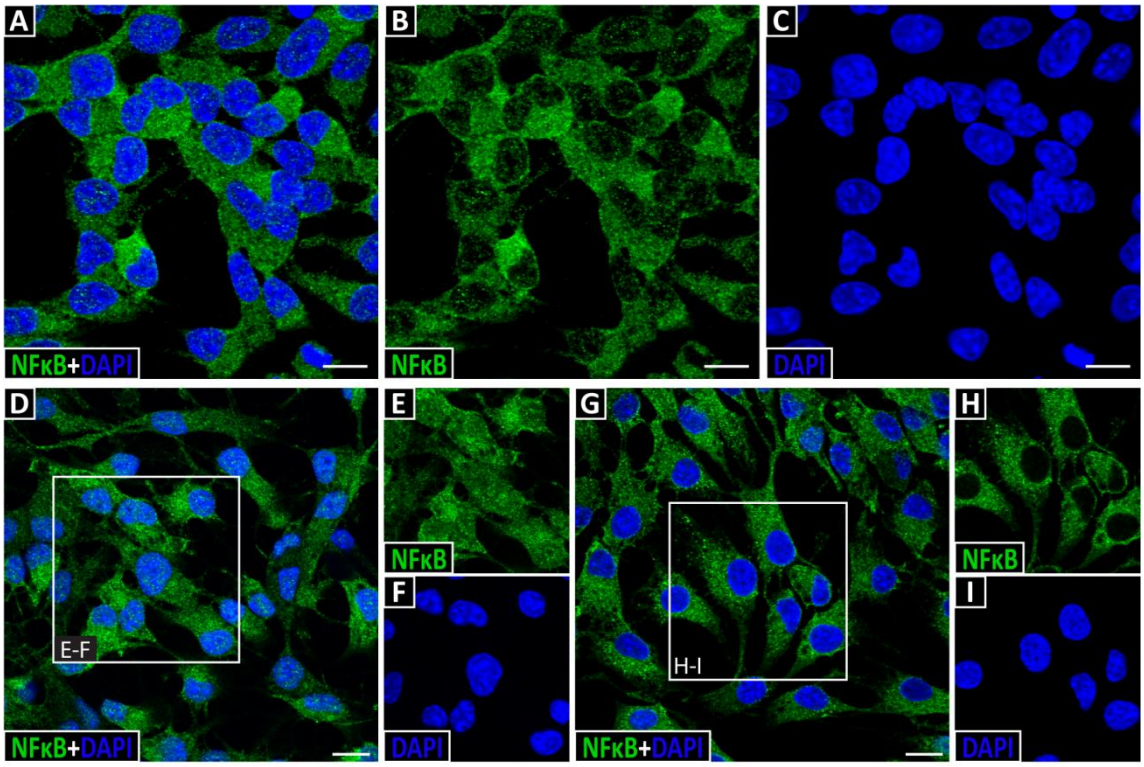

Figure 6.10 Characterisation of NFκB expression in hippocampal NPCs

Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips for 3 days prior to fixing with PFA. Immunocytochemistry was conducted against NFκB to characterise activation patterns under control conditions. Nuclear expression levels were found to vary between culture repeats; generally NFκB had punctate staining in the cytoplasm and low levels present in the nucleus (A-C). Nuclear NFκB expression levels could approach equal between cytoplasm and nucleus (D-F) or be absent from the nucleus (G-I). Nuclei were stained with DAPI. Scale bars represent 10 µm.

TNFα was used as a positive control to establish a transcription factor activation assay. NPCs were incubated with TNFα (10 ng/mL) for 30 minutes prior to fixation. Cultures were stained for NFκB and DAPI was used to mark the nucleus. Confocal microscopy was used to obtain images at a consistent exposure and Columbus image analysis software was used to calculate nuclear fluorescence intensity as an average per FOV. A significant increase in nuclear fluorescence was observed between control (Figure 6.11

A, B) and TNFα treated cells (Figure 6.11 C, D) as quantified by an independent t-test (Figure 6.11 E; [$t(28) = 5.54$, $p < 0.01$]).

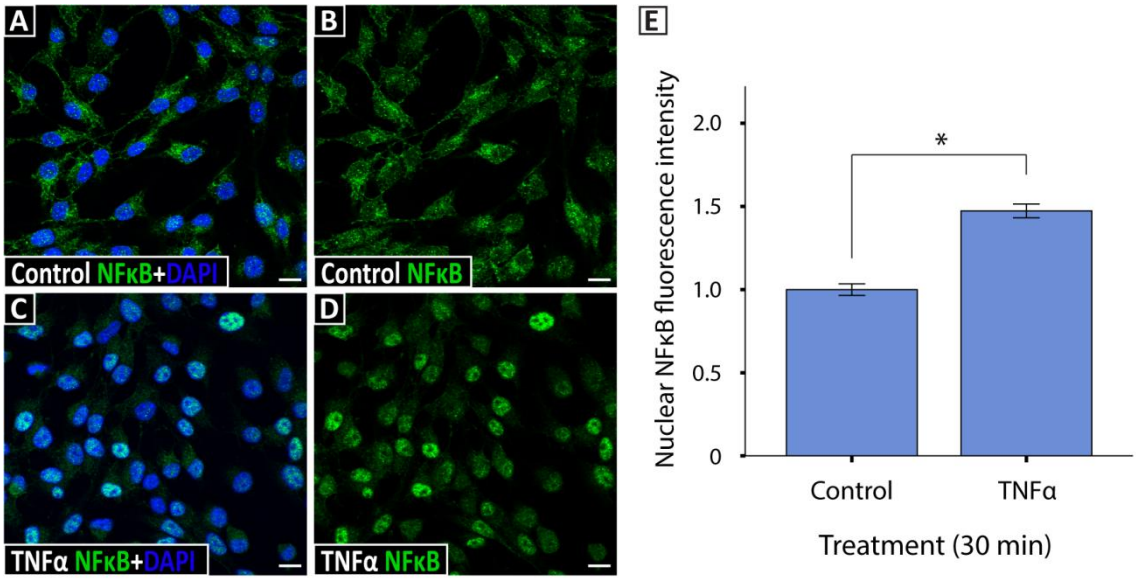

Figure 6.11 Transcription factor activation assay establishment

Hippocampal NPCs were cultured on PLO and laminin coated coverslips and incubated with TNFα (10 ng/mL) for 30 minutes prior to fixation. Control (A, B) and treated (C, D) cultures were stained for NFκB and DAPI was used to mark the nucleus. Scale bars represent 10 µm. Confocal microscopy was used to obtain images at a consistent exposure and Columbus image analysis software was used to calculate nuclear fluorescence intensity as an average per FOV. NFκB nuclear localisation is reported as an average fluorescence intensity normalised to control to calculate a relative increase in nuclear transcription factor (E). Error bars represent 1 SE, asterisk indicates significance at $p < 0.01$.

ATP was applied to hippocampal NPC cultures at 100 µM and 500 µM. Cells were fixed at 15, 30, 60 and 180 minutes after ATP application based on previously reported activation times (Ferrari et al., 1997). NFκB activation was calculated based on the mean nuclear fluorescence intensities. While an upward trend was observed, there was no significant differences between control and any time point at either concentration [*Welch's* $F(8,109) = 3.31$, $p > 0.05$].

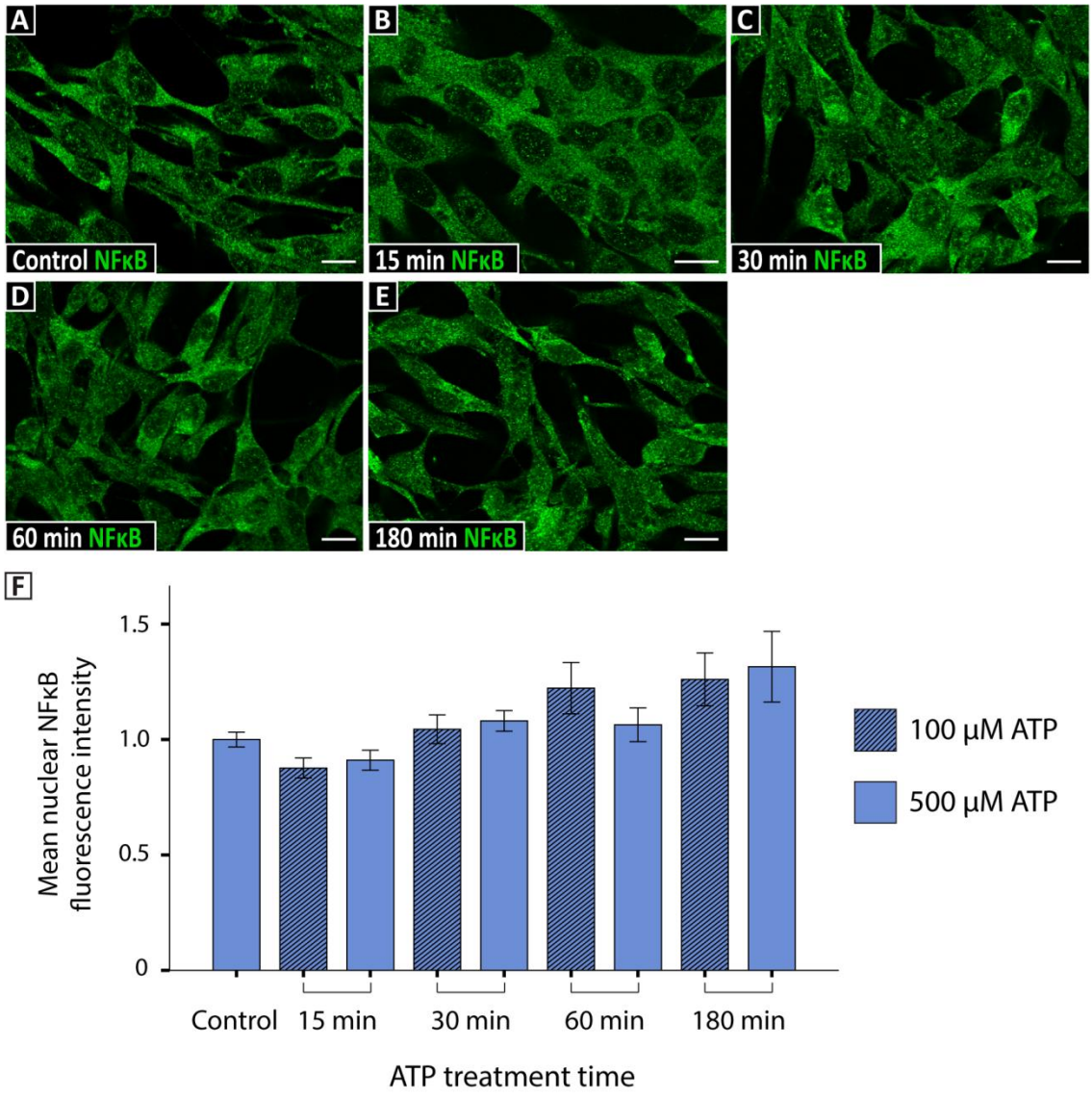

Figure 6.12 NFκB activation in response to ATP treatment

Hippocampal NPCs cultures were incubated with ATP (100 µM and 500 µM) for 15, 30, 60 and 180 minutes prior to fixation. Control (A) and treated (B-E) cultures were stained for NFκB and DAPI was used to stain the nucleus. Scale bars represent 10 µm. Nuclear (NFκB) fluorescence intensity is reported as an average per FOV normalised to the control (F). No significant increase in nuclear fluorescence intensity was detected. Error bars represent 1 SE.

6.7.2 Inhibition of NFκB in hippocampal NPCs

NFκB inhibitors BAY 11-7082 and PS-1145 were applied overnight followed by EdU application. The percentage of EdU positive cells per FOV was calculated. Neither BAY 11-7082 [$F(3,60) = 2.27$, $p = 0.090$] nor PS-1145 [$F(3,68) = 1.96$, $p = 0.128$] showed significant differences between control and treatments after two biological repeats, see Figure 6.13 A and B respectively. Preliminary experiments also showed no change in proliferation for TNFα (data not shown).

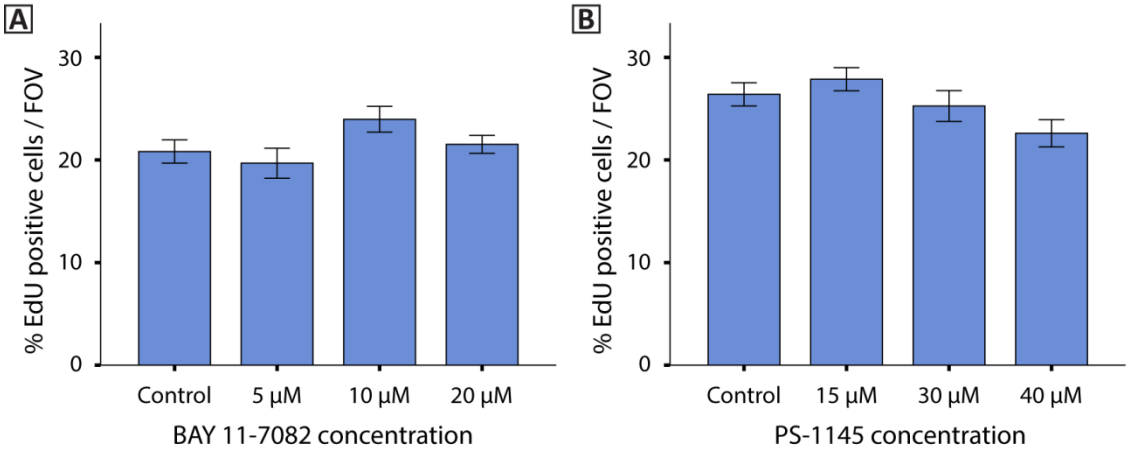

Figure 6.13 Effects of NFκB inhibition on hippocampal NPC proliferation
Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips. NFκB inhibitors BAY 11-7082 (A) and PS-1145 (B) were applied overnight. Cultures were incubated with EdU for four hours, fixed, and click chemistry conducted. Neither inhibitor caused a significant decrease in proliferation. Error bars ± 1 SE.

6.7.3 Purinergic regulation of NFATS in hippocampal NPCs

Immunocytochemistry against NFATS1-4 was conducted to determine expression patterns under control conditions. NFAT1 was observed to have diffuse punctate staining present through the cytoplasm, with low levels detected in the nucleus (Figure 6.14 A-C). NFAT2 also demonstrated punctate staining, with a concentration of

fluorescence present adjacent to the nucleus, and slightly higher intensities in the nucleus than the cytosol (Figure 6.14 D-F). NFAT3 was almost entirely restricted to the nucleus, indicating an activated state (Figure 6.14 G-I). NFAT4 was cytoplasmic, with staining occurring most intensely in the cell body, and low levels in the nucleus (Figure 6.14 J-L).

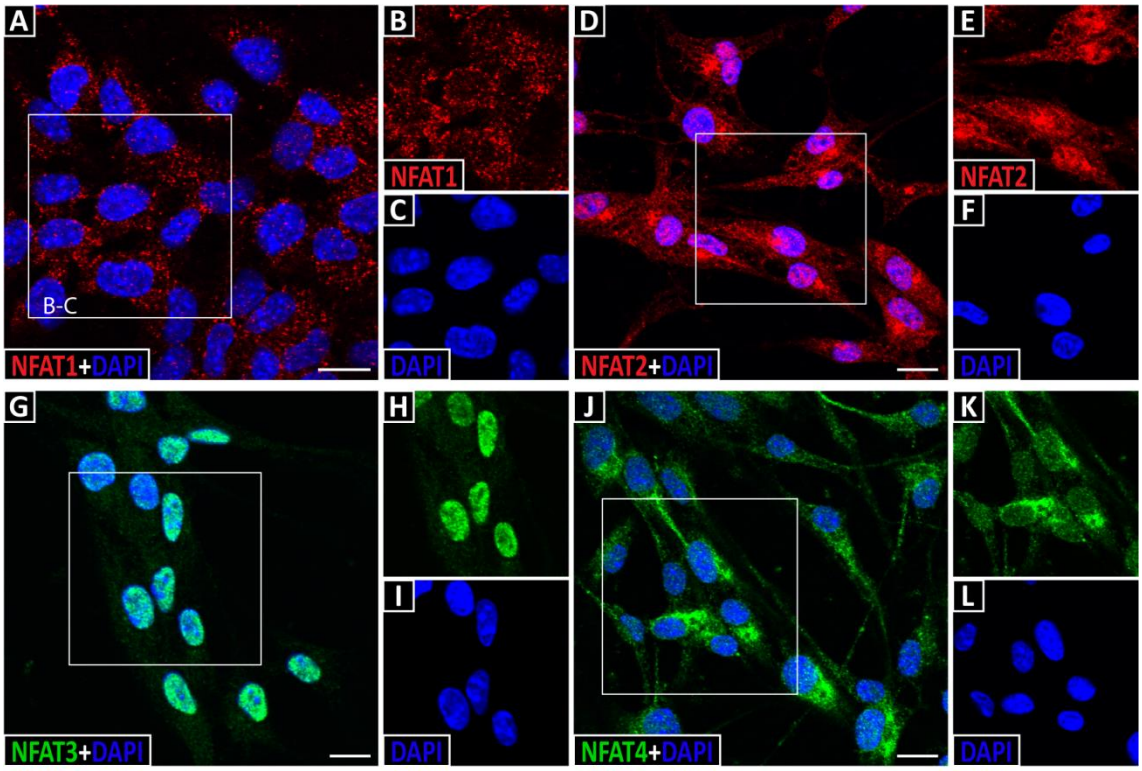

Figure 6.14 Characterisation of NFAT expression in hippocampal NPCs
Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips for 3 days prior to fixing with PFA. Immunocytochemistry was conducted against NFATS 1-4 to characterise activation patterns under control conditions. NFAT1 had diffuse punctate staining present in the cytoplasm with low levels detected in the nucleus (A-C). NFAT2 also demonstrated punctate staining with higher intensities in the nucleus than the cytosol (D-F). NFAT3 was almost entirely restricted to the nucleus (G-I). NFAT4 was cytoplasmic, with low levels in the nucleus (J-L). Nuclei were stained with DAPI. Scale bars represent 10 µm.

Possible purinergic activation of NFAT1 was assessed at multiple time points as previously described by Ferrari et al. (1999). ATP was applied to hippocampal NPC cultures at 100 µM and 500 µM. Cells were fixed at 15, 30, 60 and 180 minutes after ATP application, and NFAT activation was calculated based on the mean nuclear fluorescence intensities. Two biological replicates were conducted, and no significant differences between control and any time point at either concentration were found [*Welch's* $F(8,62) = 3.83$, $p > 0.05$]. NFATS 2 and 4 were eliminated from experiments due to poor preliminary results and lack of previously published evidence supporting the notion, and NFAT3 was omitted due to its constitutive activation.

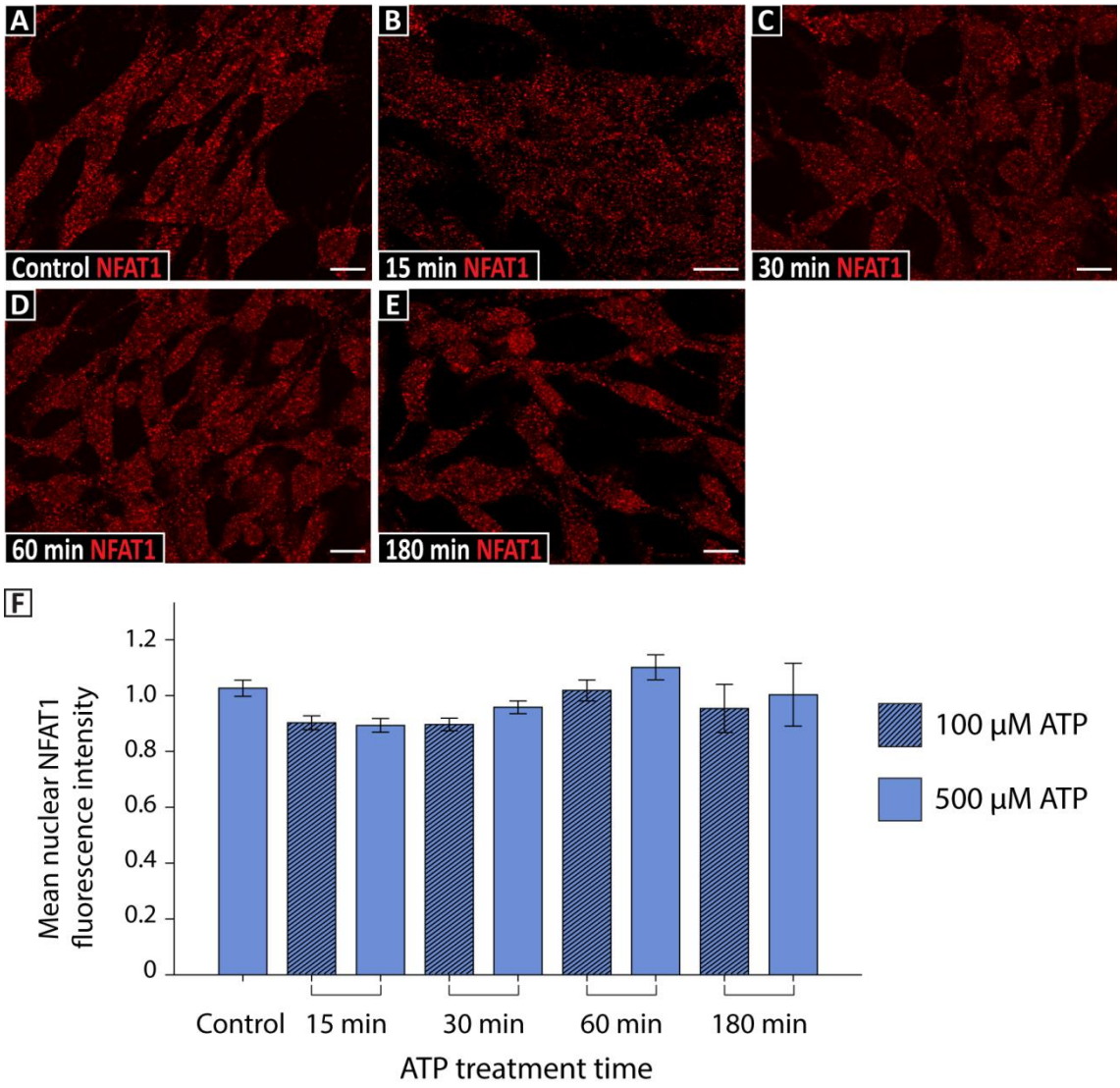

Figure 6.15 NFAT activation in response to ATP treatment

Hippocampal NPCs cultures were incubated with ATP (100 µM and 500 µM) for 15, 30, 60 and 180 minutes prior to fixation. Control (A) and treated (B-E) cultures were stained for NFAT1 and DAPI was used to stain the nucleus. Scale bars represent 10 µm. Nuclear (NFAT1) fluorescence intensity is reported as an average per FOV normalised to the control (F). No significant increase in nuclear fluorescence intensity was detected. Error bars represent 1 SE.

6.7.4 Inhibition of NFATS in hippocampal NPCs

NFAT inhibitors Cyclosporin A (which acts indirectly through inhibition of calcineurin) and the direct acting NFAT Inhibitor (VIVIT peptide) were incubated with hippocampal NPC cultures overnight, followed by EdU application. The percentage of EdU positive cells per FOV was calculated. Cyclosporin A caused a significant reduction in proliferation at 10 µM [*Welch's* $F(3,92) = 308.44$, $p < 0.01$], with proliferation decreasing by 25.65 ± 0.84% (Figure 6.16 A). Two biological repeats were conducted. Application of NFAT Inhibitor did not cause a decrease in proliferation when assayed up to 50 µM [$F(5,108) = 1.68$, $p = 0.146$], see Figure 6.16 B.

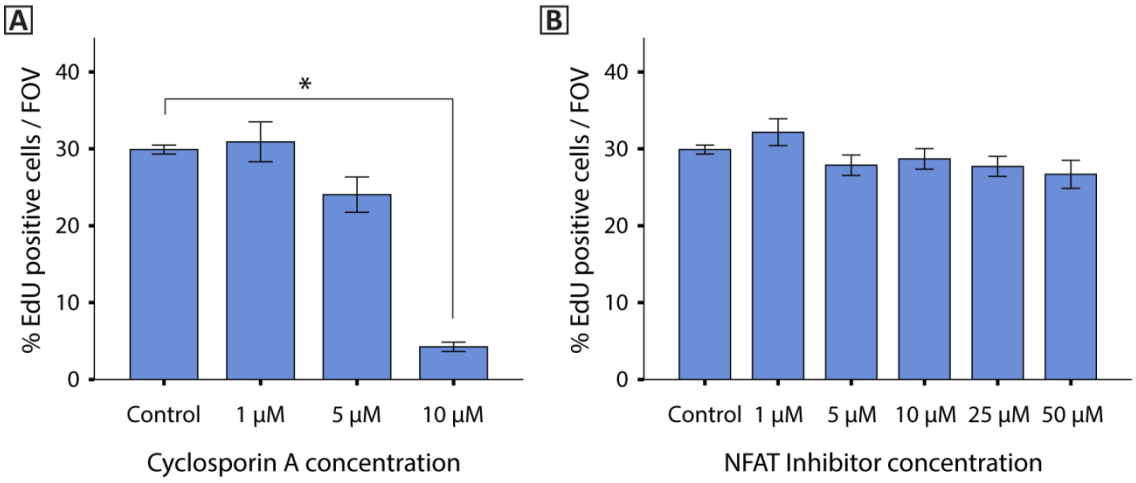

Figure 6.16 Effects of NFAT inhibition on hippocampal NPC proliferation
Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips. NFAT inhibitors Cyclosporin A and NFAT Inhibitor (VIVIT peptide) were applied overnight. Cultures were incubated with EdU for four hours, fixed, and click chemistry conducted. Cyclosporin A (A) caused a significant decrease in proliferation at 10 µM. NFAT Inhibitor (B) did not cause a decrease in proliferation. Error bars ± 1 SE, asterisk indicates $p < 0.01$.

Neither NFκB nor NFAT1 were observed to translocate to the nucleus following purinergic stimulation and application of inhibitors did not increase or decrease proliferation rates, with the exception of Cyclosporin A. Cyclosporin A is an inhibitor of calcineurin, and thus may be having off target effects. Failure to detect nuclear translocation may be due to experimental design, and is discussed further in Section 6.11. The calcium oscillations observed in these cells in response to ATP suggest that some manner of regulation at the ER level may be playing a role in purinergic decreases in proliferation. The possible roles of proteins of the endoplasmic reticulum were next examined.

6.8 EFFECTS OF ENDOPLASMIC RETICULUM FUNCTION ON PROLIFERATION

Previous work in our lab demonstrated that inhibition of proteins essential for ER functioning, such as the IP_3R receptors, significantly decreased proliferation in cells of the SVZ, and were also demonstrated to inhibit calcium oscillations in the frequently oscillating SVZ cultures (Toppinen, 2016). The effects of these inhibitors were tested in hippocampal NPCs to determine if modulating the function of various ER receptors and proteins affected proliferation rates. Numerous proteins on the ER regulate calcium storage, some of the most important being IP_3R receptors, which facilitate the release of calcium from the ER. Expression patterns of IP_3Rs were explored, as were the effects of IP_3R inhibition.

Immunocytochemistry was conducted to characterise the expression of IP_3R isotypes 1, 2 and 3 in hippocampal NPCs (Figure 6.17, A, with insert B and DAPI C). Distinct staining patterns were observed for each isotype. IP_3R_1 was localised specifically to the

nucleus (Figure 6.17 D). IP_3R_2 displayed punctate staining through the endo- and nucleoplasmic reticulum (Figure 6.17 E), while IP_3R_3 had relatively low expression levels distributed through the cytosol, with a small amount present in the nucleus (Figure 6.17 F). IP_3R_2 and IP_3R_3 were not observed to co-localise.

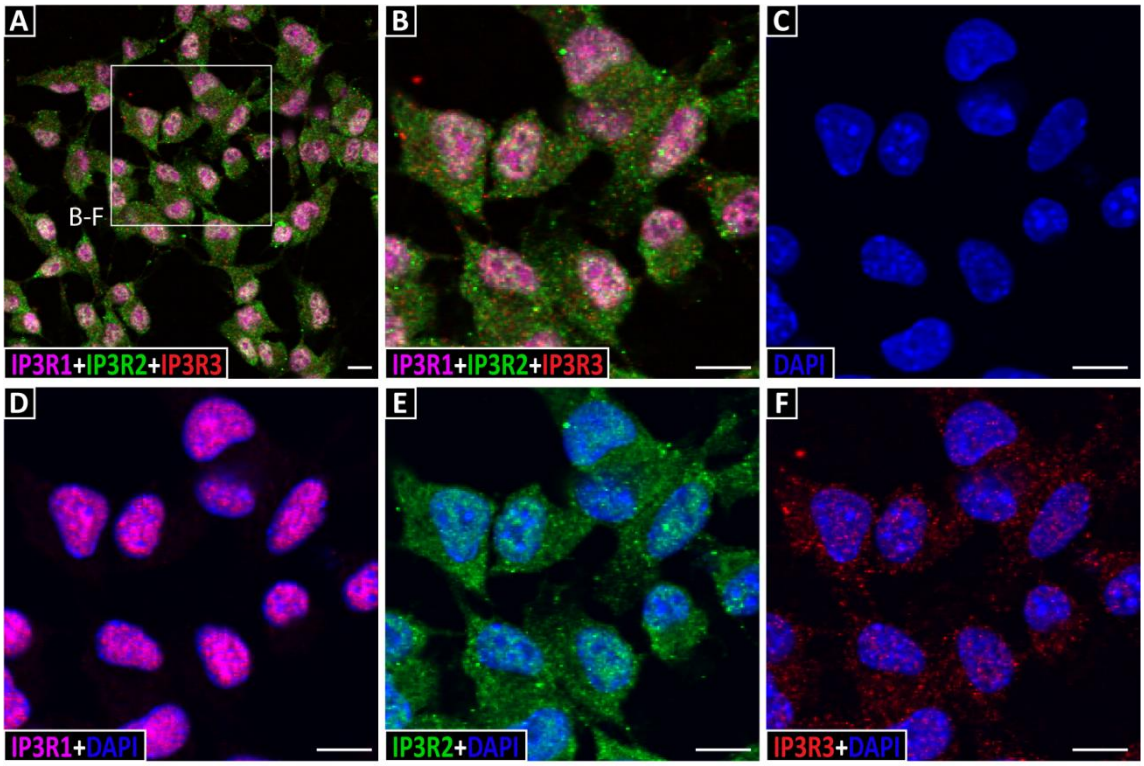

Figure 6.17 IP_3R characterisation in hippocampal NPCs

Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips for 3 days. Immunocytochemistry was conducted against IP_3R isotypes 1, 2 and 3 (A, with insert B and DAPI C). IP_3R_1 was localised to the nucleus (D). IP_3R_2 (E) and IP_3R_3 (F) displayed punctate staining through the endo- and nucleoplasmic reticulum, though did not co-localise. IP_3R_2 had higher expression levels than IP_3R_3. Nuclei were stained with DAPI. Scale bars represent 10 µm.

IP_3R inhibitors 2-APB (2-aminoethoxydiphenyl borate) and Xestospongin C were applied to hippocampal NPC cultures overnight. EdU was applied for four hours prior to fixation with PFA. Click chemistry was conducted to label dividing cells, and all cells were stained with DAPI. Random FOV were selected using the DAPI channel, images were captured and the percentage of EdU positive cells per FOV was calculated. Increasing concentrations of 2-APB decreased the proliferation in a dose dependent manner, [*Welch's* $F(3,80) = 242.67$, $p < 0.01$], see Figure 6.18 A. Xestospongin C also resulted in decreased proliferation, [$F(3,32) = 23.99$, $p < 0.01$], see Figure 6.18 B. Both 2-APB and Xestospongin C were not as potent at reducing proliferation in hippocampal NPCs when compared to previous experiments conducted in SVZ cultures, where an EC50 of approximately 15 µM was observed for 2-APB and 0.8 µM for Xestospongin C. Data is only available for one biological repeat for Xestospongin C.

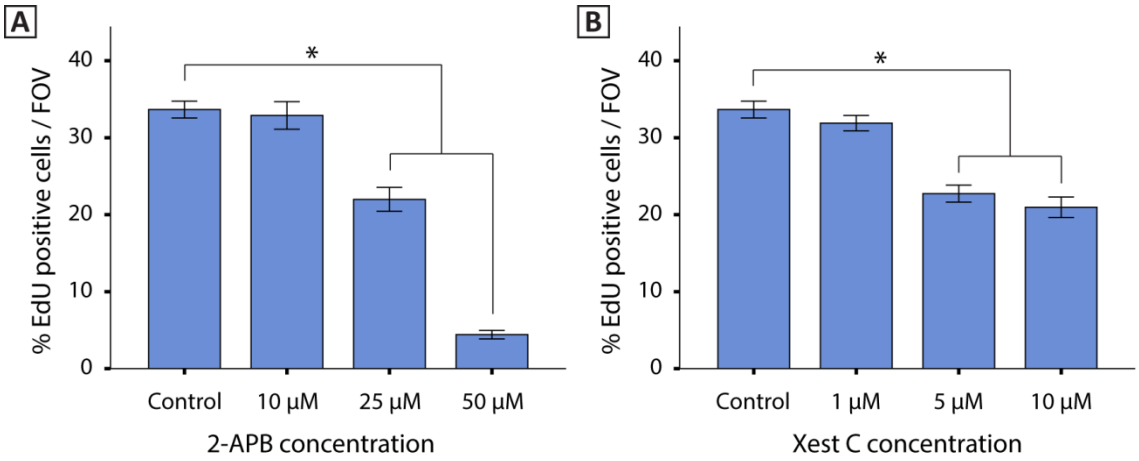

Figure 6.18 IP_3R inhibition decreases proliferation in hippocampal NPCs
Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips. IP3R inhibitors 2-APB and Xestospongin C (Xest C) were applied overnight, followed by incubation with EdU. A dose dependent decrease in percentage of EdU positive cells per FOV was observed for 2-APB at concentrations of 25 µM and 50 µM (A). Xestospongin C caused a decrease in proliferation at 5 µM and 10 µM (B). Error bars ± 1 SE, asterisk indicates $p < 0.01$.

Orai1 and STIM1 proteins also play an important role in regulating ER calcium levels; by sensing calcium depletions in the ER and coupling to activate calcium release activated calcium (CRAC) channels, they can replenish ER calcium levels. Immunocytochemistry was conducted to determine if Orai1 and STIM1 are present in hippocampal NPCs, see Figure 6.19, A-D. Punctate staining was observed for both Orai1 and STIM1, with Orai1 showing a distinct concentration of expression adjacent to the nucleus, refer to Figure 6.19 B. Interestingly, this was not observed with a second Orai1 antibody raised in rabbit against a different epitope (Figure 6.19 E-H).

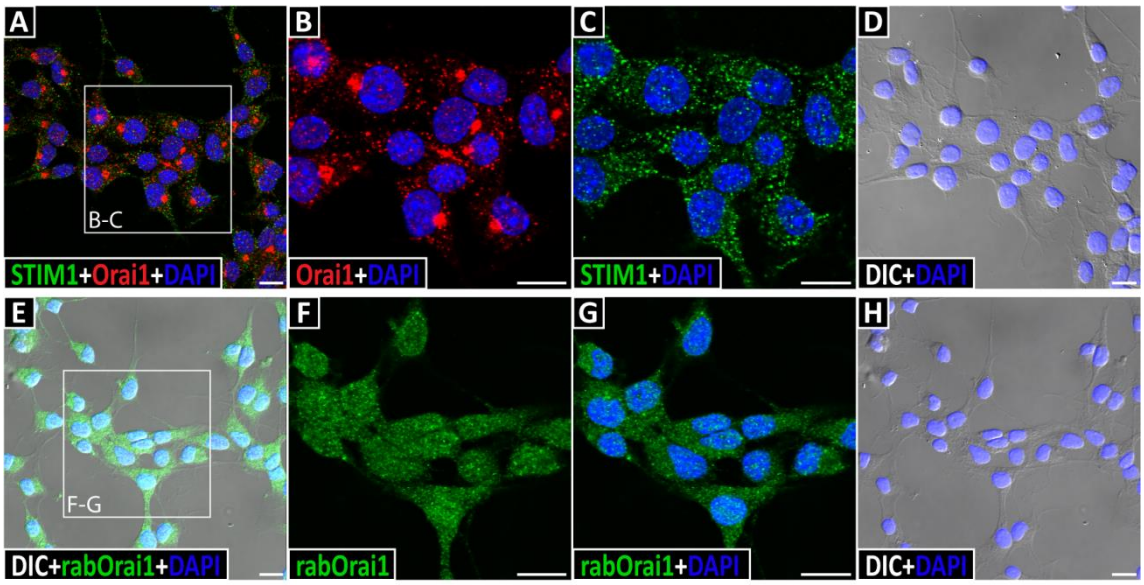

Figure 6.19 Orai1 and STIM1 characterisation in hippocampal NPCs

Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips for 3 days. Immunocytochemistry was conducted against STIM1 and Orai1 (A-D) with punctate staining patterns observed for both. Differences in staining patterns were observed between a mouse anti Orai1 (A, B) and rabbit anti Orai1 (E-H), which are raised against different epitopes. Nuclei were stained with DAPI. Scale bars represent 10 µm.

Orai1 inhibitors AnCoA4 and Cpd5J-4, and STIM1 inhibitors ML 9 and SKF 96365, were incubated with hippocampal NPC cultures overnight, followed by EdU application. The percentage of EdU positive cells per FOV was calculated. Increasing concentrations of AnCoA4 (Figure 6.20 A) decreased proliferation in a dose dependent manner, and significantly from 10 µM to 30 µM [*Welch's* $F(4,91) = 641.03$, $p < 0.01$], as did Cpd5J-4 from 100 µM to 500 µM [*Welch's* $F(5,90) = 35.37$, $p < 0.01$], see Figure 6.20 B. At concentrations above 30 µM, AnCoA4 caused complete cell cycle arrest, though cell morphology was unchanged and cell death was not apparent. Cpd5J-4 was not soluble as concentrations above 500 µM. Similar results were observed for STIM1 inhibitors. ML9 significantly decreased proliferation levels from 1 µM, [*Welch's* $F(3,91) = 48.25$, $p < 0.01$], refer to Figure 6.20 C. Application of SKF 96365 (Figure 6.20 D) decreased proliferation significantly at 5 µM and 10 µM [*Welch's* $F(3,80) = 64.18$, $p < 0.01$].

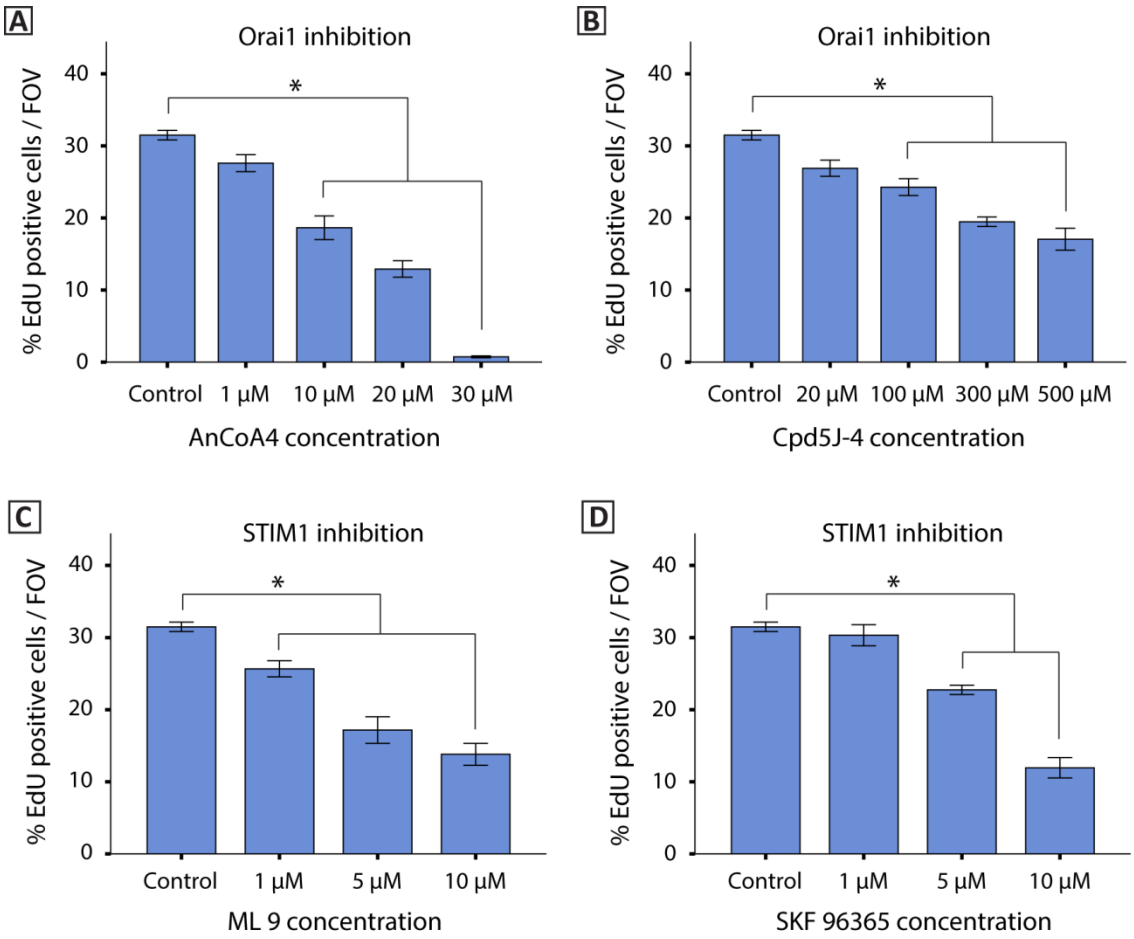

Figure 6.20 Inhibition of Orai1 and STIM1 decreases proliferation

Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips. Orai1 inhibitors AnCoA4 (A) and Cpd5J-4 (B), and STIM1 inhibitors ML9 (C) and SKF 96365 (D) were applied overnight. Cultures were incubated with EdU for four hours, fixed, and click chemistry conducted. A dose dependent decrease in percentage of EdU positive cells per FOV was observed for all Orai1 and STIM1 inhibitors. Error bars ± 1 SE, asterisk indicates $p < 0.01$.

Inhibition of IP_3R, Orai1 and STIM1 all resulted in a decrease in proliferation, indicating that ER regulation of internal calcium concentrations is vital for cell cycle progression. Given that ATP induced profound calcium oscillation activity, it is possible that the decrease in proliferation in response to purinergic signalling is due to some unknown modulation of ER function. Regulation of the ER and cellular calcium concentrations can occur through various pathways (P2X, P2Y, RTK and CRAC channels to name a few), and it is possible that purinergic activing in conjunction with other signalling pathways is responsible for the decrease in proliferation. For this reason RTK signalling was examined.

6.9 GROWTH FACTOR REGULATION OF PROLIFERATION

Receptor tyrosine kinases may also regulate calcium oscillations via PLCγ IP_3R activity. Given that no calcium dependent transcription factor activation was observed in response to purinergic signalling, the effects of EGF and bFGF were investigated, as these are present in the culture medium and exert powerful regulation over the cells. Previous data from our lab found that EGF withdrawal could modulate calcium oscillations in SVZ cultures (Toppinen, 2016). It is possible that P2X7/purinergic signalling may be combining with RTK signalling cascades to cause a decrease in proliferation. The effects of EGF and bFGF were investigated to gain an insight into the signalling mechanisms that may be present in culture.

Immunocytochemistry against EGFR and FGFR2 was conducted to determine expression patterns (Figure 6.21, A-F). FGFR2 demonstrated a concentration of staining in the cell body and was present through the processes (Figure 6.21 D). EGFR staining was punctate and dispersed evenly across the cell membrane (Figure 6.21 E).

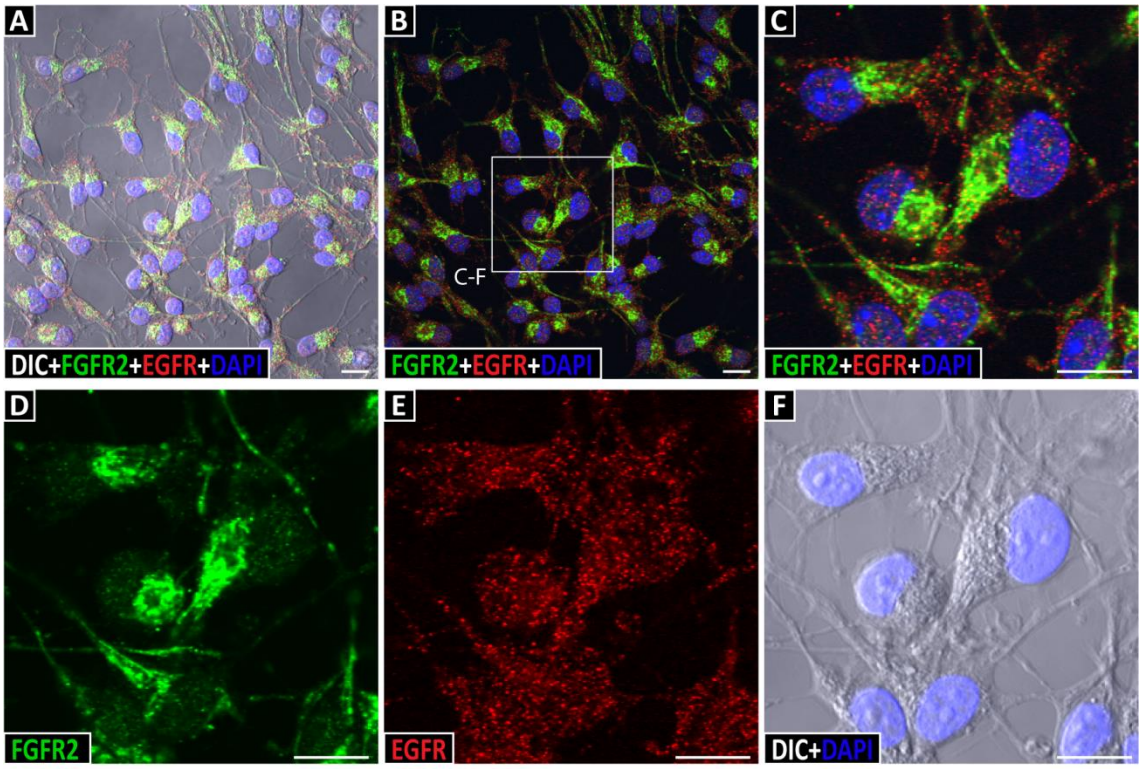

Figure 6.21 Growth factor receptor characterisation

Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips for 3 days. Immunocytochemistry was conducted against FGFR2 (A, B, and insert C, D) and EGFR (A, B, and insert C, E). DAPI and DIC are shown in F. FGFR2 demonstrated concentrated staining in the cell body and extended to the processes, while EGFR staining was punctate and dispersed evenly across the cell membrane. Nuclei were stained with DAPI, scale bars represent 10 µm.

Effects of growth factor signalling on proliferation rates were assessed by overnight growth factor withdrawal and by application of EGFR inhibitor PD 158780. Proliferation was significantly reduced by 7.30 ± 1.89% for bFGF withdrawal, 16.46 ± 1.87% for EGF withdrawal, and withdrawal of both EGF and bFGF caused a 20.47 ± 2.14% reduction, see Figure 6.22 A, [$F(3,77) = 41.20$, $p < 0.01$]. Interestingly, EGFR inhibitor PD 158780 did not affect proliferation rates, see Figure 6.22 B, [*Welch's* $F(5,102) = 2.42$, $p = 0.070$].

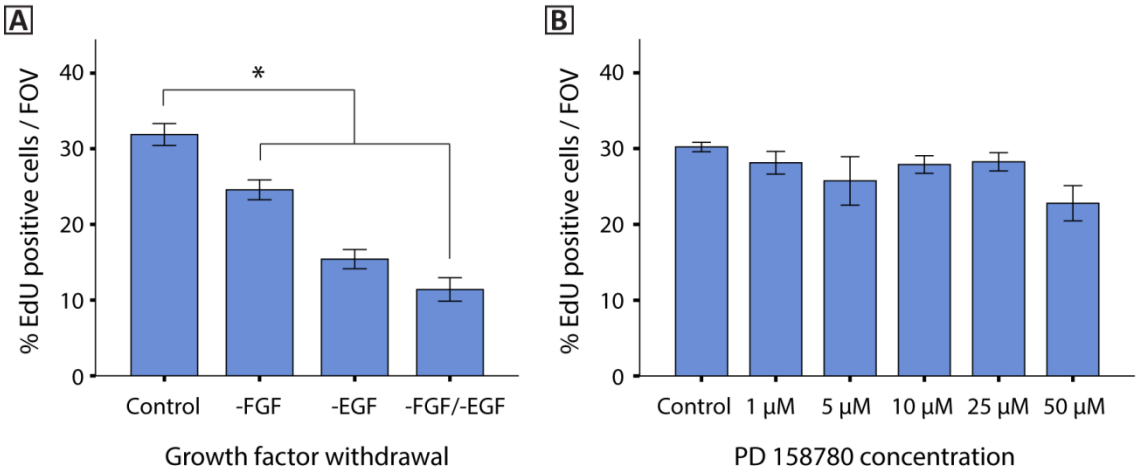

Figure 6.22 Growth factor regulation of hippocampal NPC proliferation

Hippocampal NPCs were cultured on PLO and laminin coated glass coverslips. Growth factors were withdrawn overnight (A) prior to EdU incubation. EGF inhibitor PD 158780 (B) was applied to hippocampal NPCs overnight prior to EdU incubation. Cultures were fixed and click chemistry was conducted, DAPI was used as a counterstain. A decrease in percentage of EdU positive cells per FOV was observed for bFGF withdrawal, EGF withdrawal and bFGF/EGF withdrawal. No change in proliferation was observed following PD 158780 treatment. Error bars ± 1 SE, asterisk indicates $p < 0.01$.

6.10 TRANSCRIPTION FACTOR ACTIVATION IN RESPONSE TO GROWTH FACTOR WITHDRAWAL

Activation of transcription factors in response to growth factor withdrawal was assessed. EGF and bFGF were removed for 18 hours prior to fixation, and NFκB and NFAT1 activation was calculated based on the mean nuclear fluorescence intensities per FOV. No significant differences in the nuclear localisation of NFκB were observed between the control (depicted in Figure 6.23 A, D) and bFGF withdrawal (Figure 6.23 B, E) or EGF withdrawal (Figure 6.23 C, F), see Figure 6.23 G [*Welch's* $F(3,43) = 4.32$, $p > 0.05$],. Conversely, NFAT1 (Figure 6.24 A, D) was observed to translocate to the

nucleus following both bFGF (Figure 6.24 B, E) and EGF (Figure 6.24 C, F) withdrawal [*Welch's F*(3,50) = 18.44, $p < .0.01$], see Figure 6.24 G.

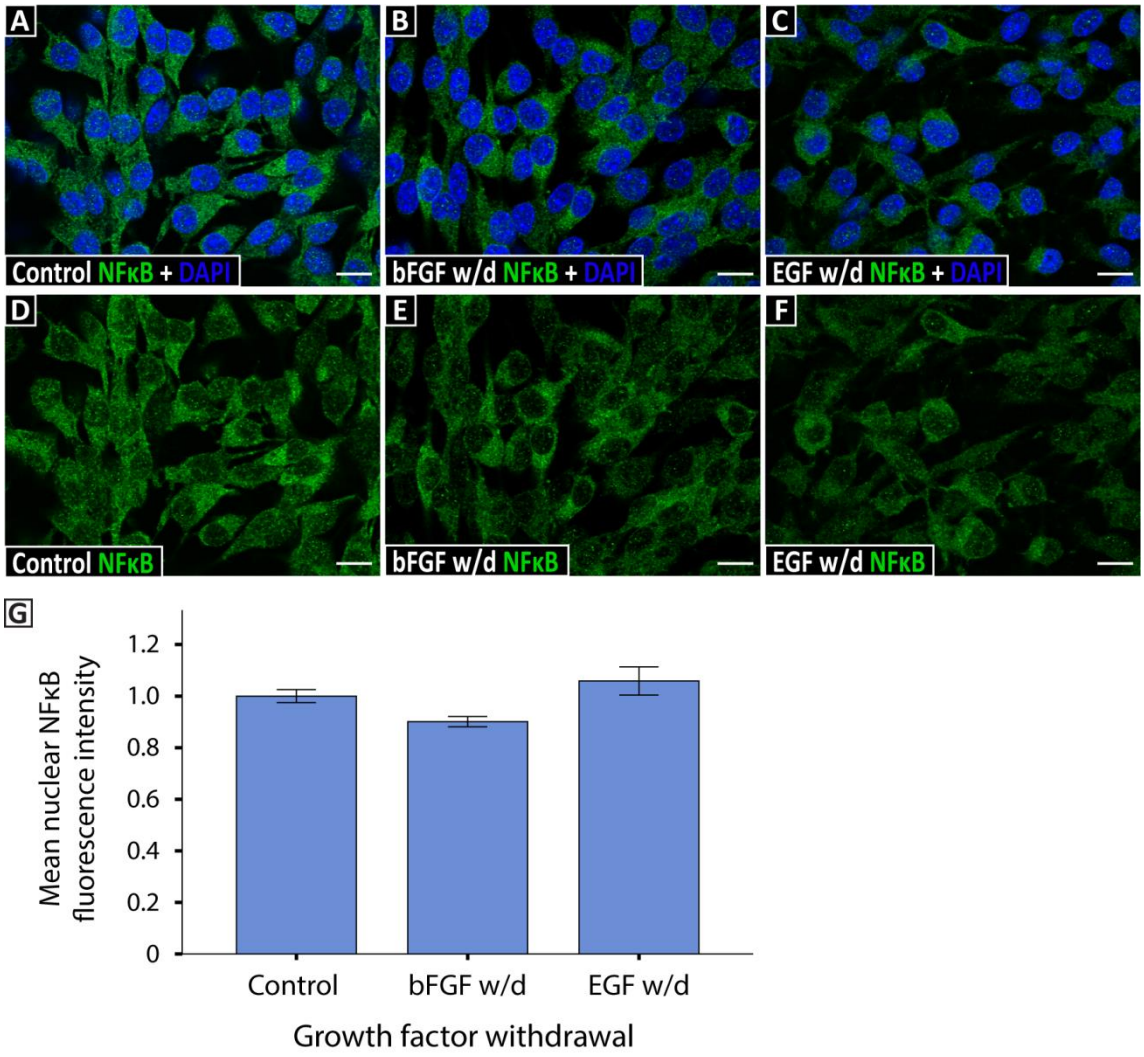

Figure 6.23 Growth factor withdrawal on NFκB activation

Hippocampal NPCs cultures were cultured for three days before growth factor withdrawal. Cells were left overnight under withdrawal conditions prior to fixing. Cells were stained for NFκB and the nuclei were stained with DAPI. Mean nuclear fluorescence intensity per FOV was calculated for control (A, D), bFGF withdrawal (B, E) and EGF withdrawal (C, F). No significant increase in nuclear fluorescence intensity was detected (G). Abbreviation: w/d, withdrawal; scale bars represent 10 μm; error bars represent 1 SE.

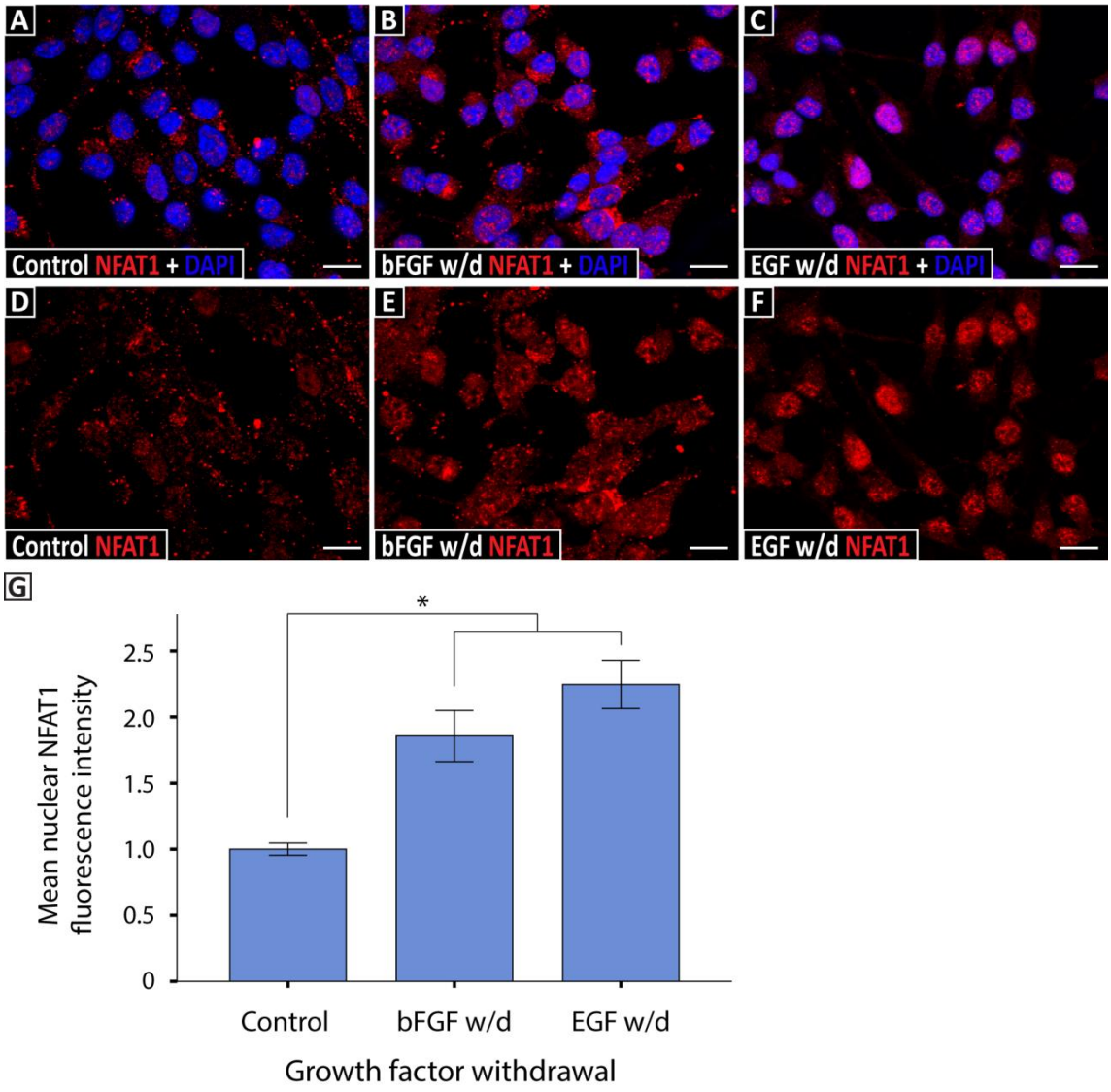

Figure 6.24 Growth factor withdrawal on NFAT1 activation

Hippocampal NPCs cultures were cultured for three days before growth factor withdrawal. Cells were left overnight under withdrawal conditions prior to fixing. Cells were stained for NFAT1 and the nuclei were stained with DAPI. Mean nuclear fluorescence intensity per FOV was calculated for control (A, D), bFGF withdrawal (B, E) and EGF withdrawal (C, F). A significant increase in mean nuclear fluorescence intensity was detected between control and both bFGF and EGF withdrawal conditions (G). Abbreviation: w/d, withdrawal; scale bars represent 10 µm; error bars represent 1 SE.

6.11 DISCUSSION

6.11.1 Purinergic signalling decreases proliferation in hippocampal NPCs

Purinergic signalling has been shown to regulate neural stem cell proliferation and this can be mediated by P2X7 receptors both *in vitro* (Glaser et al., 2014, Thompson et al., 2012, Tsao et al., 2013) and *in vivo* (Csolle et al., 2013, Sebastian-Serrano et al., 2016). Discrepancy exists with regards to the form this regulation takes; two studies using embryonic murine stem cell cultures found inhibition of P2X7 receptors using A438079 caused a decrease in cell/colony number, though Glaser et al. (2014) also observed an increase in percentage of cells in S phase with P2X7 activation, while Thompson et al. (2012) concluded that P2X7 receptors were not involved in regulating proliferation or self-renewal, but were required for survival. In accordance with Glaser's findings, Tsao and colleagues reported P2X7 activation caused an increase in percentage of cells in S phase, but observed a net decrease in proliferation rates (Tsao et al., 2013). *In vivo*, knock out mice demonstrate a greater number of proliferating cells in the hippocampal neurogenic niche when compared to wild type (Csolle et al., 2013, Sebastian-Serrano et al., 2016), supporting the notion that P2X7 receptor activation may negatively regulate proliferation rates. It is clear that further investigations are required to clarify this matter.

In the current study application of ATP was found to significantly reduce proliferation of adult hippocampal NPCs, indicating a role for P2X receptor signalling in adult NPCs. Cell death was not observed and the cells completely recovered following removal of ATP, indicating necrosis was not occurring. Application of ADP, but not UTP or UDP also resulted in a decrease in proliferation, indicating involvement of P2Y1 receptors (Jacobson et al., 2006). As ATP is only a partial agonist at P2Y1

receptors, the observed reduction in proliferation may be due to activation of multiple purinergic pathways (Hechler et al., 1998). P2X7 receptor involvement in proliferation was assessed and application of agonist BzATP caused a decreased proliferation, while P2X7 specific inhibitor A438079 attenuated ATP induced reduction, providing evidence P2X7 receptors contribute to purinergic regulation of adult NPC proliferation. P2X7 receptor knock out cultures treated with ATP did not incur reduced proliferation to the same extent as the wild type cultures did, supporting the previous data that P2X7 receptors contribute to negative regulation of proliferation. This data also supports the idea that other purinergic receptors are involved.

The decrease in proliferation was accompanied by a small but significant increase in DCX positive NPCs following ATP application; this supports findings by Tsao et al. (2013) who reported an increase in DCX mRNA levels with a similar fold increase to what was observed in the current study, as well as increases in expression levels of neuronal markers TUJ1 and MAP2. Thus, P2X7 receptor activation reduces proliferation in hippocampal NPCs and may induce neuronal differentiation. Due to time constraints further research is required to confirm this finding, and future studies include reporting effects of both P2X7 agonists and antagonists on multiple markers of differentiation. Use of knock out cultures or siRNA inhibition may also provide beneficial insights.

6.11.2 ATP induced calcium oscillations do not activate calcium dependent transcription factors

Following confirmation of a P2X7 receptor mediated decrease in proliferation rates, investigations commenced into possible molecular mechanisms governing this response. Calcium imaging revealed that application of ATP, but not ADP, UTP or

UDP, initiated calcium oscillations. These signalling events can modulate cell cycle progression in other stem and progenitor cell populations (Resende et al., 2010); through calcium oscillation decoder proteins like calmodulin and PKC the specific amplitude modulation (AM), frequency modulation (FM), or multimodal encoding of oscillations are translated to functional changes (Smedler and Uhlen, 2014). It was hypothesised that this sudden increase in oscillation events in response to ATP may result in translocation of calcium dependent transcription factors, similar to previous reports of transcription factor activation following P2X7 receptor stimulation (Ferrari et al., 1997, Ferrari et al., 1999).

Nuclear fluorescence intensities were captured using confocal microscopy and analysed using Columbus software. This method was adapted from Noursadeghi et al. (2008), and overcomes some of the drawbacks of more traditional methods of measuring transcription factor activation, such as insensitive nuclear fractionation, gel shift assays that require large scale cell culture, or costly commercial kits that utilise methods, such as ELISA or ChIP. Using this method, neither NFκB nor NFAT1 were observed to translocate to the nucleus following ATP application at the times or concentrations used. This is particularly evident when comparing staining patterns to the TNFα positive control. A possible improvement to this assay would be the inclusion of a cell cytoplasm stain, which would allow calculation of intensity ratios between nucleus and cytoplasm, and may provide a more sensitive method of analysis as any drop in cytoplasmic fluorescence would also be captured.

Inhibition of NFκB and NFAT using chemical inhibitors was conducted to establish any role the transcription factors may play in regulating proliferation the absence of purinergic signalling events. Inhibitors of NFκB and NFAT failed to alter proliferation

rates with the exception of Cyclosporin A, here used to inhibit NFAT. Cyclosporin A is an inhibitor of calcineurin, and thus may be affecting a number of different signalling pathways in addition to the dephosphorylation of NFATs.

Taken together these data indicate that the P2X7 mediated decrease in proliferation is not a result of direct NFκB or NFAT activation. Instead, the decrease may result from P2X and P2Y signals converging with other pathways like RTKs, which may induce calcium oscillations via modulation of ER proteins, thus mediating the decrease in proliferation via any number of alternate transcriptional modifications.

6.11.3 Endoplasmic reticulum proteins are essential for regulating proliferation

The ER functions as a major site of calcium storage, and in non-excitable cells provides an important source of intracellular calcium through the activation of IP_3R-dependent pathways (Berridge et al., 2003). The three IP_3Rs each have specific IP_3 and calcium binding affinities, allowing precise and dynamic regulation of the spatiotemporal aspects of calcium oscillations (Zhang et al., 2011). Immunocytochemical staining identified all three IP_3R subtypes in hippocampal NPCs, each with unique cellular localisations. Previous research in Weible Lab found inhibition of IP_3Rs diminished spontaneous calcium oscillations in SVZ cultures and reduced proliferation (Toppinen, 2016). I hypothesised that inhibition of ER proteins, such as the IP_3Rs, may inhibit the dramatic increase in both AM and FM calcium oscillations and prevent the ATP induced reduction in proliferation. However, control experiments found that inhibition of IP_3Rs alone caused a decrease in proliferation. This effect was also observed following the inhibition of Orai1 and STIM1, which replenish ER calcium stores by forming a calcium selective channel directly between the extracellular space and the ER.

Given that inhibition of IP_3R, Orai1 and STIM1 all inhibited oscillations in SVZ NPCs (Weible Lab, unpublished data), as well as decreasing proliferation in hippocampal NPCs, I concluded that ER regulation of internal calcium concentrations is vital for cell cycle progression. Altering oscillation frequency or amplitude by different methods will not encode the same outcome, and it seems that substantial deviation from any normal ER functioning can lead to conditions sub-optimal for continued proliferation. While hippocampal NPCs do not spontaneously oscillate to the same extent as SVZ NPCs, small changes in cytoplasmic calcium concentrations still occur and these are vitally important for the cells continued survival and proliferation.

6.11.4 RTK signalling can influence calcium signalling and proliferation

EGF and bFGF are potent mitogens and can mediate proliferation and cell cycle via multiple signalling pathways and are essential for maintaining stem and progenitor cell populations. EGFR regulates calcium dependent proliferation pathways via calmodulin and calcineurin, mediating numerous effector molecules, such as NFκβ and NFAT, by the presence of calcium oscillations (Dolmetsch et al., 1998). NPCs derived from the SVZ highly express EGFR; the activation of EGFR was required for calcium oscillations frequency and EGF withdrawal caused a decrease in the frequency of oscillations (Toppinen, 2016). Here hippocampal NPCs were observed to express EGFR and FGFR2. It was hypothesised that EGF and bFGF signalling may contribute to the high frequency oscillations observed in hippocampal NPCs following ATP stimulation, and may contribute to the decrease in proliferation seen following P2X7 receptor stimulation. P2X7 receptors have been shown to regulate differentiation through a PKC/ERK mediated pathway (Tsao et al., 2013), which are also downstream mediators of RTK/EGFR signalling.

In the current study, overnight growth factor withdrawal decreased proliferation in hippocampal NPCs and resulted in nuclear translocation of NFAT1, but not NFκB. Interestingly, the EGFR inhibitor PD 158780 failed to decrease proliferation, and this is possibly due to technical problems with the experiment, for example an inadequate incubation time, though due to time constraints these possibilities could not be properly explored. Future studies include determining if P2X7 receptor activation in the absence of growth factors exacerbates the decrease in proliferation and to determine if transcription factor activation levels are increased by purinergic signalling.

6.11.5 Conclusion

This evidence suggests P2X7 receptors have a role in signal transduction by calcium influx, and can regulate adult NPC proliferation and possibly differentiation by an as yet unknown mechanism. This may be a physiological response of NPCs to a cell death event, where neighbouring cells (who experience only a decrease in proliferation) can initiate lineage elaboration pathways as a compensatory mechanism to replace lost cells. It is probable that the decrease in proliferation observed in NPCs occurs through a variety of downstream mechanisms regulated by complex calcium oscillation signalling. Studying the mechanisms that regulate basal ER and RTK function is the first step to understanding how P2X7 receptors may incite these changes. These data demonstrate the multidimensional properties of the P2X7 receptor and must be thoroughly explored to fully appreciate the roles P2X7 receptors may play in the adult neurogenic niches.

7.0 GENERAL DISCUSSION

7.1 P2X7 RECEPTORS PLAY MULTIPLE ROLES IN THE ADULT HIPPOCAMPAL NEUROGENIC NICHE

7.1.1 Functional P2X7 receptors are present in the hippocampal dentate gyrus

Evidence presented here is the first to provide a detailed functional analysis of P2X7 receptors expressed by progenitor cells of the adult hippocampal neurogenic niche. Previous literature has focused on the expression of P2X7 receptors by murine and human embryonic stem cells (Delarasse et al., 2009, Tsao et al., 2013, Lovelace et al., 2015), and in adult SVZ neural progenitors (Messemer et al., 2013). The presence of P2X7 receptors in the hippocampus has been reported in adult rats (Hogg et al., 2004) and in juvenile mice (Rozmer et al., 2017); these reports focused on membrane potential and electrical excitability in response to ATP and BzATP, and utilised P2X7 specific antagonists.

The data presented here significantly expands on prior findings and demonstrates that the P2X7 receptor is present in adult NPCs derived from the dentate gyrus using immunochemistry and western blot, and shows that they are functional by calcium influx and ethidium uptake assays. These methods confirm the presence of a full length protein as opposed to the Δ-C splice variant with a truncated C-terminus also reported in the brain (Cheewatrakoolpong et al., 2005). These data also demonstrate that adult hippocampal NPCs are able to utilise P2X7 receptor activation in response to high

levels of ATP to induce transmembrane pore formation, highlighting the vulnerability of adult NPCs to membrane disruption following inflammatory events, see Figure 7.1.

Functional P2X7 receptors in adult hippocampal NPCs may have significant implications during an ischemic event, as high amounts of ATP are released from necrotic cells, and this may cause prolonged receptor activation leading to cell death. These findings are in agreement with results observed in embryonic NPCs, where P2X7 receptor activation can lead to membrane disruption and necrotic cell death (Delarasse et al., 2009). A number of recent studies have demonstrated the conferral of neuroprotection by modulation of P2X7 receptor activity in ischemic brain injury, leading some to postulate that epilepsy and stroke may be treated by targeting P2X7 receptors as a means of therapeutic intervention in cerebrovascular diseases (reviewed in Sperlagh and Illes, 2014). Many of these reports surmise the basic principle that inhibiting P2X7 receptors can prevent pore formation and secondary cell death caused by the inflammation of the primary insult. The findings presented here provide a more detailed understanding of the functional roles of P2X7 receptors in this niche, and offers valuable insight into the potential of P2X7 receptors as a therapeutic target for treating some forms of ischemic brain injury.

7.1.2 Phagocytosis in the neurogenic niche

During adult neurogenesis the niche must remain permissive towards proliferation, while carefully controlling population numbers. The progenitor cell pool is tightly regulated via signalling mechanisms including PCD within the niche, as well as at the cell's target destination. Both professional phagocytes (microglia) and non-professional phagocytes contribute to the removal of these naturally occurring cell corpses; resident neural precursors and neuroblasts have been demonstrated to engulf the debris of

neighbouring apoptotic cells via innate phagocytosis. In their study, Lu et al. (2011) demonstrated DCX positive adult NPCs to have a non-professional phagocytic role within both the hippocampal and sub ventricular neurogenic niches. Recently, human embryonic neuroblasts with high expression of both DCX and P2X7 receptors demonstrated the greatest phagocytic capability of developing CNS cells, and P2X7 receptors were identified as the scavenger receptor responsible for the observed phagocytosis (Lovelace et al., 2015).

A defining feature of P2X7 receptors is their ability to mediate phagocytosis, and in the present study, it was confirmed that adult NPCs derived from the hippocampus are capable of phagocytosis via P2X7 receptors, despite low levels of DCX expression. Phagocytosis was inhibited by the presence of ATP, which by activating the ion channel function of the receptor dissociates the carboxyl terminus of P2X7 from the underlying cytoskeleton, thereby abolishing its phagocytic function (refer to Figure 7.1). These data suggest P2X7 receptors in the adult dentate gyrus possess the ability to phagocytose and may have a role in maintaining the neurogenic niche by facilitating engulfment of neighbouring apoptotic NPCs.

7.1.3 P2X7 receptors may regulate proliferation in the neurogenic niche

ATP has been shown to regulate neural stem cell proliferation and this can be mediated by P2X7 receptors (Glaser et al., 2014, Thompson et al., 2012, Tsao et al., 2013). Application of ATP and BzATP was found to significantly reduce proliferation of adult hippocampal NPCs, and this was attenuated by pre-incubation with P2X7 receptor specific inhibitor. P2X7 knock out cultures treated with ATP did not demonstrate as significant a reduction in proliferation as did the wild type cultures, supporting our previous data. This suggests P2X7 receptors can contribute to purinergic regulation of

adult NPC proliferation, either alone or in conjunction with other purinergic receptors, such as P2Y1 receptors, which have also been reported in NPCs (Illes et al., 2013). This finding provides a new and interesting perspective of the regulation of progenitor pools in the adult hippocampus, and may have significant implications in our current understanding of the role of P2X7 receptor signalling in the adult brain. For example, it is possible that P2X7 receptors function via different mechanisms that are dependent on the extracellular environment, as well as the stage of progenitor development. Activation by low concentrations of extracellular ATP may act to regulate proliferation via calcium signalling whereas at higher concentrations, signalling could lead to cell death through transmembrane pore formation. In the absence of ATP, P2X7 receptors can facilitate phagocytosis of apoptotic cells. Figure 7.1 below demonstrates these concepts and highlights the roles P2X7 receptors may play in the adult neurogenic niches.

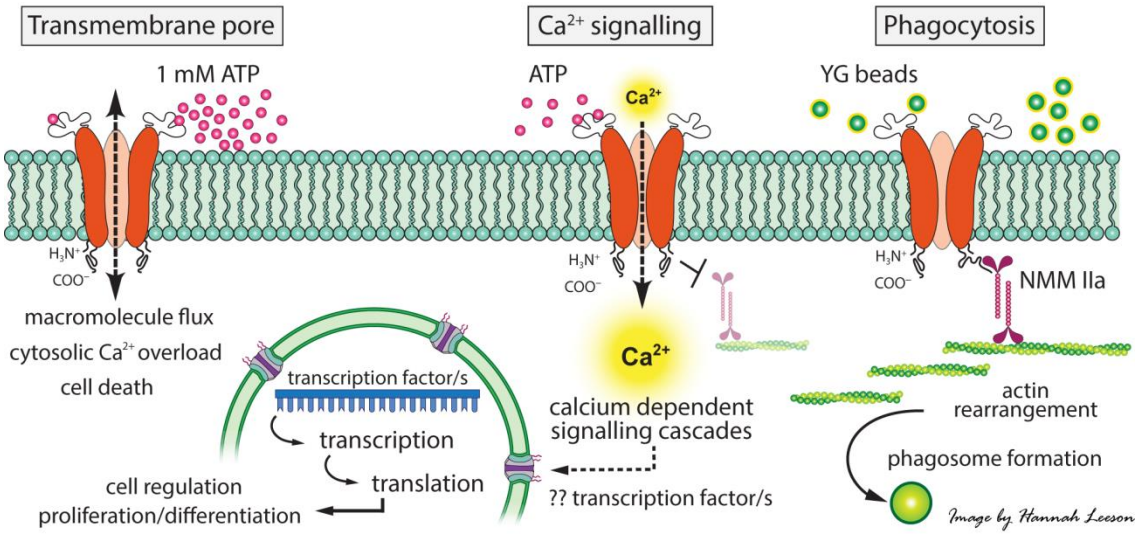

Figure 7.1 The multiple roles of P2X7 receptors in adult hippocampal NPCs

P2X7 receptors have at least three distinct roles in adult hippocampal NPCs, these being transmembrane pore formation, calcium signalling and phagocytosis. When extracellular ATP concentrations are high (in the millimolar range) P2X7 receptors form a large transmembrane pore that causes cell death by macromolecule flux and cytosolic calcium overload (North, 2002, Surprenant et al., 1996). In the presence of lower concentrations of ATP and a cation channel opens, allowing calcium into the cell (Sperlagh et al., 2006). This activates calcium dependent signalling cascades, and can regulate cellular functions (Ferrari et al., 1999). In the absence of ATP, the C terminus of P2X7 receptors interacts with the heavy chain of non-muscle myosin IIa (NMM IIa), and can facilitate phagocytosis by actin rearrangement (Gu et al., 2010, Yamamoto et al., 2013). This interaction with NMM IIa dissociates in the presence of ATP.

7.2 MULTIPLE CALCIUM SIGNALLING PATHWAYS COMBINE TO REGULATE PROLIFERATION IN NEURAL PROGENITOR CELLS

Investigations into possible signalling mechanisms that mediate the P2X7 receptor induced reduction in proliferation found that application of ATP resulted in calcium oscillations. These oscillation events can modulate a vast array of biological functions including cell cycle progression, and decoder proteins like calmodulin and PKC work to

translate the specific AM and FM oscillations in order to mediate downstream signalling cascades, thus effecting functional changes (Smedler and Uhlen, 2014). These different encodings may lead to the nuclear import of transcription factors, and P2X7 receptors have been previously reported to cause activation of NFκB and NFAT (Ferrari et al., 1997, Ferrari et al., 1999). The NFAT family of transcription factors are heavily involved in the transcription of genes involved in the immune response, such as cytokines (Rao et al., 1997), while also playing vital roles in cell proliferation, differentiation, development and adaptation (Horsley and Pavlath, 2002). In this study transcription factor activation assays did not detect a significant difference in nuclear import of NFκB and NFAT in response to ATP, though positive control assays were able to demonstrate these transcription factors in their activated states. It was concluded that P2X7 receptors mediate the proliferation decrease via an alternate signalling method than those specifically studied here and may even act to modify other regulatory pathways like P2Y signalling and RTKs.

Investigations into possible co-regulatory pathways revealed modulation of ER proteins IP_3R, Orai1 and STIM1 all decreased the proliferation in hippocampal NPCs. It was concluded that ER regulation of internal calcium concentrations is required for cell cycle progression, and that altering ER protein function can lead to dysregulation of the cell cycle. EGF and bFGF signalling was also investigated, as P2X7 receptors have been shown to act through a PKC/ERK mediated pathway (Tsao et al., 2013), also downstream mediators of RTK/EGFR signalling. Withdrawal of growth factors decreased proliferation in hippocampal NPCs and resulted in nuclear translocation of NFAT1, but not NFκB. These studies have provided a better understanding of the roles P2X7 receptors are able to play within the adult hippocampal neurogenic niche.

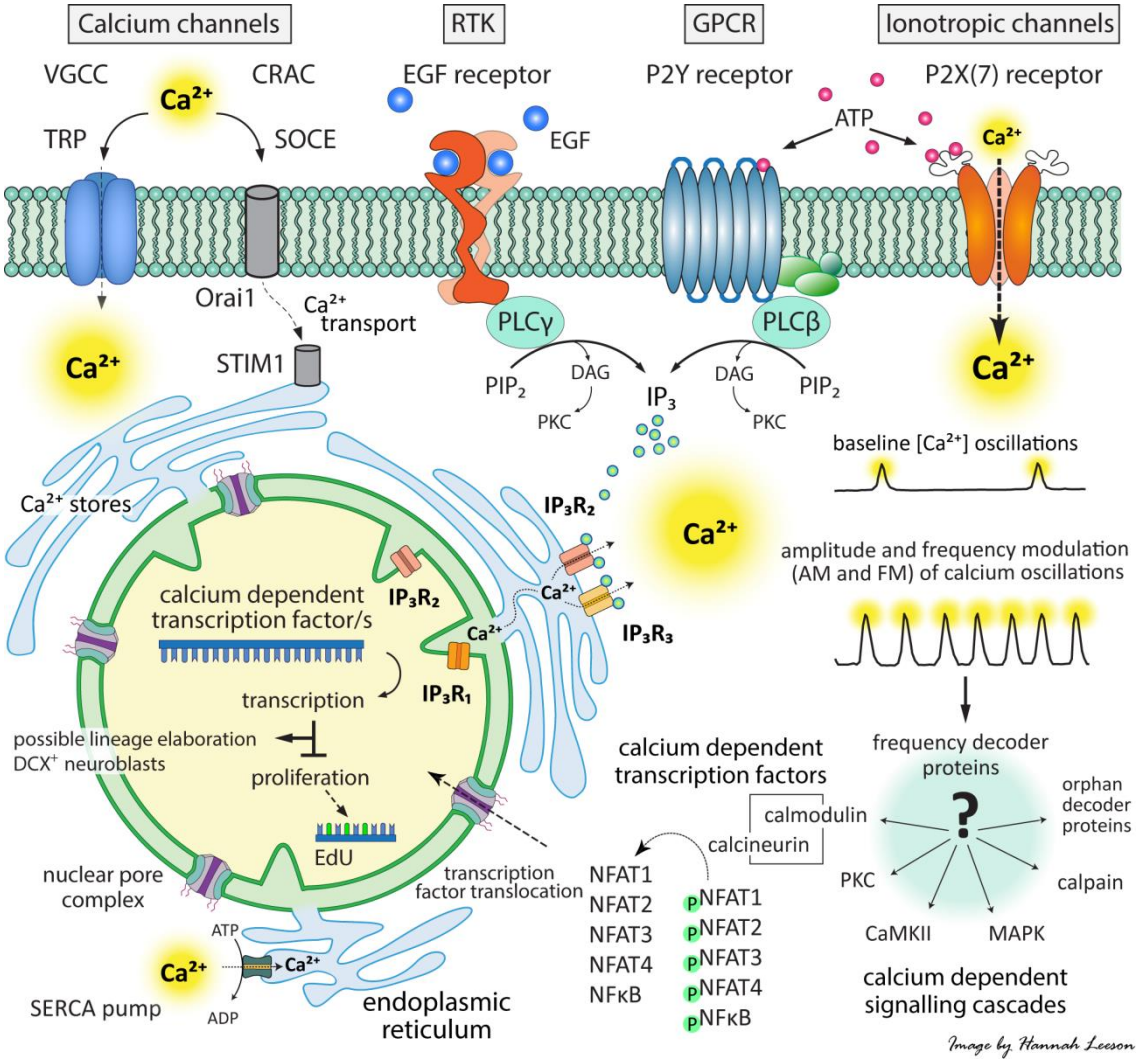

Figure 7.2 Calcium signalling mechanisms regulating hippocampal NPCs

Calcium is a powerful and versatile signalling molecule that can regulate NPCs via multiple mechanisms. Many proteins on the cell surface facilitate calcium influx and signalling, chief among these are VGCC and CRAC channels, the RTK IP_3R pathway, GPCRs, and ionotropic channels, which include P2X7 receptors. VGCC and CRAC channels are activated by changes in calcium concentrations to restore homeostasis (Atlas, 2010). RTKs, such as EGF receptors, signal to IP_3Rs on the ER to release calcium to the cytoplasm (Berridge, 2009). GPCRs including P2Y receptors can also act on IP_3Rs, while ionotropic channels, such as P2X receptors, allow direct influx of calcium to the cytoplasm (Abbracchio et al., 2009). These mechanisms can converge to regulate oscillations in cytoplasmic calcium concentrations. Decoder proteins can convert these oscillations to biological changes by regulation of calcium dependent transcription factors (Smedler and Uhlen, 2014). P2X7 receptors can decrease proliferation through an unknown mechanism, and possibly regulates lineage

elaboration (Diaz-Hernandez et al., 2008, Glaser et al., 2014). More investigations are required to determine the signalling mechanism involved. Abbreviations: VGCC, voltage gated calcium channel; CRAC, calcium release activated calcium (channels); TRP, transient receptor potential; SOCE, store operated calcium entry; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; PLC, phospholipase C; PIP, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3(R), inositol triphosphate (receptor); PKC, protein kinase C; GPCR, G protein coupled receptor; SERCA, sarco/endoplasmic reticulum calcium ATPase.

8.0 FUTURE DIRECTIONS

8.1 ANALYSIS OF THE P2X7 KNOCK OUT MOUSE

The brain of the P2X7 knock out mouse line is yet to be fully characterised in terms of gross anatomy. Specifically, any differences in the size, volume, and structure of the hippocampus between wild type and knock out could provide some insight into how this receptor may impact the long term function of NPCs. As P2X7 receptors are heavily involved in immune function and have the potential to regulate cell death events, it is possible that significant differences might occur in areas of the brain that are naturally subject to cell death events, such as the neurogenic niches. While the knock out is not lethal, it has been noted that P2X7 knock out mice appears to succumb to age abnormally fast (Gu laboratory, unpublished data), and proper assessment of this possibility may also provide fascinating insights into the functioning of this receptor, and open up new avenues of investigation. Proliferation rates of $P2X7^{-/-}$ NPCs *in vivo* should also be assessed. In this study, it was noted that cultured NPCs seemed susceptible to fluctuations in proliferation between passages, making it difficult to draw conclusions regarding differences in proliferation between wild type and knock out mice. By conducting *in vivo* EdU analysis, effects of the knock out on proliferation rates within the neurogenic niches can be assessed.

Confirmation of splice variant absence should be conducted in the future. P2X7 knock out mice from both Pfizer (generated by Solle et al., 2001) and GlaxoSmithKline

(generated by Chessell et al., 2005) have been demonstrated to express some truncated splice variants in select areas (Bartlett et al., 2014). While the P2X7$^{-/-}$ NPCs used in this study were determined by PCR to lack the full P2X7 gene, as well as being functionally inactive by ethidium uptake assays, the uncertainty surrounding these mice models may be cause for concern when utilising them for *in vivo* assays.

8.2 ROLES OF P2X7 FOLLOWING INFLAMMATION *IN VIVO*

In vivo investigations focusing on the impacts of inflammation specifically on NPCs is the next major experimental project to be undertaken. Other studies have detailed roles for P2X7 in recovery following induced status epilepticus (Engel et al., 2012, Rozmer et al., 2017), it is now important that we look specifically at the NPCs of the adult brain to confirm the roles that P2X7 receptors may have in the presence of inflammation. Importantly, the recovery and proliferation rates of hippocampal NPCs should be focused on. The potential for P2X7 receptor inhibitors to be used as a therapeutic has been established following kainic acid treatment (Engel et al., 2012); this should be expanded to include other sources of inflammation, such as pilocarpine to induce status epilepticus, or manual obstruction or occlusion of blood flow to induce ischemic conditions representative of stoke. Receptor activation under these conditions needs to be assessed for calcium influx, pore formation, and cell death events, in the presence and absence of inhibitors. Knock out mouse strains may also prove useful for these investigations.

8.3 SIGNALLING PATHWAYS DOWNSTREAM OF P2X7 RECEPTOR ACTIVATION

Further study is required to fully determine the signalling pathways initiated by P2X7 receptor activation. Some of these assays may include activation and inhibition of P2X7 receptors in combination with the inhibition of various ER and RTK pathways, some of which were investigated here. Synergistic assays are complex but more accurately reflect the conditions *in vivo*, and would be necessary to determine the effects of any convergent signalling. To determine if there are any specific transcription factors activated by P2X7 signalling, nuclear localisation assays could be conducted with a wider scope to also investigate the calcium independent transcription factors that may be present. A possible improvement to this assay would be the use of fluorescently tagged transcription factors in conjunction with a cytoplasm stain, which would allow calculation of intensity ratios between nuclei and cytoplasm using live-cell fluorescent microscopy. This may provide a more sensitive method of analysis as any drop in cytoplasmic fluorescence would also be captured in real time. Commercially available ChIP or ELISA kits could also be used to screen a large number of known transcription factors for any activation. Alternatively, transcriptomic approaches such as microarrays and RNA sequencing could provide a high throughput approach to determining activated genes downstream of P2X7. A greater understanding of the molecular mechanisms and targeted genes may provide ways to modulate P2X7 receptor signalling in targeted areas without systemic modulation of the receptor. Alternate methods of modulating activity may also arise should synergistic activity be detected. These investigations could provide the knowledge required for the advancement of P2X7 as a therapeutic target.

9.0 FINAL CONCLUSIONS

The data presented here demonstrate that functional P2X7 receptors are expressed by adult NPCs of the hippocampus and that there are at least three distinct functions P2X7 receptors may play depending on the conditions present in the extracellular environment. The first is that of a transmembrane pore; P2X7 receptors can induce cell death in the presence of inflammation and high concentrations of extracellular ATP, and this presents a potential therapeutic target for reducing the neural damage that occurs with stroke, epilepsy or other ischemic injuries. Secondly, in the absence of ATP, P2X7 receptors can function as a scavenger receptor and may play a role in the removal of apoptotic bodies by facilitating phagocytosis, and this has significant implications in our current understanding of adult neurogenesis. Finally, as a cation channel, P2X7 receptors are able to facilitate signal transduction via calcium, thus regulating biological processes, such as proliferation and differentiation. These alternate roles of P2X7 signalling have diverse functions, and elucidating these mechanisms is an important step in providing a better understanding as to how P2X7 receptors regulate neurogenesis in the adult brain.

10.0 REFERENCES

ABBRACCHIO, M. P., BURNSTOCK, G., VERKHRATSKY, A. & ZIMMERMANN, H. 2009. Purinergic signalling in the nervous system: an overview. *Trends Neurosci,* 32**,** 19-29.

ADINOLFI, E., CIRILLO, M., WOLTERSDORF, R., FALZONI, S., CHIOZZI, P., PELLEGATTI, P., CALLEGARI, M. G., SANDONA, D., MARKWARDT, F., SCHMALZING, G. & DI VIRGILIO, F. 2010. Trophic activity of a naturally occurring truncated isoform of the P2X7 receptor. *FASEB J,* 24**,** 3393-404.

AGGLETON, J. P. 2012. Multiple anatomical systems embedded within the primate medial temporal lobe: implications for hippocampal function. *Neurosci Biobehav Rev,* 36**,** 1579-96.

AGLEY, C. C., VELLOSO, C. P., LAZARUS, N. R. & HARRIDGE, S. D. 2012. An image analysis method for the precise selection and quantitation of fluorescently labeled cellular constituents: application to the measurement of human muscle cells in culture. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society,* 60**,** 428-438.

ALTMAN, J. & DAS, G. D. 1965. Autoradiographic and Histological Evidence of Postnatal Hippocampal Neurogenesis in Rats. *Journal of Comparative Neurology,* 124**,** 319-&.

ANNENKOV, A. 2014. Receptor tyrosine kinase (RTK) signalling in the control of neural stem and progenitor cell (NSPC) development. *Mol Neurobiol,* 49**,** 440-71.

ARGUELLO, A. A., FISCHER, S. J., SCHONBORN, J. R., MARKUS, R. W., BREKKEN, R. A. & EISCH, A. J. 2009. Effect of chronic morphine on the dentate gyrus neurogenic microenvironment. *Neuroscience,* 159**,** 1003-10.

ASHWELL, K. W. & MAI, J. K. 1997. Developmental expression of the CD15 epitope in the hippocampus of the mouse. *Cell Tissue Res,* 289**,** 17-23.

ATKINSON, L., BATTEN, T. F., MOORES, T. S., VAROQUI, H., ERICKSON, J. D. & DEUCHARS, J. 2004. Differential co-localisation of the P2X7 receptor subunit with vesicular glutamate transporters VGLUT1 and VGLUT2 in rat CNS. *Neuroscience,* 123**,** 761-8.

ATLAS, D. 2010. Signaling role of the voltage-gated calcium channel as the molecular on/off-switch of secretion. *Cell Signal,* 22**,** 1597-603.

BABU, H., CHEUNG, G., KETTENMANN, H., PALMER, T. D. & KEMPERMANN, G. 2007. Enriched monolayer precursor cell cultures from micro-dissected adult mouse dentate gyrus yield functional granule cell-like neurons. *PloS one,* 2.

BABU, H., CLAASEN, J. H., KANNAN, S., RUNKER, A. E., PALMER, T. & KEMPERMANN, G. 2011. A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. *Front Neurosci,* 5**,** 89.

BARTLETT, R., STOKES, L. & SLUYTER, R. 2014. The P2X7 receptor channel: recent developments and the use of P2X7 antagonists in models of disease. *Pharmacol Rev,* 66**,** 638-75.

BASSO, A. M., BRATCHER, N. A., HARRIS, R. R., JARVIS, M. F., DECKER, M. W. & RUETER, L. E. 2009. Behavioral profile of P2X7 receptor knockout mice in animal models of depression and anxiety: relevance for neuropsychiatric disorders. *Behav Brain Res,* 198**,** 83-90.

BAYER, R., FRANKE, H., FICKER, C., RICHTER, M., LESSIG, R., BUTTNER, A. & WEBER, M. 2015. Alterations of neuronal precursor cells in stages of human adult neurogenesis in heroin addicts. *Drug Alcohol Depend,* 156**,** 139-49.

BERRIDGE, M. J. 1997. The AM and FM of calcium signalling. *Nature,* 386**,** 759-60.

BERRIDGE, M. J. 2003. Cardiac calcium signalling. *Biochemical Society Transactions,* 31**,** 930-933.

BERRIDGE, M. J. 2009. Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta,* 1793**,** 933-40.

BERRIDGE, M. J., BOOTMAN, M. D. & RODERICK, H. L. 2003. Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol,* 4**,** 517-29.

BIANCHI, B. R., LYNCH, K. J., TOUMA, E., NIFORATOS, W., BURGARD, E. C., ALEXANDER, K. M., PARK, H. S., YU, H., METZGER, R., KOWALUK, E., JARVIS, M. F. & VAN BIESEN, T. 1999. Pharmacological characterization of recombinant human and rat P2X receptor subtypes. *Eur J Pharmacol,* 376**,** 127-38.

BLASCHKE, A. J., STALEY, K. & CHUN, J. 1996. Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. *Development,* 122**,** 1165-74.

BRAUN, S. M., PILZ, G. A., MACHADO, R. A., MOSS, J., BECHER, B., TONI, N. & JESSBERGER, S. 2015. Programming Hippocampal Neural Stem/Progenitor Cells into Oligodendrocytes Enhances Remyelination in the Adult Brain after Injury. *Cell Rep,* 11**,** 1679-85.

BROWN, J. P., COUILLARD-DESPRES, S., COOPER-KUHN, C. M., WINKLER, J., AIGNER, L. & KUHN, H. G. 2003. Transient expression of doublecortin during adult neurogenesis. *J Comp Neurol,* 467**,** 1-10.

BRUEL-JUNGERMAN, E., LAROCHE, S. & RAMPON, C. 2005. New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment. *Eur J Neurosci,* 21**,** 513-21.

BURGESS, N., MAGUIRE, E. A. & O'KEEFE, J. 2002. The human hippocampus and spatial and episodic memory. *Neuron,* 35**,** 625-41.

BURNSTOCK, G. 1972. Purinergic nerves. *Pharmacol Rev,* 24**,** 509-81.

BURNSTOCK, G. 2002. Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol,* 22**,** 364-73.

BURNSTOCK, G. 2013. Introduction to purinergic signalling in the brain. *Adv Exp Med Biol,* 986**,** 1-12.

BURNSTOCK, G. 2016. An introduction to the roles of purinergic signalling in neurodegeneration, neuroprotection and neuroregeneration. *Neuropharmacology,* 104**,** 4-17.

CAMERON, H. A. & MCKAY, R. D. 2001. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *The Journal of comparative neurology,* 435**,** 406-417.

CAMPBELL, N. R., FERNANDES, C. C., HALFF, A. W. & BERG, D. K. 2010. Endogenous signaling through alpha7-containing nicotinic receptors promotes maturation and integration of adult-born neurons in the hippocampus. *J Neurosci,* 30**,** 8734-44.

CAO, X., LI, L. P., QIN, X. H., LI, S. J., ZHANG, M., WANG, Q., HU, H. H., FANG, Y. Y., GAO, Y. B., LI, X. W., SUN, L. R., XIONG, W. C., GAO, T. M. & ZHU, X. H. 2013. Astrocytic adenosine 5'-triphosphate release regulates the proliferation of neural stem cells in the adult hippocampus. *Stem Cells,* 31**,** 1633-43.

CAPELA, A. & TEMPLE, S. 2006. LeX is expressed by principle progenitor cells in the embryonic nervous system, is secreted into their environment and binds Wnt-1. *Dev Biol,* 291**,** 300-13.

CHADWICK, M. J., HASSABIS, D., WEISKOPF, N. & MAGUIRE, E. A. 2010. Decoding individual episodic memory traces in the human hippocampus. *Curr Biol,* 20**,** 544-7.

CHAMBERS, R. A. 2013. Adult hippocampal neurogenesis in the pathogenesis of addiction and dual diagnosis disorders. *Drug Alcohol Depend,* 130**,** 1-12.

CHAZOTTE, B. 2011. Labeling the plasma membrane with TMA-DPH. *Cold Spring Harb Protoc,* 2011**,** pdb prot5622.

CHEEWATRAKOOLPONG, B., GILCHREST, H., ANTHES, J. C. & GREENFEDER, S. 2005. Identification and characterization of splice variants of the human P2X7 ATP channel. *Biochem Biophys Res Commun,* 332**,** 17-27.

CHESSELL, I. P., HATCHER, J. P., BOUNTRA, C., MICHEL, A. D., HUGHES, J. P., GREEN, P., EGERTON, J., MURFIN, M., RICHARDSON, J., PECK, W. L., GRAHAMES, C. B., CASULA, M. A., YIANGOU, Y., BIRCH, R., ANAND, P. & BUELL, G. N. 2005. Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain,* 114**,** 386-96.

CHO, K. O., LYBRAND, Z. R., ITO, N., BRULET, R., TAFACORY, F., ZHANG, L., GOOD, L., URE, K., KERNIE, S. G., BIRNBAUM, S. G., SCHARFMAN, H. E., EISCH, A. J. & HSIEH, J. 2015. Aberrant hippocampal neurogenesis contributes to epilepsy and associated cognitive decline. *Nat Commun,* 6**,** 6606.

CHOI, H. B., RYU, J. K., KIM, S. U. & MCLARNON, J. G. 2007. Modulation of the purinergic P2X7 receptor attenuates lipopolysaccharide-mediated microglial activation and neuronal damage in inflamed brain. *J Neurosci,* 27**,** 4957-68.

CHUNG, K. M. & YU, S.-W. W. 2013. Interplay between autophagy and programmed cell death in mammalian neural stem cells. *BMB reports,* 46**,** 383-390.

CLELLAND, C. D., CHOI, M., ROMBERG, C., CLEMENSON, G. D., JR., FRAGNIERE, A., TYERS, P., JESSBERGER, S., SAKSIDA, L. M., BARKER, R. A., GAGE, F. H. & BUSSEY, T. J. 2009. A functional role for adult hippocampal neurogenesis in spatial pattern separation. *Science,* 325**,** 210-3.

COLLO, G., NEIDHART, S., KAWASHIMA, E., KOSCO-VILBOIS, M., NORTH, R. A. & BUELL, G. 1997. Tissue distribution of the P2X7 receptor. *Neuropharmacology,* 36**,** 1277-83.

CORNING. 2012, 2013. *Corning Matrigel Matrix Frequently Asked Questions* [Online]. Available: http://csmedia2.corning.com/LifeSciences//media/pdf/faq_DL_026_Corning_Matrigel_Matrix.pdf [Accessed].

COUILLARD-DESPRES, S., WINNER, B., SCHAUBECK, S., AIGNER, R., VROEMEN, M., WEIDNER, N., BOGDAHN, U., WINKLER, J., KUHN, H.

G. & AIGNER, L. 2005. Doublecortin expression levels in adult brain reflect neurogenesis. *Eur J Neurosci,* 21**,** 1-14.

CSOLLE, C., BARANYI, M., ZSILLA, G., KITTEL, A., GOLONCSER, F., ILLES, P., PAPP, E., VIZI, E. S. & SPERLAGH, B. 2013. Neurochemical Changes in the Mouse Hippocampus Underlying the Antidepressant Effect of Genetic Deletion of P2X7 Receptors. *PLoS One,* 8**,** e66547.

DANZER, S. C. 2012. Depression, stress, epilepsy and adult neurogenesis. *Exp Neurol,* 233**,** 22-32.

DAVID, D. J., SAMUELS, B. A., RAINER, Q., WANG, J. W., MARSTELLER, D., MENDEZ, I., DREW, M., CRAIG, D. A., GUIARD, B. P., GUILLOUX, J. P., ARTYMYSHYN, R. P., GARDIER, A. M., GERALD, C., ANTONIJEVIC, I. A., LEONARDO, E. D. & HEN, R. 2009. Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. *Neuron,* 62**,** 479-93.

DAYER, A. G., FORD, A. A., CLEAVER, K. M., YASSAEE, M. & CAMERON, H. A. 2003. Short-term and long-term survival of new neurons in the rat dentate gyrus. *The Journal of comparative neurology,* 460**,** 563-572.

DE LA ROSA, E. J. & DE PABLO, F. 2000. Cell death in early neural development: beyond the neurotrophic theory. *Trends Neurosci,* 23**,** 454-8.

DEKKERS, M. P. & BARDE, Y. A. 2013. Programmed cell death in neuronal development. *Science,* 340**,** 39-41.

DELARASSE, C., GONNORD, P., GALANTE, M., AUGER, R., DANIEL, H., MOTTA, I. & KANELLOPOULOS, J. M. 2009. Neural progenitor cell death is induced by extracellular ATP via ligation of P2X7 receptor. *J Neurochem,* 109**,** 846-57.

DIAZ-HERNANDEZ, M., DEL PUERTO, A., DIAZ-HERNANDEZ, J. I., DIEZ-ZAERA, M., LUCAS, J. J., GARRIDO, J. J. & MIRAS-PORTUGAL, M. T. 2008. Inhibition of the ATP-gated P2X7 receptor promotes axonal growth and branching in cultured hippocampal neurons. *J Cell Sci,* 121**,** 3717-28.

DIAZ, S. L., NARBOUX-NEME, N., TROWBRIDGE, S., SCOTTO-LOMASSESE, S., KLEINE BORGMANN, F. B., JESSBERGER, S., GIROS, B., MAROTEAUX, L., DENERIS, E. & GASPAR, P. 2013. Paradoxical increase in survival of newborn neurons in the dentate gyrus of mice with constitutive depletion of serotonin. *Eur J Neurosci,* 38**,** 2650-8.

DIEZ-ZAERA, M., DIAZ-HERNANDEZ, J. I., HERNANDEZ-ALVAREZ, E., ZIMMERMANN, H., DIAZ-HERNANDEZ, M. & MIRAS-PORTUGAL, M. T. 2011. Tissue-nonspecific alkaline phosphatase promotes axonal growth of hippocampal neurons. *Mol Biol Cell,* 22**,** 1014-24.

DIXON, C. J., BOWLER, W. B., FLEETWOOD, P., GINTY, A. F., GALLAGHER, J. A. & CARRON, J. A. 1997. Extracellular nucleotides stimulate proliferation in MCF-7 breast cancer cells via P2-purinoceptors. *Br J Cancer,* 75**,** 34-9.

DOLMETSCH, R. E., XU, K. & LEWIS, R. S. 1998. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature,* 392**,** 933-6.

DOMERCQ, M., PEREZ-SAMARTIN, A., APARICIO, D., ALBERDI, E., PAMPLIEGA, O. & MATUTE, C. 2010. P2X7 receptors mediate ischemic damage to oligodendrocytes. *Glia,* 58**,** 730-40.

DRANOVSKY, A. & HEN, R. 2006. Hippocampal neurogenesis: regulation by stress and antidepressants. *Biol Psychiatry,* 59**,** 1136-43.

DRANOVSKY, A., PICCHINI, A. M., MOADEL, T., SISTI, A. C., YAMADA, A., KIMURA, S., LEONARDO, E. D. & HEN, R. 2011. Experience dictates stem cell fate in the adult hippocampus. *Neuron,* 70**,** 908-23.

DUARTE-GUTERMAN, P., YAGI, S., CHOW, C. & GALEA, L. A. 2015. Hippocampal learning, memory, and neurogenesis: Effects of sex and estrogens across the lifespan in adults. *Horm Behav,* 74**,** 37-52.

EHNINGER, D. & KEMPERMANN, G. 2008. Neurogenesis in the adult hippocampus. *Cell Tissue Res,* 331**,** 243-50.

EISCH, A. J., BARROT, M., SCHAD, C. A., SELF, D. W. & NESTLER, E. J. 2000. Opiates inhibit neurogenesis in the adult rat hippocampus. *Proc Natl Acad Sci U S A,* 97**,** 7579-84.

ENGEL, T., GOMEZ-VILLAFUERTES, R., TANAKA, K., MESURET, G., SANZ-RODRIGUEZ, A., GARCIA-HUERTA, P., MIRAS-PORTUGAL, M. T., HENSHALL, D. C. & DIAZ-HERNANDEZ, M. 2012. Seizure suppression and neuroprotection by targeting the purinergic P2X7 receptor during status epilepticus in mice. *FASEB J,* 26**,** 1616-28.

ERIKSSON, P. S., PERFILIEVA, E., BJÖRK-ERIKSSON, T., ALBORN, A. M., NORDBORG, C., PETERSON, D. A. & GAGE, F. H. 1998. Neurogenesis in the adult human hippocampus. *Nature medicine,* 4**,** 1313-1317.

ERNST, A., ALKASS, K., BERNARD, S., SALEHPOUR, M., PERL, S., TISDALE, J., POSSNERT, G., DRUID, H. & FRISEN, J. 2014. Neurogenesis in the striatum of the adult human brain. *Cell,* 156**,** 1072-83.

FAIN, J. N. & BERRIDGE, M. J. 1979. Relationship between hormonal activation of phosphatidylinositol hydrolysis, fluid secretion and calcium flux in the blowfly salivary gland. *Biochem J,* 178**,** 45-58.

FANG, K. M., YANG, C. S., SUN, S. H. & TZENG, S. F. 2009. Microglial phagocytosis attenuated by short-term exposure to exogenous ATP through P2X receptor action. *J Neurochem,* 111**,** 1225-37.

FERRARI, D., STROH, C. & SCHULZE-OSTHOFF, K. 1999. P2X7/P2Z purinoreceptor-mediated activation of transcription factor NFAT in microglial cells. *J Biol Chem,* 274**,** 13205-10.

FERRARI, D., WESSELBORG, S., BAUER, M. K. & SCHULZE-OSTHOFF, K. 1997. Extracellular ATP activates transcription factor NF-kappaB through the P2Z purinoreceptor by selectively targeting NF-kappaB p65. *J Cell Biol,* 139**,** 1635-43.

FIEBICH, B. L., AKTER, S. & AKUNDI, R. S. 2014. The two-hit hypothesis for neuroinflammation: role of exogenous ATP in modulating inflammation in the brain. *Frontiers in cellular neuroscience,* 8**,** 260.

FILIPPOV, V., KRONENBERG, G., PIVNEVA, T., REUTER, K., STEINER, B., WANG, L. P., YAMAGUCHI, M., KETTENMANN, H. & KEMPERMANN, G. 2003. Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. *Mol Cell Neurosci,* 23**,** 373-82.

FORSTER, E., ZHAO, S. & FROTSCHER, M. 2006. Laminating the hippocampus. *Nat Rev Neurosci,* 7**,** 259-67.

FRANKE, H., GUNTHER, A., GROSCHE, J., SCHMIDT, R., ROSSNER, S., REINHARDT, R., FABER-ZUSCHRATTER, H., SCHNEIDER, D. & ILLES, P. 2004. P2X7 receptor expression after ischemia in the cerebral cortex of rats. *J Neuropathol Exp Neurol,* 63**,** 686-99.

FRIELINGSDORF, H., SIMPSON, D. R., THAL, L. J. & PIZZO, D. P. 2007. Nerve growth factor promotes survival of new neurons in the adult hippocampus. *Neurobiol Dis,* 26**,** 47-55.

FRISCHAUF, I., SCHINDL, R., DERLER, I., BERGSMANN, J., FAHRNER, M. & ROMANIN, C. 2008. The STIM/Orai coupling machinery. *Channels (Austin),* 2**,** 261-8.

FUCHS, Y. & STELLER, H. 2011. Programmed cell death in animal development and disease. *Cell,* 147**,** 742-58.

FUKUDA, S., KATO, F., TOZUKA, Y., YAMAGUCHI, M., MIYAMOTO, Y. & HISATSUNE, T. 2003. Two distinct subpopulations of nestin-positive cells in adult mouse dentate gyrus. *J Neurosci,* 23**,** 9357-66.

FULGENZI, A., TICOZZI, P., GABEL, C. A., DELL'ANTONIO, G., QUATTRINI, A., FRANZONE, J. S. & FERRERO, M. E. 2008. Periodate oxidized ATP (oATP) reduces hyperalgesia in mice: involvement of P2X7 receptors and implications for therapy. *Int J Immunopathol Pharmacol,* 21**,** 61-71.

GAGE, F. H., RAY, J. & FISHER, L. J. 1995. Isolation, characterization, and use of stem cells from the CNS. *Annu Rev Neurosci,* 18**,** 159-92.

GE, S., YANG, C. H., HSU, K. S., MING, G. L. & SONG, H. 2007. A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. *Neuron,* 54**,** 559-66.

GEIBIG, C. S., KEINER, S. & REDECKER, C. 2012. Functional recruitment of newborn hippocampal neurons after experimental stroke. *Neurobiol Dis,* 46**,** 431-9.

GEIL, C. R., HAYES, D. M., MCCLAIN, J. A., LIPUT, D. J., MARSHALL, S. A., CHEN, K. Y. & NIXON, K. 2014. Alcohol and adult hippocampal neurogenesis: promiscuous drug, wanton effects. *Prog Neuropsychopharmacol Biol Psychiatry,* 54**,** 103-13.

GLASER, T., CAPPELLARI, A. R., PILLAT, M. M., ISER, I. C., WINK, M. R., BATTASTINI, A. M. & ULRICH, H. 2012. Perspectives of purinergic signaling in stem cell differentiation and tissue regeneration. *Purinergic Signal,* 8**,** 523-37.

GLASER, T., DE OLIVEIRA, S. L., CHEFFER, A., BECO, R., MARTINS, P., FORNAZARI, M., LAMEU, C., JUNIOR, H. M., COUTINHO-SILVA, R. & ULRICH, H. 2014. Modulation of mouse embryonic stem cell proliferation and neural differentiation by the P2X7 receptor. *PLoS One,* 9**,** e96281.

GLEESON, J. G., LIN, P. T., FLANAGAN, L. A. & WALSH, C. A. 1999. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron,* 23**,** 257-71.

GOLDMAN, S. A. & NOTTEBOHM, F. 1983. Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. *Proc Natl Acad Sci U S A,* 80**,** 2390-4.

GOMES, F. C., PAULIN, D. & MOURA NETO, V. 1999. Glial fibrillary acidic protein (GFAP): modulation by growth factors and its implication in astrocyte differentiation. *Braz J Med Biol Res,* 32**,** 619-31.

GORITZ, C. & FRISEN, J. 2012. Neural stem cells and neurogenesis in the adult. *Cell Stem Cell,* 10**,** 657-9.

GOTZ, M. & HUTTNER, W. B. 2005. The cell biology of neurogenesis. *Nat Rev Mol Cell Biol,* 6**,** 777-88.

GRIMM, I., MESSEMER, N., STANKE, M., GACHET, C. & ZIMMERMANN, H. 2009. Coordinate pathways for nucleotide and EGF signaling in cultured adult neural progenitor cells. *J Cell Sci,* 122**,** 2524-33.

GRIMM, I., ULLSPERGER, S. N. & ZIMMERMANN, H. 2010. Nucleotides and epidermal growth factor induce parallel cytoskeletal rearrangements and migration in cultured adult murine neural stem cells. *Acta Physiol (Oxf),* 199**,** 181-9.

GU, B. J., LOVELACE, M. D., WEIBLE, M. W., 2ND, ALLEN, D. G., EAMEGDOOL, S. S., CHAN-LING, T. & WILEY, J. S. 2015. P2X7 is an archaic scavenger receptor recognizing apoptotic neuroblasts in early human neurogenesis. *Receptor Clin Invest,* 2**,** e699.

GU, B. J., RATHSAM, C., STOKES, L., MCGEACHIE, A. B. & WILEY, J. S. 2009. Extracellular ATP dissociates nonmuscle myosin from P2X(7) complex: this dissociation regulates P2X(7) pore formation. *Am J Physiol Cell Physiol,* 297**,** C430-9.

GU, B. J., SAUNDERS, B. M., JURSIK, C. & WILEY, J. S. 2010. The P2X7-nonmuscle myosin membrane complex regulates phagocytosis of nonopsonized particles and bacteria by a pathway attenuated by extracellular ATP. *Blood,* 115**,** 1621-1631.

GU, B. J., SAUNDERS, B. M., PETROU, S. & WILEY, J. S. 2011. P2X(7) is a scavenger receptor for apoptotic cells in the absence of its ligand, extracellular ATP. *Journal of immunology (Baltimore, Md. : 1950),* 187**,** 2365-2375.

GU, B. J., SUN, C., FULLER, S., SKARRATT, K. K., PETROU, S. & WILEY, J. S. 2014. A quantitative method for measuring innate phagocytosis by human monocytes using real-time flow cytometry. *Cytometry A,* 85**,** 313-21.

GU, Y., ARRUDA-CARVALHO, M., WANG, J., JANOSCHKA, S. R., JOSSELYN, S. A., FRANKLAND, P. W. & GE, S. 2012. Optical controlling reveals time-dependent roles for adult-born dentate granule cells. *Nat Neurosci,* 15**,** 1700-6.

GULBRANSEN, B. D., BASHASHATI, M., HIROTA, S. A., GUI, X., ROBERTS, J. A., MACDONALD, J. A., MURUVE, D. A., MCKAY, D. M., BECK, P. L., MAWE, G. M., THOMPSON, R. J. & SHARKEY, K. A. 2012. Activation of neuronal P2X7 receptor-pannexin-1 mediates death of enteric neurons during colitis. *Nat Med,* 18**,** 600-4.

HASTINGS, N. B. & GOULD, E. 1999. Rapid extension of axons into the CA3 region by adult-generated granule cells. *J Comp Neurol,* 413**,** 146-54.

HECHLER, B., VIGNE, P., LEON, C., BREITTMAYER, J. P., GACHET, C. & FRELIN, C. 1998. ATP derivatives are antagonists of the P2Y1 receptor: similarities to the platelet ADP receptor. *Mol Pharmacol,* 53**,** 727-33.

HECKERS, S. 2001. Neuroimaging studies of the hippocampus in schizophrenia. *Hippocampus,* 11**,** 520-8.

HEINRICH, A., ANDO, R. D., TURI, G., ROZSA, B. & SPERLAGH, B. 2012. K+ depolarization evokes ATP, adenosine and glutamate release from glia in rat hippocampus: a microelectrode biosensor study. *Br J Pharmacol,* 167**,** 1003-20.

HEWAVITHARANA, T., DENG, X., SOBOLOFF, J. & GILL, D. L. 2007. Role of STIM and Orai proteins in the store-operated calcium signaling pathway. *Cell Calcium,* 42**,** 173-82.

HILL, A. S., SAHAY, A. & HEN, R. 2015. Increasing Adult Hippocampal Neurogenesis is Sufficient to Reduce Anxiety and Depression-Like Behaviors. *Neuropsychopharmacology,* 40**,** 2368-78.

HOGG, R. C., CHIPPERFIELD, H., WHYTE, K. A., STAFFORD, M. R., HANSEN, M. A., COOL, S. M., NURCOMBE, V. & ADAMS, D. J. 2004. Functional maturation of isolated neural progenitor cells from the adult rat hippocampus. *Eur J Neurosci,* 19**,** 2410-20.

HORSLEY, V. & PAVLATH, G. K. 2002. NFAT: ubiquitous regulator of cell differentiation and adaptation. *J Cell Biol,* 156**,** 771-4.

HUNT, J., CHENG, A., HOYLES, A., JERVIS, E. & MORSHEAD, C. M. 2010. Cyclosporin A has direct effects on adult neural precursor cells. *J Neurosci,* 30**,** 2888-96.

IARIA, G., PETRIDES, M., DAGHER, A., PIKE, B. & BOHBOT, V. D. 2003. Cognitive strategies dependent on the hippocampus and caudate nucleus in human navigation: variability and change with practice. *J Neurosci,* 23**,** 5945-52.

ILLES, P., KHAN, T. M. & RUBINI, P. 2017. Neuronal P2X7 Receptors Revisited: Do They Really Exist? *J Neurosci,* 37**,** 7049-7062.

ILLES, P., MESSEMER, N. & RUBINI, P. 2013. P2Y receptors in neurogenesis. *Wiley Interdisciplinary Reviews: Membrane Transport and Signaling,* 2**,** 43-48.

INOUE, K. 2008. Purinergic systems in microglia. *Cell Mol Life Sci,* 65**,** 3074-80.

ISO, H., SIMODA, S. & MATSUYAMA, T. 2007. Environmental change during postnatal development alters behaviour, cognitions and neurogenesis of mice. *Behav Brain Res,* 179**,** 90-8.

IWANO, T., MASUDA, A., KIYONARI, H., ENOMOTO, H. & MATSUZAKI, F. 2012. Prox1 postmitotically defines dentate gyrus cells by specifying granule cell identity over CA3 pyramidal cell fate in the hippocampus. *Development,* 139**,** 3051-62.

JACOBSON, K. A., COSTANZI, S., JOSHI, B. V., BESADA, P., SHIN, D. H., KO, H., IVANOV, A. A. & MAMEDOVA, L. 2006. Agonists and antagonists for P2 receptors. *Novartis Found Symp,* 276**,** 58-68; discussion 68-72, 107-12, 275-81.

JESSBERGER, S. & KEMPERMANN, G. 2003. Adult-born hippocampal neurons mature into activity-dependent responsiveness. *Eur J Neurosci,* 18**,** 2707-12.

JESSBERGER, S. & PARENT, J. M. 2015. Epilepsy and Adult Neurogenesis. *Cold Spring Harb Perspect Biol,* 7.

JIANG, L. H., BALDWIN, J. M., ROGER, S. & BALDWIN, S. A. 2013. Insights into the Molecular Mechanisms Underlying Mammalian P2X7 Receptor Functions and Contributions in Diseases, Revealed by Structural Modeling and Single Nucleotide Polymorphisms. *Front Pharmacol,* 4**,** 55.

JIANG, L. H., MACKENZIE, A. B., NORTH, R. A. & SURPRENANT, A. 2000. Brilliant blue G selectively blocks ATP-gated rat P2X(7) receptors. *Mol Pharmacol,* 58**,** 82-8.

JIN, K., WANG, X., XIE, L., MAO, X. O. & GREENBERG, D. A. 2010. Transgenic ablation of doublecortin-expressing cells suppresses adult neurogenesis and worsens stroke outcome in mice. *Proc Natl Acad Sci U S A,* 107**,** 7993-8.

JOHNS, P. R. & EASTER, S. S., JR. 1977. Growth of the adult goldfish eye. II. Increase in retinal cell number. *J Comp Neurol,* 176**,** 331-41.

JUN, H., MOHAMMED QASIM HUSSAINI, S., RIGBY, M. J. & JANG, M. H. 2012. Functional role of adult hippocampal neurogenesis as a therapeutic strategy for mental disorders. *Neural Plast,* 2012**,** 854285.

JURSIK, C., SLUYTER, R., GEORGIOU, J. G., FULLER, S. J., WILEY, J. S. & GU, B. J. 2007. A quantitative method for routine measurement of cell surface P2X7 receptor function in leucocyte subsets by two-colour time-resolved flow cytometry. *J Immunol Methods,* 325**,** 67-77.

KACZMAREK-HAJEK, K., LORINCZI, E., HAUSMANN, R. & NICKE, A. 2012. Molecular and functional properties of P2X receptors--recent progress and persisting challenges. *Purinergic Signal,* 8**,** 375-417.

KAECH, S. & BANKER, G. 2006. Culturing hippocampal neurons. *Nat Protoc,* 1**,** 2406-15.

KAPUR, N., MIGNERY, G. A. & BANACH, K. 2007. Cell cycle-dependent calcium oscillations in mouse embryonic stem cells. *Am J Physiol Cell Physiol,* 292**,** C1510-8.

KEMPERMANN, G. & GAGE, F. H. 2002. Genetic determinants of adult hippocampal neurogenesis correlate with acquisition, but not probe trial performance, in the water maze task. *Eur J Neurosci,* 16**,** 129-36.

KEMPERMANN, G., GAST, D. & GAGE, F. H. 2002. Neuroplasticity in old age: sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Ann Neurol,* 52**,** 135-43.

KEMPERMANN, G., JESSBERGER, S., STEINER, B. & KRONENBERG, G. 2004. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci,* 27**,** 447-52.

KEMPERMANN, G., KUHN, H. G. & GAGE, F. H. 1998. Experience-induced neurogenesis in the senescent dentate gyrus. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 18**,** 3206-3212.

KETTENMANN, H., HANISCH, U.-K. K., NODA, M. & VERKHRATSKY, A. 2011. Physiology of microglia. *Physiological reviews,* 91**,** 461-553.

KIM, E. J., BATTISTE, J., NAKAGAWA, Y. & JOHNSON, J. E. 2008. Ascl1 (Mash1) lineage cells contribute to discrete cell populations in CNS architecture. *Mol Cell Neurosci,* 38**,** 595-606.

KIM, J. E., KIM, D. S., JIN RYU, H., IL KIM, W., KIM, M. J., WON KIM, D., YOUNG CHOI, S. & KANG, T. C. 2013. The effect of P2X7 receptor activation on nuclear factor-kappaB phosphorylation induced by status epilepticus in the rat hippocampus. *Hippocampus,* 23**,** 500-14.

KIM, J. E., RYU, H. J. & KANG, T. C. 2011. P2X7 receptor activation ameliorates CA3 neuronal damage via a tumor necrosis factor-alpha-mediated pathway in the rat hippocampus following status epilepticus. *J Neuroinflammation,* 8**,** 62.

KNOTH, R., SINGEC, I., DITTER, M., PANTAZIS, G., CAPETIAN, P., MEYER, R. P., HORVAT, V., VOLK, B. & KEMPERMANN, G. 2010. Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. *PLoS One,* 5**,** e8809.

KRONENBERG, G., REUTER, K., STEINER, B., BRANDT, M. D., JESSBERGER, S., YAMAGUCHI, M. & KEMPERMANN, G. 2003. Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. *J Comp Neurol,* 467**,** 455-63.

LE STUNFF, H., AUGER, R., KANELLOPOULOS, J. & RAYMOND, M. N. 2004. The Pro-451 to Leu polymorphism within the C-terminal tail of P2X7 receptor impairs cell death but not phospholipase D activation in murine thymocytes. *J Biol Chem,* 279**,** 16918-26.

LEASURE, J. L. & DECKER, L. 2009. Social isolation prevents exercise-induced proliferation of hippocampal progenitor cells in female rats. *Hippocampus,* 19**,** 907-12.

LEE, I. & KESNER, R. P. 2004. Differential contributions of dorsal hippocampal subregions to memory acquisition and retrieval in contextual fear-conditioning. *Hippocampus,* 14**,** 301-10.

LEMMON, M. A. & SCHLESSINGER, J. 2010. Cell signaling by receptor tyrosine kinases. *Cell,* 141**,** 1117-34.

LEUNER, B., CAPONITI, J. M. & GOULD, E. 2012. Oxytocin stimulates adult neurogenesis even under conditions of stress and elevated glucocorticoids. *Hippocampus,* 22**,** 861-8.

LI, Y., MU, Y. & GAGE, F. H. 2009. Development of neural circuits in the adult hippocampus. *Curr Top Dev Biol,* 87**,** 149-74.

LIN, J. H., TAKANO, T., ARCUINO, G., WANG, X., HU, F., DARZYNKIEWICZ, Z., NUNES, M., GOLDMAN, S. A. & NEDERGAARD, M. 2007. Purinergic signaling regulates neural progenitor cell expansion and neurogenesis. *Dev Biol,* 302**,** 356-66.

LIU, Y., CHEN, G. Q., LIU, B. Y., CHEN, Q., QIAN, Y. M., QIN, S. S., LIU, C. L. & XU, C. S. 2017. P2X7 receptor in the hippocampus is involved in gp120-induced cognitive dysfunction. *Genet Mol Res,* 16.

LOVELACE, M. D., GU, B. J., EAMEGDOOL, S. S., WEIBLE, M. W., 2ND, WILEY, J. S., ALLEN, D. G. & CHAN-LING, T. 2015. P2X7 receptors mediate innate phagocytosis by human neural precursor cells and neuroblasts. *Stem Cells,* 33**,** 526-41.

LU, Z., ELLIOTT, M. R., CHEN, Y., WALSH, J. T., KLIBANOV, A. L., RAVICHANDRAN, K. S. & KIPNIS, J. 2011. Phagocytic activity of neuronal progenitors regulates adult neurogenesis. *Nat Cell Biol,* 13**,** 1076-83.

LUGERT, S., BASAK, O., KNUCKLES, P., HAUSSLER, U., FABEL, K., GOTZ, M., HAAS, C. A., KEMPERMANN, G., TAYLOR, V. & GIACHINO, C. 2010. Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell,* 6**,** 445-56.

MALBERG, J. E., EISCH, A. J., NESTLER, E. J. & DUMAN, R. S. 2000. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci,* 20**,** 9104-10.

MAMBER, C., KOZAREVA, D. A., KAMPHUIS, W. & HOL, E. M. 2013. Shades of gray: The delineation of marker expression within the adult rodent subventricular zone. *Prog Neurobiol,* 111**,** 1-16.

MANJI, H. K., MOORE, G. J., RAJKOWSKA, G. & CHEN, G. 2000. Neuroplasticity and cellular resilience in mood disorders. *Mol Psychiatry,* 5**,** 578-93.

MCEOWN, K. & TREIT, D. 2013. Alpha2 GABAA receptor sub-units in the ventral hippocampus and alpha5 GABAA receptor sub-units in the dorsal hippocampus mediate anxiety and fear memory. *Neuroscience,* 252**,** 169-77.

MERKLE, F. T. & ALVAREZ-BUYLLA, A. 2006. Neural stem cells in mammalian development. *Curr Opin Cell Biol,* 18**,** 704-9.

MERKLE, F. T., TRAMONTIN, A. D., GARCIA-VERDUGO, J. M. & ALVAREZ-BUYLLA, A. 2004. Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc Natl Acad Sci U S A,* 101**,** 17528-32.

MESHI, D., DREW, M. R., SAXE, M., ANSORGE, M. S., DAVID, D., SANTARELLI, L., MALAPANI, C., MOORE, H. & HEN, R. 2006. Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. *Nat Neurosci,* 9**,** 729-31.

MESSEMER, N., KUNERT, C., GROHMANN, M., SOBOTTKA, H., NIEBER, K., ZIMMERMANN, H., FRANKE, H., NORENBERG, W., STRAUB, I., SCHAEFER, M., RIEDEL, T., ILLES, P. & RUBINI, P. 2013. P2X7 receptors at adult neural progenitor cells of the mouse subventricular zone. *Neuropharmacology,* 73**,** 122-37.

MING, G. L. & SONG, H. J. 2005. Adult neurogenesis in the mammalian central nervous system. *Annual Review of Neuroscience,* 28**,** 223-250.

MING, G. L. & SONG, H. J. 2011. Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions. *Neuron,* 70**,** 687-702.

MIRAS-PORTUGAL, M. T., SEBASTIAN-SERRANO, A., DE DIEGO GARCIA, L. & DIAZ-HERNANDEZ, M. 2017. Neuronal P2X7 Receptor: Involvement in Neuronal Physiology and Pathology. *J Neurosci,* 37**,** 7063-7072.

MONIF, M., REID, C. A., POWELL, K. L., SMART, M. L. & WILLIAMS, D. A. 2009. The P2X7 receptor drives microglial activation and proliferation: a trophic role for P2X7R pore. *J Neurosci,* 29**,** 3781-91.

MURGIA, M., HANAU, S., PIZZO, P., RIPPA, M. & DI VIRGILIO, F. 1993. Oxidized ATP. An irreversible inhibitor of the macrophage purinergic P2Z receptor. *J Biol Chem,* 268**,** 8199-203.

NADEL, L. & MOSCOVITCH, M. 1997. Memory consolidation, retrograde amnesia and the hippocampal complex. *Curr Opin Neurobiol,* 7**,** 217-27.

NEUMANN, H., KOTTER, M. R. & FRANKLIN, R. J. 2009. Debris clearance by microglia: an essential link between degeneration and regeneration. *Brain,* 132**,** 288-95.

NILSSON, M., PERFILIEVA, E., JOHANSSON, U., ORWAR, O. & ERIKSSON, P. S. 1999. Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *J Neurobiol,* 39**,** 569-78.

NORTH, R. A. 2002. Molecular physiology of P2X receptors. *Physiological reviews,* 82**,** 1013-1067.

NOURSADEGHI, M., TSANG, J., HAUSTEIN, T., MILLER, R. F., CHAIN, B. M. & KATZ, D. R. 2008. Quantitative imaging assay for NF-kappaB nuclear translocation in primary human macrophages. *J Immunol Methods,* 329**,** 194-200.

OLIVEIRA, S. L., PILLAT, M. M., CHEFFER, A., LAMEU, C., SCHWINDT, T. T. & ULRICH, H. 2013. Functions of neurotrophins and growth factors in neurogenesis and brain repair. *Cytometry A,* 83**,** 76-89.

OYA, S., YOSHIKAWA, G., TAKAI, K., TANAKA, J., HIGASHIYAMA, S., SAITO, N., KIRINO, T. & KAWAHARA, N. 2008. Region-specific proliferative response of neural progenitors to exogenous stimulation by growth factors following ischemia. *Neuroreport,* 19**,** 805-9.

PAPP, L., VIZI, E. S. & SPERLAGH, B. 2004. Lack of ATP-evoked GABA and glutamate release in the hippocampus of P2X7 receptor-/- mice. *Neuroreport,* 15**,** 2387-91.

PAPP, L., VIZI, E. S. & SPERLAGH, B. 2007. P2X7 receptor mediated phosphorylation of p38MAP kinase in the hippocampus. *Biochem Biophys Res Commun,* 355**,** 568-74.

PARVATHENANI, L. K., TERTYSHNIKOVA, S., GRECO, C. R., ROBERTS, S. B., ROBERTSON, B. & POSMANTUR, R. 2003. P2X7 mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. *J Biol Chem,* 278**,** 13309-17.

PENALOZA, C., LIN, L., LOCKSHIN, R. A. & ZAKERI, Z. 2006. Cell death in development: shaping the embryo. *Histochem Cell Biol,* 126**,** 149-58.

PERIASAMY, M. & KALYANASUNDARAM, A. 2007. SERCA pump isoforms: their role in calcium transport and disease. *Muscle Nerve,* 35**,** 430-42.

PITKANEN, A., KELLY, J. L. & AMARAL, D. G. 2002. Projections from the lateral, basal, and accessory basal nuclei of the amygdala to the entorhinal cortex in the macaque monkey. *Hippocampus,* 12**,** 186-205.

RAO, A., LUO, C. & HOGAN, P. G. 1997. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol,* 15**,** 707-47.

RESENDE, R. R., ADHIKARI, A., DA COSTA, J. L., LORENCON, E., LADEIRA, M. S., GUATIMOSIM, S., KIHARA, A. H. & LADEIRA, L. O. 2010. Influence of spontaneous calcium events on cell-cycle progression in embryonal carcinoma and adult stem cells. *Biochim Biophys Acta,* 1803**,** 246-60.

REYNOLDS, B. A. & WEISS, S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science,* 255**,** 1707-10.

REZAIE, P. & MALE, D. 1999. Colonisation of the developing human brain and spinal cord by microglia: a review. *Microsc Res Tech,* 45**,** 359-82.

RIDET, J. L., MALHOTRA, S. K., PRIVAT, A. & GAGE, F. H. 1997. Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci,* 20**,** 570-7.

ROLLS, E. T. 2015. Limbic systems for emotion and for memory, but no single limbic system. *Cortex,* 62**,** 119-57.

ROZMER, K., GAO, P., ARAUJO, M. G., KHAN, M. T., LIU, J., RONG, W., TANG, Y., FRANKE, H., KRUGEL, U., FERNANDES, M. J. & ILLES, P. 2017. Pilocarpine-Induced Status Epilepticus Increases the Sensitivity of P2X7 and P2Y1 Receptors to Nucleotides at Neural Progenitor Cells of the Juvenile Rodent Hippocampus. *Cereb Cortex.*

RYU, J. K., CHOI, H. B., HATORI, K., HEISEL, R. L., PELECH, S. L., MCLARNON, J. G. & KIM, S. U. 2003. Adenosine triphosphate induces proliferation of human neural stem cells: Role of calcium and p70 ribosomal protein S6 kinase. *J Neurosci Res,* 72**,** 352-62.

RYU, J. R., HONG, C. J., KIM, J. Y., KIM, E. K., SUN, W. & YU, S. W. 2016. Control of adult neurogenesis by programmed cell death in the mammalian brain. *Mol Brain,* 9**,** 43.

SAHAY, A., SCOBIE, K. N., HILL, A. S., O'CARROLL, C. M., KHEIRBEK, M. A., BURGHARDT, N. S., FENTON, A. A., DRANOVSKY, A. & HEN, R. 2011. Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature,* 472**,** 466-70.

SANCHES, G., DE ALENCAR, L. S. & VENTURA, A. L. 2002. ATP induces proliferation of retinal cells in culture via activation of PKC and extracellular signal-regulated kinase cascade. *Int J Dev Neurosci,* 20**,** 21-7.

SANCHO-TELLO, M., VALLES, S., MONTOLIU, C., RENAU-PIQUERAS, J. & GUERRI, C. 1995. Developmental pattern of GFAP and vimentin gene expression in rat brain and in radial glial cultures. *Glia,* 15**,** 157-66.

SANTARELLI, L., SAXE, M., GROSS, C., SURGET, A., BATTAGLIA, F., DULAWA, S., WEISSTAUB, N., LEE, J., DUMAN, R., ARANCIO, O., BELZUNG, C. & HEN, R. 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science,* 301**,** 805-9.

SCHMIDT-HIEBER, C., JONAS, P. & BISCHOFBERGER, J. 2004. Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. *Nature,* 429**,** 184-7.

SEBASTIAN-SERRANO, A., ENGEL, T., DE DIEGO-GARCIA, L., OLIVOS-ORE, L. A., ARRIBAS-BLAZQUEZ, M., MARTINEZ-FRAILES, C., PEREZ-DIAZ,

C., MILLAN, J. L., ARTALEJO, A. R., MIRAS-PORTUGAL, M. T., HENSHALL, D. C. & DIAZ-HERNANDEZ, M. 2016. Neurodevelopmental alterations and seizures developed by mouse model of infantile hypophosphatasia are associated with purinergic signalling deregulation. *Hum Mol Genet,* 25**,** 4143-4156.

SEO, D. O., CARILLO, M. A., CHIH-HSIUNG LIM, S., TANAKA, K. F. & DREW, M. R. 2015. Adult Hippocampal Neurogenesis Modulates Fear Learning through Associative and Nonassociative Mechanisms. *J Neurosci,* 35**,** 11330-45.

SERI, B., GARCIA-VERDUGO, J. M., MCEWEN, B. S. & ALVAREZ-BUYLLA, A. 2001. Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J Neurosci,* 21**,** 7153-60.

SIERRA, A., ENCINAS, J. M., DEUDERO, J. J., CHANCEY, J. H., ENIKOLOPOV, G., OVERSTREET-WADICHE, L. S., TSIRKA, S. E. & MALETIC-SAVATIC, M. 2010. Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell,* 7**,** 483-95.

SMEDLER, E. & UHLEN, P. 2014. Frequency decoding of calcium oscillations. *Biochim Biophys Acta,* 1840**,** 964-9.

SOLLE, M., LABASI, J., PERREGAUX, D. G., STAM, E., PETRUSHOVA, N., KOLLER, B. H., GRIFFITHS, R. J. & GABEL, C. A. 2001. Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem,* 276**,** 125-32.

SONG, H. J., STEVENS, C. F. & GAGE, F. H. 2002. Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. *Nat Neurosci,* 5**,** 438-45.

SONG, J., SUN, J., MOSS, J., WEN, Z., SUN, G. J., HSU, D., ZHONG, C., DAVOUDI, H., CHRISTIAN, K. M., TONI, N., MING, G. L. & SONG, H. 2013. Parvalbumin interneurons mediate neuronal circuitry-neurogenesis coupling in the adult hippocampus. *Nat Neurosci,* 16**,** 1728-30.

SORGE, R. E., TRANG, T., DORFMAN, R., SMITH, S. B., BEGGS, S., RITCHIE, J., AUSTIN, J. S., ZAYKIN, D. V., VANDER MEULEN, H., COSTIGAN, M., HERBERT, T. A., YARKONI-ABITBUL, M., TICHAUER, D., LIVNEH, J., GERSHON, E., ZHENG, M., TAN, K., JOHN, S. L., SLADE, G. D., JORDAN, J., WOOLF, C. J., PELTZ, G., MAIXNER, W., DIATCHENKO, L., SELTZER, Z., SALTER, M. W. & MOGIL, J. S. 2012. Genetically determined P2X7 receptor pore formation regulates variability in chronic pain sensitivity. *Nat Med,* 18**,** 595-9.

SOUTHWELL, D. G., PAREDES, M. F., GALVAO, R. P., JONES, D. L., FROEMKE, R. C., SEBE, J. Y., ALFARO-CERVELLO, C., TANG, Y., GARCIA-VERDUGO, J. M., RUBENSTEIN, J. L., BARABAN, S. C. & ALVAREZ-BUYLLA, A. 2012. Intrinsically determined cell death of developing cortical interneurons. *Nature,* 491**,** 109-13.

SPALDING, K. L., BERGMANN, O., ALKASS, K., BERNARD, S., SALEHPOUR, M., HUTTNER, H. B., BOSTROM, E., WESTERLUND, I., VIAL, C., BUCHHOLZ, B. A., POSSNERT, G., MASH, D. C., DRUID, H. & FRISEN, J. 2013. Dynamics of hippocampal neurogenesis in adult humans. *Cell,* 153**,** 1219-27.

SPERLAGH, B. & ILLES, P. 2014. P2X7 receptor: an emerging target in central nervous system diseases. *Trends Pharmacol Sci,* 35**,** 537-47.

SPERLAGH, B., KOFALVI, A., DEUCHARS, J., ATKINSON, L., MILLIGAN, C. J., BUCKLEY, N. J. & VIZI, E. S. 2002. Involvement of P2X7 receptors in the

regulation of neurotransmitter release in the rat hippocampus. *J Neurochem,* 81**,** 1196-211.

SPERLAGH, B., VIZI, E. S., WIRKNER, K. & ILLES, P. 2006. P2X7 receptors in the nervous system. *Prog Neurobiol,* 78**,** 327-46.

SPRITZER, M. D., CURTIS, M. G., DELOACH, J. P., MAHER, J. & SHULMAN, L. M. 2016. Sexual interactions with unfamiliar females reduce hippocampal neurogenesis among adult male rats. *Neuroscience,* 318**,** 143-56.

SPRITZER, M. D. & GALEA, L. A. 2007. Testosterone and dihydrotestosterone, but not estradiol, enhance survival of new hippocampal neurons in adult male rats. *Dev Neurobiol,* 67**,** 1321-33.

SQUIRE, L. R. & ALVAREZ, P. 1995. Retrograde amnesia and memory consolidation: a neurobiological perspective. *Curr Opin Neurobiol,* 5**,** 169-77.

SQUIRE, L. R. & WIXTED, J. T. 2011. The cognitive neuroscience of human memory since H.M. *Annu Rev Neurosci,* 34**,** 259-88.

STEINER, B., KLEMPIN, F., WANG, L., KOTT, M., KETTENMANN, H. & KEMPERMANN, G. 2006. Type-2 cells as link between glial and neuronal lineage in adult hippocampal neurogenesis. *Glia,* 54**,** 805-14.

STEINER, B., ZURBORG, S., HORSTER, H., FABEL, K. & KEMPERMANN, G. 2008. Differential 24 h responsiveness of Prox1-expressing precursor cells in adult hippocampal neurogenesis to physical activity, environmental enrichment, and kainic acid-induced seizures. *Neuroscience,* 154**,** 521-9.

STEVENS, H. E., JIANG, G. Y., SCHWARTZ, M. L. & VACCARINO, F. M. 2012. Learning and memory depend on fibroblast growth factor receptor 2 functioning in hippocampus. *Biol Psychiatry,* 71**,** 1090-8.

STRANAHAN, A. M., KHALIL, D. & GOULD, E. 2006. Social isolation delays the positive effects of running on adult neurogenesis. *Nat Neurosci,* 9**,** 526-33.

STRIEDINGER, K., MEDA, P. & SCEMES, E. 2007. Exocytosis of ATP from astrocyte progenitors modulates spontaneous Ca2+ oscillations and cell migration. *Glia,* 55**,** 652-62.

SUH, H., CONSIGLIO, A., RAY, J., SAWAI, T., D'AMOUR, K. A. & GAGE, F. H. 2007. In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. *Cell Stem Cell,* 1**,** 515-28.

SUN, G. J., ZHOU, Y., ITO, S., BONAGUIDI, M. A., STEIN-O'BRIEN, G., KAWASAKI, N. K., MODAK, N., ZHU, Y., MING, G. L. & SONG, H. 2015a. Latent tri-lineage potential of adult hippocampal neural stem cells revealed by Nf1 inactivation. *Nat Neurosci,* 18**,** 1722-4.

SUN, W., WINSECK, A., VINSANT, S., PARK, O. H., KIM, H. & OPPENHEIM, R. W. 2004. Programmed cell death of adult-generated hippocampal neurons is mediated by the proapoptotic gene Bax. *J Neurosci,* 24**,** 11205-13.

SUN, X., SUN, X., LIU, T., ZHAO, M., ZHAO, S., XIAO, T., JOLKKONEN, J. & ZHAO, C. 2015b. Fluoxetine enhanced neurogenesis is not translated to functional outcome in stroke rats. *Neurosci Lett,* 603**,** 31-6.

SURPRENANT, A., RASSENDREN, F., KAWASHIMA, E., NORTH, R. A. & BUELL, G. 1996. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science,* 272**,** 735-8.

TAKAMURA, N., NAKAGAWA, S., MASUDA, T., BOKU, S., KATO, A., SONG, N., AN, Y., KITAICHI, Y., INOUE, T., KOYAMA, T. & KUSUMI, I. 2014. The effect of dopamine on adult hippocampal neurogenesis. *Prog Neuropsychopharmacol Biol Psychiatry,* 50**,** 116-24.

TANG, Y. & ILLES, P. 2017. Regulation of adult neural progenitor cell functions by purinergic signaling. *Glia,* 65**,** 213-230.

THOMPSON, B. A., STORM, M. P., HEWINSON, J., HOGG, S., WELHAM, M. J. & MACKENZIE, A. B. 2012. A novel role for P2X7 receptor signalling in the survival of mouse embryonic stem cells. *Cell Signal,* 24**,** 770-8.

TOMIDA, T., HIROSE, K., TAKIZAWA, A., SHIBASAKI, F. & IINO, M. 2003. NFAT functions as a working memory of Ca2+ signals in decoding Ca2+ oscillation. *EMBO J,* 22**,** 3825-32.

TONELLI, F. M., SANTOS, A. K., GOMES, D. A., DA SILVA, S. L., GOMES, K. N., LADEIRA, L. O. & RESENDE, R. R. 2012. Stem cells and calcium signaling. *Adv Exp Med Biol,* 740**,** 891-916.

TOPPINEN, K. M. 2016. *EGFR activates NFAT3 through frequency modulated Ca^{2+} oscillations to regulate the proliferation of transit-amplifying (type C) cells in the adult SVZ.* Doctor of Philosophy, Griffith University.

TSAO, H. K., CHIU, P. H. & SUN, S. H. 2013. PKC-dependent ERK phosphorylation is essential for P2X7 receptor-mediated neuronal differentiation of neural progenitor cells. *Cell Death Dis,* 4**,** e751.

TSUKIMOTO, M., MAEHATA, M., HARADA, H., IKARI, A., TAKAGI, K. & DEGAWA, M. 2006. P2X7 receptor-dependent cell death is modulated during murine T cell maturation and mediated by dual signaling pathways. *J Immunol,* 177**,** 2842-50.

TU, M. T., LUO, S. F., WANG, C. C., CHIEN, C. S., CHIU, C. T., LIN, C. C. & YANG, C. M. 2000. P2Y(2) receptor-mediated proliferation of C(6) glioma cells via activation of Ras/Raf/MEK/MAPK pathway. *Br J Pharmacol,* 129**,** 1481-9.

ULLRICH, A. & SCHLESSINGER, J. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell,* 61**,** 203-12.

ULRICH, H., ABBRACCHIO, M. P. & BURNSTOCK, G. 2012. Extrinsic purinergic regulation of neural stem/progenitor cells: implications for CNS development and repair. *Stem Cell Rev,* 8**,** 755-67.

VAN PRAAG, H. 2008. Neurogenesis and exercise: past and future directions. *Neuromolecular Med,* 10**,** 128-40.

VAN PRAAG, H., KEMPERMANN, G. & GAGE, F. H. 1999. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci,* 2**,** 266-70.

VAN PRAAG, H., SCHINDER, A. F., CHRISTIE, B. R., TONI, N., PALMER, T. D. & GAGE, F. H. 2002. Functional neurogenesis in the adult hippocampus. *Nature,* 415**,** 1030-4.

VENNA, V. R., XU, Y., DORAN, S. J., PATRIZZ, A. & MCCULLOUGH, L. D. 2014. Social interaction plays a critical role in neurogenesis and recovery after stroke. *Transl Psychiatry,* 4**,** e351.

VERKHRATSKY, A., PANKRATOV, Y., LALO, U. & NEDERGAARD, M. 2012. P2X receptors in neuroglia. *Wiley Interdiscip Rev Membr Transp Signal,* 1.

VIDEBECH, P. & RAVNKILDE, B. 2004. Hippocampal volume and depression: a meta-analysis of MRI studies. *Am J Psychiatry,* 161**,** 1957-66.

VIRGILIO, F., CERUTI, S., BRAMANTI, P. & ABBRACCHIO, M. P. 2009. Purinergic signalling in inflammation of the central nervous system. *Trends in neurosciences,* 32**,** 79-87.

VIRGINIO, C., MACKENZIE, A., RASSENDREN, F. A., NORTH, R. A. & SURPRENANT, A. 1999. Pore dilation of neuronal P2X receptor channels. *Nat Neurosci,* 2**,** 315-21.

VON ALLMEN, D. Y., WURMITZER, K., MARTIN, E. & KLAVER, P. 2013. Neural activity in the hippocampus predicts individual visual short-term memory capacity. *Hippocampus,* 23**,** 606-15.

VON BOHLEN UND HALBACH, O. 2011. Immunohistological markers for proliferative events, gliogenesis, and neurogenesis within the adult hippocampus. *Cell and Tissue Research,* 345**,** 1-19.

WAKE, H., MOORHOUSE, A. J., JINNO, S., KOHSAKA, S. & NABEKURA, J. 2009. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. *J Neurosci,* 29**,** 3974-80.

WALKER, T. L. & KEMPERMANN, G. 2014. One Mouse, Two Cultures: Isolation and Culture of Adult Neural Stem Cells from the Two Neurogenic Zones of Individual Mice. *Journal of Visualized Experiments : JoVE*.

WANG, D. J., HUANG, N. N. & HEPPEL, L. A. 1992. Extracellular ATP and ADP stimulate proliferation of porcine aortic smooth muscle cells. *J Cell Physiol,* 153**,** 221-33.

WANG, X., ARCUINO, G., TAKANO, T., LIN, J., PENG, W. G., WAN, P., LI, P., XU, Q., LIU, Q. S., GOLDMAN, S. A. & NEDERGAARD, M. 2004. P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat Med,* 10**,** 821-7.

WEISSMAN, T. A., RIQUELME, P. A., IVIC, L., FLINT, A. C. & KRIEGSTEIN, A. R. 2004. Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. *Neuron,* 43**,** 647-61.

WEST, M. J., SLOMIANKA, L. & GUNDERSEN, H. J. 1991. Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. *Anat Rec,* 231**,** 482-97.

WILEY, J. S. & GU, B. J. 2012. A new role for the P2X7 receptor: a scavenger receptor for bacteria and apoptotic cells in the absence of serum and extracellular ATP. *Purinergic Signal,* 8**,** 579-86.

WILEY, J. S., SLUYTER, R., GU, B. J., STOKES, L. & FULLER, S. J. 2011. The human P2X7 receptor and its role in innate immunity. *Tissue Antigens,* 78**,** 321-32.

WITTING, A., MULLER, P., HERRMANN, A., KETTENMANN, H. & NOLTE, C. 2000. Phagocytic clearance of apoptotic neurons by Microglia/Brain macrophages in vitro: involvement of lectin-, integrin-, and phosphatidylserine-mediated recognition. *J Neurochem,* 75**,** 1060-70.

WU, P. Y., LIN, Y. C., CHANG, C. L., LU, H. T., CHIN, C. H., HSU, T. T., CHU, D. & SUN, S. H. 2009. Functional decreases in P2X7 receptors are associated with retinoic acid-induced neuronal differentiation of Neuro-2a neuroblastoma cells. *Cell Signal,* 21**,** 881-91.

YAMAMOTO, M., KAMATSUKA, Y., OHISHI, A., NISHIDA, K. & NAGASAWA, K. 2013. P2X7 receptors regulate engulfing activity of non-stimulated resting astrocytes. *Biochem Biophys Res Commun,* 439**,** 90-5.

YAU, S. Y., LI, A. & SO, K. F. 2015. Involvement of Adult Hippocampal Neurogenesis in Learning and Forgetting. *Neural Plast,* 2015**,** 717958.

YOSHIMIZU, T. & CHAKI, S. 2004. Increased cell proliferation in the adult mouse hippocampus following chronic administration of group II metabotropic

glutamate receptor antagonist, MGS0039. *Biochem Biophys Res Commun,* 315**,** 493-6.

YU, Q., GUO, Z., LIU, X., OUYANG, Q., HE, C., BURNSTOCK, G., YUAN, H. & XIANG, Z. 2013. Block of P2X7 receptors could partly reverse the delayed neuronal death in area CA1 of the hippocampus after transient global cerebral ischemia. *Purinergic Signal,* 9**,** 663-75.

ZHANG, K., LIU, J., YOU, X., KONG, P., SONG, Y., CAO, L., YANG, S., WANG, W., FU, Q. & MA, Z. 2016. P2X7 as a new target for chrysophanol to treat lipopolysaccharide-induced depression in mice. *Neurosci Lett,* 613**,** 60-5.

ZHANG, S., FRITZ, N., IBARRA, C. & UHLEN, P. 2011. Inositol 1,4,5-trisphosphate receptor subtype-specific regulation of calcium oscillations. *Neurochem Res,* 36**,** 1175-85.

ZHENG, J., JIANG, Y. Y., XU, L. C., MA, L. Y., LIU, F. Y., CUI, S., CAI, J., LIAO, F. F., WAN, Y. & YI, M. 2017. Adult Hippocampal Neurogenesis along the Dorsoventral Axis Contributes Differentially to Environmental Enrichment Combined with Voluntary Exercise in Alleviating Chronic Inflammatory Pain in Mice. *J Neurosci,* 37**,** 4145-4157.

11.0 APPENDICES

11.1 APPENDIX 1: IMMUNOCHEMISTRY SECONDARY CONTROLS

Secondary controls were conducted to ensure absence of non-specific binding of the secondary antibodies. Samples were prepared as usual, and the primary antibody was omitted. Controls were conducted at each experiment, and included antibodies conjugated to Cy3 (IgG H+L), Cy5 (IgG H+L and IgG1), or Alexa Fluor 488 (IgG H+L), 594 (IgG H+L, IgG1, and IgM) or 647 (IgG H+L and IgG1). Representative images for immunohistochemistry are depicted in Figure 11.1 A and B, and immunocytochemistry in Figure 11.1 C and D.

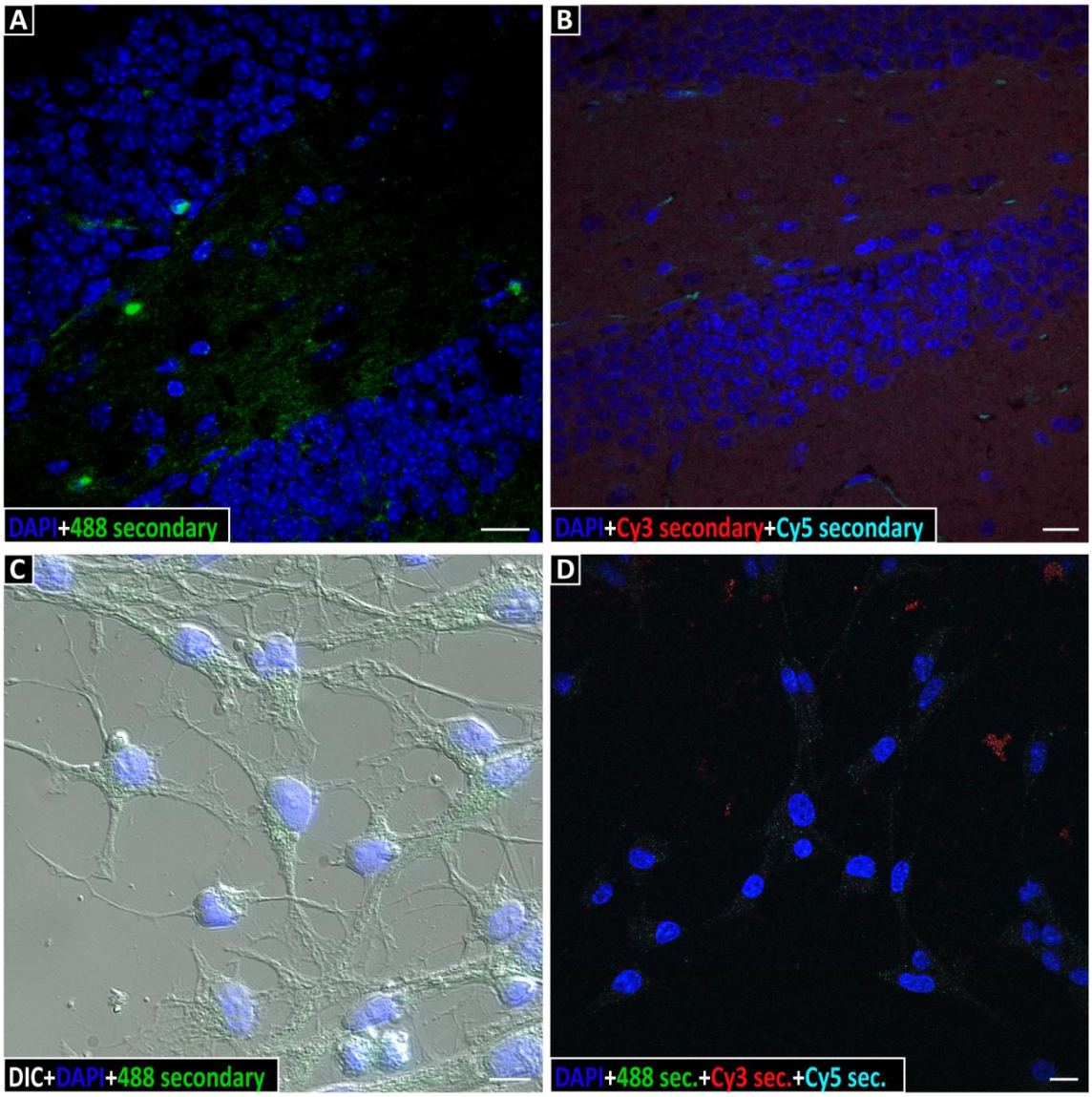

Figure 11.1 Immunochemistry secondary controls

Samples were prepared for immunochemistry as usual, and the primary antibody was omitted. Controls were conducted at each experiment, and included antibodies conjugated to Cy3 (IgG H+L), Cy5 (IgG H+L and IgG1), or Alexa Fluor 488 (IgG H+L), 594 (IgG H+L, IgG1, and IgM) or 647 (IgG H+L and IgG1). Representative images for immunohistochemistry are depicted in A and B, and immunocytochemistry in C and D. Nuclei were labelled with DAPI. Scale bar represents 20 µm for immunohistochemistry samples (A and B) and 10 µm for immunocytochemistry samples (C and D).

11.2 APPENDIX 2: GROWTH FACTOR CONCENTRATIONS PRESENT IN CORNING MATRIGEL MATRIX

Matrigel is a reconstituted basement membrane prepared from a mouse sarcoma rich in extracellular matrix proteins, containing approximately 60% laminin, 30% collagen IV and 8% entactin. The remaining 2% consists of various growth factors, listed below.

Table 11.1 Growth factor concentrations in Matrigel

Amounts of Growth Factors (GF) Present in Corning Matrigel Matrix vs. Growth Factor Reduced (GFR) Corning Matrigel Matrix			
Growth Factor	**Range of GF Concentration in Corning Matrigel Matrix**	**Average GF Concentration in Corning Matrigel Matrix**	**Typical GF Concentration in GFR Corning Matrigel Matrix**
EGF	0.5-1.3 ng/mL	0.7 ng/mL	< 0.5 ng/mL
bFGF	< 0.1-0.2 pg/mL	n.a.*	n.d.**
NGF	< 0.2 ng/mL	n.a.*	< 0.2 ng/mL
PDGF	5-48 pg/mL	12 pg/mL	< 5 pg/mL
IGF-1	11-24 ng/mL	16 ng/mL	5 ng/mL
TGF-β	1.7-4.7 ng/mL	2.3 ng/mL	1.7 ng/mL

*** n.a. – not applicable**

**** n.d. – not determined**

This information has been sourced from the suppliers website (Corning, 2012, 2013).

11.3 APPENDIX 3: NPCS UNDERGO MEMBRANE BLEBBING FOLLOWING EXPOSURE TO ATP

A canonical feature of P2X7 receptors is their ability to form a large transmembrane pore. This pore allows macromolecule exchange and can result in membrane blebbing and cell death. Application of the agonist ATP (1 mM ATP) resulted in the formation of a transmembrane pore, measurable by ethidium bromide fluorescence once intercalated with DNA (Figure 5.8). Live cell microscopy experiments were conducted to visualise the effects of 1 mM ATP on cell integrity. Membrane blebbing was observed following a one hour exposure (Figure 11.2), indicating a loss of membrane potential consistent with the effects of P2X7 pore formation and subsequent macromolecule exchange.

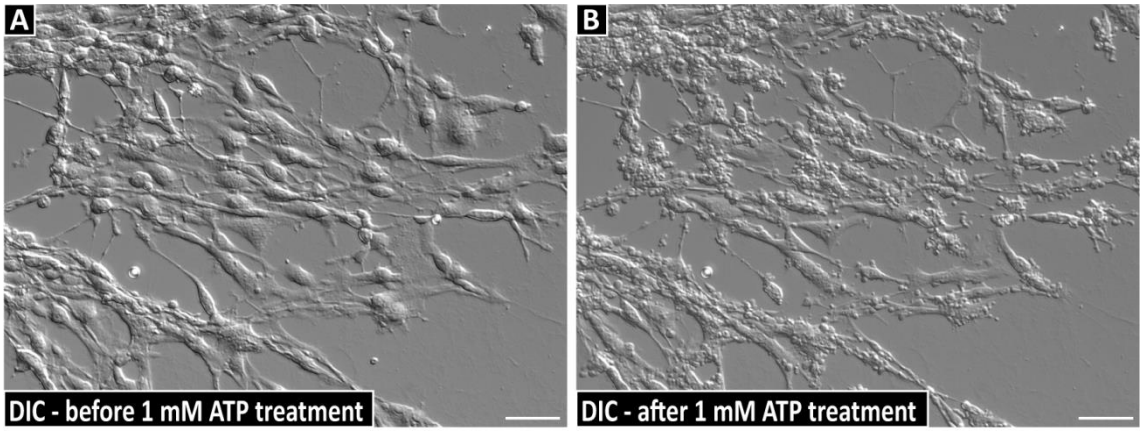

Figure 11.2 NPCs exposed to ATP undergo membrane blebbing

Hippocampal NPCs were plated onto PLO and laminin coated glass coverslips and cultured in proliferation medium for three days. The cells were placed in a live cell microscope and treated with 1 mM ATP and imaged over a one hour time period. A indicates the first image captured immediately prior to ATP addition, while B depicts the NPCs at the end of the incubation time period. Membrane blebbing and a loss of integrity can be seen. Scale bars 20 µm.

11.4 APPENDIX 4: SVZ NEURAL PROGENITOR CELLS DERIVED FROM P2X7 KNOCK OUT MICE DO NOT HAVE FUNCTIONAL P2X7 RECEPTORS

A canonical feature of P2X7 receptors is their ability to form a large transmembrane pore. Ethidium bromide uptake was used to assess the transmembrane pore-forming ability of the P2X7 receptors in SVZ NPCs derived from wild type vs knock out mice, and was measured using time resolved flow cytometry (Figure 11.3). Application of ATP (1 mM ATP) at the 40 second mark (indicated by arrow) resulted in the formation of a transmembrane pore, measurable by ethidium bromide fluorescence once intercalated with DNA. This effect was not observed in the knock out NPCs, demonstrating absence of function P2X7 receptors.

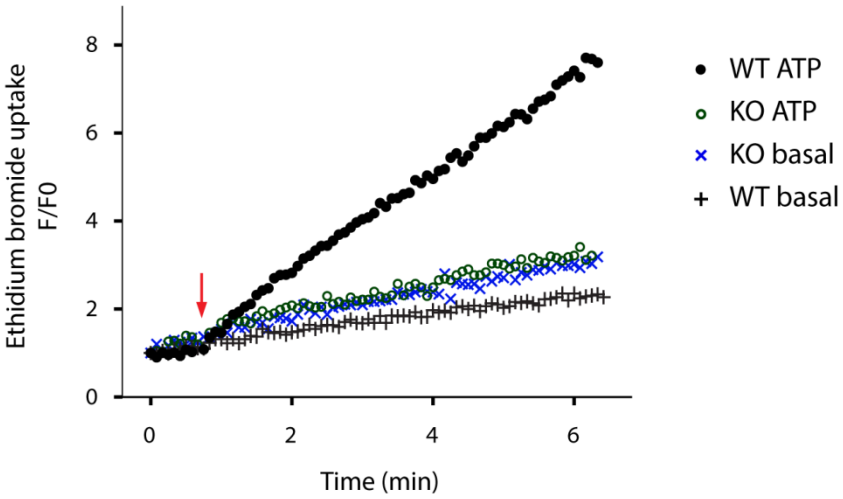

Figure 11.3 P2X7 KO derived NPCs do not have functional P2X7 receptors
Presence of P2X7 receptors was assessed by pore formation, measurable by ethidium bromide uptake. ATP (1 mM ATP) was applied at the 40 second mark (indicated by arrow) and resulted in ethidium bromide uptake in the wild type culture only. This demonstrates absence of functional P2X7 receptors in the culture derived from knock out mice.