Investigating The Regulation of Adult Hippocampal Neurogenesis: Endogenous and Exogenous Cues

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Abstract

The discovery of stem and progenitor cells capable of ongoing neurogenesis in the adult mammalian brain has raised hope that we will one day be able to harness their intrinsic regenerative capacity following injury. Development of such therapeutic strategies relies on a comprehensive understanding of the underlying regulation of the neurogenic process. To this end, I show, in this thesis, that cultured post-natal hippocampal neural progenitor cells (NPCs) express a specific repertoire of connexins (Cx), a family of channel forming proteins critical for communication prior to the development of functional chemical synapses. I show that this pattern of Cx expression, specifically Cx43 and Cx45, is modulated by interaction with the extracellular matrix component laminin providing evidence of extracellular matrix-cell interaction in the regulation of intrinsic Cx expression and function in postnatal NPCs. In adult brain, I show, for the first time, that Cx45 localizes to all cell types of the neuronal lineage with the exception of the type 3 doublecortin (DCX)-positive NPCs. Using a loss of function approach, I show that this expression is required for the normal proliferation of type 1 nestin and glial fibrillary acidic protein-positive stem like NPCs but not for the differentiation or survival of their progeny in the adult hippocampus. With respect to exogenous pharmacological cues that influence hippocampal neurogenesis, this thesis also demonstrates that chronic treatment with a sub-set of selective serotonin reuptake inhibitor antidepressants, fluoxetine and escitalopram, increases the proliferation but not the survival of adult NPCs in healthy, non-depressed mice. Further, standard post-operative analgesia with the opiate buprenorphine inhibits the proliferation of

DCX-positive adult NPCs and increases the survival of their progeny. Finally, over the course of the research for this thesis, it became clear that exposing research animals to even very subtle environmental changes can influence the basal neurogenic process. Ultimately this work further highlights the exquisite sensitivity of the regulation of what is already recognized to be a highly dynamic process and provides important insight into the neurogenic process that can be used to inform future therapeutic development and application.

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List of Abbreviations

ANOVA	analysis of variance
AQP4	aquaporin-4
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BrdU	5-bromo-2-deoxyuridine
Cdk	cyclin dependent kinase
CldU	5-chloro-2-deoxyuridine
CFC	contextual fear conditioning
CNS	central nervous system
Cx	connexin
DCX	doublecortin
DG	dentate gyrus
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DIV	days *in vitro*
dNTPs	deoxynucleotides
DT	diphtheria toxin
DTA	diphtheria toxin A subunit
DTR	diphtheria toxin receptor
EC	extracellular loop
ECM	extracellular matrix
ECT	electroconvulsive therapy
EE	enriched environment
EGF	epidermal growth factor
ER	estrogen receptor
ES	embryonic stem cells
FACS	fluorescent activated cell sorting
FAK	focal adhesion kinase
FBS	fetal bovine serum
FFA	flufenamic acid
FGF-2	fibroblast growth factor 2
FST	forced swim test
GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCL	granule cell layer
GCV	ganciclovir
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GJIC	gap junctional intercellular communication
GR	glucocorticoid receptor
GRA	18α-glycyrrhetinic acid
GZA	glycyrrhizic acid
HPA	hypothalamic-pituitary-adrenal axis
HSV-TK	herpes simplex virus thymidine kinase
KO	knockout

IC	intracellular loop
ICV	intracerebroventricular
IdU	5'-iodo-2-deoxyuuridine
IL-1β	interleukin-1β
IHC	immunohistochemistry
i.p.	intraperitoneal
ISH	*in situ* hybridization
KOR	κ opioid receptor
LSM	laser scanning microscope
LY	lucifer yellow
MAM	methylazoxymethanol acetate
MAP-2	microtubule associated protein 2
MMP	matrix metalloproteinases
MOR	mu opioid receptor
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MWM	morris water maze
NB	neuroblast
NCAM	neural cell adhesion molecule
NeuN	neuronal nuclei
NG2	chondroitin sulphate proteoglycan
NPC	neural progenitor cell
NSC	neural stem cell
NSF	novelty suppressed feeding
OB	olfactory bulb
OPC	oligodendrocyte progenitor cell
ORL-1	opioid like receptor-1
PCNA	proliferation cell nuclear antigen
PET	positron emission tomography
PH3	phospho-histone H3
PI3K	phosphatidylinositol-3 kinase
RD	rhodamine B isothiocyanate-dextran
RG	radial glia
RMS	rostral migratory stream
s.c.	subcutaneous
SEM	standard error of the mean
SGZ	subgranular zone
SSRI	selective serotonin reuptake inhibitor
SVZ	subventricular zone
TAM	tamoxifen
TAPS	transient amplifying cells
TF	Transcription Factor
TM	transmembrane
TST	tail suspension test
TUJ1	class III β-Tubulin

TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
YFP	yellow fluorescent protein
VEGF	vascular endothelial growth factor

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Chapter 1 – General introduction

1.1 Adult neurogenesis – a history

Neurogenesis, defined as the process of generating functional neurons from stem and progenitor cells, was long thought to be restricted to the embryonic and early postnatal period of mammalian development (Ramon y Cajal, 1913). The first suggestion that this could be an ongoing process in human adults was published in the 1940's (Globus and Kuhlenbeck, 1944). It was ultimately, a series of seminal studies by Altman, that clearly demonstrated the generation of new neurons in the postnatal rat hippocampus, neocortex and olfactory bulb for the first time (Altman, 1969; Altman and Das, 1965, 1966; Messier et al., 1958). These reports were initially overlooked receiving relatively little attention, perhaps due to the lack of functional implications and almost certainly due to the difficulty associated with labeling and characterizing these cells. Sporadic reports through the late 70's and 80's demonstrated that newly born neurons in the hippocampus survived for extended periods of time, appeared to receive synaptic input, and extended axons to the expected targets yet with unknown functional implications (Kaplan and Bell, 1983; Kaplan and Hinds, 1977; Stanfield and Trice, 1988). The first real evidence of a functional role for adult neurogenesis was demonstrated by researchers examining seasonal song learning in songbirds (Nottebohm, 2004). Three critical findings (functional and technical in nature) catapulted the field to where it stands today: first was the isolation of adult rodent, and later human, neural stem cells and the development of protocols that allowed for their culture and manipulation *in vitro* (Kukekov et al., 1999; Reynolds and Weiss,

1992). Second, bromodeoxyuridine (BrdU), a synthetic thymidine analogue, was introduced and shown, when injected into a living subject and incorporated in the DNA of cells in S phase, to be later detectable using immunohistochemistry (IHC) (Gratzner, 1982). Third, application of BrdU for birth-dating of mammalian neurons lead to the identification of ongoing neurogenesis in all mammals examined including, most excitingly, humans (Eriksson et al., 1998). Debate continues today as to whether these new neurons arise from a true neural stem cell (NSC) population, with an unlimited capacity for self-renewal and multipotentiality, or a neural progenitor cell (NPC) population, with a finite capacity for self renewal and restricted fate potential (Bull and Bartlett, 2005; Walker et al., 2008). Nevertheless this discovery that the brain has a regenerative capacity, albeit limited, led to great hope that it may be possible to harness these cells to replace lost neurons following injury, illness, and over the course of aging. Development of such therapeutic strategies requires an intimate understanding of all steps of this intricate process and has been the focus of a great many researchers since these early discoveries.

1.2 Adult Neurogenesis – the locations, the cells, and the process

There are two regions within the uninjured adult mammalian brain that are capable of generating functional neurons; the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Figure 1.1A) (Gage, 2000). The neurogenic process in both locations recapitulates that seen during development (with some adult

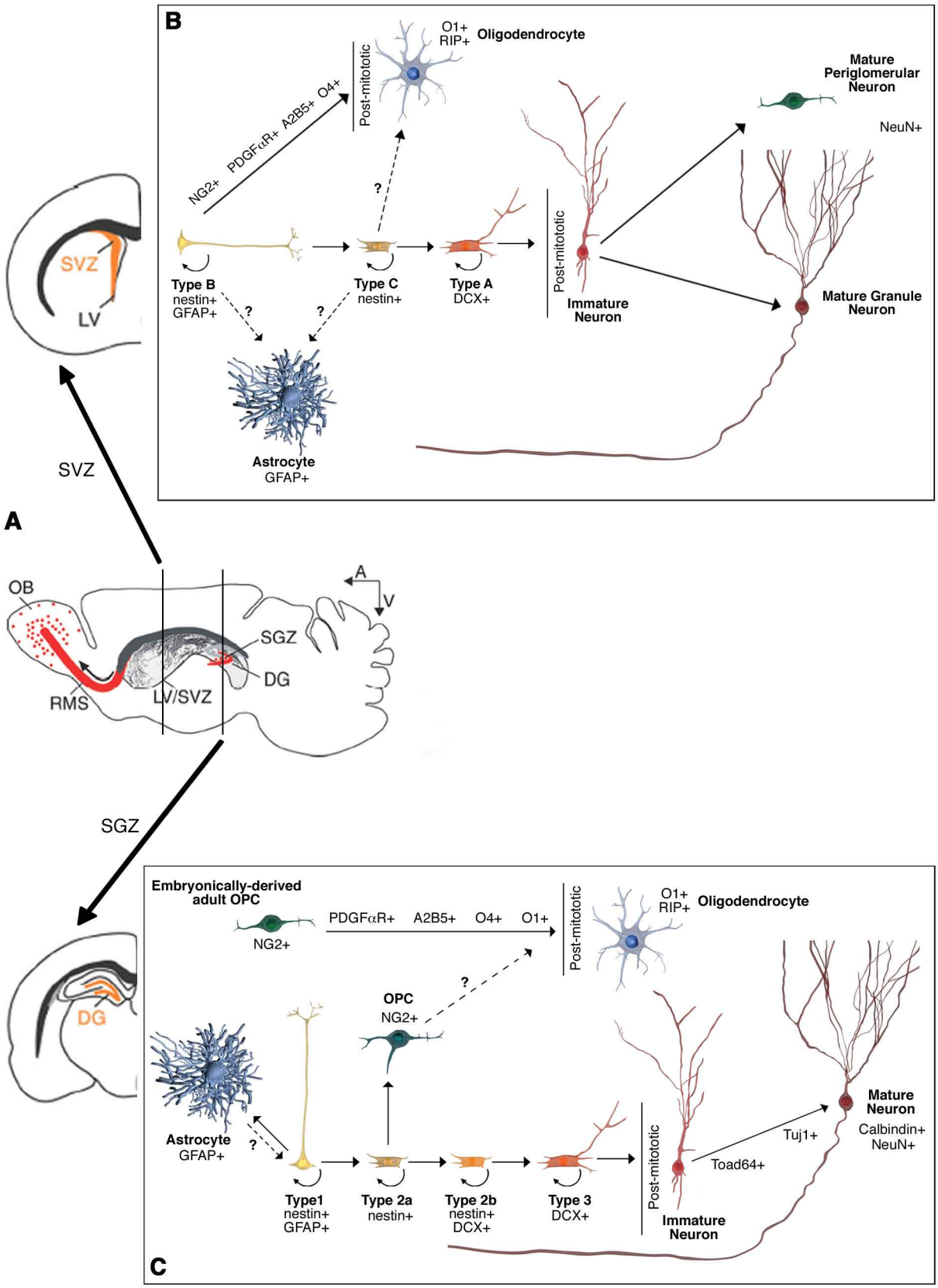
B
O1+
RIP+
Oligodendrocyte
Post-mitotic
NG2+
PDGFαR+ A2B5+ O4+
?
Mature
Periglomerular
Neuron
NeuN+
SVZ
LV
Type B
nestin+
GFAP+
?
?
Type C
nestin+
Type A
DCX+
Post-mitotic
Immature
Neuron
Mature Granule
Neuron
Astrocyte
GFAP+
SVZ
A
OB
A
V
SGZ
DG
RMS
LV/SVZ
SGZ
DG
Embryonically-derived
adult OPC
NG2+
PDGFαR+
A2B5+
O4+
O1+
Post-mitotic
O1+
RIP+
Oligodendrocyte
?
OPC
NG2+
Astrocyte
GFAP+
?
Type1
nestin+
GFAP+
Type 2a
nestin+
Type 2b
nestin+
DCX+
Type 3
DCX+
Post-mitotic
Immature
Neuron
Tuj1+
Toad64+
Mature
Neuron
Calbindin+
NeuN+
C

Figure 1.1

Figure 1.1 - The adult SVG and SGZ support neurogenesis and gliogenesis.

A) This diagram depicts a sagittal mouse brain section highlighting both the SVZ and SGZ, along with coronal views of each region. These are the two regions within the adult brain that have significant neurogenic capacity. This process is followed using a series of antigenic lineage markers that are depicted in panels B and C. B) In the SVZ radial glia-like type B cells can self renew or give rise to either glia or increasingly committed neurogenic precursors, type C. These type C cell in turn give rise to type A neuroblasts which migrate along the RMS to the OB where they terminally differentiate (Alvarez-Buylla and Lim, 2004). C) Within the SGZ type 1 cells are the putative stem cells of the niche, they can self-renew and give rise to both glia and increasingly committed neuronal and glial precursors with restricted self-renewal capacity. First, type 2a cells may give rise to oligodendrocytic precursors or type 2b NPCs, which give rise to type 3 NPCs. The type 3 cells then exit the cell cycle becoming immature neurons and in turn terminally differentiate into mature granule neurons (Kempermann et al., 2004).

refinements) and proceeds in a stepwise fashion that includes the proliferation, fate specification, migration of increasingly lineage restricted progeny followed by the maturation, integration and ultimately survival of these adult born neurons (reviewed in Duan et al., 2008b and Ming and Song, 2011). The nomenclature used to describe the process varies by niche and each will be discussed individually, focusing on the hippocampus, as it is the area of interest for this thesis. These classification systems rely on morphological characteristics along with the expression pattern of specific proteins (referred to collectively as lineage markers in this capacity) to differentiate between cell populations throughout the maturation process. As our understanding of the neurogenic process evolves, the list of lineage markers used to categorize these cells expands including, for example, cytoskeletal components, growth factor receptors and transcription factors. The classical markers used within this thesis will be outlined here but by no means are meant as an exhaustive list.

In the SVZ (Figure 1.1B) the putative stem cells are radial glia-like cells also known as type B cells. Type B cells are characterized by their expression of the intermediate filament proteins nestin and glial fibrillary acidic protein (GFAP). Found adjacent to the ependymal cells lining the ventricle, they have a roughly triangular soma with a short apical cilium that often contacts the ventricle lumen and a long basal process that contacts the local vasculature. Type B cells give rise to transient amplifying type C cells which no longer express the glial marker GFAP and in turn give rise to neuroblasts, also known as type A cells. Type A cells express doublecortin (DCX), a microtubule associate protein, and are the

migratory cells that travel along the rostral migratory stream (RMS) towards the olfactory bulb (OB). Upon their arrival in the OB, neuroblasts migrate tangentially to the appropriate cell layer, undergo terminal differentiation and synaptic integration primarily as inhibitory GABAergic granule or periglomerular neurons with a small subset becoming dopaminergic periglomerular neurons or glutamatergic juxtaglomerular neurons (Alvarez-Buylla and Lim, 2004; Brill et al., 2009; Doetsch et al., 1999). Generation of new neurons in this region is critical for the maintenance of the olfactory bulb.

Several nomenclature and classification systems for hippocampal neurogenesis have been proposed and co-exist within the literature (Ables et al., 2010; Encinas et al., 2011; Kempermann et al., 2004). The model proposed by Kempermann is adhered to in this thesis (with the exception of Chapter 4 where the Ables 2010 model is used) and will be described in more detail here (Figure 1.1C) (Kempermann et al., 2004). As with the SVZ, neurogenesis in the adult hippocampus starts with the putative stem cells of the region, which are radial glia-like type 1 cells. Type 1 cells are found in a niche located between the hilus and the granule cell layer (GCL) of the DG known as the SGZ and have a distinctive triangular soma with a single process that projects through the GCL. These type 1 cells express nestin and GFAP and are generally quiescent, with as little as 1% of their population actively cycling, at any given time (Encinas et al., 2011). Type 1 cells are multipotent, able to generate glia as well as more committed neuronal precursors (Steiner et al., 2004). It is through asymmetric division that type 1 cells give rise to non-radial progenitor cells. Upon the loss of

GFAP, these nestin-positive cells are referred to as type 2a cells. They then gain expression of DCX and are known as type 2b cells (Encinas et al., 2011; Encinas et al., 2006). Transition to a type 3 phenotype occurs with the loss of nestin expression, migration into the inner GCL, and adoption of a rounded morphology and extension of short processes. Type 2a, 2b and 3 cells are all highly proliferative with each stage being increasingly committed to the neuronal lineage (Encinas et al., 2011; Filippov et al., 2003; Fukuda et al., 2003; Kempermann et al., 2004; Kronenberg et al., 2005). The type 3 stage is one of the critical regulatory points for the neurogenic process. Following a robust expansion of the progenitor cell pool through these highly proliferative stages, it is at this point that there is a significant loss of cells thereby regulating the final number of immature neurons generated (Kempermann et al., 2003; Kempermann et al., 2004). Type 3 progenitor cells ultimately step out of the cell cycle and terminally differentiate into postmitotic immature neurons, temporally characterized by a decline in DCX expression and the expression of class III β-Tubulin (Tuj1) and calretinin (Kempermann et al., 2003; Kempermann et al., 2004). Finally, these immature neuronal markers are replaced by expression of markers of mature glutamatergic granule neurons such as the neuronal nuclei protein (NeuN), glutamate, and the calcium binding protein, calbindin (Kempermann et al., 2004). The murine neurogenic process takes on average three to four days following activation of type 1 cells to progress through to the DCX positive type 3 phenotype (Kempermann et al., 2004; Ming and Song, 2005). Markers of mature neurons are generally seen between day 14 and 21

(Kempermann et al., 2004; Lledo et al., 2006; Ming and Song, 2005). The presence of mature neuronal markers does not, however, denote the completion of maturation (Kempermann et al., 2004). A full four to eight weeks is required for integration into the existing circuitry through further development of axonal and dendritic projections, formation and maturation of synaptic connections, and development of electrophysiological properties of functional granule neurons such that they become indistinguishable from their existing GCL neighbors (Jessberger and Kempermann, 2003; Mongiat and Schinder, 2011; van Praag et al., 2002). It is during this integration phase that there is the second critical regulatory point wherein a selection process occurs such that cells that do not form functional or beneficial connections are eliminated; this selection process is finalized by the 4-week time point and number of cells remains consistent thereafter (Kempermann et al., 2003; Tashiro et al., 2006).

1.3 Methodologies for assessing neurogenesis

The study of neurogenesis has historically been technically challenging, with each newly developed technique seemingly catapulting the field forward. Established and developing techniques to study neurogenesis both *in vivo* and *in vitro* will be outlined here and are summarized in Figure 1.2.

1.3.1 *In Vitro* and *Ex Vivo* Techniques

Neural stem cells can be isolated both from the adult rodent (Reynolds and Weiss, 1992) and humans (Kukekov et al., 1999). These cells can be expanded and manipulated in culture; all the while maintaining their multipotency or differentiated using well defined protocols (Figure 1.2D). The relative ease of

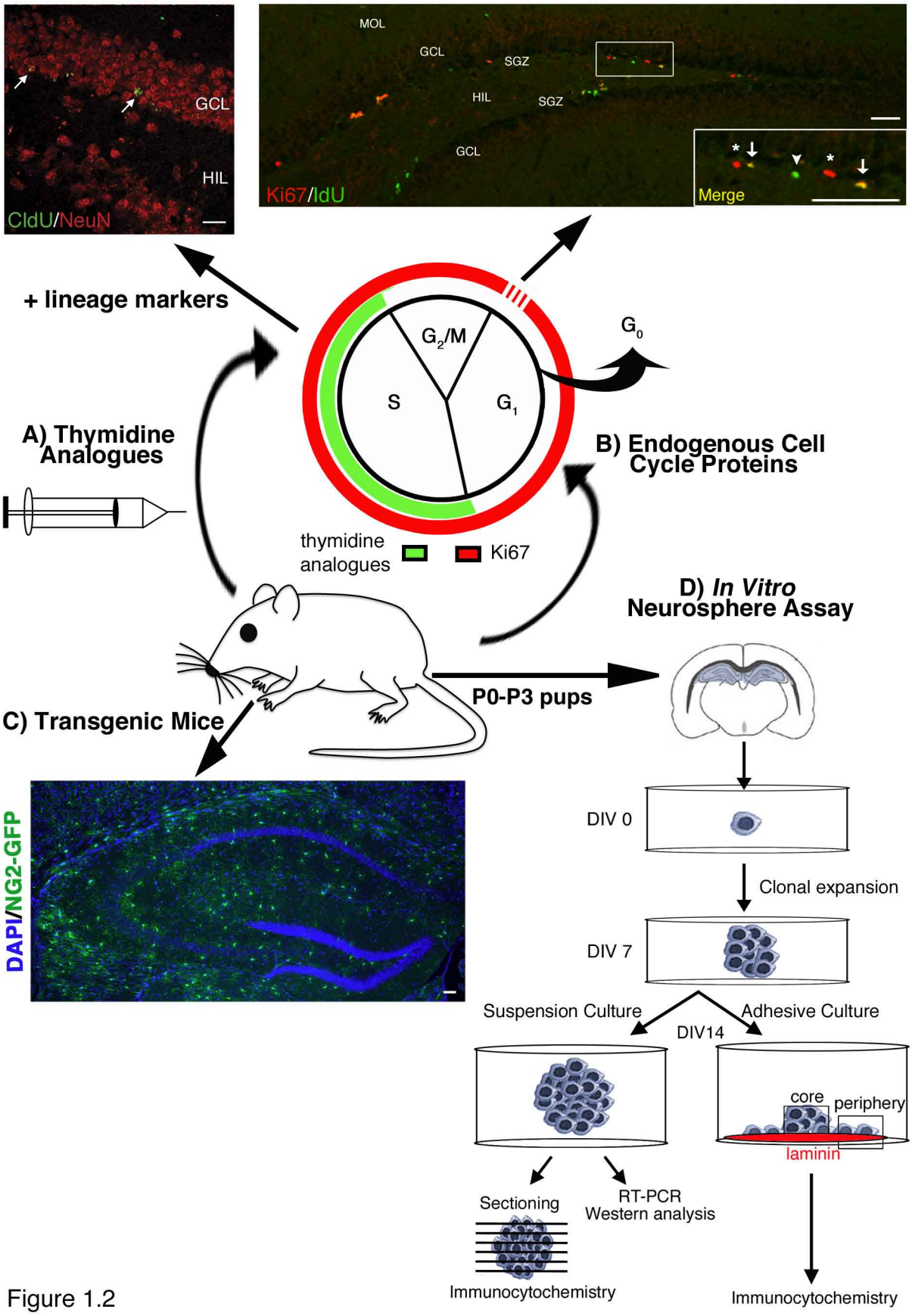
GCL
HIL
CldU/NeuN
MOL
GCL
SGZ
HIL
SGZ
GCL
Ki67/IdU
Merge
+ lineage markers
G2/M
S
G1
G0
A) Thymidine Analogues
B) Endogenous Cell Cycle Proteins
thymidine analogues
Ki67
C) Transgenic Mice
P0-P3 pups
D) In Vitro Neurosphere Assay
DAPI/NG2-GFP
DIV 0
Clonal expansion
DIV 7
Suspension Culture
Adhesive Culture
DIV14
core
periphery
laminin
Sectioning
RT-PCR
Western analysis
Immunocytochemistry
Immunocytochemistry

Figure 1.2

Figure 1.2 - Commonly used methodologies for studying neurogenesis.

This diagram depicts methodologies that were used within this thesis to study neurogenesis. **A)** Thymidine analogues are the primary means used to label proliferating cells within the brain as they are incorporated into cells during S phase. Sacrifice within 24 hours of injection is used to assess proliferation while longer time points are used to assess maturation and survival. These markers may be used in conjunction with antigenic lineage markers to identify the stage of maturation of a given cell at the time of sacrifice. The micrograph shows CldU (green) labeled cells, which have matured into NeuN (red) positive granule neuron 28 days after labeling. **B**) Endogenous cell cycle proteins may be used in place of thymidine analogues to identify proliferation cells in the neurogenic niches or in conjunction with them study cell cycle progression of proliferative cells. Ki67, depicted here, is most commonly used and is expressed throughout the cell cycle except in early G_1. Immunofluorescence shown here depicts a dentate gyrus double labeled for IdU (green) and Ki67 (red) where cells in G_0 are only positive for IdU, G_1+G_2/M are positive for only Ki67 and those in S phase are double positive (yellow). **C)** Transgenic mice are frequently used to follow neurogenesis. Shown here is a coronal section from an NG2CreER^{T2} x ROSA-Tomato mouse following treatment with tamoxifen, which induces recombination within NG2+ cells resulting in the expression of the GFP protein in them. **D)** For the *in vitro* study of neurogenesis the model used is the neurosphere assay, depicted in this schematic. Briefly hippocampi are dissected from P0-P3 mouse pups and dissociated into a single cell suspension. These cell are then grown in suspension with bFGF and EGF where they form neurospheres, cells may continue to be grown in suspension culture and used for immunocytochemistry, RNA and protein analysis or plated onto adhesive substrates and induced to differentiate which may be assessed by immunocytochemistry. All scale bars are 50μm.

their culture and high level of control the researcher has on their environment allows for the precise investigation of the intrinsic and extrinsic factors which regulate neurogenesis. In fact, it is the culture of stem cells that forms the basis of our ability to identify their defining features (i.e. – to demonstrate that they are capable of self renewal and are multipotent), which cannot be rapidly determined in adult brain.

NPC cultured from the adult CNS grow preferentially in defined media supplemented with specific growth factors in both suspension as neurospheres and attached to defined matrices (Gottlieb, 2002). Cells may be plated directly from dissociated tissue (Reynolds and Weiss, 1992) or partially purified (Palmer et al., 1999; Rietze et al., 2001). The most commonly used growth factors used to maintain self-renewal in NPC culture are epidermal growth factor (EGF) and basic fibroblast growth factor (FGF-2). NPC culture is done in two principal manners: (1) neurosphere culture where a single NPC plated on a non-adherent substrate proliferates to form a suspended cluster known as a neurosphere (Reynolds and Weiss, 1992) or (2) cells are plated on adhesive substrates such as laminin (Ray et al., 1993). Cultured NPCs can then be used to study neurogenesis in the tightly controlled culture environment or expanded for transplantation back into a living animal (Philips et al., 2001; Song et al., 2002). NPC culture has allowed for many important discoveries about the neurogenic process. We must, however, remember that these cells have been removed from their niche within the brain, which we also know provides a great many instructive cues likely absent from the culture environment, and thus whenever

possible we must try and confirm our *in vitro* findings in the living animal. This need is highlighted by the very development of the technique itself, which has allowed researchers to clearly demonstrate the multipotentiality of SGZ progenitors *in vitro* where as *in vivo* studies alone would have led us to conclude that they were restricted to generating primarily neurons (Kempermann et al., 2004; Reynolds and Weiss, 1992; Song et al., 2002).

It is also possible to study NPCs in their natural niche using slice culture where it is thought that the neurogenic microenvironment remains largely intact and unlike neurosphere culture allows for the analysis of the integration of maturing cells into the existing circuitry (Noraberg et al., 2005; Raineteau et al., 2004). Acute slice culture is routinely used to assess the electrophysiological properties of progenitor cells and is the predominant application of *ex vivo* approaches in the study of adult neurogenesis (Ming and Song, 2005; Overstreet et al., 2004; van Praag et al., 2002). While it is thought that slice culture better replicates the microenvironment of the neurogenic niche there are technical limitations associated with the longer term organotypic culture necessary to follow the neurogenic process, particularly the differentiation, survival and integration aspects. It is primarily the reduction in neuronal differentiation and a concomitant shift toward glial lineages seen following long term culture that limits its use (Namba et al., 2007). In both the case of the NPC culture and slice culture the standard techniques discussed below may be applied to study neurogenesis.

1.3.2 In Vivo Techniques

I - Thymidine and analogues

Exogenous markers of DNA synthesis were the first and remain the primary means of labeling NPCs and their progeny (Altman and Das, 1965; Eisch and Mandyam, 2007; Vega and Peterson, 2005). The seminal studies that identified neurogenesis in adult mammals used tritiated [H^3] thymidine, visualized using autoradiography to identify actively dividing cells (Altman, 1969; Altman and Das, 1965, 1966). It was, however, the introduction of halogenated thymidine analogues, BrdU and later analogues iododeoxyuridine (IdU) and cholorodeoxyuridine (CldU) (Gratzner, 1982; Vega and Peterson, 2005), which are visualized using IHC thereby eliminating the dangers of radiation associated with [H^3]-thymidine that revolutionized the study of neurogenesis and became a standard technique in the field (Figure 1.2A).

Systemically administered thymidine and its analogues readily cross the blood brain barrier (BBB) via the nucleoside transporter where they are incorporated into the new strand of DNA of any cell in the S phase of the cell cycle (Spector and Johanson, 2007). These compounds are most often administered via intraperitoneal (i.p.) injection but may also be given orally or through subcutaneous implantation of osmotic minipumps to deliver it systemically or regionally when combined with intraventricular canulation. By varying the dosage, frequency, combination of analogues administered, and the time point examined it is possible to quantitatively analyze the proliferation, differentiation, migration and survival of NPCs and their progeny. A single

injection, at a concentration high enough to saturate the actively dividing cells, is the most common administration paradigm reported (Landgren and Curtis, 2011). Experimental animals are generally sacrificed 1 min to 24 hrs following the injection in order to examine proliferation or 28 days post injection to examine survival (Eisch and Mandyam, 2007; Taupin, 2007b). There are however a great many well established administration/sacrifice paradigms that carefully consider the population of cells to be studied (i.e. – differential labeling of the almost quiescent type 1 vs. rapidly proliferating type 3 cells) and the data which the researcher plans to collect (i.e. – variations in the cell cycle vs. migration defects) (reviewed in Taupin, 2007b).

While widely used, thymidine analogue labeling does have certain limitations. BrdU being the oldest and most routinely used thymidine analogue has the greatest body of research about it and will be discussed here, however, the same considerations apply to all analogues. BrdU cannot be used to analyze living cells as it visualization requires tissue fixation and DNA denaturation prior to immunocytochemistry. Also its labeling is limited to the nucleus and thus requires the use of confocal microscopy (Z-stacks) to accurately confirm colocalization with cell markers (Rakic, 2002). BrdU labeling is diluted beyond detectable levels after 3 cell divisions that may skew interpretations of the data generated with it (Hayes and Nowakowski, 2002). First used as a chemosensitizing agent, BrdU can be toxic and mutagenic, leading to concerns that its administration may in and of itself affect neurogenesis or our interpretation of it (Bannigan, 1985; Ross et al., 2008). Furthermore we must

remember that BrdU is not a marker of cell division but rather DNA synthesis and as such other processes may lead to labeling with BrdU including DNA repair and cell cycle re-entry associated with certain apoptotic events (Kruman, 2004). Finally given that BrdU enters the brain via transporters in the BBB, it is necessary to control for the integrity of the BBB in experimental conditions (Spector and Johanson, 2007). While refinement of the dosage/administration paradigms and the inclusion of the appropriate experimental controls have led to a general acceptance that the protocols used today are safe and label strictly proliferating NPCs (Landgren and Curtis, 2011), we must take care to consider these possibilities when interpreting our data and increasingly incorporate complementary approaches to confirm results generated with these techniques (Bauer and Patterson, 2005; Cooper-Kuhn and Kuhn, 2002; Taupin, 2007b).

Ii - Cell cycle proteins

One of the primary means of overcoming the issues with thymidine analog administration is to use endogenous cell cycle proteins (Figure 1.2B). The cell cycle, which consists of: gap 1 (G_1), when the DNA prepares for replication, synthesis (S), when the DNA is replicated, gap 2 (G_2), when the cell prepares for physical division and mitosis (M), when the cell physically divides, is regulated by a well documented series of protein-protein interactions (Harper and Brooks, 2005). These proteins vary in the number of cell cycle stages during which they are expressed, their phosphorylation state, and, in some cases, their subcellular localization according to cell cycle stage. Using one or more of these proteins alone, or in combination with an exogenous S phase marker, is extremely useful

in the analysis of proliferation and cell cycle kinetic of NPCs. Furthermore these markers allow for the study of neurogenesis in human tissue postmortem without prior manipulation of the living patient (Reif et al., 2006). While the list of cell cycle proteins that could be used for such analyses is extensive (for a review see (Eisch and Mandyam, 2007)), the three most commonly reported proteins will be discussed here: proliferating cell nuclear antigen (PCNA), Ki-67 and phosphohistone-H3 (PH3) all of which have been used to study neurogenesis in the human brain (Curtis et al., 2011).

PCNA is a protein subunit of the DNA polymerase delta and epsilon that can be detected by IHC or *in situ* hybridization (ISH) in the nucleus (Miyachi et al., 1978). It is expressed throughout the cell cycle at varying levels, peaking in S phase and as such has been used as a marker of proliferation in the adult mammalian brain (Linden et al., 1992; Zacchetti et al., 2003). One must however be careful as it may also be seen in G_0 due to its long half-life (20 h) (Zacchetti et al., 2003). Furthermore given its role in DNA synthesis, PCNA expression may also be evident during DNA repair or cell death, much like BrdU can be incorporated into cells undergoing DNA repair/damage (Paunesku et al., 2001).

Ki-67 is a nuclear protein that is expressed throughout the cell cycle with the exception of early G_1 and G_0 (Zacchetti et al., 2003). Despite its strong correlation to proliferating cells it is only in recent years that evidence supporting its functional role in cell cycle progression are beginning to emerge (reviewed in Ross and Hall, 1995). The number of Ki-67 positive cells in the adult hippocampus is comparable to the number of PCNA positive cells and generally

two times that of BrdU labeled cells (when examined shortly following BrdU injection), as is expected given that thymidine analogues only label cells in S phase (Jinno, 2011; Kee et al., 2002). One advantage of Ki-67 over both BrdU and PCNA is its absence in cells undergoing DNA repair (Zacchetti et al., 2003). Further studies are, however, needed to confirm if it is expressed by all proliferating cells in the mammalian brain and if its expression varies between or even within a given neurogenic niche (Eisch and Mandyam, 2007).

Histone H3 is part of the histone octamer and is phosphorylated (PH3) during late G_2 and M phases (Hendzel et al., 1997). PH3 is now routinely used to label proliferating cells in the adult hippocampus, exploiting its strong association with the DNA during mitosis to distinguish between the individual phases (Del Bigio, 1999; Eisch and Mandyam, 2004; Mandyam et al., 2004).

The use of endogenous cell cycle markers to identify cycling cells in neurogenic niches is not without limitations. As was highlighted when defining the individual proteins, it is important to know the details of your marker (i.e. – its regulation, half life, localization, phosphorylation state, etc…) and the antibody (i.e. – specificity for human vs. rodent protein isoforms, phospho-specific, optimal antigen retrieval steps, etc…) being using to detect it. Also, we cannot forget that these markers will not label the post-mitotic progeny of the proliferating cells or identify the specific lineage to which these cells belong. Both of these analyses require the use of additional protein and/or morphological markers, which were discussed in section 1.2 (Figure 1.1A and B). It is important to remember when immunostaining for these endogenous proteins that their antigenicity may be

affected by the fixation method selected and in the case of human sample, the postmortem delay until fixation (Boekhoorn et al., 2006; Liu et al., 2008; Sierra et al., 2011). Furthermore when quantifying endogenous cell cycle markers, it is important to use unbiased stereology and if colocalization with lineage markers is desired this must be combined with confocal microscopy, in order to have an accurate assessment of the experimental measure (Sierra et al., 2011). When these relevant technical considerations are accounted for endogenous cellular proteins represent an invaluable tool in the study of neurogenesis.

Iii - Transgenic Mice

The development of transgenic mice is an exciting and ever expanding area of study that is generating a significant amount of data in regards to understanding the neurogenic process in addition to producing innovative experimental tools and models in which to study neurogenesis (Figure 1.2C). Due to the great number of mouse lines generated in recent years it would be impractical to outline them all here but they have been recently reviewed by Dhaliwal (Dhaliwal and Lagace, 2011). The general classifications of model types, their uses and limitations will however be outlined here.

a) Constitutive reporter mice - The earliest transgenics generated were mice that allowed for the visualization, characterization and isolation of specific cellular populations in the adult brain. Generally these mice have the regulatory elements of a cell type specific gene (e.g. – nestin) and a reporter gene (e.g. – lacZ or green fluorescent protein, GFP) to mark a desired cell type. These constructs are injected into embryonic stem cells leading to random insertion into

the mouse genome. This may however lead to lack of expression or ectopic expression due to positional effects (Picciotto and Wickman, 1998). To circumvent this problem researchers now use more specific targeting strategies to direct the desired genetic manipulation (Duchala et al., 1996; Hofker, 2003; Kim et al., 2010). These targeted approaches may also be used to knockout a gene of interest in a specific cell type replacing the target gene (or exon) with the reporter gene construct. Understanding the regulatory elements of the cell type specific gene whose promoter is being employed is very important in this approach. The use of the nestin promoter illustrates this well. Nestin is an intermediate filament protein expressed by type 1 and 2a NPCs in the SGZ and frequently used in CNS specific transgenic mouse lines, it is also expressed by non-neural tissue (Lendahl et al., 1990; Wiese et al., 2004). In order to restrict the expression of a reporter gene to the CNS using the nestin promoter, one must include the enhancer element from the second intron of the rat nestin gene (Zimmerman et al., 1994). Another consideration when designing transgenic mouse lines is the localization of the marker. Most often these mice display whole cell cytoplasmic expression of the selected fluorescent marker which while usefully for many applications (i.e. - morphological analysis) may not be optimal for others (i.e. – for quantification) where the inclusion of a nuclear localization signal may be desirable. Further limitations of these mice include the length of expression of the marker gene. Theoretically the marker is expressed only as long as the promoter driving it is active, this makes these mice useful for the visualization, characterization and quantification of the specific cell type labeled

along with short term fate mapping or for isolation of these specific cells (i.e. – using fluorescent activated cell sorting, FACS). In practice, the half-life of the reporter protein may lead to its persistence and temporally indistinct results, as has been observed in several adult neurogenesis studies (Brill et al., 2009; Regan et al., 2007). There also remain unanswered questions about the potential toxicity of the reporter gene products themselves. While generally considered to be biologically inert there have been reports of GFP toxicity in embryonic development (Torbett, 2002).

b) Conditional and inducible transgenic mice – Building on this basic transgenic mouse technology was the introduction of the Cre/loxP system (Orban et al., 1992). A conditional transgenic mouse generally has the bactriophage Cre recombinase under the control of a cell or tissue specific promoter and a second gene of interest flanked by loxP sites or in the case of a reporter gene a stop codon flanked by loxP sites upstream of the reporter gene. Following activation of the selected promoter, Cre recombinase is expressed and excises the loxP-flanked region resulting in its knockout if a gene of interest is selected or expression of the reporter if an upstream stop codon is removed. This change is permanent, resulting in the loss of the gene of interest or expression of the marker gene in the specified cell population and its progeny. Use of this approach has been somewhat limited within the neurogenesis field as the majority of the specific promoters researcher's use to study adult neurogenesis also play critical roles in embryonic development making it difficult to distinguish the developmental vs. adult effects these manipulations are having on

neurogenesis. Nevertheless the use of a GFAP-Cre mouse crossed onto a lacZ reporter line led to the one of the key studies confirming that adult born neurons are generated from GFAP positive neurogenic stem cells (Garcia et al., 2004b).

Two major inducible systems have been developed to allow for recombination as described above to either knockout or in a gene at a specific time point, although others do exist (Saunders, 2011; Yamamoto et al., 2001). First is the inducible Cre system where the expression of Cre recombinase is once again under the control of a specific promoter, it is however, fused to the ligand binding region of an estrogen responsive (ER) element. When treated with an estrogen ligand such as tamoxifen (TAM), the TAM binds to the ER and allows for translocation of the Cre to the nucleus where it can act on loxP sites (Feil et al., 1996; Indra et al., 1999). Second is the Tet-off system, in which a selected promoter controls the expression of tTA and the second transgene has the gene of interest under the control of the tTA-responsive TetOp promoter (Gossen and Bujard, 1992). In this system the gene of interest or marker is expressed as normal when the tTA binds to the TetOP promoter but when tetracycline or doxycycline are administered tTA cannot bind to the TetOp promoter and expression of the downstream genes are turned off. This system has also been manipulated to generate a Tet-On system in which addition of doxycycline results in the expression of a gene of interest (Urlinger et al., 2000). The Tet system is particularly advantageous as it allows for a reversible manipulation simply by starting or stopping doxycycline treatment.

Both of these systems have been used to fate map many stages of the neurogenesis process by labeling a population of cells (i.e. - nestin or doublecortin (DCX) expressing cells) to allow for researchers to follow the proliferation, differentiation and survival of their progeny and also to investigate the role that specific genes play in the neurogenic process by knocking them out (Beech et al., 2004; Lagace et al., 2008; Lagace et al., 2007). In several instances multiple labs have generated similar models to investigate the same processes and yet have highly variable results. One such example are the NestinCreER^{T2} marker mice, in some cases labeling has been restricted to one or the other of the SVZ/RMS or SGZ, and in another cases it has been found in both, and in yet other lines expression of the marker has also been found in non-neurogenic regions of the brain (Burns et al., 2007; Chen et al., 2009; Lagace et al., 2007). These models are somewhat difficult to compare for a variety of reasons, including but not limited to: variability in recombination efficiency, differing protocol used to or induce expression of the Cre recombinase, variations in the design of the constructs used to generate the mice, location of the transgene insertion and background mouse strain selected (Dhaliwal and Lagace, 2011). However, these differences can be used to a researchers advantage as studying one or more models in combination may allow for specific discoveries about individual cellular populations or brain regions more easily than dissecting apart the differences from a mouse model where all populations are affected.

As our understanding of genetic manipulation grows the mice which can be generated seems almost endless. Beyond the use of transgenic mice to mark cells or investigate the effects of gene ablation on neurogenesis come its investigation under pathological or injury conditions. Ongoing studies are subjecting marker mouse lines to injury models (i.e. - stroke, traumatic brain injury or epilepsy) or crossing them onto disease models (i.e. – Alzheimer or Parkinson's disease) (Winner et al., 2011). Perhaps even more exciting is the application of these technologies to investigate the functional implications of neurogenesis. In order to definitively address this question there is the need to be able to eliminate and/or increase neurogenesis. Much of this work to date has been done using gamma irradiation or cytotoxic agents such as methylazoxymethanol acetate (Saxe et al., 2007; Shors et al., 2002). These approaches are however rife with concerns regarding the off-target effects of such harsh treatments which target all dividing cells in the body, making a genetic approach to the ablation of neurogenesis appealing. Several researchers have used the Herpes simplex virus thymidine kinase (HSV-TK) to ablate dividing NPCs upon treatment with ganciclovir (GCV) under the control of various NPC targeted promoters (i.e. – GFAP, nestin, DCX) (Garcia et al., 2004b; Jin et al., 2010; Saxe et al., 2007; Singer et al., 2011; Yu et al., 2008). Several other groups have employed the inducible Cre/loxP system. To specifically ablate NPCs researchers have expressed of diphtheria toxin or its receptor under the control of the nestin promoter (Arruda-Carvalho et al., 2011; Buch et al., 2005; Imayoshi et al., 2008). Similarly this approach has been used to increase

neurogenesis by knocking out the pro-apoptotic protein BAX in nestin positive populations thereby reducing cell death of NPCs (Dupret et al., 2008; Sahay et al., 2011). Each of these approaches (summarized in Table 1.1) has generated valuable data regarding not only the process of neurogenesis but also the involvement of neurogenesis in learning, memory and increasingly emotion. These mouse lines do however require further development to overcome problems associated with fertility, systemic illness caused by the inducing compounds and poor recombination efficiency resulting in incomplete ablation (reviewed in Dhaliwal and Lagace, 2011). It is however clear that the possible explorations using such approaches are almost endless.

iv - ^{14}C labeling to follow neurogenesis in post-mortem human brain

Given the ultimate clinical impact that the study of neurogenesis aims to have, understanding neurogenesis in humans is critical but technically very challenging. Use of ^{14}C birth dating of cells in post-mortem tissue takes advantage of the dramatic global increase of ^{14}C in the atmosphere due to nuclear weapons testing and exponential decrease after 1963. This technique has been validated and could be used to retrospectively birth date cells (Spalding et al., 2005).

v – Visualizing neurogenesis in living subjects

All of the techniques described to this point have been used to generate static analyses in fixed tissue. While monumental discoveries have been made using these techniques, it is becoming increasingly apparent that neurogenesis is

Table 1.1 – Genetic models to increase or decrease adult hippocampal neurogenesis

Gene	Specific promoter	Effect on Neurogenesis	Reference
HSV-Tk	GFAP	Decrease	Bush et al. (1998) Garcia et. al. (2004)
	Nestin	Decrease	Deng et al. (2009) Yu et al. (2008) ^
	DCX	Decrease	Jin et al. (2010)
TRE-Bax*	Nestin-rtTA	Decrease	Dupret et. al. (2008)
iBax (Takeuchi et al., 2005)	NestinCreER^{T2}	Increase	Sahay et al. (2011)
NSE-DTA (Kobayakawa et al., 2007)	NestinCreER^{T2} (Imayoshi et al., 2006)	Decrease	Imayoshi et al. (2008)
DTA (Brockschnieder et al., 2006)	NestinCreER^{T2} (Lagace et al., 2007)	Decrease	D.Lagace (personal communication)
iDTR (Buch et al., 2005)	NestinCreER^{T2} (Imayoshi et al., 2006)	Decrease	Arruda-Carvalho et al. (2011)

^ HSV-Tk lacking a fragment between the first and second codon
* Tetracycline Response Element (TRE)-Bax mice have a TRE responsive minimal CMV (CytoMegaloVIrus) promoter which drives the expression of Bax

a dynamic process with an almost endless set of modulators that would benefit from analyses in real-time within the same subject. Therefore there is a real push to generate minimally invasive protocols that would allow for the monitoring of neurogenesis within a living subject. Not only is this of great interest for the scientific community seeking to understand the neurogenic process in model species, it would also allow for a clearer understanding of the process in humans which at present is limited to investigation in post-mortem tissue. Furthermore i*n vivo* imaging is essential in order to be able to implement and monitor cell replacement/regenerative therapies.

Optical imaging has evolved significantly in recent years and when used in combination with transgenic and retroviral approaches to label NPCs, it is a very powerful tool in preclinical studies. Given the abundance of marker mice expressing fluorescent proteins in existence today researchers sought a means to image those proteins directly within the living animal. Unfortunately the shallow tissue penetration for the existing imaging platforms, in addition to the high auto-fluorescence of biological tissue precludes this possibility at present. The focus has instead turned to an alternate strategy using bioluminescent luciferases. In rodents engineered to express the firefly luciferase under the control of a specific promoter or injected with a retrovirus expressing the luciferase, following anesthesia and the administration of the luciferase's substrate, D-luciferin, imaging using an extremely sensitive photodetector in a dark chamber allows for visualization of the expressing cells. Both the retroviral and transgenic approach to label NSCs for their visualization in an intact living

animal have proven successful (Couillard-Despres and Aigner, 2011; Couillard-Despres et al., 2008). The lack of endogenous bioluminescence allows for highly sensitive detection; however, due to the depth of the neurogenic structures in the brain there is a significant scattering of the light emitted from luciferase expressing cells reducing its resolution. Furthermore the distribution and enzyme kinetics for the luciferase and its substrate must be considered in the interpretation of results from these models.

Clinically a technique developed using an existing imaging platform would be most desirable and expedient. As such techniques using magnetic resonance imaging (MRI) and positron emission tomography (PET) are the current focus. MRI offers the best morphological resolution available to date in voxels of 78 x 78 x 370 μm within rodent CNS structures *in vivo* (Weber et al., 2006). Initially it was hypothesized that volume based analysis of structures with ongoing neurogenesis could be used as a surrogate measure of neurogenic rate. Depressed patients do in fact display reduced hippocampal volume on MRI (Sheline et al., 1999); however, postmortem studies suggest that this is due to cellular shrinkage rather than neurogenic changes (Muller et al., 2001). Also if one considers the approximately 36 million cells that are in the human hippocampus relative to the approximately 600 that are added each day it seems unlikely that overall volume measurements of the hippocampus will be informative (Czeh and Lucassen, 2007). Finally this approach would not allow for tracking neurogenesis at the level of a single cell.

These limitations have lead to the search for a marker that could be used to label proliferating cells and their progeny, that could be visualized using MRI or PET and are biologically inert. Iron oxide can be used as a dark contrast agent for MRI (Norman et al., 1992) and has been shown to be engulfed by NPCs *in vitro* (Hoehn et al., 2002). At present the use of this technique to study neurogenesis *in vivo* is limited by the need for *ex vivo* loading of iron oxide into cells for transplantation or its stereotaxic injection into the brain. Other serious limitations include problems with imaging at the site of injection, the fact that iron oxide labeling is permanent and its toxicity remains unknown, our inability to distinguish between intra- vs. extra-cellular iron particles and the possibility of extracellular particles being taken up by other cells, such as microglia, leading to misinterpretations about neurogenesis (reviewed in Couillard-Despres and Aigner, 2011).

PET scanning is based on the concentration of radioactively labeled molecules at specific sites. Recently a report demonstrated *in vivo* detection of proliferation in the neurogenic niches of the rat brain using [^{18}F]-FLT, a thymidine analogue (Rueger et al., 2010). While exciting there are some issues to resolve prior to widespread use of this technique which include the lack of signal intensity differences between neurogenic niches despite differing rates of neurogenesis and the inability to identify the origins of the labeled cells. Nevertheless this could be an exciting breakthrough in the study of neurogenesis in human patients.

1.4 Adult Neurogenesis – endogenous and exogenous regulation

Adult neurogenesis is a tightly regulated process with a host of physiological, pathological and pharmacological cues differentially affecting specific stages of the process and ultimately shaping the final outcome (Encinas et al., 2006; Kempermann, 2006; Kronenberg et al., 2003). It is this regulation that is the central focus of this thesis. I will discuss aspects of the endogenous regulation of basal neurogenesis, along with both positive and negative exogenous regulators of neurogenesis relevant to the specific aims addressed in this study.

1.4.1 The role of the microenvironment in regulating NPC fate

Neural stem and progenitor cells have been found outside of the SGZ and SVZ in the uninjured adult mammalian brain; however, these niches do not support the generation of neurons (Gage, 2000). In the granule layer of the dentate gyrus and the olfactory bulb (the terminus for differentiated progeny born from NPCs in the SGZ and SVZ respectively), we see specification of progenitors into primarily neurons with some astrocytes. However, both resident NPCs and those transplanted from these neurogenic niches to ectopic locations consistently differentiate into glia under basal conditions (Seidenfaden et al., 2006; Shihabuddin et al., 2000). When proliferating cells from these “gliogenic” regions are transplanted into the SGZ or SVZ, they demonstrate capacity to generate mature neurons (Seidenfaden et al., 2006; Shihabuddin et al., 2000). This highlights the importance of the niche or microenvironment in which the NPCs are found for neurogenesis.

The “stem cell-permissive” niches, both neurogenic and gliogenic, are composed of a diverse population of cells including immature and mature neurons, astrocytes, oligodendrocytes, microglia, NPCs, extracellular matrix components and a significant vasculature. The SGZ is depicted in Figure 1.3. Each of these instructive cells and all of the tissue architecture likely contribute to the regulation of either a neurogenic or gliogenic program both through direct cell to cell contact/communication and the release of soluble factors, particularly from the astrocytes and endothelial cells surrounding the vasculature (Leventhal et al., 1999; Palmer et al., 2000; Shen et al., 2004; Song et al., 2002). While each of these components could be discussed in great detail I will focus on the role of connexin-mediated communication and extracellular matrix (ECM) interactions in the regulation of the neurogenic process, as they are two of the experimental foci in this thesis. For a recent overview of the regulatory role of the neurogenic niche as a whole, please refer to (Miller and Gauthier-Fisher, 2009).

i) Connexin mediated control of neurogenesis

An important method by which NPCs receive external environmental cues and communicate with neighboring cells is through connexin-mediated communication (Figure 1.4). Connexins are a multigene family with 20 known members in the mouse and 21 in humans. Gene names are classified according to homology and order of discovery (i.e., murine Gja1 and human GJA1 were the first alpha connexin to be identified and code for the protein connexin 43 (Cx43)) (Sohl and Willecke, 2003). The original connexin protein naming convention grouped family members based on overall homology but has now been replaced

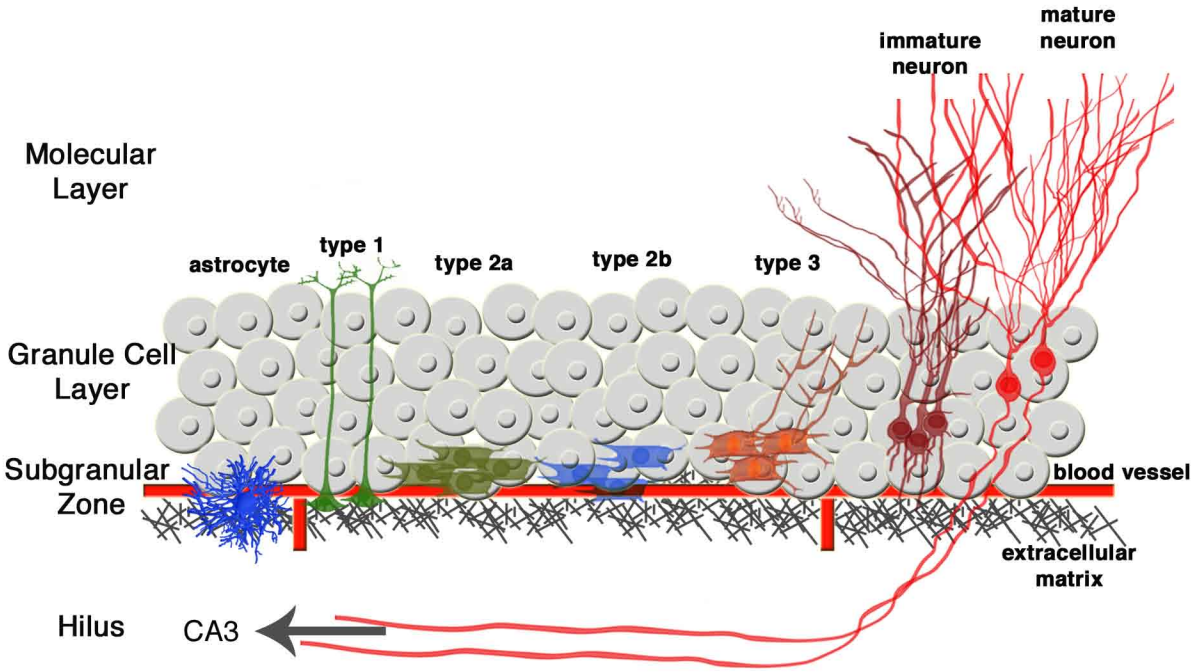
mature
neuron
immature
neuron
Molecular
Layer
astrocyte
type 1
type 2a
type 2b
type 3
Granule Cell
Layer
Subgranular
Zone
blood vessel
extracellular
matrix
Hilus
CA3

Figure 1.3

Figure 1.3 – The SGZ niche.

This schematic depicts the diverse population of cells and overall environment found in the SGZ that play a crucial role in dictating stem cell fate. These cells include the most common mature granule neurons along with astrocytes, microglia and oligodendrocytes. This is in addition to the putative stem cells of the area, type 1 radial glia-like cells and their more mature and neuronally committed progeny including type 2a, 2b and 3 cells which ultimately give rise to post-mitotic immature neurons. There is also a rich ECM, providing structural/adhesive elements in addition to important effects on intracellular signaling in addition to a significant vascular component within the region which not only brings external cues to the niche but whose component cells also secretes relevant instructive factors.

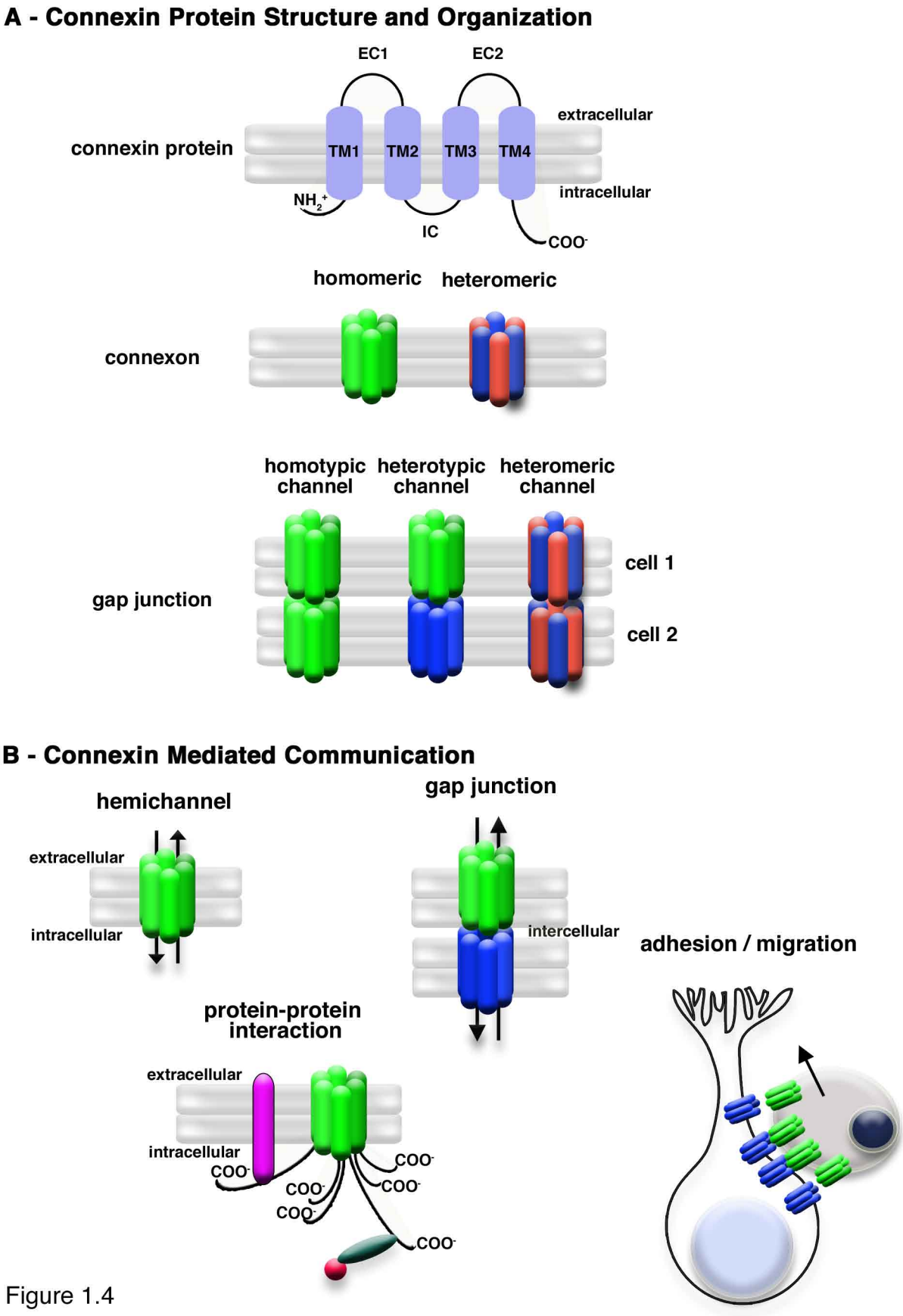
A - Connexin Protein Structure and Organization
EC1
EC2
connexin protein
TM1
TM2
TM3
TM4
extracellular
intracellular
NH2+
IC
COO-
homomeric
heteromeric
connexon
homotypic channel
heterotypic channel
heteromeric channel
gap junction
cell 1
cell 2
B - Connexin Mediated Communication
hemichannel
extracellular
intracellular
gap junction
intercellular
adhesion / migration
protein-protein interaction
extracellular
intracellular
COO-

Figure 1.4

Figure 1.4 – Connexin protein structure, organization and communication.

A) Connexin proteins consist of an intracellular N terminal tail, four transmembrane domains connected by two extracellular loops and an intracellular loop and an intracellular C terminal tail. Six connexin proteins hexamerize to form a connexon, which may be homomeric, consisting of a single connexin type or heteromeric with more than one connexin family member. Two connexons in adjacent membranes can dock to form a gap junction, if it is made up of a single connexin it is referred to as a homotypic channel, if the two connexons are made up of different connexins it is a heterotypic channel and if it includes heteromeric connexons it is a heteromeric channel. B) Connexins are known to contribute to communication in four ways to date. First, hemichannels consisting of unpaired connexons in a membrane allow for the passage of molecules up to 1.6kDa between the intra- and extracellular spaces. Second, gap junctions allow for the passage of such molecules between the intracellular space to two docked cells. Third, connexins also have channel independent signaling functions through protein-protein interactions that occur predominantly at their C terminal tail and fourth through adhesive properties that contribute to cellular migration.

by a system using predicted molecular weight (i.e., connexin 45 (Cx45) has a predicted molecular weight of 45kDa) (Sohl and Willecke, 2003). Table 1.2 provides a complete list of all murine and human connexins identified and their homologies as we currently understand them.

An individual connexin protein has 4 membrane spanning hydrophobic regions, two extracellular loops, an intracellular loop as well as an N and C terminal tail (Figure 1.4) (reviewed in Goodenough et al., 1996). The greatest divergence between family members is seen in the intracellular loop and C termini. Six connexin proteins hexamerize to form a connexon. These "channels" may be made up of a single connexin family member, named a homomeric connexon, or different connexins, heteromeric. When found in a non-junctional membrane a connexon is also known as a hemichannel or single membrane channel allowing for communication between the cell's cytosol and the extracellular environment of the cell (i.e., pore-forming properties). When connexons in two adjacent cells dock through interaction of their extracellular loops, they form an intercellular channel between these adjacent cells, which allows for movement of ions, small metabolites and second messengers up to 1.6 kDa in size from one cell to another (reviewed in Evans and Martin, 2002 and Zlomuzica et al., 2010). Connexins of the same type can dock in a homotypic channel. Two different homomeric channels can form a heterotypic channel. Finally, two heteromeric channels can dock forming a heteromeric channel (Sohl et al., 2005). It should, however, be noted that not all connexon pairs will form functional channels. The clustering of multiple connexin channels form the

Table 1.2 – Human and mouse connexin genes and proteins

Human Connexin	Previous Human Gene Name	Gene Name (Human/Mouse)	Previous Mouse Gene Name	Mouse Connexin
Cx43	nc	GJA1 / Gja1	nc	Cx43
Cx46	nc	GJA3 / Gja3	nc	Cx46
Cx37	nc	GJA4 / Gja4	nc	Cx37
Cx40	nc	GJA5 / Gja5	nc	Cx40
---	---	Gja6	nc	Cx33
Cx50	nc	GJA8 / Gja8	nc	Cx50
Cx59	GJA10	GJA9	---	---
Cx62	---	GJA10 / Gja10	---	Cx57
Cx32	nc	GJB1 / Gjb1	nc	Cx32
Cx26	nc	GJB2 / Gjb2	nc	Cx26
Cx31	nc	GJB3 / Gjb3	nc	Cx31
Cx30.3	nc	GJB4 / Gjb4	nc	Cx30.3
Cx31.1	nc	GJB5 / Gjb5	nc	Cx31.1
Cx30	nc	GJB6 / Gjb6	nc	Cx30
Cx25	nc	GJB7	---	---
Cx45	GJA7	GJC1 / Gjc1	Gja7	Cx45
Cx47	GJA12	GJC2 / Gjc2	Gja12	Cx47
Cx31.3	GJE1	GJC3 / Gjc3	Gje1	Cx29
Cx36	GJA9	GJD2 / Gjd2	Gja9	Cx36
Cx31.9	GJA11	GJD3 / Gjd3	Gja111	Cx30.2
Cx40.1	---	GJD4 / Gjd4	---	Cx39
Cx23	---	GJE1/ Gje1	---	Cx23

nc – no change

morphologically distinct gap junction plaque detectable by freeze fracture replica immunogold labeling (FRIL) (Kamasawa et al., 2006). In addition to their pore and channel forming properties, connexins may be involved in signaling processes through protein-protein interactions, which primarily occur at the intracellular C terminal tail, as well as through the formation of adhesion domains (reviewed in Mroue et al., 2011 and Prochnow and Dermietzel, 2008). Specificity of communication (both channel-dependent and independent) is dictated by specific connexin composition, post-translational modification, namely phosphorylation, as well as assembly and disassembly, with both hemichannel and gap junctions known to be voltage-sensitive, and regulated by intracellular pH and extracellular calcium concentration (Rackauskas et al., 2010).

Not unexpectedly given the number of connexin isoforms, at least one family member is expressed in most tissue and cell types of the body. The presence of multiple isoforms in a single tissue is thought to confer a mechanism for functional compensation in the case of genetic mutation or deletion (Bedner et al., 2011). The high level of homology between the family members has, however, resulted in significant cross-reactivity of antibodies and RNA probes which in addition to the lack of knockout controls within early studies in the field have lead to discrepant reports, making it difficult at times to truly define the connexin make up of a tissue (Bennett et al., 1999; Fowler et al., 2009). Presently, it is generally accepted that in the adult brain, neurons express Cx36 and Cx45, oligodendrocytes Cx29, Cx32 and Cx47, astrocytes Cx26, Cx30 and

Cx43, and activated microglia Cx36 (Dobrenis et al., 2005; Mese et al., 2007). Compatible homotypic connexons form functional homotypic (Cx36, neuron-neuron; neuron-microglia and Cx45, neuron-neuron) and heterotypic gap junction channels (Cx32-Cx26; Cx32-Cx30; Cx47-Cx43 oligodendrocyte-astrocyte), as determined by FRIL, dye transfer and electrophysiological investigations, which when examined at a more global level lead to the formation of cellular syncytiums within the CNS (Altevogt and Paul, 2004; Nagy et al., 2004; Orthmann-Murphy et al., 2008; Rash et al., 2001).

While chemical transmission is the most commonly discussed form of transmission in the adult brain the metabolic and electrical transmission contributed by gap junctions are equally important, notably during development. This is likely why the role of gap junctions in the CNS and neurogenesis, in particular, has been most extensively characterized during brain development prior to establishment of functional chemical transmission. Numerous studies both *in vivo* and *in vitro* show strong coupling in proliferating progenitor cells that is downregulated with differentiation, supporting a role for intercellular communication in the maintenance of proliferation and direction of differentiation (Bittman et al., 1997; Duval et al., 2002; Rozental et al., 1998). Cells within the developing ventricular zone/neocortex express Cx26 and 43, amongst others, and allow for coupling between neural precursors and radial glia cells with their expression decreasing after the peak period of embryonic neurogenesis (Nadarajah et al., 1997). The expression of these two connexins are also differentially regulated across the cell cycle, with no coupling being observed

between Cx26 and Cx43 connexons suggesting a compartmentalization of cells depending on their expression of connexins. Furthermore pharmacological blockade of these gap junctions results in an arrest of the cell cycle (Bittman et al., 1997; Bittman and LoTurco, 1999). As traditional gap junction biology suggests, this intercellular coupling likely directs proliferation and differentiation by creating gradients of intercellular signaling molecules, such as second messengers and small metabolites that provide important positional information to the embryo (Bruzzone and Dermietzel, 2006). However, single membrane channels also play a role in regulating proliferation within the developing ventricular zone where they are required for the initiation of calcium waves in NPCs, without which there is an arrest of proliferation (Weissman et al., 2004). A third property of gap junctions has also been implicated in the embryonic neurogenic process. Elias et al. demonstrated that Cx26 and 43 in radial glia within the developing ventricular zone are also necessary for the proper migration of developing neurons to the cortical plate, independent of intercellular communication but rather as a result of the adhesive properties of the gap junctions allowing the developing neuron in essence to crawl up the radial glia process by stabilizing its leading process (Elias et al., 2007).

It remains unclear whether these connexin-mediated functions are recapitulated during adult neurogenesis. Until recently even the expression profile of connexins in adult NPCs had not been described. As part of this thesis, our lab has examined the expression profile of connexins at both the mRNA and protein level in postnatal hippocampal derived neurospheres (Imbeault et al.,

2009). Both the mRNA and protein of Cx26, 29, 30, 37, 40, 43, 45 and 47 were detected in these neurospheres and this profile was changed upon the induction of differentiation, suggesting that they may be involved in this dynamically regulated process. My contribution to this paper is presented in Chapter 2.

Further, albeit still limited, understanding into the role of connexins in the regulation of adult neuro- and gliogenesis has also come from examination of naturally occurring genetic mutations along with murine gene deletion models. Cx32 mutations in humans lead to X-linked Charcot-Marie-Tooth disease, a demyelinating peripheral neuropathy (Bergoffen et al., 1993). Interestingly, in single Cx29, 32 or 47 knockout mice, only very mild, if any, CNS myelination deficits, are seen; however, Cx32/47 double knockout and Cx29/32/47 triple knockout mice show severe myelination defects and develop a tremor and seizures which result in death by the sixth postnatal week (Imbeault, 2010; Menichella et al., 2003). Our laboratory has shown that Cx32 is expressed in NG2+ and PDGFαR+ oligodendrocyte progenitor cells (OPCs) in the adult mouse hippocampal dentate gyrus and that turnover of the NG2+ population is increased in these mice (Melanson-Drapeau et al., 2003). Further unpublished work subsequent to this study showed that Cx32 knockout mice are able to effectively replace injured hippocampal pyramidal cells following excitotoxic injury leading to a restoration of behavioral indices, presumably by altering OPC cell fate (Melanson-Drapeau, 2006). These studies formed the framework for our interest in the continued study of the role of connexins in adult neurogenesis and its manipulation for potential therapeutic intervention.

Since beginning my PhD studies, the astrocytic connexins, 30 and 43, have been examined by others in relation to hippocampal neurogenesis. Not only do astrocytes play an important role in directing most aspects of neuronal maturation but the resident stem cell population in the hippocampus is radial glia-like and express all three astrocytic connexins, 26, 30 and 43 (Kunze et al., 2009). In fact single cell RT-PCR coupled with the injection of a tracer dye in these radial glia-like cells within the intact adult hippocampal dentate gyrus have provided the first demonstration of gap junctional cellular coupling of adult NPCs in this area. Unpublished data from our laboratory shows that a loss of Cx30 alone reduces the number of proliferating DCX+ neuroblasts in this area (Toeg, 2008). While others have shown that the loss of both Cx30 and 43, which leads to a complete absence of astrocyte and radial glial cell coupling, reduced hippocampal NPC proliferation by 80%, ultimately reducing the number of newborn neurons generated (Kunze et al., 2009). These results lend further credence to the hypothesis that connexin mediated communication is critical for the regulation of adult hippocampal neurogenesis.

Of the greatest interest in the study of the involvement of connexins in neurogenesis are the neuronal connexins themselves, Cx36 and 45. In contrast to other cell and tissue types, there is minimal gap junctional coupling between mature neurons and these two connexins display different coupling properties (Sohl et al., 2005). Furthermore, they do not appear to be expressed within the same neuronal subtypes within the hippocampus, Cx36 restricted to parvalbumin-positive interneurons and Cx45 reported in NeuN-positive pyramidal

neurons of the CA3 region of the hippocampus, suggesting that they do not have the same localization and thus redundancy or potential for compensation following mutation or gene ablation, although *de novo* compensatory expression cannot be ruled out (Condorelli et al., 2000; Hormuzdi et al., 2001; Maxeiner et al., 2003; Peinado et al., 1993; Rash et al., 2000; Rash et al., 2001). Both of these connexins are regulated across development, peaking during the period of embryonic corticogenesis (or shortly thereafter) and display dramatically reduced and regionally restricted expression in the mature adult (Cina et al., 2007; Hormuzdi et al., 2001). Thus it has been suggested that the neuronal connexins play a key role in the developmental process but, following the development of mature synaptic connections, they are no longer critical for neuronal communication (Cina et al., 2007). Their involvement in adult neurogenesis has not however been evaluated. Our laboratory first examined Cx36 as it is considered to be the predominant neuronal connexin. Using Cx36 knockout mice, a previous MSc student demonstrated that the loss of Cx36 does not affect hippocampal NPC proliferation or survival under basal conditions or following excitotoxic injury. She did however observe an increased resistance to cell death in these mice suggesting that Cx36 mediated signaling may play a role in neuronal vulnerability to injury (Sorbara, 2009). Underlying mechanisms are currently the focus of colleagues in my laboratory.

In contrast to Cx36 there is relatively little known about Cx45 within the CNS. Its mRNA expression profile has been reported using a lacZ reporter mouse, and protein based on mouse lines where Cx45 is c terminally tagged with

eGFP (Maxeiner et al., 2003; von Maltzahn et al., 2009; Zlomuzica et al., 2010). These reports describe the presence of the Cx45 mRNA and protein predominantly in the CA3 field of the hippocampus but do not discuss the dentate gyrus. Using the Willecke Cx45eGFP mouse it has been reported that Cx45 is expressed by the neurogenic transient amplifying progenitor cells and neuroblasts of the SVZ along with the ependymal cells which line the lateral ventricles, where it partially co-localizes with Cx43 (von Maltzahn et al., 2009; Zlomuzica et al., 2010). The Cx45eGFP mouse was however unable to rescue the embryonic lethality of the constitutive Cx45 knockout mouse, which occurs due to cardiac defects. Thus despite seemingly proper membrane localization of the protein, gap junctions formed with the fusion protein are likely functionally impaired (von Maltzahn et al., 2009). Using a neuron targeted Cx45 knockout mouse Zolumzica *et al.* have demonstrated that the loss of Cx45 results in impaired novel object recognition, despite normal exploratory behavior and place recognition (Zlomuzica et al., 2010). Electrophysiological recording from these mice also revealed reduced amplitude of kainate-induced γ-oscillations in the CA3 and a larger full width maximum in the CA1, which is suggestive of a slight desynchronization of neurons in these mice (Axmacher et al., 2006). Synchronous neuronal activity within the hippocampus is thought to be important for the formation of long-term memory, the primary hypothesized function for adult neurogenesis in uninjured tissue. This, in combination with the behavioral deficits and localization of Cx45 to NPC subtypes in the SVZ, suggest that it

could play an important role in adult hippocampal neurogenesis: a hypothesis that will be investigated in this thesis.

ii) Extracellular Matrix

Another important means by which cells detect external environmental signals is through receptor-mediated interactions with the ECM. The system is uniquely responsive thanks to the heterogeneous nature of ECM components and specific patterns of receptor expression resulting in a finely tuned response to specific conditions. Fibronectin, for instance, is predominantly known to suppress stem cell proliferation and differentiation; while laminin, is most permissive to these processes although this can vary significantly according to stem cell type and experimental system studied (Andressen et al., 2005; von der Mark and Ocalan, 1989). In the developing embryonic brain, the interaction of $\alpha 1\beta 6$ integrin receptors and laminin define specific proliferative units within the ventricular zone, furthermore the interaction of $\beta 1$ integrin receptors and laminin directs the migration of neuroblasts generated in this embryonic ventricular zone along the RMS to the OB, a regulatory event which is maintained in the post-natal adult brain (Belvindrah et al., 2007; Lathia et al., 2007). Intriguingly, both of these events are also associated with specific patterns of gap junctional coupling, as was discussed in the previous section, suggesting that ECM interactions could be involved in the regulation of Cx expression to achieve these events.

The manner in which the ECM components affect gene expression and post-transcriptional signaling cascades to regulate stem cell proliferation, migration and differentiation in the adult brain are not yet fully understood.

Specific patterns of ECM cleavage, through the action of matrix metalloproteinases (MMPs), are thought to underlie the involvement of ECM in directing the neurogenic process. In the brain it has been shown that a spatiotemporally regulated pattern of MMP-9 expression in the cerebellum relates to granule cell migration during development. MMP-2 contributes to the breakdown of ECM barriers that impede astrocyte movement while MMP-2 and 3 play a role in dendro-axonal growth of neurons in the developing cortex (Ogier et al., 2006; Vaillant et al., 2003; Zuo et al., 1998). Further, MMP-3 and 9 play a role in hippocampal dependent learning (Meighan et al., 2006). While direct evidence for involvement of MMP mediated breakdown of the ECM in the adult neurogenic process *in vivo* is limited, it is clear that they are abundantly expressed by neural stem cells supporting the hypothesis that their role in adult neurogenesis and gliogenesis recapitulates that seen during development (Frolichsthal-Schoeller et al., 1999). Another important function of the ECM is to modulate the response to growth factors through the direct binding of growth factors and functional modulation of receptor tyrosine kinases. These interactions have been shown to promote migration, proliferation and survival in various stem cells (Alam et al., 2007; Rahman et al., 2005; Wijelath et al., 2006). Finally, and likely best characterized, is the activation of specific signaling cascades in response to cell attachment to the ECM. The currently identified cascades predominantly result in the cytoskeletal reorganization required for migration and center on the focal adhesion kinase (FAK). In addition to its role in migration FAK may regulate NPC proliferation and/or differentiation through

activation of mitogen-activated protein kinase (MAPK) or phosphatidylinositol-3 kinase (PI3K) pathways (Wojcik-Stanaszek et al., 2011).

The literature discussed here strongly support a role for the ECM in modulating the embryonic neurogenic process under physiological conditions. While not outlined here, there is a significant body of literature suggesting that the same is true in the injured adult brain; however, despite a clear role in the developing brain, mechanistic details are lacking and it remains to be seen if these ECM mediated mechanisms of regulating neurogenesis are recapitulated in the adult brain. Given the notable overlap in the role of gap junctions and specific ECM interactions in regulating neurogenesis within the developing neocortex, I sought in this thesis to determine if there is a specific effect of ECM interactions on the expression of connexins, my interest being Cx43 and 45 specifically, in postnatal hippocampus using an *in vitro* approach.

1.4.2 Negative regulators of neurogenesis

Aging is the best-characterized negative neurogenic stimulus and is considered universal, affecting all studied species (Cameron and McKay, 1999; Kempermann et al., 1998b; Leuner et al., 2007). In fact decreased neurogenesis has been hypothesized to underlie the cognitive decline that is commonly associated with aging (Cameron and McKay, 1999; Montaron et al., 2006). While researchers agree that the final result of aging is a decrease in baseline neurogenesis and neurogenic response to positive stimuli, there is debate as to how this occurs (Encinas and Sierra, 2011). The longest held and most prominent hypothesis has been that stem cells remain in the DG throughout the

life of the animal but become senescent, loosing mitotic capacity, with age (Hattiangady and Shetty, 2008). There is, however, a hypothesis, with recently emerging evidence, that stem cells in the DG undergo activation-dependent depletion, where following activation they undergo an average of three rapid asymmetric divisions and then terminally differentiate into mature astrocytes (Encinas et al., 2011). The mechanisms that underlie the age dependent decrease in neurogenesis, regardless of the hypothesis you favor, are largely unknown but likely reflect a contribution from age related changes in the neurogenic niche including, but not limited to: chronic inflammation, increased adrenal corticosteroids and decreased growth factors along with the well characterized age dependent telomere shortening and oxidative DNA damage (reviewed in Encinas and Sierra, 2011 and Lee et al., 2011).

While aging is regarded as the most robust and universal negative regulator of neurogenesis there are a great many stimuli that have been shown to have negative effects on some or all of the stages of the neurogenic process. They include a variety of neurodegenerative and psychiatric diseases, traumatic injuries, physical and psychological stressors along with drugs of abuse. This introduction will however be limited to the effects of anxiety/depression and opiates on neurogenesis as they are central focuses of this thesis (Figure 1.5).

i) Opiates

Opiates are a class of compounds that are constituents or derivatives of the opium poppy plant. Opiates are predominantly used for analgesia, although

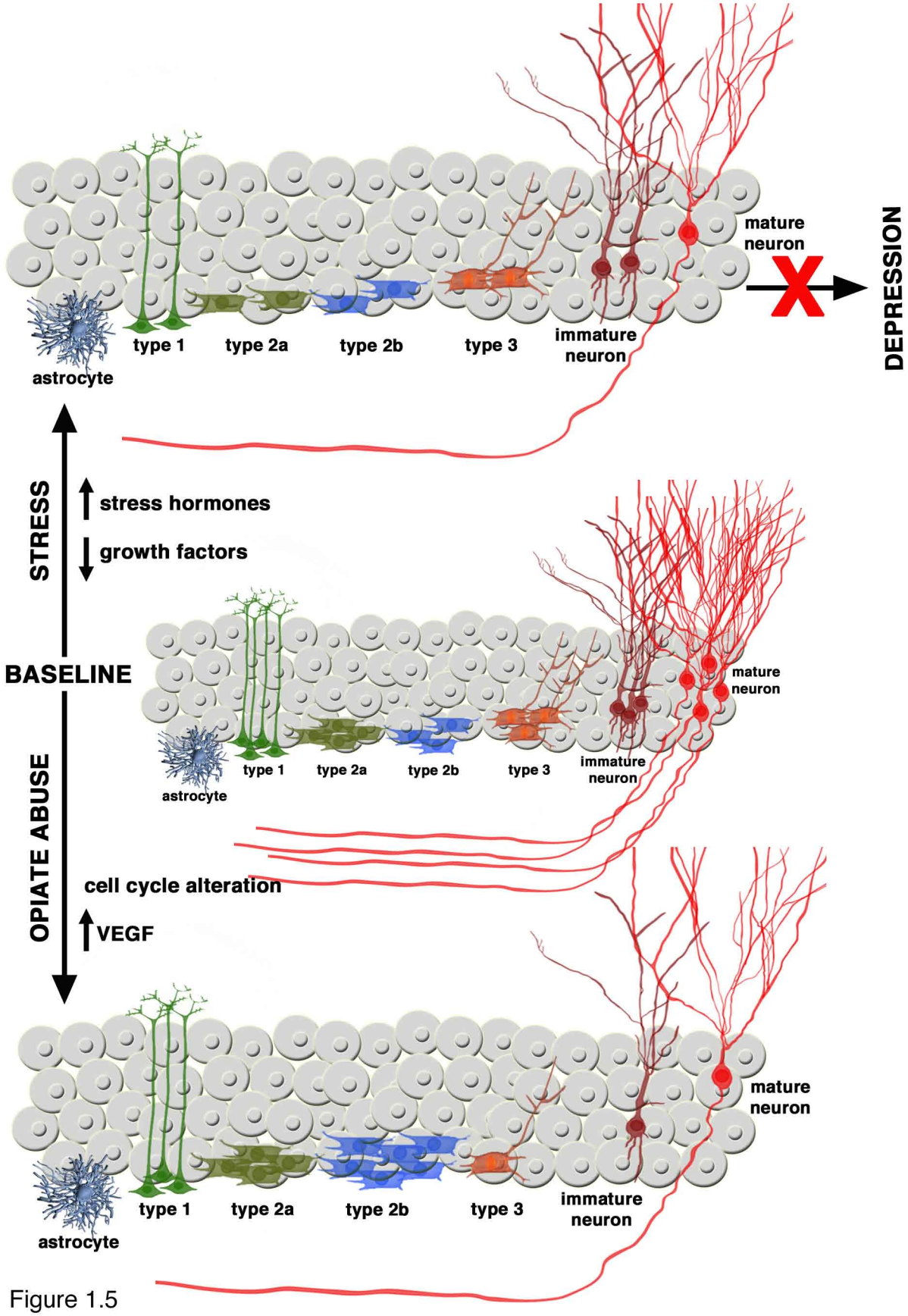
mature neuron
DEPRESSION
type 1
type 2a
type 2b
type 3
immature neuron
astrocyte
STRESS
stress hormones
growth factors
BASELINE
mature neuron
type 1
type 2a
type 2b
type 3
immature neuron
astrocyte
OPIATE ABUSE
cell cycle alteration
VEGF
mature neuron
type 1
type 2a
type 2b
type 3
immature neuron
astrocyte

Figure 1.5

Figure 1.5 – Negative regulators of neurogenesis: stress and opiate abuse.

Stress results in an increase in circulating stress hormones and a decrease in the expression of growth factors leading to an overall decrease in the proliferation of NPCs and number of mature neurons generated from this process. Interestingly, these changes alone are do not result in depression in animal models. Opiate abuse has been shown to alter the cell cycle of proliferating NPCs and increase the expression of VEGF within the hippocampus leading to the reduced proliferation of type 3 DCX+ NPCs, an accumulation of the preceding type 2b cells, and fewer mature neuronal progeny.

some family members are now being administered “off-label” to treat addiction and mood disorders (Davis, 2007; Howland, 2010; Likar et al., 2007). Beyond their conventional uses opiates represent the third most commonly abused class of drugs in the world (Chawla, 2011).

Addiction is the most expensive neuropsychiatric disorder due not only to the cost of healthcare but also the loss of productivity and crime associated with it (Uhl and Grow, 2004). It is a chronic, relapsing disease that is accompanied by neurological impairments including deficits in cognition, motivation, attention, behavioral disinhibition, mood disorders, impulsiveness and aggressiveness. Opiate abusers most often display impairments in executive and memory functions, which can persist for years despite abstinence from drug taking (Ersche et al., 2006). These findings lead researchers to hypothesize that long-term negative effects may occur in the brains of drug takers, particularly in those structures associated with learning and memory such as the hippocampus. Further supporting this was the knowledge that opiates negatively regulate neural progenitors *in utero* and in early development but little was known with respect to their impact on the adult brain (Reznikov et al., 1999; Sargeant et al., 2008a).

The study of the impact of opiates on hippocampal neurogenesis in the adult brain since 2000 has lead to the consensus that chronic exposure to opiates leads to impaired neurogenesis in rodents (reviewed in Eisch and Harburg, 2006; Sargeant et al., 2008b). The studies published to date focus on morphine and use forced administration paradigms either via subcutaneous (s.c) pellet implantation or i.p. injection; however a more pertinent model of abuse

intravenous (i.v.) self-administration of the opiate, heroin, also reduces progenitor cell proliferation in the SGZ (Arguello et al., 2008) (Eisch et al., 2000; Fischer et al., 2008; Kahn et al., 2005; Mandyam et al., 2004). Regardless of the administration paradigm used, decreased proliferation and survival of NPCs are only observed following a chronic treatment which leads to a high, relatively steady blood morphine level and dependence on the drug (Fischer et al., 2008). Cell cycle phase analysis of BrdU positive cells in chronic morphine-treated mice revealed a reduction in the number of cells in S phase 24 hours following treatment accompanied by an increase in the number of apoptotic cells at this time point (Arguello et al., 2008). By 96 hours following treatment, BrdU positive cells showed premature mitosis due to shortened G_2/M phase but no changes in cell death (Mandyam et al., 2004). Taken together, these findings suggest that, following the initial exposure to morphine, actively dividing cells are more susceptible to its effects but, with longer exposure, cell cycle is affected. A more detailed lineage analysis revealed a decrease in the number of type 3 DCX^+ progenitor cells paired with an increase in the preceding type 2b stage with morphine treatment both of which correspond to the overall decrease in mature neurons seen 28 days after treatment (Arguello et al., 2008; Eisch et al., 2000; Mandyam et al., 2004). Interestingly these changes in proliferation seem to be reversible following withdrawal from the drug and may even lead to a rebound increase in proliferation (Kahn et al., 2005).

The mechanisms by which opiates elicit their negative effects on neurogenesis remain uncertain. Opiates are known to activate the hypothalamic-

pituitary-adrenal (HPA) axis leading to an increase in corticosteroids, which can decrease neurogenesis in and of themselves (Gould et al., 1992; Majewska, 2002). HPA activation does not appear to underlie the decreased neurogenesis observed with morphine treatment as the phenotype persists following adrenalectomy with corticosterone replacement to stabilize circulating stress hormone levels (Eisch et al., 2000). The neurogenic niche, is an important regulator of the neurogenic response as it is rich in vasculature and both pro- and anti- proliferative factors such as brain derived neurotrophic factor (BDNF) and interleukin-1β (IL-1β) respectively (Song et al., 2002). An examination of the protein levels for these latter factors and their receptors in the DG of control and morphine-treated mice revealed no changes following treatment (Arguello et al., 2009). Expression of vascular endothelial growth factor (VEGF), but not its receptor, is increased following 96 hrs of treatment, when NPC proliferation is decreased (Arguello et al., 2009). This finding is counterintuitive due to the pro-proliferative effects most commonly attributed to VEGF. VEGF is however also know to have angiogenic properties which likely underlay its role here, as the increase in VEGF following morphine treatment correlates with an increase in vessel perimeter and area (Arguello et al., 2009). While it remains necessary to confirm causality, the authors suggest that these changes are occurring as a compensatory change in an effort to aid the remaining NPCs to survive following opiate treatment. Yet another possible explanation for the effects of morphine on neurogenesis is a direct action on the stem and progenitor cells themselves. While it remains unknown in what subtype of NPCs, if any, the mu opioid

receptor (MOR) is expressed *in vivo,* it does appear to be likely as expression is detected in cultured adult hippocampal NPCs with functional response to MOR ligands, including morphine (Persson et al., 2003). Furthermore MOR knockout mice have enhanced survival of new neurons and an increase in GCL neuronal number as a result, (Harburg et al., 2007). It is tempting to speculate that a differential expression of this receptor may underlie the cell type-specific effects of morphine on neurogenesis, but further research is required in this area (Garcia et al., 2004a).

As a relatively new area of study, the investigation of the effects of opiates on neurogenesis has been limited to the context of drug abuse. Certainly drug abuse is a significant societal problem; however, these compounds are administered far more frequently in a clinical setting for their analgesic properties and as such it is important to investigate their impact on neurogenesis in this far more prevalent context. Furthermore, researchers have yet to tackle what mechanistic contribution these neurogenic changes are making to the cognitive and emotional effects seen following opiate administration (be it legal or not). Insight gained from such studies is crucial and could fundamentally alter how opiates are administered.

ii) Anxiety and Depression – is it all just stress?

Mental health disorders including: anxiety, affective and depressive disorders, are responsible for a significant financial, societal and personal burden today (Andrews et al., 2000). Despite the numerous treatment options available there are still a great many patients who go untreated, experience minimal

treatment efficacy or relapse following treatment prompting researchers to continue developing new and better treatment options (Gartlehner et al., 2007). Such research is limited by our relatively poor understanding of the biological underpinnings of this surprisingly heterogeneous collection of diseases. While diagnosed as different diseases, anxiety and depression share many of the same symptoms and complex yet overlapping etiologies that may include genetic, epigenetic, neuroanatomical, environmental and experiential factors (reviewed in Petrik et al., 2011). The most prominent hypotheses regarding the underlying biology of anxiety and depressive disorders center on the dysfunction of neurotransmitters, endocrine signaling and the inflammatory response (Brown et al., 2004; Catena-Dell'Osso et al., 2011; Harris and Seckl, 2011; Krishnan and Nestler, 2008; Luscher et al., 2011). It is clear that a single factor is insufficient to explain a mental health disorder and, given the inadequacies of the available treatments, other underlying factors should be considered.

The neurogenesis hypothesis of depression originated in the 1990's based on a series of correlative findings that came to light in this era. First it was demonstrated that stress and stress hormones, which are both co-morbid with depression, decrease hippocampal neurogenesis and increase cell death in rodents (Gould et al., 1992; Gould et al., 1991a; Gould et al., 1991b). Serotonin depletion, the most commonly hypothesized cause of depression, was also shown to decrease hippocampal neurogenesis (Brezun and Daszuta, 1999). Furthermore, an MRI-based study also showed a decrease in hippocampal volume in depressed human patients which could be correlated with the length of

time the patient had been depressed (Sheline et al., 1999). It was these findings, combined with the demonstration that chronic antidepressant treatment increased hippocampal neurogenesis, that contributed to the 'neurogenesis hypothesis of depression' (Duman et al., 1999; Malberg et al., 2000). This hypothesis, in its original form, stated that a) decreased neurogenesis results in depression and b) that effective treatment of depression requires hippocampal neurogenesis. Since the initial hypothesis, its scope has grown to include anxiety, post-traumatic stress disorder and in some instances other psychiatric disorders. This unifying hypothesis was highly attractive as it has a large body of correlative evidence behind it and it could explain and/or integrate all of the existing hypothesized etiologies for depression/anxiety including the most frustrating phenomena in the treatment of depression – the 3-4 week lag before antidepressant therapies display clinical effects. This is a time line which could be explained by the roughly 28 days required for the generation of an adult neuron from a stem cell and functional integration into the existing neuronal circuitry (Kempermann et al., 2004). Since the year 2000, research in this field has expanded significantly and increasingly tried to move beyond correlative studies to find firm causative evidence. Petrik et al. recently reviewed the literature published since 2000 on the neurogenesis hypotheses of depression (Petrik et al., 2011). Of the 21 published studies discussed 19 found no change in over 75% of the mood-related behavioral tests examined when neurogenesis was ablated in naïve non-stressed mice. Similarly there was no change in mood related behavioral testing when neurogenesis was ablated in stressed-mice (a

model of depression). Certainly there does appear to be at least a minor effect of ablating neurogenesis on depression/anxiety behavioural testing but much more work will be required to determine if this is due to the timing, extent, and/or specificity of the ablation achieved or, in fact, a true causal relationship. Finally, clinical evidence demonstrates that tryptophan depletion/reintroduction can be used to precipitate/remit depressive symptoms in humans on a time scale far too short for significant neurogenic changes (Bell et al., 2001; Spillmann et al., 2001). This argues strongly that impaired neurogenesis is not the principal causal factor for anxiety or depressive disorders. For a time this lead many researchers to largely eliminate the first postulate from the neurogenesis hypothesis of depression; however, studies published within the last year have begun to uncover subtle but intriguing connections that may lead to a significant refinement rather than elimination of this first point (see section 1.5.2 for a discussion pertaining to this data). Despite neurogenesis not being the principal root cause of depression or anxiety it remains clear that there is an association between stress which is tightly associated with such disorders and modulation of the neurogenic process acting as a vulnerability or precipitating factor and as such should not be overlooked (Snyder et al., 2011).

Stress is associated with an activation of the fight or flight response in which the body shifts the utilization of its resources to those processes necessary to maintain life often to the detriment of other biological processes. In this context, the negative impacts of stress hormones have long been recognized. In the simplest of examples, glucocorticoids have been shown to impair the proliferation

and promote the death of numerous cell types including NSCs (Chen, 2004; Gould et al., 1992; McConkey et al., 1989; te Pas et al., 2000). Gould's group was one of the first to demonstrate that a behavioral model of stress and/or exogenous administration of stress hormones could decrease hippocampal cell proliferation and survival while increasing cell death in rodents (Gould et al., 1992; Gould et al., 1991a; Gould et al., 1991b). These finding have since been extended to a multitude of other stress paradigms, life stages and species (Coe et al., 2003; Gould et al., 1997; Gould et al., 1998; Jaako-Movits et al., 2006; Malberg and Duman, 2003; Mirescu and Gould, 2006; Mirescu et al., 2006). While there may be some subtle differences in the response to different stressors particularly with regards to the control of the stressor and the sex of the stressed animal, the highly similar results from all of these studies suggest that stress-mediated impairment of neurogenesis may in fact be a common characteristic amongst mammals (Falconer and Galea, 2003; Van der Borght et al., 2005; Westenbroek et al., 2004).

Activation of the HPA axis and the resultant increase in stress hormones is thought to be the principal mechanism by which the stressor causes the reduction in neurogenesis. Adrenalectomy, which eliminates circulating corticosterone, increases neurogenesis, while treatment with exogenous corticosterone inhibits the proliferation of hippocampal progenitor cells (Cameron and Gould, 1994; Gould et al., 1992). In fact, it is hypothesized that long-term exposure to stress hormones may, at least in part, explain the strong decline in neurogenesis seen with aging (Cameron and McKay, 1999). In support of a

direct mechanism of action on neural progenitors, a specific expression pattern of the adrenal steroid receptors (mineralocorticoid (MR) and glucocorticoids (GR)) has been shown. A subset of type 1, 2a and 3 cells express GR but not MR, while all mature DG neurons express both receptors (Garcia et al., 2004a). Intriguingly, all type 2b and calretitin positive immature neurons are devoid of adrenal steroid receptors (Garcia et al., 2004a). The type 2b cell types are most important with regards to the expansion on the NPC pool, having a robust capacity for expansion and the quantitative regulation of the surviving mature neurons with relatively few type 3 cells making the transition to the immature neuron stage. Therefore the absence of the MRs and GRs in these cell types may be crucial in allowing them to undergo these important regulatory stages undisturbed. GR expression may actually be a mechanism by which type 3 cells are marked for apoptotic degradation as approximately 50% of them transiently expresse this receptor, although this hypothesis has yet to be proven (Garcia et al., 2004a). Furthermore the receptor expression pattern shifts with age towards increased expression in all cell types with an absence of expression in the type 3 cells and *de novo* expression in the immature neuron stage, potentially explaining their increased vulnerability to corticosteroids (Garcia et al., 2004a).

The downstream signaling pathways from these receptor activation events that result in the decreased proliferation have not been fully explored. It is however likely that they contribute to a slowing or arrest of the cell cycle as it has been shown that GR activation in NPCs increases the levels of p27Kip1, a cell cycle inhibitor and decreases the levels of cyclinD1, a key regulator of the G_1/S

transition (Heine et al., 2004; Sundberg et al., 2006). Additionally, stress hormones have been shown to increase glutamate release in the hippocampus which has in turn been associated with decreased cell proliferation (Abraham et al., 1998), suggesting that an indirect mechanism of action may also contribute to the net effects of stress on neurogenesis. Paradoxically, exercise, learning and enriched environment, all of which strongly promote neurogenesis, are also known to increase glucocorticoid levels (Droste et al., 2003; Kempermann et al., 1998a; Kempermann et al., 2010; Leuner et al., 2004; Moncek et al., 2004). While seemingly contradictory, it is clear that these stimuli impart many effects on the organism beyond the changes in glucocorticoids and the positives must therefore outweigh the negative effects imparted by the activation of the HPA axis. Ultimately this highlights the emerging picture of the complexity of how glucocorticoids regulate neurogenesis and supports continued research in this field.

1.4.3 Positive regulators of neurogenesis

It has been recognized since the early 1950's that exposure to an enriched environment (EE) has long term positive effects on learning and memory (Hymovitch, 1952). Initially these studies focused on the effects of genes vs. experience on brain development and revealed a wide variety of dynamic changes within the brain in response to EE (Rosenzweig and Bennett, 1996). The first demonstration of its effects on adult hippocampal neurogenesis was made in 1997, when it was published that mice living in an enriched environment had more hippocampal granule cells (Kempermann et al., 1997).

Since that time EE has become known as the most robust and reproducible positive regulator of neurogenesis. Aside from some minor effects due to rodent strain, EE has been shown to positively influence neurogenesis across the life span of mice, with aged mice revealing a relatively larger positive effect than young mice, even with very short exposures to the environment (Kempermann et al., 1998a; Kempermann et al., 1998b; Leal-Galicia et al., 2008). EE is considered to be a setting that increases the motor, cognitive, social and sensory stimulation of the animal. These changes are achieved by housing rodents in larger cages supplemented with toys, tunnels and most often running wheels within larger social groups. It was not until recently that it became clear that the exercise and cognitive stimulation have different effects on the neurogenic process (Fabel et al., 2009; Olson et al., 2006). The laboratory setting allows for an artificial separation of exercise (running wheel) from cognitive enrichment (larger social groups, larger cage, tube network for exploration and nesting material) that is not possible in nature. Such studies have revealed that exercise promotes the proliferation of progenitor cells, type 2 nestin+ positive cells specifically, while the enriched environment promotes survival of the progenitor cell progeny and that exposure to both stimuli has an additive impact on the positive neurogenic outcomes (Fabel and Kempermann, 2008; Fabel et al., 2009; Kronenberg et al., 2003; Olson et al., 2006). Interestingly the molecular changes identified to date, primarily increased expression of growth factors, which are thought to underlay the pro-neurogenic effects are common to both stimuli, thus it remains unclear how exactly their differential outcomes are conferred (Olson et

al., 2006). This dissection of the proneurogenic effects by stimulus observed in early EE studies was one of the earliest to demonstrate a specific effect of a stimulus on an individual stage of the neurogenic processes and a specific cell type in particular. This observation serves as an important reminder that a global study comprising of all stages of neurogenesis should always be completed as progenitor cell proliferation, the most commonly examined measure of neurogenesis, may not reflect the whole story.

Unlike negative neurogenic stimuli, there are fewer pro-neurogenic stimuli known beyond exercise and enriched environment. Even fewer still are those stimuli that generate mature, correctly localized, functional granule neurons. Antidepressant therapies are one such stimulus that has garnered great attention over the past decade and are a central focus of this thesis (Figure 1.6).

i) Antidepressant therapy

As introduced in section 1.4.2ii, the idea that antidepressants act as positive modulators of neurogenesis first emerged in the late 1990s (Duman et al., 1999; Malberg et al., 2000). Since this initial observation all classes of antidepressants, including electroconvulsive therapy (ECT), pharmacological and behavioral therapies, have been shown to positively impact upon one or more aspect of the neurogenic process, as well as rescuing the hippocampal impairments seen in depression models (Drew and Hen, 2007; Madsen et al., 2000; Perera et al., 2007; Santarelli et al., 2003). Interestingly these effects, at least with pharmacological therapies, are limited to the SGZ, suggesting a

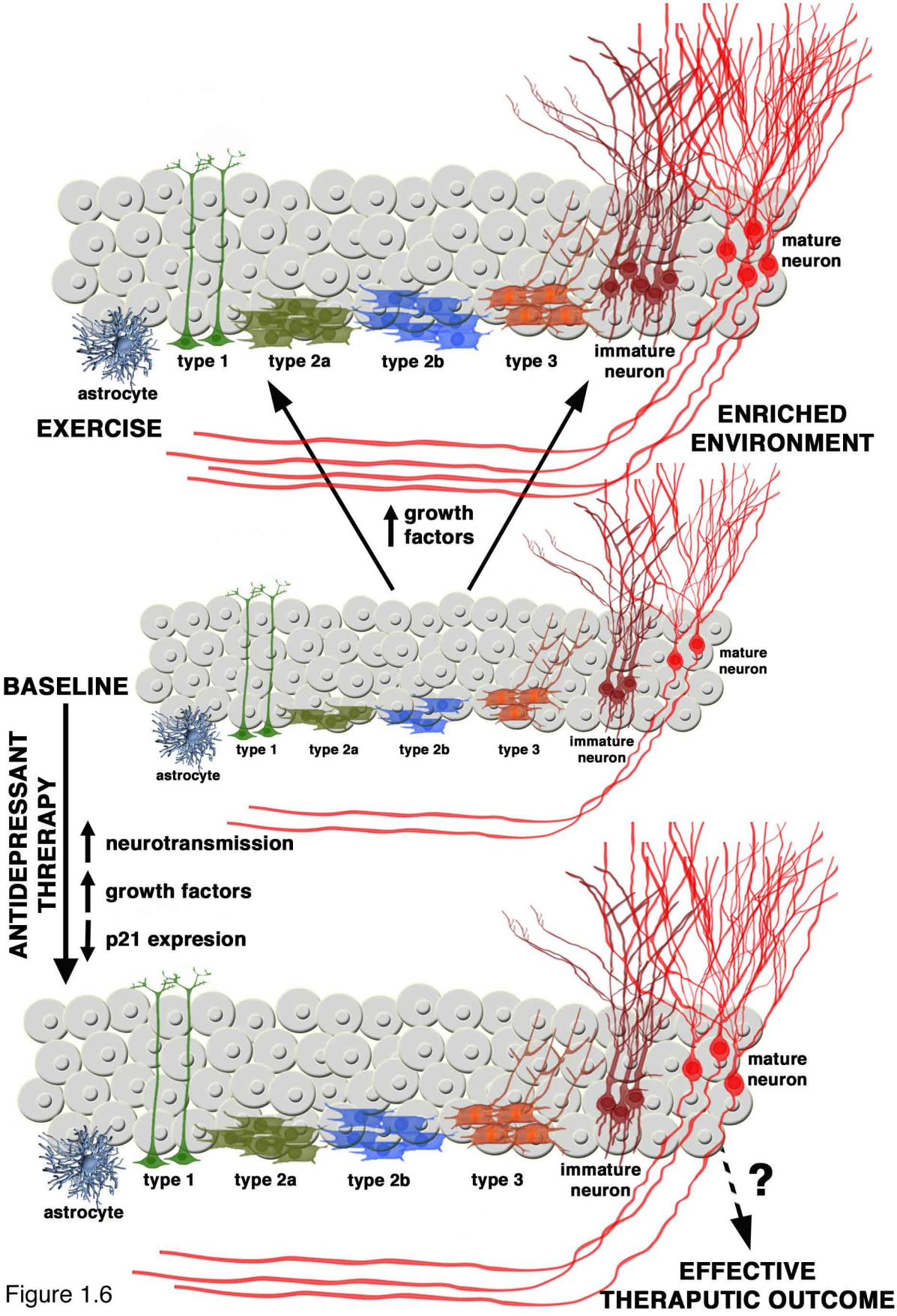
mature neuron
immature neuron
type 1
type 2a
type 2b
type 3
astrocyte
EXERCISE
ENRICHED ENVIRONMENT
growth factors
BASELINE
ANTIDEPRESSANT THRERAPY
neurotransmission
growth factors
p21 expresion
?
EFFECTIVE THERAPUTIC OUTCOME

Figure 1.6

Figure 1.6 – Positive regulators of neurogenesis: exercise, enriched environment and antidepressant treatment.

The most consistent pro-neurogenic stimulus is environmental enrichment. The exercise component underlies the increase in NPC proliferation generated while the cognitive/social enrichment leads to the increase in survival of the mature neurons. Increases in growth factor expression are at the heart of both of these effects but how this results in differential effects between the two stimuli remains unclear. Many pharmacological and behavioural paradigms that lead to effective antidepressant therapy in rodents have been shown to increase proliferation of NPCs, most often type 2b or 3 cells, and in some instances survival of their progeny. This is thought to occur not only through the expected increase in neurotransmission but also through increase expression of growth factors decrease expression of p21, a cell cycle protein which blocks the G_1 to S phase transition. It remains unclear if this effect is specific to certain antidepressant drugs or families of drugs as well as if these effects on neurogenesis are simply coincident with treatment or required for effective therapy.

specific hippocampal mechanism of action (Keilhoff et al., 2006; Santarelli et al., 2003). This contributed to the second postulate of the neurogenesis hypothesis of depression, which posits that effective treatment of depression requires hippocampal neurogenesis.

Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed class of antidepressant and as a result most studied with regards to their impact on neurogenesis. Of this class of drugs, fluoxetine, also known as prozac has been examined in the greatest depth. Chronic, but not acute, fluoxetine treatment has been shown to increase hippocampal progenitor cell proliferation and, arguably, survival in both rodents and nonhuman primates, although it does not alter the proportion of surviving cells that become neurons or glia (Kodama et al., 2004; Malberg et al., 2000; Marcussen et al., 2008; Perera et al., 2007; Santarelli et al., 2003). It has also been shown to reverse the hippocampal progenitor loss induced by several models of depression (Alonso et al., 2004; Chen et al., 2006; Malberg and Duman, 2003; Santarelli et al., 2003). A study investigating the specific population of NPCs affected by fluoxetine identified increased symmetric division of amplifying NPCs (type 2a cells) while a second study suggest that fluoxetine acts to accelerate the maturation of immature DCX+ neuroblasts/immature neurons (Encinas et al., 2006; Wang et al., 2008a). Several other SSRI family members: citalopram, escitalopram, paroxetine, fluvoxamine and sertaline have also been shown to have similarly positive effects on the neurogenic process, most often conferring an increase in NPC proliferation, although other neurogenic parameters have not for the most

part been examined (Duan et al., 2008a; Jaako-Movits et al., 2006; Jayatissa et al., 2006; Lau et al., 2007; Peng et al., 2008; Qiu et al., 2007; Wang et al., 2008b; Yoshimizu and Chaki, 2004). These findings do remain somewhat controversial as there are reports for several of these SSRIs suggesting that some, or even all, of their effects on neurogenesis are animal strain or stress model-specific and may therefore not be important with regards to the treatment of depression in human patients (Alahmed and Herbert, 2008; Huang et al., 2008; Kuipers et al., 2006; Petersen et al., 2008).

The most convincing and direct evidence for the importance of neurogenesis in the treatment of depression arose from Santrelli's 2003 paper that questioned the requirement for hippocampal neurogenesis in response to fluoxetine treatment for the effective treatment of depression. They addressed this by ablating the NPCs in specific brain regions using X-Ray irradiation, which in the SGZ lead to an 85% reduction in BrdU labeled cells. In contrast to their sham irradiated counterparts the mice with reduced neurogenic potential no longer showed an increase in BrdU-labeled cells following fluoxetine or imipramine treatment. Furthermore the irradiated and antidepressant treated mice no longer showed behavioral improvements in the novelty suppressed feeding test in a normal healthy condition or coat score when a model of depression was induced using the chronic unpredictable stress paradigm (Santarelli et al., 2003). Given the harsh nature in which the progenitor cells were ablated, an extensive series of control experiments were conducted. They all supported the notion that unexpected side effects were not responsible for the

lack of effective antidepressant action seen. The authors of this study therefore concluded that antidepressant mediated neurogenesis is required for effective therapy (Santarelli et al., 2003). These results have since been reproduced in several instances using both rodent and nonhuman primates with both pharmacological and behavioural therapies (Bruel-Jungerman et al., 2005; Perera et al., 2011; Schloesser et al., 2010; Surget et al., 2008; Wang et al., 2008a).

In contrast to these reports is a study that used the cytotoxic agent methylazoxymethanol (MAM) to eliminate NPCs in an effort to avoid the harsh effects of irradiation (Bessa et al., 2009). Following 6 weeks of chronic mild stress (CMS), mice treated with fluoxetine, imipramine, or two novel treatments showed behavioural improvements in the sucrose consumption and novelty suppressed feeding tests regardless of MAM treatment (Bessa et al., 2009). The forced swim test did, however, show that ablation of NPCs prevented the anxiety improving benefits of AD treatment (Bessa et al., 2009). Similarly, when neurogenesis was ablated using an inducible nestin-specific thymidine kinase genetic model, mice were able to respond normally to imipramine treatment in the tail suspension test (Singer et al., 2009). Contradictory studies have also been seen in the case of behavioural paradigms conferring antidepressant like effects (Meshi et al., 2006).

When behavioural tests from all studies examining the outcomes of antidepressant therapy following the ablation of neurogenesis are considered, just over half (approx 55%) support the requirement for neurogenesis in effective

therapy (Petrik et al., 2011). The correlation is higher when examining certain behavioural tests, particularly the novelty suppressed feeding test, leading some researchers to suggest that there are neurogenesis-dependent and independent effects of antidepressant therapy (David et al., 2009). While the differences between genetic background, ablation technique, depression model, and behavioural testing protocols used in each study make it very difficult to truly compare between them, it is abundantly clear that this is a highly controversial topic and with fewer than 20 published studies that directly address the requirement for neurogenesis in effective therapy it is still a field in its infancy. Therefore further rigorous study of the impact of antidepressants, not only on all aspects of NPC biology (proliferation, specification, migration, and survival), but also in clarifying the role of antidepressant elicited neurogenesis on the behavioural outcomes of these therapies is required and this thesis will contribute to this goal.

Moving beyond the current lack of consensus of the impact of antidepressants on neurogenesis and their role in therapeutic outcomes, it is not yet clear how SSRIs might signal changes in proliferation, specification, migration and/or survival in hippocampal NPCs. The predominant hypothesis focuses on neurotrophic factors, most often BDNF. Direct infusion of BDNF into the brain has antidepressant-like and pro-neurogenic effects, albeit with some unwanted and possibly confounding side effects (Duman and Monteggia, 2006; Madsen et al., 2000; Nibuya et al., 1995; Siuciak et al., 1997). Furthermore, treatment with many classes of antidepressant treatment increase hippocampal BDNF levels

which appear to be required for positive therapeutic outcomes (Pilar-Cuellar et al., 2012). When the BDNF receptor, TrkB, is genetically ablated in nestin positive cells, fluoxetine's beneficial behavioural effects are lost (Li et al., 2008). While these effects are generally thought to be related to BDNF's effects on NPC proliferation, further work seeks to clarify its effects on maturation and synaptic integration. Serotonin (5-HT) receptor activation/inhibition itself has also been shown to modulate NPC proliferative indices (Banasr et al., 2004; Jha et al., 2008; Klempin et al., 2010; Radley and Jacobs, 2002). A key player when discussing changes in cell division and turnover are cell cycle proteins themselves. Of particular importance to hippocampal neurogenesis is p21, a cyclin dependent kinase (Cdk) inhibitor, which plays an important role in restraining the proliferation of NPCs by blocking the G_1 to S phase transition. p21 is expressed in hippocampal NPCs, most prominently in type 2 and 3 cells of the hippocampus and its expression is downregulated by chronic treatment with several classes of antidepressants thereby stimulating proliferation (Pechnick et al., 2011; Pechnick et al., 2008). One question that is always raised when looking at NPCs is: How is this regulation occurring? Unlike mature neurons, NPCs do not have mature, functional, classical chemical synapses, as demonstrated by their unique electrophysiological profiles, despite an increasing awareness that they do express more receptors and transporters than originally thought. As previously discussed, one means by which these immature cells can communicate with each other and their surrounding niche is through connexin-mediated communication. Intriguingly, treatment with fluoxetine (and

antipsychotics which have $5HT_{1A}$ receptor antagonist properties) increase Cx43 expression (Fatemi et al., 2008). Furthermore, loss of the aquaporin-4 (AQP4) gene, which results in a downregulation of Cx43, impairs neurogenesis in cultured NSCs and knockout mice under basal conditions. Both *in vitro* and *in vivo* there is also an inability to respond to fluoxetine treatment in AQP4 knockouts (Kong et al., 2008; Kong et al., 2009). This suggests that modulation of connexin expression may represent a novel target of antidepressants which contributes to their regulation of neurogenesis and that these changes in connexin expression and/or function might also underlie some of the electrophysiological changes noted in fluoxetine-treated hippocampi (Wang et al., 2008a).

1.5 Functional Outcomes of Neurogenesis

The question of why and what adult neurogenesis is doing, has long been at the forefront of many researcher's minds but has been exceedingly difficult to test experimentally. The development of methods to specifically ablate or increase neurogenesis (discussed in section 1.3.1iv) has been an exciting development within the field and allows for these questions to begin to be addressed. Prior to the development of such techniques much of what was known was inferred from anatomical study along with brain lesion models and pharmacological manipulation of the hippocampus.

Anatomically it has long been recognized that the dorsal and ventral hippocampus have largely different neuronal connectivity patterns. At both the anatomical and molecular level it has now been accepted that the hippocampus

can be divided into the dorsal, intermediate and ventral sections and these divisions appear to be well conserved amongst mammals (Burwell, 2000; Dong et al., 2009; Moser and Moser, 1998; Swanson and Cowan, 1977). Functionally it is also becoming increasingly clear that they regulate distinct processes.

Since the study of patient H.M., whose medial temporal lobe was removed as a treatment for severe epilepsy and who subsequently lost most of his memory, learning and memory have been the most recognized functions of the hippocampus (Scoville and Milner, 1957). While not all memory is hippocampal-dependent, lesions have been associated with impairments in episodic, spatial, and contextual memory (Fanselow and Dong, 2010). The dorsal hippocampus appears to be crucial for these functions as lesions affecting as little as 25% of total dorsal structure impairs acquisition of a spatial memory task, while ventral lesions have no effect (Moser et al., 1995). This holds true not only in rodents but also in human patients (Maguire et al., 1998; Maguire et al., 2006).

The attribution of emotional regulation as one of the functions of the hippocampus arose from the recognition of its involvement in the limbic circuit and evidence from the nonhuman primate studies showing severe emotional disturbances upon the removal of the medial temporal lobe (Kluver and Bucy, 1937). Also the role of the hippocampus as a strong regulator of the HPA axis, which controls the stress response, suggested its involvement in emotional regulation (Dedovic et al., 2009). Rodents with ventral hippocampal lesions have a decreased corticosterone response in stress paradigms and show reduced anxiety in the elevated plus maze and light/dark box (Kjelstrup et al., 2002).

Not all behavioural studies have been as “cut and dry” with respect to specific dorsal vs. ventral roles of the hippocampus, notably contextual fear conditioning, but one must consider that this paradigm has both a significant memory (spatial context) and emotional component (fear of the aversive stimulus). This is also not totally unexpected or counter to the hypothesized functional separation of the individual regions as there is still significant interconnectivity between the subregions of the hippocampus (Fanselow and Dong, 2010). Furthermore studies which report ambiguity or combined effects on both memory and emotional outcomes often involve injuries that extend, at least partially, into the intermediate zone of the hippocampus which is rich in connections between the two poles (Fanselow and Dong, 2010). As a result, research with respect to how these functions may involve adult hippocampal neurogenesis within each region will be discussed below.

1.5.1 Neurogenesis in the dorsal hippocampus – role in learning and memory

Given the long-standing recognition that the dorsal hippocampus is crucial for learning and memory, it is not surprising that these were the first hypothesized functional correlates of hippocampal neurogenesis (Gould et al., 1999). The Morris water maze (MWM) is likely the best-known hippocampal-dependent learning and memory test and is a measure of spatial learning (Morris, 1984). In the MWM mice must learn the location of a submerged platform within a pool over the course of several days. Following the acquisition phase their knowledge of the platform location is tested by removing it and assessing the amount of time they spend in the quadrant where the platform was

previously located. Hippocampal neurogenesis does appear to be functionally related to spatial learning and memory as decreasing neurogenesis either by natural aging or x-ray irradiation results in significant impairments in the MWM test (Marlatt et al., 2012; Rola et al., 2004). Conversely, increasing neurogenesis through wheel running attenuates the age related decline in MWM performance although increasing basal levels of neurogenesis in a young animal does not alter performance (Marlatt et al., 2012; Sahay et al., 2011).

Contextual fear conditioning (CFC) is another hippocampal-dependent learning task in which an aversive stimulus, such as a foot shock, and a spatial context are paired during a training phase and later tested. Franland's group used a genetic approach to label newly born neurons during the training phase of the CFC task, they then inducibly ablated these labeled cells using a toxogenic approach following which these mice no longer demonstrated a fear of the aversive stimulus (Arruda-Carvalho et al., 2011). Similarly, when a genetic approach was used to increase neurogenesis by overexpressing the anti-apoptotic protein Bax, mice had an increased level of context discrimination in this task (Sahay et al., 2011).

Perhaps of the most interest in relation to hippocampal neurogenesis is the hippocampus's involvement in pattern separation. Pattern separation is described as the ability to distinguish between closely related but distinct pieces of information and it has been suggested that the more similar two pieces of information are the greater the role for neurogenesis for separating them cognitively (Sahay et al., 2011). Initially it was computational modeling that

supported a role for neurogenesis in the generation of new memories and their involvement in pattern separation; in recent year however this proposed role has been assessed in a series of functional analyses (Aimone et al., 2010, 2011; Aimone et al., 2006). Ablating neurogenesis via X-ray or genetic manipulation, decreases pattern separation in both a touch screen test for two-choice spatial discrimination task and a classic spatial discrimination task (Clelland et al., 2009; Tronel et al., 2012). Conversely, increasing neurogenesis, through genetic manipulation or running, was able to increase pattern separation ability of mice, in either a contextual fear learning paradigm or a touch screen task (Creer et al., 2010; Sahay et al., 2011).

These data certainly support the notion that ongoing adult neurogenesis plays a functional role is the formation of memories. Furthermore these new neurons seem to be of the greatest importance when it comes to distinguishing highly similar memories. This involvement role of new neurons in distinguishing between memories is thought to be due to their higher excitability than mature cells. This lower threshold for excitability likely leads to their encoding of additional information thus producing an overall richer data store allowing generating this highly specific discrimination (Opendak and Gould, 2011; Wojtowicz, 2012).

1.5.2 Neurogenesis in the ventral hippocampus – role in affective disorders

Beyond the recognition that the ventral hippocampus is involved in emotional regulation, behavioural research done to examine the cognitive functions of the hippocampus also emphasizes that most tests rely on an

emotional component to drive the rodent to complete the desired task (i.e., the desire to escape the cold water in MWM testing; fear and/or physical discomfort in CFC or similar pattern separation tasks). This leads to the suggestion that even the 'cold cognitive' functions of the dorsal hippocampus, which do require neurogenesis, may be influenced by the 'warm emotional' components of the ventral hippocampus and possibly neurogenesis in this region. This seems likely given that research is beginning to recognize that some stimuli, such as agomelatine treatment, preferentially regulate proliferation and/or survival of NPCs within specific regions of the hippocampus (Banasr et al., 2006). Such an effect is similar to that observed in depressed human patients treated with SSRIs who show increased NPC proliferation preferentially in the anterior region of the hippocampus (Boldrini et al., 2009).

Functionally, the involvement of neurogenesis in emotional outcomes has been in doubt given the overwhelming evidence that ablating neurogenesis does not lead to a depressed or anxious phenotype as previously discussed (Petrik et al., 2011). Snyder and colleagues have, however, just shown that newborn neurons in the hippocampus appear to be crucial in the normal regulation of the HPA axis (Snyder et al., 2011). Using either an inducible genetic ablation model or X-ray irradiation to inhibit adult hippocampal neurogenesis, they show the classic neurodendocrine phenotype of depression (Snyder et al., 2011). While mice lacking neurogenesis had a normal corticosterone response to both moderate and severe restraint stress, they took longer to return to baseline levels after the stressor than controls and were not susceptible to dexamethasone

suppression (Snyder et al., 2011). Upon behavioural testing they also displayed depression-like symptoms in tests of despair and anhedonia (Snyder et al., 2011). Thus the authors argue that newborn hippocampal neurons play an important role in regulating stress reactions by shutting down the HPA axis activation (Snyder et al., 2011). Given that stress hormones themselves negatively regulate neurogenesis, this could generate a feedback loop wherein there is decrease in basal neurogenesis resulting in an increase in stress responsiveness, thereby contributing to the development of depression (Snyder et al., 2011). While exciting this study is not without controversy. Another recent report indicated that ablating neurogenesis did not influence the suppression of the HPA axis by dexamethasone (Surget et al., 2011). It is important to note that these two studies used different timelines for dexamethasone testing and stress paradigms, the latter being a 7 week CMS paradigm, suggesting that the neurogenesis dependent regulation of the HPA axis may be specific to acute, intense stressors rather than under basal day-to-day living or mild stress conditions (Anacker and Pariante, 2012).

While further study is certainly warranted it is clear that ongoing adult hippocampal neurogenesis plays a functional role in the learning, memory and emotional functions of the hippocampus. It will be exciting to see if neurogenesis within distinct subregions of the hippocampus can be definitively linked to individual functional measures once even more specific means of selectively ablating neurogenesis are developed.

1.6 Overarching goal and objectives

The discovery of adult neurogenesis in the human brain brought with it great hope that it may be possible to harness these cells for regenerative purposes following injury, illness and ageing (Eriksson et al., 1998; Taupin, 2007a, c). While the individual steps in the neurogenic processes have been well characterized, overall the underlying regulatory events remain poorly understood (Doetsch et al., 1999; Kempermann et al., 2004). Many stem cell-based therapies are currently under development, some of which have even reached clinical testing; however, a comprehensive understanding of the regulation of neurogenesis is required if the full therapeutic potential of these cells is to be realized (Aboody et al., 2011).

The overarching goal of this thesis is to further the understanding of the regulation of adult hippocampal neurogenesis. To this end I sought to characterize the effect of two endogenous and two exogenous pharmacological cues on adult hippocampal neurogenesis through the following four aims (Figure 1.7):

1) Identify the repertoire of connexins expressed by adult hippocampal progenitor cells and potential for regulation by cell-extracellular matrix interactions (Chapter 2).
2) Assess the impact of connexin expression, specifically Cx45, on adult hippocampal NPC proliferation, specification, and survival using an *in vivo* loss of function approach (Chapter 3).
3) Establish whether opiate analgesia alters hippocampal neurogenesis (Chapter 4).

4) Assess regulation of adult hippocampal neurogenesis by a sub-set of selective serotonin reuptake inhibitors (SSRI) (Chapter 5).

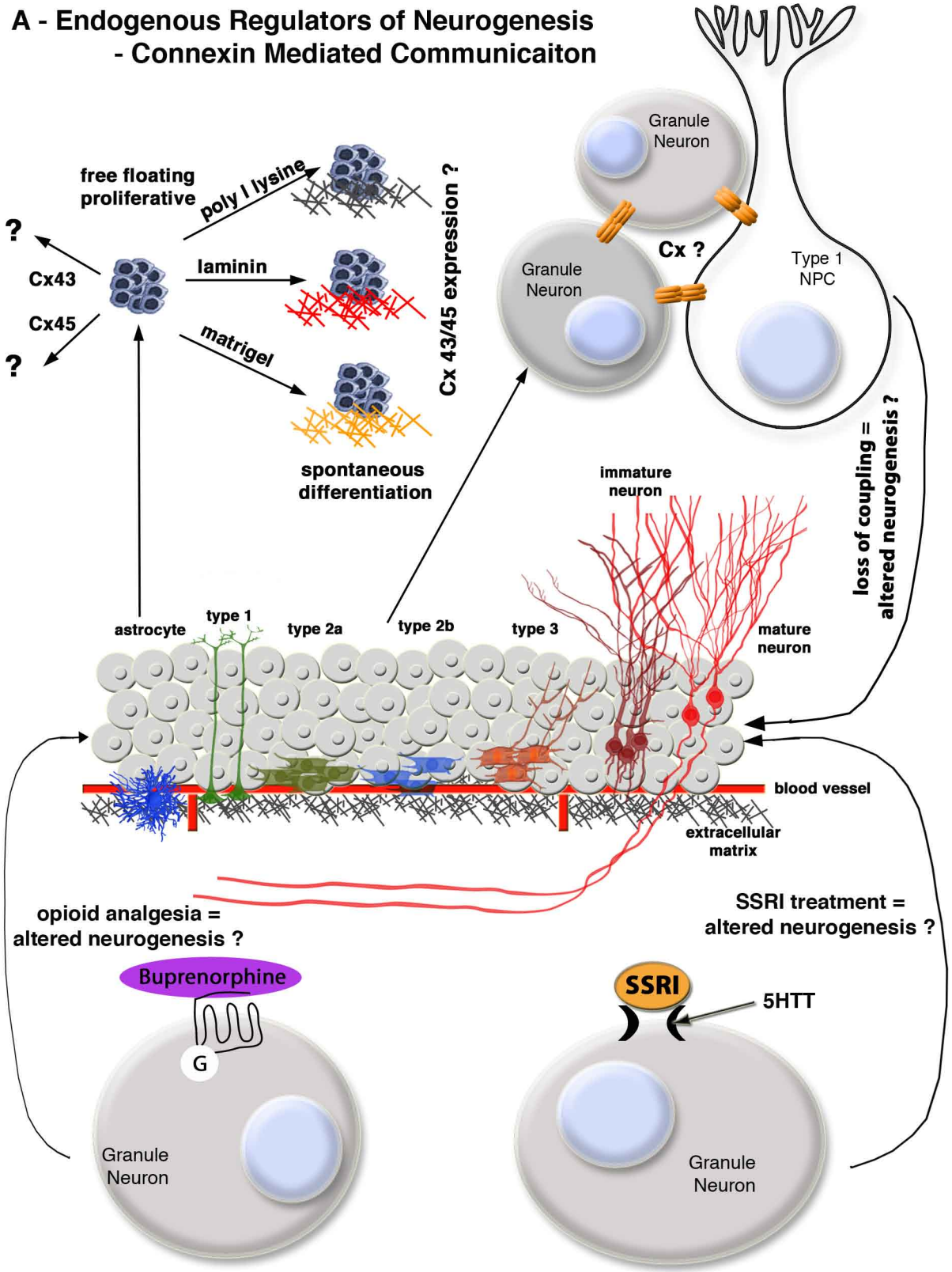
A - Endogenous Regulators of Neurogenesis
- Connexin Mediated Communicaiton
free floating
proliferative
poly l lysine
laminin
matrigel
Cx43
Cx45
?
?
spontaneous
differentiation
Cx 43/45 expression ?
Granule
Neuron
Granule
Neuron
Cx ?
Type 1
NPC
loss of coupling =
altered neurogenesis ?
immature
neuron
astrocyte
type 1
type 2a
type 2b
type 3
mature
neuron
blood vessel
extracellular
matrix
opioid analgesia =
altered neurogenesis ?
SSRI treatment =
altered neurogenesis ?
Buprenorphine
G
SSRI
5HTT
Granule
Neuron
Granule
Neuron
B - Exogenous Pharmacological Regulators of Neurogenesis

Figure 1.7

Figure 1.7 - Schematic depicting the specific aims examined within this thesis.

A) Investigating the role of an endogenous regulators of neurogenesis, connexin mediated communication. First, I asked which connexins are expressed in adult NPCs, focusing on Cx43 and 45, and how is this expression profile affected by plating on extracellular matrix components that lead to spontaneous differentiation. This was addressed using the *in vitro* neurosphere assay. Second, I examined the previously unknown expression pattern of Cx45 in the hippocampal dentate gyrus and determined its role in regulating adult neurogenesis using a loss of function mouse model. B) Investigating the role of exogenous clinically relevant pharmacological regulators of neurogenesis. First, I asked how treatment with opiate analgesia and second, a group of selective serotonin reuptake inhibitors used to treat depression, affect adult hippocampal neurogenesis *in vivo*.

1.7 References

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Chapter 2 – Connexin 43 and 45 are expressed by adult hippocampal progenitor cells *in vitro* and protein expression and function is regulated by extracellular matrix interactions[*]

2.1 Objectives of this study

1) Determine the repertoire of connexins that are expressed by adult hippocampal progenitor cells

2) Determine the cell type in which each connexin is expressed

3) Determine if connexin protein expression and function is altered by extracellular matrix interactions

2.2 Statement of author contributions

This publication was a collaborative effort by the connexin researchers in the Bennett laboratory under SALB's direction. SI and LGG were the study coordinators. SI, LGG, and AP designed the experiments with SALB. All of the Cx43 and Cx45 investigations were performed by AP, including the reverse transcriptase-polymerase chain reaction (RT-PCR) assessing mRNA expression and immunofluorescence localizing protein, along with the comparison of Cx43 expression with different matrices. SI, CDS and RD performed the lineage analysis. CDS performed the immnofluorescent analysis of Cx36-/- neurospheres. LM assisted in the functional communication analysis. HDT completed the Cx30 analyses. SI and LGG performed all other experiments.

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2.3 The extracellular matrix controls gap junction protein expression and function in postnatal hippocampal neural progenitor cells

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2.4 Summary

Gap junction protein and extracellular matrix signalling systems act in concert to influence developmental specification of neural stem and progenitor cells. It is not known how these two signalling systems interact. Here, we examined the role of ECM components in regulating connexin expression and function in postnatal hippocampal progenitor cells. **Results:** We found that Cx26, Cx29, Cx30, Cx37, Cx40, Cx43, Cx45 and Cx47 mRNA and protein but only Cx32 and Cx36 mRNA are detected in distinct neural progenitor cell populations cultured in the absence of exogenous ECM. Multipotential Type 1 cells express Cx26, Cx30, and Cx43 protein. Their Type 2a progeny but not Type 2b and 3 neuronally committed progenitor cells additionally express Cx37, Cx40, and Cx45. Cx29 and Cx47 protein is detected in early oligodendrocyte progenitors and mature oligodendrocytes respectively. Engagement with a laminin substrate markedly increases Cx26 protein expression, decreases Cx40, Cx43, Cx45, and Cx47 protein expression, and alters subcellular localization of Cx30. These changes are associated with decreased neurogenesis. Further, laminin elicits the appearance of Cx32 protein in early oligodendrocyte progenitors and Cx36 protein in immature neurons. These changes impact upon functional connexin-mediated hemichannel activity but not gap junctional intercellular communication. **Conclusions:** Together, these findings demonstrate a new role for extracellular matrix-cell interaction, specifically laminin, in the regulation of intrinsic connexin expression and function in postnatal neural progenitor cells.

2.5 Introduction

Juxtacrine signaling mechanisms, specifically cell-extracellular matrix (ECM) interactions and gap junctional intercellular communication (GJIC), act in concert to influence developmental specification of neural stem and progenitor cells (NPCs). The interaction of laminins with α1ß6 integrin receptors segregates proliferating units in the embryonic ventricular zone from migrating units in the overlying cortex (Lathia et al., 2007) while GJIC within these ECM-defined boundaries ensures synchronous cellular activity between cells destined to become functional domains (Peinado, 2001; Peinado et al., 1993; Russo et al., 2008). Similarly, the interaction of laminin with receptors containing ß1 integrin directs radial migration of proliferating neuroblasts from the embryonic ventricular zone and along the adult rostral migratory pathway (Belvindrah et al., 2007; Lathia et al., 2007) while the presence of gap junction connexin proteins facilitates migration of neuroblasts along the axial processes of their radial glial guides during cortical lamination (Cina et al., 2009; Elias et al., 2007; Fushiki et al., 2003). Thus, available data suggest that ECM and connexin-mediated signaling systems act synergistically to direct NPC specialization and migration.

Gap junction communication occurs through intercellular channels formed by connexins (Simon and Goodenough, 1998). Oligomerization of six connexins forms a hemichannel (connexon) when inserted into non-junctional plasma membranes (Stout et al., 2004). Connexons allow for the regulated passage of ions and small molecules (≤ 1 kDa) between the cytoplasm and the extracellular space (Stout et al., 2004). Alignment and docking of two connexons in adjacent

cells creates an intercellular channel enabling direct cell-cell communication and possibly adhesion. Clusters of intercellular channels make up the morphologically defined gap junction (Simon and Goodenough, 1998). There are 20 different connexin proteins in mouse; 21 in humans (Sohl and Willecke, 2004). Fourteen (Cx26, Cx29, Cx30, Cx30.2, Cx31.1, Cx31.9, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx47, Cx57) are detected in embryonic and/or adult central nervous system (Dere et al., 2008; Kreuzberg et al., 2008; Rouach et al., 2002; Vandecasteele et al., 2006) but only select connexin combinations are capable of oligomerization, adhesion, and functional communication.

The impact of ECM components upon connexin expression in NPCs has not been tested. This is an important issue because NPCs are routinely expanded as neurospheres in the absence of exogenous ECM while functional assessment of cell-cell signaling pathways often involves replating on adhesive substrates. Understanding the changes in NPC phenotype in 2D and 3D culture systems is necessary if we are to rigorously test and validate potential strategies involving NPC expansion and specification *in vitro* (Pampaloni et al., 2007). It has already been demonstrated, in other cell types, that connexin expression and intercellular communication is strongly influenced by laminin-integrin interactions (Guo et al., 2001; Isakson et al., 2006; Lampe et al., 1998). In this study, we sought to identify the connexins intrinsically expressed by postnatal NPC populations and determine whether exposure to laminin or simple adhesion alters connexin expression and connexin-mediated GJIC and/or hemichannel activity. We show that subsets of NPCs exhibit a unique connexin profile and that this

profile is altered by laminin but not poly-L-lysine impacting upon functional channel activity.

2.6 Methods

2.6.1 Mice

Breeding pairs of $Cx29^{-/-}$, $Cx36^{-/-}$, $Cx37^{-/+}$, $Cx40^{-/-}$, $Cx45^{F/F}$, and $Cx47^{-/-}$ animals were generated by our laboratories as described (Altevogt et al., 2002; Deans et al., 2001; Menichella et al., 2003; Nishii et al., 2003; Simon et al., 1997; Simon et al., 1998). $Cx32^{-/-}$ and $Cx30^{-/-}$ animals (Nelles et al., 1996; Teubner et al., 2003) were kindly provided by Dr. Klaus Willecke (University of Bonn). Nestin-Cre recombinase transgenics (Berube et al., 2005) were kindly provided by Dr. Ruth Slack (University of Ottawa). Each strain was backbred for 3-12 generations into a C57BL/6 lineage. All null-mutant mice were compared to congenic wild-type (WT) littermates. Mice were kept on a 12h light-dark cycle and allowed food and water *ad libitum*. Mice were genotyped using the primer pairs listed in Table 2.1. All experimental protocols were approved by the Animal Care Committee of the University of Ottawa according to guidelines set forth by the Canadian Council on Animal Care.

2.6.2 Neurosphere suspension culture

All chemical reagents were obtained from Sigma-Aldrich (St.-Louis, MO, USA) and all cell culture reagents were obtained from Invitrogen (Burlington, ON, Canada) unless otherwise stated. NPCs were cultured as described in

Table 2.1 - Genotyping protocols

Gene	*Reaction Conditions*[a]	*Cycling & Amplicon sizes*
Cx29	*Cx29F: 5'-ATCTGTGCTGTGCTATTTGGAGT-3'* *Cx29R: 5'-ACAGGTTGTGCTGCCAATAC-3'* *lacZF: 5'-CCGACGGCACGCTGATTGAAG-3'* *lacZR: 5'-ATGCGGTCGCGTTCGGTTGC-3'* *Primer concentrations: 10 ng/μL each* *Volume of tail DNA: 5 μL* *Final reaction volume: 20 μL*	*95°C 5 m* *30 cycles:* *94°C 15 s* *66°C 15 s* *72°C 80 s* *(+ allele=700 bp)* *(- allele=1100 bp)*
Cx30	*F: 5'-GGTACCTCCTACTAATTAGCTTGG-3'* *lacZR: 5'-AGCGAGTAACAACCCGTCGGATTC-3'* *WTR: 5'-AGGTGGTACCCATTGTAGAGGAAG-3'* *Primer concentrations: 8 ng/μL each* *Volume of tail DNA: 1 μL* *Final reaction volume: 25 μL*	*94°C 5 m* *35 cycles:* *92°C 45 s* *60°C 45 s* *72°C 45 s* *72°C 10 m* *(+ allele=544 bp)* *(- allele=460 bp)*
Cx32	*F: 5'-ATACACCTTGCTCAGTGGCGTGAATCGGCA-3'* *R: 5'-TCATTCTGCTTGTATTCAGGTGAGAGGCGG-3'* *Primer concentrations: 8 ng/μL each* *Volume of tail DNA: 2.5 μL* *Final reaction volume: 12.5 μL*	*95°C 10 m* *30 cycles:* *95°C 60 s* *67°C 60 s* *72°C 60 s* *(+ allele=750 bp)*
	F: 5'-TCTTACTCCACACAGGCATAGAGTGTCTGC-3' *R: 5'-TCATTCTGCTTGTATTCAGGTGAGAGGCGG-3'* *Primer concentrations: 8 ng/μL each* *Volume of tail DNA: 2.5 μL* *Final reaction volume: 12.5 μL*	*95°C 10 m* *30 cycles:* *95°C 60 s* *67°C 60 s* *72°C 60 s* *(- allele=1300 bp)*
Cx36	*F: 5'-AGCGGAGGGAGCAAACGAGAAG-3'* *R: 5'-CTGCCGAAATTGGGAACACTGAC-3'* *Primer concentrations: 8 ng/μL each* *Volume of tail DNA: 5 μL* *Final reaction volume: 25 μL*	*94°C 60s* *30 cycles:* *94°C 15 s* *69°C 15 s* *72°C 45 s* *(+ allele=533 bp)*
	PLAPF: 5'-GGTGAACCGCAACTGGTACT-3' *PLAPR: 5'-CCCACCTTGGCTGTAGTCAT-3'* *Primer concentrations: 0.5 μM each* *Volume of tail DNA: 5 μL* *Final reaction volume: 20 μL*	*95°C 15m* *35 cycles:* *95°C 30s* *63°C 90s* *72°C 2m* *72°C 15m* *(- allele=187 bp)*
Cx37[b]	*F: 5'-TGCTAGACCAGGTCCAGGAAC-3'* *neoR: 5'-AGAGGCTATTCGGCTATGACT-3'* *WTR: 5'-GTCCCTTCGTGCCTTTATCTC-3'* *Primer concentrations: 125 nM each* *Volume of tail DNA: 1.5 μL* *Final reaction volume: 20 μL*	*94°C 3 m* *30 cycles:* *94°C 30 s* *63°C 30 s* *72°C 90 s* *72°C 5 m* *(+ allele=750 bp)* *(- allele=1300 bp)*
Cx40[c]	*F: 5'-TGGAGCCACAGTTGCAATGGT-3'*	*94°C 3 m*

	neoR: 5'-GCACGAGACTAGTGAGACGTG-3' *WTR: 5'-TCTCTGACTCCGAAAGGCAAG-3'* *Primer concentrations: 20 ng/μL each* *Volume of tail DNA: 2 μL* *Final reaction volume: 30 μL*	*30 cycles:* *94°C 30 s* *64°C 30 s* *72°C 30 s* *72°C 4 m* *(+ allele=270 bp)* *(- allele=470 bp)*
Cx45[d]	*CreTK139F: 5'-ATTTGCCTGCATTACCGGTC-3'* *CreTK141R: 5'-ATCAACGTTTTGTTTTCGGA-3'* *Primer concentrations: 4 ng/μL each* *Volume of tail DNA: 2.5 μL* *Final reaction volume: 12.5 μL*	*94°C 5m* *30 cycles:* *94°C 30 s* *56°C 30 s* *72°C 60 s* *(Cre transgene=300 bp)*
	Cx45F: 5'-CTTGGCTTCCTTAATTACTTTA-3' *Cx45R: 5'-CTTCCCTACAAATGTCGAATG-3'* *neoR: 5'-AGGGGACGAAGACAGTAT3'* *Primer concentrations: 0.8 μg/μL each* *Volume of tail DNA: 2.5 μL* *Final reaction volume: 12.5 μL*	*94°C 5m* *30 cycles:* *94°C 30 s* *56°C 30 s* *72°C 60 s* *(+ allele=570 bp)* *(Flx allele=610 bp* *(- allele=820 bp)*
Cx47[e]	*F: 5'-AAGGCTGGTGCTGCTGGAAT-3'* *R: 5'-TGACCACCGTCTTGCCATCA-3'* *Primer concentrations: 0.4 μM each* *Volume of tail DNA: 5 μL* *Final reaction volume: 25 μL*	*95°C 5 m* *35 cycles:* *95°C 15 s* *67°C 15 s* *72°C 3 m* *(+ allele=1100 bp)*
	F: 5'-TCGCATTGTCTGAGTAGGTGTC-3' *R: 5'-CAGAGTTCCTCTGCACAGAGAT-3'* *Primer concentrations: 10 ng/μL each* *Volume of tail DNA: 5 μL* *Final reaction volume: 20 μL*	*95°C 15 m* *35 cycles:* *95°C 30 s* *64°C 90 s* *72°C 2 m* *72°C 15m* *(- allele=2200 bp)*

[a]*Unless otherwise stated, PCR reactions contain 1X Advantage 2 PCR buffer, 0.8 mM dNTPs and 0.4 μL of Advantage 2 Taq polymerase (Clontech)*
[b]*Final concentration of dNTPs is 0.2 mM.*
[c]*Final concentration of dNTPs is 0.2 mM. Reaction uses Titanium Taq Polymerase in 1X Titanium PCR Buffer*
[d]*Reactions use Titanium Taq Polymerase in 1X Titanium PCR Buffer.*
[e]*For the + allele, final Advantage 2 PCR buffer concentration is 0.8X.*

(Bennett and Melanson-Drapeau, 2003) with some modifications. Briefly, cells were isolated from the hippocampi of postnatal day 0 - 2 (P0-P2) mouse pups. Animals were sacrificed by lethal injection with Euthansol (Schering-Plough Canada Inc., Pointe-Claire, QC, Canada) and 500 µm sections between bregma -1.7 mm and -2.2 mm were prepared on a VT1000S vibratome (Leica Microsystems Inc.) in ice-cold artificial cerebral spinal fluid (26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM D-glucose, 100 U/ml penicillin and 100 µg/ml streptomycin). Hippocampi from n=2-6 pups were pooled for each culture. Under a Leica MZ6 dissecting microscope, hippocampi were removed and dissected free of blood vessels and choroid plexus. Hippocampi were minced with a scalpel and enzymatically dissociated in: 26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$, 3.2 mM $MgCl_2$, 10 mM D-glucose, 1% penicillin/streptomycin, 0.1 % neural protease, 0.01 % papain, and 0.01% DNase I for 45 min at 37°C. Single cells were resuspended in expansion media (Dulbecco's modified Eagle's medium F12 (DMEM/F12), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1X B27 supplement, 20 ng/ml human recombinant epidermal growth factor (EGF) and 10 ng/ml basic fibroblast growth factor (FGF-2)) in 60 mm Petri dishes (Fisher Scientific, Nepean, ON, Canada). Cell viability was established by Trypan Blue hemacytometer counts and cells plated at a density of $2.5x10^5$ viable cells/dish. This protocol typically yields 200 viable neurospheres/60 mm dish cultured in suspension. Cultures were maintained at 37°C in a 5% CO_2 atmosphere with fresh EGF and FGF-2 added every two days for eight days *in vitro* (DIV).

2.6.3 Laminin, Matrigel, and Poly-L-Lysine Treatment

On DIV 8, neurospheres were plated in 10 cm tissue culture dishes containing laminin (15 µg/mL), Matrigel (Invitrogen), or poly-L-lysine (100 µg/ml)-coated glass coverslips (Corning, NY, USA) and cultured in expansion media supplemented with EGF and FGF-2 or in differentiation media: DMEM/F12, 2 mM L-glutamine, 1 mM sodium pyruvate, 200mM D-glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1X N2 supplement containing EGF and FGF-2 or 0.5 µM retinoic acid and 0.5% fetal bovine serum. The latter condition promotes astroglial specification (Song et al., 2002). Cells were not dissociated to maintain the 3D topography of the cultures. Neurospheres adhered to each substrate and began to grow as monolayer colonies within 24 h of transfer. To maintain NPC proliferation, half the volume of media was removed and replenished every 2 days with fresh addition of EGF and FGF-2 to final concentrations of 10 and 20 ng/ml for 6 DIV (total 14 DIV). Some cultures were pulsed with 20 µg/mL 5'-bromo-2-deoxyuridine (BrdU, Roche Diagnostics, Laval, QC) 24 h prior to plating on laminin to determine the percentage of actively dividing cells in suspension that generated mature progeny. Cultures exposed to exogenous matrix or adhesive substrates were compared to cultures maintained in suspension in the same media supplemented with EGF and FGF-2. All immunocytochemistry and western analysis were performed on DIV 14.

2.6.4 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from WT, $Cx29^{-/-}$, $Cx30^{-/-}$, and $Cx45^{-/-}$

neurospheres and from adult and/P2 WT, Cx32$^{-/-}$, Cx36$^{-/-}$, Cx37$^{-/-}$, Cx40$^{-/-}$, and Cx47$^{-/-}$ mouse brain using Trizol Reagent (Invitrogen). RNA was collected from one dish per experiment. Each assessment was confirmed in duplicate or triplicate. Total RNA was treated with DNaseI (Promega, Madison, WI, USA). First-strand synthesis was performed using pdN_6 (Promega) random primers and Superscript II RT (BD Biosciences, San Jose, CA, USA) according to manufacturer's recommendations. PCR was performed using the primers and conditions listed in Table 2.2. The PCR reaction contained, in a final volume of 25 µl: 1X PCR buffer, 0.8 mM dNTPs, 1X Advantage 2 Polymerase (Clontech, Cambridge, ON, Canada). All reactions were carried out using the following cycling parameters: 94^{o}C for 5 min, 35 cycles of 94^{o}C for 25 sec, 59^{o}C for 50 sec, and 72^{o}C for 1 min 45 sec, and a final step at 72^{o}C for 7 min in a Whatman Biometra T-Gradient thermocycler (Montreal-Biotech Inc., Kirkland, QC, Canada).

2.6.5 Western analysis

Neurospheres were washed in PBS (10 mM PBS: 0.154M NaCl, 0.0028M NaH_2PO_4, 0.0072M Na_2HPO_4, pH 7.2), pelleted, and resuspended in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mM sodium fluoride, 1 mM sodium orthovanadate, 50 µg/ml aprotinin, and 1 mg/mL phenylmethylsulphonylfluoride in PBS). Where Western analysis of cultures grown on laminin substrate is indicated, adherent cultures were washed with PBS and RIPA buffer added directly to plates. Cells were collected using a

Table 2.2 - RT-PCR protocols[a]

Gene	Primer Sequence	Primer Concentration	Amplicon length
Cx26 (GJB2)	F: 5'-GGATGTGGCAGTCAGTATCA R: 5'-TCTTGGCAGGAAGAAGTGTC	0.5 µM	368 bp
Cx29 (GJC3)	F: 5'-GGTTTTCGGCAATGAT R: 5'-AGAAGCTTGAGGCTTTTAGC	4 ng/µL	278 bp
Cx30 (GJB6)	F: 5'-GCCAGGGTGCAAGAACGTCTGC R: 5'-GGCATGGTTGGGTGGTTTCTC	10 ng/µL	535 bp
Cx32 (GJB1)[b]	F: 5'-GTGGCGTGAATCGGCACTCTAC R: 5'-CTCCGCCACGTTGAGGATAATG	10 ng/µL	593 bp
Cx36 (GJD2)	F: 5'-AGCGGAGGGAGCAAACGAGAAG R: 5'-CTGCCGAAATTGGGAACACTGAC	10 ng/µL	533 bp
Cx37 (GJA4)	F: 5'-AGAGCGGTTGCGGCAGAAAGAGG R: 5'-TGGATGAGAGCCCGTTGTAGGTG	10 ng/µL	551 bp
Cx40 (GJA5)	F: 5'-TTTGGCCAAGTCACGGCAGGG R: 5'-TTGTCACTGTGGTAGCCCTGAGG	4 ng/µL	311 bp
Cx43 (GJA1)	F: 5'-CCTGCCGCAATTACAACAAG R: 5'-AAGGTCGCTGATCCACGATA	10 ng/µL	201 bp
Cx45 (GJC1)[b]	F: 5'-GAGGTGGGCTTTCTAATAGGGCAG R: 5'-ATGGGGGTTGTTTTGGTGATGG	10 ng/µL	528 bp
Cx47 (GJC2)	F: 5'-GCTGGAGGAGATCCACAATCATTC R: 5'-GTGTGGAGATGACCACTATCTGGA	10 ng/µL	233 bp
GAPDH[b]	F: 5'-TGGTGCTGAGTATGTCGTGGAGT R: 5'-AGTCTTCTGAGTGGCAGTGATGG	0.2 µM	292 bp

[a]All reactions contained 1 µL of RT product, 0.8 mM dNTPs, 1X PCR Buffer, 1X Advantage 2 Taq Polymerase in a reaction volume of 25 µL except Cx32 and GAPDH reactions.
[b]1X Titanium Taq Polymerase was used.

cell scraper (Melanson-Drapeau et al., 2003), incubated on ice for 30 min, then centrifuged at 13 400 x g for 30 min. Protein was isolated from mouse brain homogenized in RIPA buffer using a Tissue Tearor (Biospec Products Inc., Bartlesville, OK, USA). Protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's protocol. Protein samples of 30 µg were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Boucher and Bennett, 2003). Membranes were blocked at room temperature for 1 h with 1% casein in PBS. Primary and secondary antibodies are listed in Table 2.3. Immunoreactivity was visualized using the SuperSignal West Pico Chemilunescent Substrate kit (Pierce Biotechnology Inc., Rockford, IL, USA). All assessments were performed in duplicate.

2.6.6 Immunocytochemistry

Neurospheres expanded in suspension or grown on laminin were fixed in 3.7% formaldehyde solution in PBS for 20 min. Following extensive washes in PBS, neurospheres were cryoprotected for 24 h with 15% sucrose in PBS + 0.001% NaN_3, flash-frozen, and serially sectioned (10 µm thickness) using a cryostat (Leica CM1900), Immunocytochemistry was carried out as previously described (Boucher and Bennett, 2003; Stout et al., 2002). Antibodies are listed in Table 2.3 and were diluted in 3% bovine serum albumin + 0.3% Triton X-100 in PBS. Hoechst 33258 (1 µg/mL) was used as a nuclear counterstain. For suspension cells, the number of connexin$^+$ cells was expressed as the

Table 2.3 - Primary and secondary antibodies used in this study

Antibody	Type	Species	Source	Dilution Immuno	Dilution Western	Dilution Flow
Cx26	Polyclonal	Rabbit	Zymed	1:25	1:100	---
Cx29	Polyclonal	Rabbit	Dr.David Paul	1:20	---	---
Cx30	Monoclonal	Mouse	Zymed	1:50	---	1:25
Cx30	Polyclonal	Rabbit	Zymed	---	---	1:25
Cx32	Monoclonal	Mouse	Zymed	1 µg/mL	1:250	---
Cx36	Polyclonal	Rabbit	Zymed	5 µg/mL	1:100	---
Cx37	Polyclonal	Rabbit	Dr. Alex Simon	1:200	---	---
Cx40	Polyclonal	Rabbit	Zymed	1:50	---	---
Cx43	Monoclonal	Mouse	Chemicon	1:100	---	1:1000
Cx43	Polyclonal	Rabbit	Zymed	1:100	---	---
Cx45	Polyclonal	Rabbit	Chemicon	1:1000	---	---
Cx47	Polyclonal	Rabbit	Dr. David Paul	1:250	---	---
Actin	Monoclonal	Mouse	Sigma	---	1:1000	---
β-galactosidase	Polyclonal	Rabbit	Chemicon	1:1500	---	---
BrdU	Monoclonal	Mouse	Roche	6 µg/mL	---	---
DCX	Polyclonal	Guinea Pig	Chemicon	1:4000	---	---
GFAP	Polyclonal	Rabbit	Sigma	1:100	---	---
GFAP	Cy-3 conjugated	Mouse	Sigma	1:800	---	---
GFAP	Monoclonal	Rat	Zymed	1:40	---	1:25
Nestin	Monoclonal	Mouse	Chemicon	1:50	---	1:50
NCAM	Monoclonal	Mouse	Sigma	1:400	---	---
NG2	Polyclonal	Rabbit	Chemicon	1:200	---	---
NeuN	Monoclonal	Mouse	Chemicon	1:100	---	---
PDGFαR	Monoclonal	Rat	BD	1:300		
RIP	Monoclonal	Mouse	Chemicon	1:1000	---	---
TuJ1 (βIII-tubulin)	Monoclonal	Mouse	Research Diagnostics	1:250	---	---
Mouse IgG	Cy3-conjugated	Donkey	Jackson	1:800	---	---
Rabbit IgG	Cy3-conjugated	Donkey	Jackson	1:600	---	---
Mouse IgG	FITC-conjugated	Donkey	Jackson	1:100	---	---
Rabbit IgG	FITC-conjugated	Donkey	Jackson	1:100	---	1:50
Rat IgG	FITC-conjugated	Donkey	Jackson	1:80	---	---
Mouse IgG	HRP-conjugated	Donkey	Jackson	---	1:2000	---
Rabbit IgG	HRP-conjugated	Donkey	Jackson	---	1:5000	---
Mouse IgG	Cy5-conjugated	Goat	Invitrogen	---	---	1:50
Rat IgG	RPE-conjugated	Goat	Serotec	---	---	1:10
Rabbit IgG	AMCA-conjugated	Goat	Jackson	1:100	---	---
Mouse IgG	AMCA-conjugated	Donkey	Jackson	1:100	---	---

percentage of Hoechst$^+$ nuclei counted in serial sections of 10-50 neurospheres. For cultures plated on laminin, immunocytochemistry was performed directly on laminin-coated glass-coverslips. Connexin$^+$ cells/Hoechst$^+$ nuclei were established in five fields per neurosphere calculated for 5-10 neurospheres per connexin (n=25-50 fields per condition). Because cell density in the sphere core was such that individual cells could not be quantified or verified to be in contact with the laminin, cell counts were performed over five defined peripheral cell fields shot at 40X magnification. All cell images were taken with a Leica DMXRA2 epifluorescence microscope and analyzed using Openlab software v5.05 (Improvision, Lexington, MA, USA).

2.6.7 Flow Cytometry

Cultures were expanded as neurospheres for 14 DIV before being suspended in 2% formaldehyde in 10 mM PBS and triturated to achieve a single cell suspension. Following two PBS washes and gentle centrifugation at 2000 rpm for 5 min, NPCs were suspended in PFN (2% fetal bovine serum, 0.1% NaN_3, 0.18% saponin in 10 mM PBS). Cell suspensions were separated into 1.5 ml microfuge tubes at a concentration of 1 x 10^6 cells per tube. Primary or isotype control antibodies were added directly to cell suspensions at appropriate concentrations followed by incubation at room temperature for 30 min on a shaker. Each sample was washed twice by the addition of 0.8 ml of PFN and gentle centrifugation. Secondary antibodies were added to cell suspensions at appropriate concentrations followed by incubation at room temperature for 30

min on a shaker and washed as described above. Cells were resuspended in 0.5 ml of PFN and analyzed using a Beckman Coulter FC500 Flow Cytometer (Beckman Coulter Canada Inc., Mississauga, ON) and Beckman Coulter CXP software.

2.6.8 GJIC and hemichannel assay

Dye uptake and dye transfer assays were assessed on 10 neurospheres/condition in two separate experiments per condition. For quantification, four fields were photographed per sphere. For dye coupling assays, photomicrographs represented two fields within the core of the neurosphere and two fields at the periphery where cells were in direct apposition and thus potentially coupled. To image cells within the core in direct contact with matrix, micrographs were obtained using a Leica DMIR epifluorescent inverted microscope equipped with a QICAM digital camera (Quorum Technologies, Guelph, ON, Canada) and captured using OpenLab software v5.05. For hemichannel assays, all four fields were shot along the periphery and were taken with a Leica DMXRA2 epifluorescence microscope and analyzed using Openlab software (v5.05). Cultures expanded in suspension as neurospheres for 8 DIV were plated on coverslips in expansion media containing (a) EGF and FGF-2 for 1 DIV to assess function prior to laminin-induced changes in connexin expression, (b) EGF and FGF-2 for 6 DIV to assess connexin function after laminin-induced changes, and (c) in differentiation media for 6 DIV supplemented with RA (0.5 μM) and FBS (0.5%) to promote glial differentiation. Hemichannel

activity was assessed by comparing uptake of connexin channel-permeant Lucifer yellow (LY, 457 Da) and channel-impermeant rhodamine B isothiocyanate-dextran (RD, 10,000 Da) in the presence or absence of 50 μM flufenamic acid (FFA) or 100 μM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) as previously described (Boucher and Bennett, 2003). Mechanical stimulation with glass microbeads was used to trigger hemichannel opening (Dere et al., 2008; Kreuzberg et al., 2008; Rouach et al., 2002; Vandecasteele et al., 2006). Cells positive for both LY and RD were excluded from the measurements to control for LY uptake resulting from loss of membrane integrity. For all assays, the number of LY^+/RD^- cells is expressed as a percentage of the total number of cells per microscopic field ± standard error of the mean (SEM). Dye transfer between cells indicative of GJIC was assessed by scrape loading in the presence or absence of the intercellular channel blocker 18α-glycyrrhetinic acid (GRA, 100 μM) or its inactive analog glycyrrhizic acid (GZA, 100 μM) as previously described (Altevogt and Paul, 2004; Teubner et al., 2003). Dye transfer was quantified by counting the number of LY^+/RD^- cells emanating from a LY^+/RD^+ cell adjacent to the scrape line. Data are expressed as the mean number of LY^+/RD^- cells ± SEM.

2.6.9 Statistics

Data were analyzed by analysis of variance (ANOVA) followed by *post hoc* Tukey tests or Student's *t* test where appropriate.

2.7 Results

2.7.1 ECM effects on postnatal NPC culture

To characterize neurosphere composition, we analyzed marker expression in suspension and in cultures plated on a variety of adhesive substrates by immunofluorescence (Figure 2.1). Although each neurosphere is likely derived from a single NPC, progeny spontaneously adopt distinct developmental lineages in culture (Figure 2.2a). We found neurospheres cultured in the absence of exogenous ECM were primarily composed of nestin$^+$ cells (Figure 2.2i). Cells co-expressing the Type 1 NPC marker GFAP localized to the periphery of neurospheres (Figure 2.2b). Type 2a cells expressing nestin only were found in the core of cultures (Figure 2.2b). DCX$^+$, NCAM$^+$, and TuJ1$^+$ neuroblasts and immature neurons were detected in clusters throughout the neurosphere structure (Figure 2.2c-e). NG2$^+$ and PDGFαR$^+$ oligodendrocyte progenitor cells (OPCs) and RIP$^+$ oligodendrocytes were found at the periphery of neurospheres with rare cells detected in the core (Figure 2.2f-h). When cultures were plated on laminin, we observed a significant increase in the percentage of cells that retained a nestin$^+$ NPC identity and a significant decrease in the percentage of cells that specified to neuroblasts, immature neurons, and/or OPCs (Figure 2.2i). No change in the number of astrocytes was detected but a small increase in RIP$^+$ oligodendrocytes was observed in laminin-plated cultures (Figure 2.2i).

2.7.2 Intrinsic connexin mRNA expression

To identify the connexins expressed by postnatal progenitor cells in the

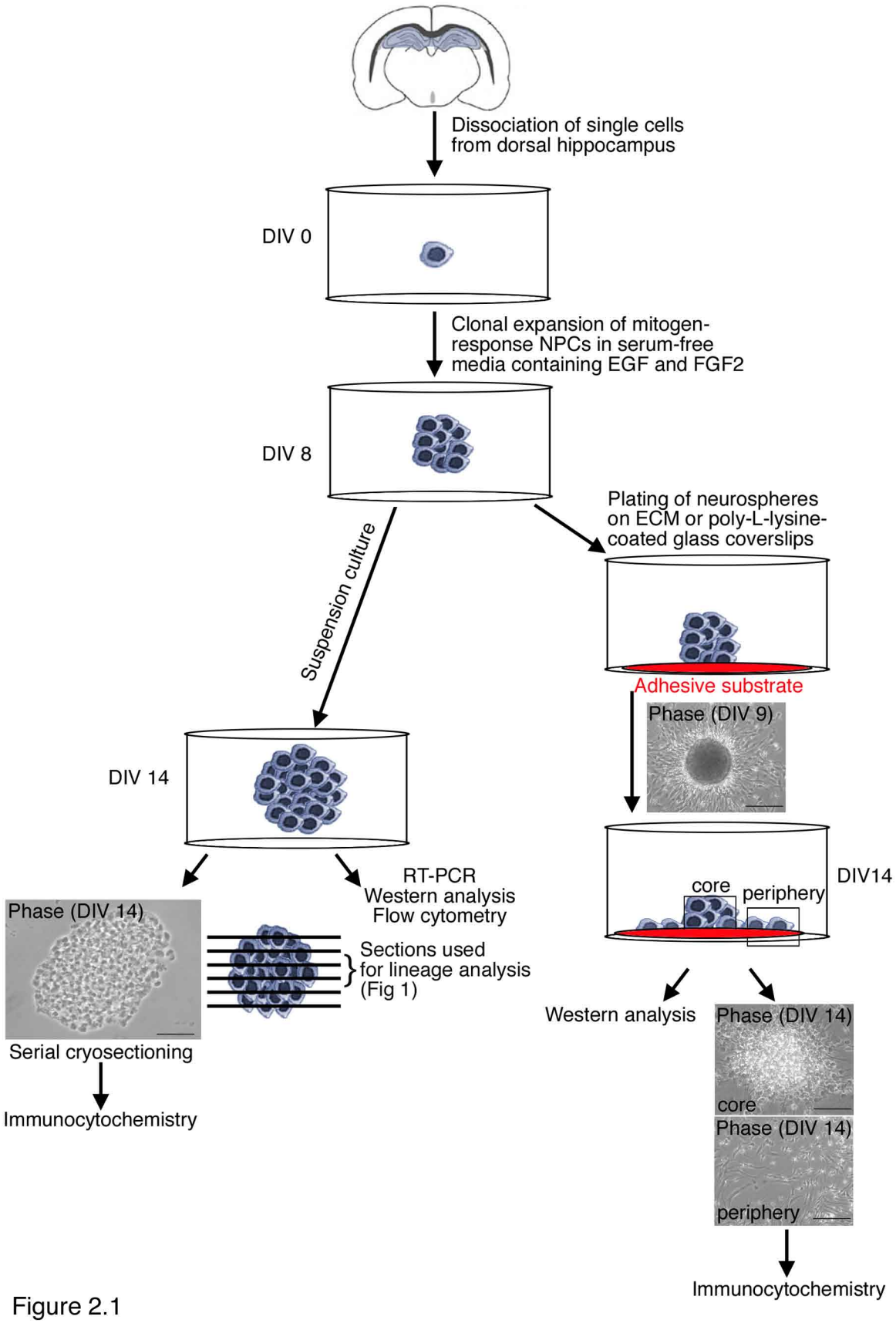
Dissociation of single cells
from dorsal hippocampus
DIV 0
Clonal expansion of mitogen-
response NPCs in serum-free
media containing EGF and FGF2
DIV 8
Plating of neurospheres
on ECM or poly-L-lysine-
coated glass coverslips
Suspension culture
Adhesive substrate
Phase (DIV 9)
DIV 14
RT-PCR
Western analysis
Flow cytometry
DIV14
core
periphery
Phase (DIV 14)
Sections used
for lineage analysis
(Fig 1)
Western analysis
Phase (DIV 14)
core
Phase (DIV 14)
periphery
Serial cryosectioning
Immunocytochemistry
Immunocytochemistry

Figure 2.1

Figure 2.1 - Schematic representation of the neurosphere culture protocol and analysis.

A graphic depiction of the protocol employed to culture and analyze neurospheres in the presence and absence of laminin.

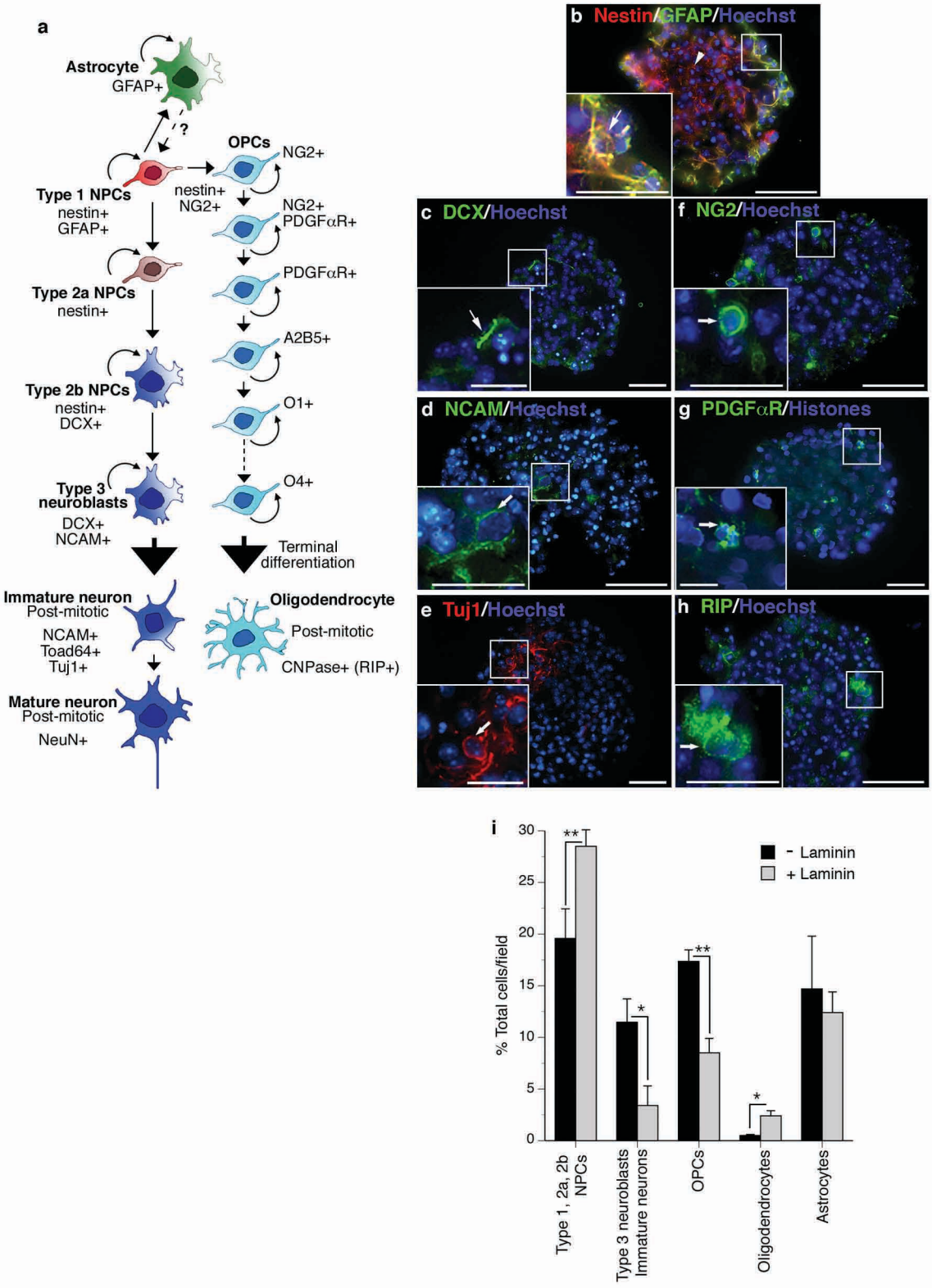
a
Astrocyte
GFAP+
?
OPCs
NG2+
Type 1 NPCs
nestin+
GFAP+
nestin+
NG2+
NG2+
PDGFαR+
Type 2a NPCs
nestin+
PDGFαR+
A2B5+
Type 2b NPCs
nestin+
DCX+
O1+
Type 3
neuroblasts
DCX+
NCAM+
O4+
Terminal
differentiation
Immature neuron
Post-mitotic
NCAM+
Toad64+
Tuj1+
Oligodendrocyte
Post-mitotic
CNPase+ (RIP+)
Mature neuron
Post-mitotic
NeuN+
b Nestin/GFAP/Hoechst
c DCX/Hoechst
f NG2/Hoechst
d NCAM/Hoechst
g PDGFαR/Histones
e Tuj1/Hoechst
h RIP/Hoechst
i
- Laminin
+ Laminin
% Total cells/field
30
25
20
15
10
5
0
**
*
**
*
Type 1, 2a, 2b
NPCs
Type 3 neuroblasts
Immature neurons
OPCs
Oligodendrocytes
Astrocytes

Figure 2.2

Figure 2.2 - Postnatal hippocampal-derived neurospheres are composed of subpopulations of progenitor and immature cell types.

Schematic of spontaneous specification over the course of neurosphere expansion *in vitro*. Pertinent lineage markers are indicated (a). Representative digital micrographs depicting antigenic lineage analysis of NPC populations through the central sections of serial cryosections of neurospheres cultured in the absence of laminin are depicted in b-h. Scale bars, 50 μm, insets 25 μm. Type 1 NPCs expressing GFAP and nestin localized to the periphery of spheres (b, arrows and inset); Type 2a NPCs expressing nestin were found toward the centre of the core (b, arrowhead). Type 3 neuroblasts (c,d, arrows and inset) and immature neurons (d,e, arrows and inset) were found in clusters throughout serial sections. $NG2^+$ (f, arrows and inset) and $PDGF\alpha R^+$ (g, arrows and inset) OPCs and RIP^+ oligodendrocytes (h, arrows and inset) tended to be found along the neurosphere periphery. Culture on a laminin matrix increased the percentage of NPCs that retained a $nestin^+$ NPC identity and decreased the percentage of cells that specified to neuroblasts, immature neurons, and OPCs (i). Data represent mean of 5-15 sections counted over n=5-10 cultures/condition $\pm$ standard error of measurement (SEM). *p<0.05, **p<0.01, ANOVA, *post-hoc* Tukey test.

absence of exogenous ECM we performed RT-PCR. Fourteen connexins (Cx26, Cx29, Cx30, Cx30.2, Cx31.1, Cx31.9, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx47, Cx57) are expressed in the mammalian CNS (Deans et al., 2001). Transcripts for ten of these connexins (Cx26, Cx29, Cx30, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, and Cx47) were detected in neurosphere culture (Figure 2.3a-j). Cx29, Cx30, and Cx36 expression was further confirmed by analysis of the reporter gene products present in null-mutant lines. In $Cx29^{-/-}$ and $Cx30^{-/-}$ mice, the lacZ open reading frame replaces the connexin coding sequences (Filippov et al., 2003) whereas a bicistronic reporter cassette containing lacZ, an internal ribosome entry site, and placental alkaline phosphatase replaces the connexin coding sequence in $Cx36^{-/-}$ mice (Csete, 2005; Pampaloni et al., 2007; Ruiz et al., 2008). b-galactosidase expression was readily detected in neurosphere cultures from these three null-mutant lines (Figure 2.3b,c,e).

2.7.3 Differential expression of connexins in subsets of NPCs

We used immunofluorescence to localize connexins to NPC subtypes grown in the absence of exogenous laminin (Fig 2.4-2.8). For all connexins except Cx26, cultures from null-mutant mice served as negative controls to ensure antibody specificity. For Cx26, where deletion is embryonic lethal (Boucher and Bennett, 2003), antibody specificity was determined by Western blot (Figure 2.6a, inset). Four distinct connexin protein profiles were detected:

(I) Cx30 (Figure 2.4a) and Cx43 (Figure 2.4d) were expressed by the majority of cells (>60%) throughout the structure of the sphere. Punctate membrane-

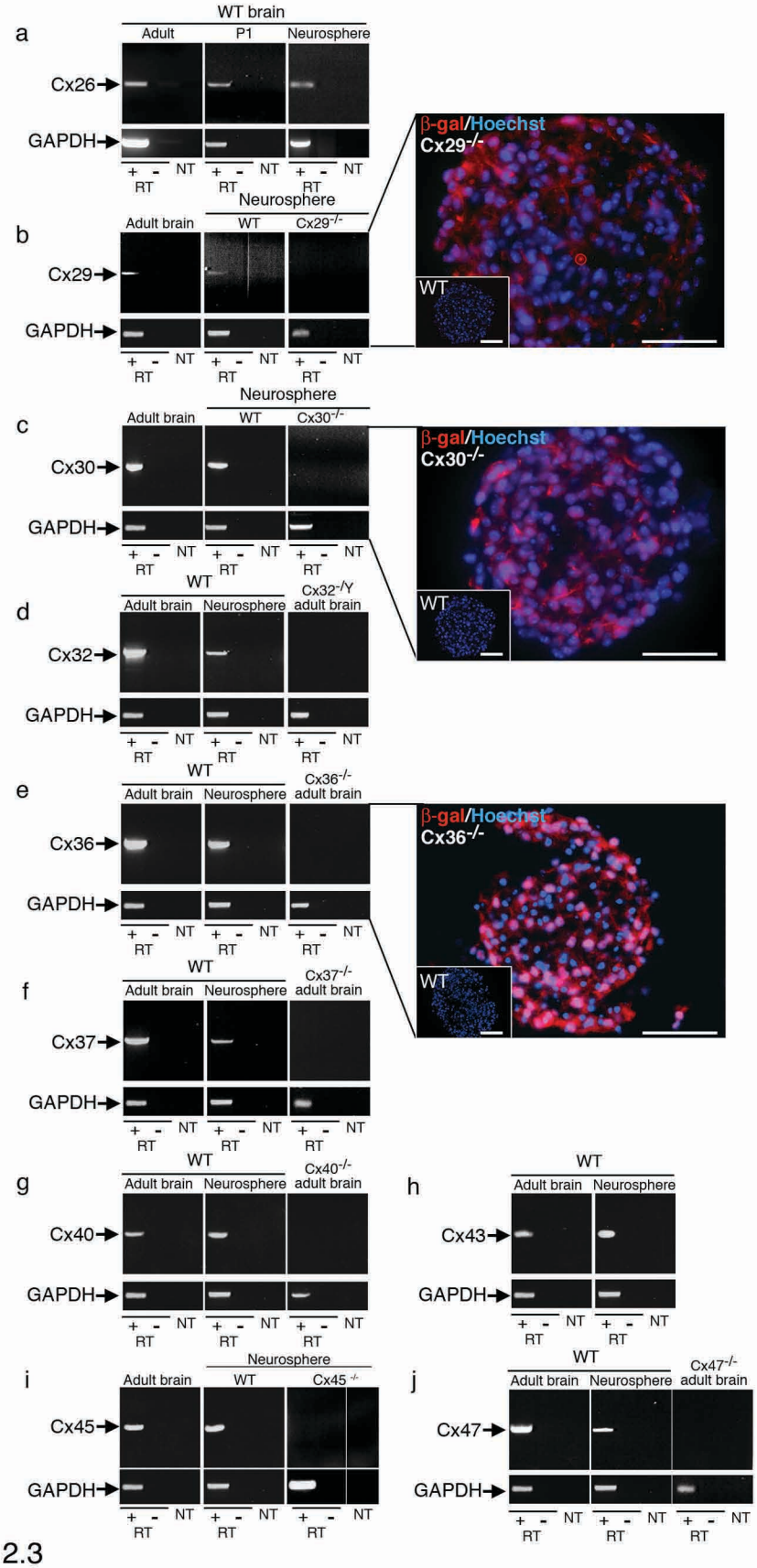
a
WT brain
Adult
P1
Neurosphere
Cx26
GAPDH
+ - NT
RT
b
Neurosphere
Adult brain
WT
Cx29-/-
Cx29
β-gal/Hoechst
Cx29-/-
WT
c
Cx30
Cx30-/-
β-gal/Hoechst
Cx30-/-
d
Cx32
Cx32-/Y
adult brain
e
Cx36
Cx36-/-
adult brain
β-gal/Hoechst
Cx36-/-
f
Cx37
Cx37-/-
adult brain
g
Cx40
Cx40-/-
adult brain
h
Cx43
i
Cx45
Cx45 -/-
j
Cx47
Cx47-/-
adult brain

Figure 2.3

Figure 2.3 - Connexin mRNA expression in postnatal hippocampal neurospheres cultured in the absence of exogenous ECM.

RT-PCR analysis of Cx26 (a), Cx29 (b), Cx30 (c), Cx32 (d), Cx36 (e), Cx37 (f), Cx40 (g), Cx43 (h), Cx45 (i), and Cx47 (j) of random-primed RNA extracted from 100-150 pooled neurosphere cultures (+RT). GAPDH was amplified to confirm template integrity. Positive controls were: Total RNA isolated from adult and/or P1 WT whole brain. Negative controls included RT-PCR reactions performed on total RNA from null-mutant neurospheres ($Cx29^{-/-}$, $Cx30^{-/-}$, and $Cx45^{-/-}$) or adult brain ($Cx32^{-/Y}$, $Cx36^{-/-}$, $Cx40^{-/-}$, $Cx37^{-/-}$, and $Cx47^{-/-}$), and reactions processed in the absence of template (NT). Potential contamination by genomic DNA was assessed by omitting the RT enzyme from the reactions (-RT). In a, c, and e, panels depict neurosphere cryosections derived from $Cx29^{-/-}$, $Cx30^{-/-}$, and $Cx36^{-/-}$ animals processed for β-galactosidase immunocytochemistry. The LacZ gene replaces the connexin coding sequence in each of these null-mutant animals. Insets are antibody controls demonstrating WT sections are negative for the β-galactosidase marker. Scale bars, 50 μm.

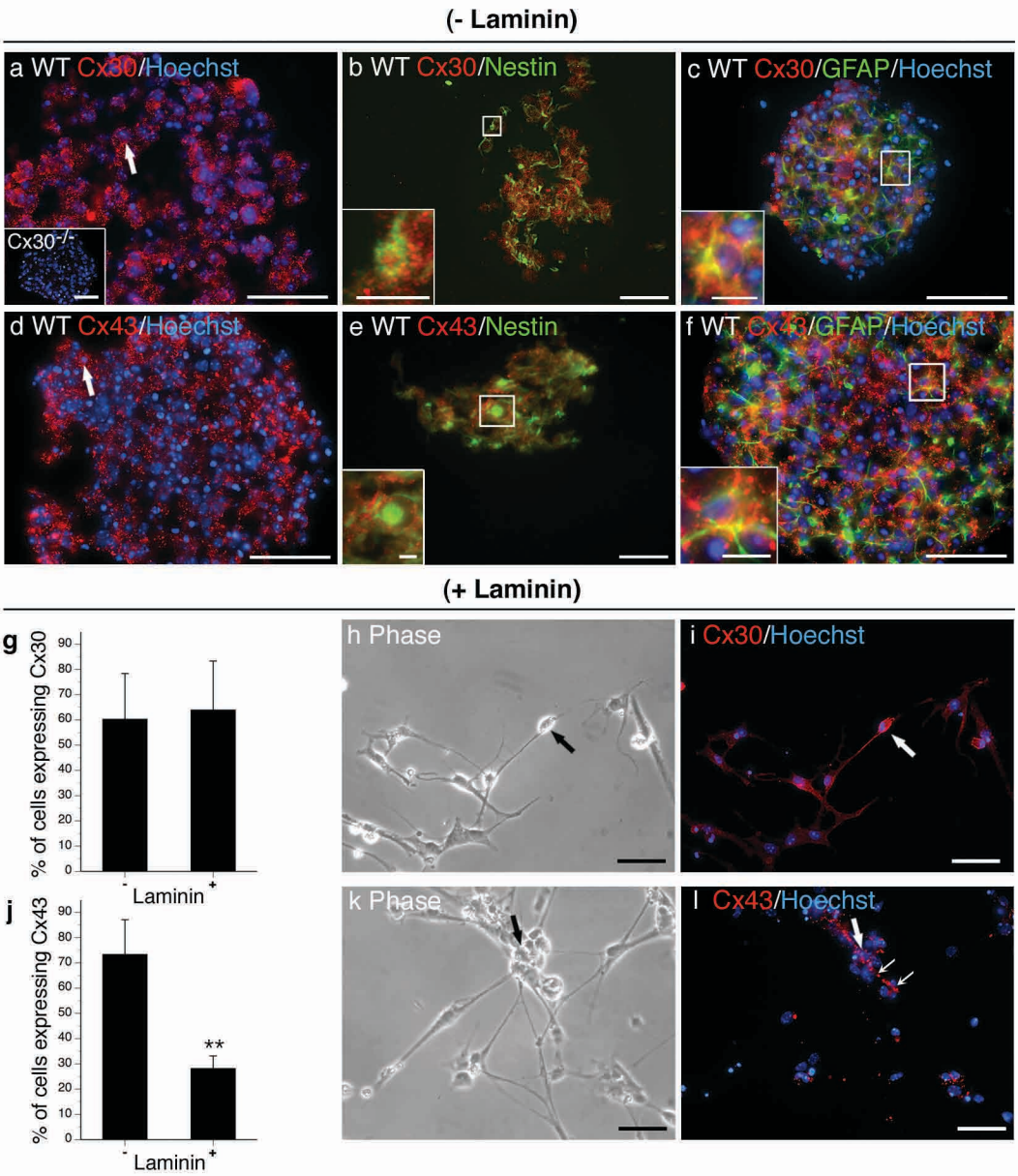
(- Laminin)
a WT Cx30/Hoechst
Cx30-/-
b WT Cx30/Nestin
c WT Cx30/GFAP/Hoechst
d WT Cx43/Hoechst
e WT Cx43/Nestin
f WT Cx43/GFAP/Hoechst
(+ Laminin)
g
% of cells expressing Cx30
Laminin
h Phase
i Cx30/Hoechst
j
% of cells expressing Cx43
**
Laminin
k Phase
l Cx43/Hoechst

Figure 2.4

Figure 2.4 - Cx30 and Cx43 are expressed by Type 1 and Type 2a NPCs and are responsive to laminin.

Cx30 and Cx43 was detected in the majority of cells within neurospheres (a,d, arrows) and localized to cells expressing nestin (b,e, insets) and GFAP (c,f, insets). Inset in (a) demonstrates lack of Cx30 immunostaining on null-mutant neurospheres confirming antibody specificity. Adherence to laminin did not impact on the frequency of $Cx30^{+}$ cells (g) although a change in subcellular localization was observed (compare punctate membrane staining (a, arrows) with diffuse cytoplasmic staining (i, arrows)). A decrease in the frequency of $Cx43^{+}$ cells (arrows) was detected following culture on laminin (j). Cx43 was present at the plasma membrane between cells in direct apposition in the presence (a) or absence (b, small arrows) of laminin. Scale bars, 50 μm, insets in a,b,c,f, 25 μm, inset in e 10 μm. Data represent mean of 5-15 sections (- laminin) or field (+ laminin) counted over n=5-10 cultures/condition $\pm$ SEM (g,j). **p<0.01 Student's *t*-test.

associated immunoreactivity characteristic of gap junction labelling was evident in nestin$^+$ and GFAP$^+$ cells (Figure 2.4b-f). We used flow cytometry to quantify Cx30 expression in nestin$^+$/GFAP$^+$ Type 1 NPCs, nestin$^+$ Type 2a NPCs, and GFAP$^+$ astrocytes. We found that all of the Type 1 NPCs (93$\pm$6%) and the vast majority of Type 2a NPCs (83$\pm$2%) expressed Cx30 (Figure 2.5). Surprisingly, few if any committed astrocytes were Cx30$^+$ (<0.1%) (Figure 2.5). These results were further substantiated by triple-labeling for Cx30, Cx43, and GFAP (Figure 2.6). Cx43 but not Cx30 was detected in all GFAP$^+$ cell types suggesting ubiquitous localization of Cx43 in GFAP$^+$ NPCs and astroglia but a more restricted distribution of Cx30 to subsets of multipotential Type 1 GFAP$^+$ NPCs.

(II) Cx26 (Figure 2.7a), Cx29 (Figure 2.8a), Cx37 (Figure 2.8c), and Cx40 (Figure 2.8e) were expressed in a minority of cells (~10%) localizing to both the sphere core and periphery in the absence of laminin. Cx26 and Cx29 exhibited membrane-associated staining but rarely in apposition with neighboring cells expressing the same connexin (Figure 2.7a, 2.8a arrows, 2.7b, 2.8b inset). Cx26 localized to nestin$^+$ and GFAP$^+$ Type 1 NPCs (Figure 2.7b,c) at the periphery of the sphere and nestin$^+$ Type 2a NPCs within the core (Figure 2.7b) as well as GFAP$^+$/nestin$^-$ astrocytes (Figure 2.7b,c). Cx29 localized to PDGFαR$^+$ OPCs (Figure 2.8b, inset). Cx37 and Cx40 labeling appeared to be largely intracellular (Figure 2.8c, e, arrows) suggesting these connexins are not forming gap junction channels at the plasma membrane. Both Cx37 and Cx40 were expressed primarily by nestin$^+$ Type 2a cells (Figure 2.8d, f, insets).

(III) Cx45 (Figure 2.9a) and Cx47 (Figure 2.9c) were expressed by rare

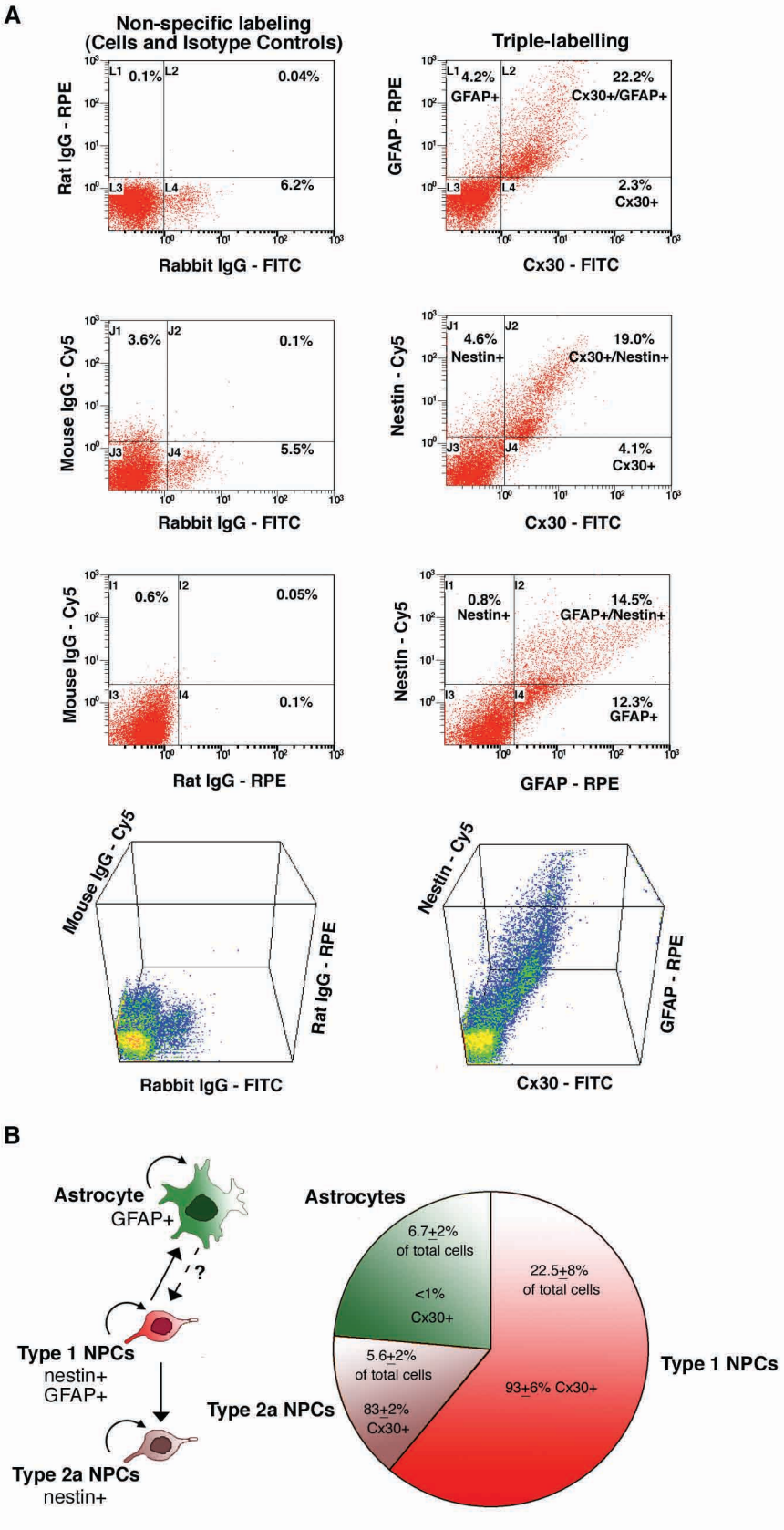
A
Non-specific labeling
(Cells and Isotype Controls)
Triple-labelling
Rat IgG - RPE
Rabbit IgG - FITC
0.1%
0.04%
6.2%
GFAP - RPE
Cx30 - FITC
4.2%
GFAP+
22.2%
Cx30+/GFAP+
2.3%
Cx30+
Mouse IgG - Cy5
3.6%
0.1%
5.5%
Nestin - Cy5
4.6%
Nestin+
19.0%
Cx30+/Nestin+
4.1%
Cx30+
0.6%
0.05%
0.1%
0.8%
Nestin+
14.5%
GFAP+/Nestin+
12.3%
GFAP+
B
Astrocyte
GFAP+
?
Type 1 NPCs
nestin+
GFAP+
Type 2a NPCs
nestin+
Astrocytes
6.7±2%
of total cells
<1%
Cx30+
22.5±8%
of total cells
5.6±2%
of total cells
83±2%
Cx30+
93±6% Cx30+
Type 2a NPCs
Type 1 NPCs

Figure 2.5

Figure 2.5 - Cx30 is expressed by Type 1 and 2a NPCs.

(a) Hippocampal NPCs cultured as neurospheres were dissociated on DIV 14 and triple-labelled for Cx30, nestin, and GFAP. Two and three dimensional histograms from a representative flow cytometry analysis are presented for both isotype control (left panel) and experimental (right panel) reactions. The quadrant percentages represent the percentage of total cells expressing corresponding cell markers. (b) Quantitative analysis of Cx30 expression in Type 1 (GFAP+/nestin+) NPCs, Type 2a (GFAP-/nestin+) NPCs, and committed astrocytes (GFAP+/nestin-). The percentage of cells co-expressing Cx30 was calculated divided by the total percentage of each cell type by the total percentage of cells within each cell type expressing Cx30 in triple-labelling studies. Data are presented as mean$\pm$SEM and represent the average of three independent flow cytometric analyses/cultures.

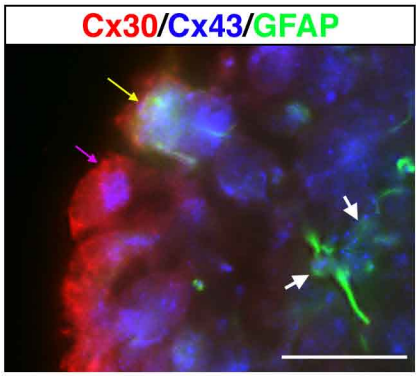

Figure 2.6

Figure 2.6 – Cx43 but not Cx30 is detected in all GFAP$^+$ cell types.

A 10 µm cyrosection through a neurosphere harvested on DIV 14 triple-labelled for Cx30, Cx43, and GFAP is presented. Yellow arrow points to cells expressing GFAP/Cx30/ and Cx43. Purple arrow identifies cells expressing Cx43 and Cx30 but not GFAP. White arrows indicate cells expressing GFAP and Cx43 but not Cx30. Scale bar, 50 µm.

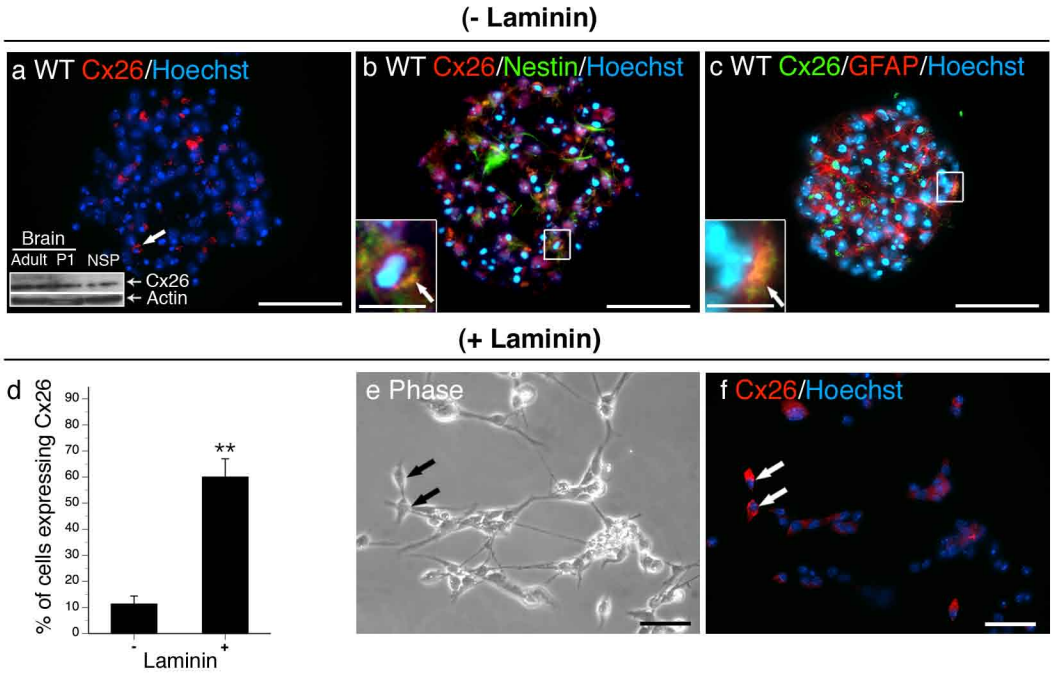
(- Laminin)
a WT Cx26/Hoechst
Brain
Adult P1 NSP
← Cx26
← Actin
b WT Cx26/Nestin/Hoechst
c WT Cx26/GFAP/Hoechst
(+ Laminin)
d
% of cells expressing Cx26
90
80
70
60
50
40
30
20
10
0
**
-
+
Laminin
e Phase
f Cx26/Hoechst

Figure 2.7

Figure 2.7 – Cx26 is expressed Type 1 and Type 2a NPCs and is responsive to laminin.

Cx26 was expressed by a small population of cells within neurospheres (a, arrow) and localizes to both cells expressing nestin (b, inset and arrow) and GFAP (c, inset and arrow). Inset in (a) depicts western blotting on protein derived from adult, P1 and neurospheres cultured in the absence of laminin to confirm antibody specificity. A marked increase in the frequency of $Cx26^{+}$ cells was detected when cultured on laminin (d,e,f, arrows). Data represent mean of 5-15 sections (- laminin) or field (+ laminin) counted over n=5-10 cultures/condition $\pm$ SEM. (d). Scale bars, 50 μm, insets, 25 μm. **p<0.01 Student's *t*-test.

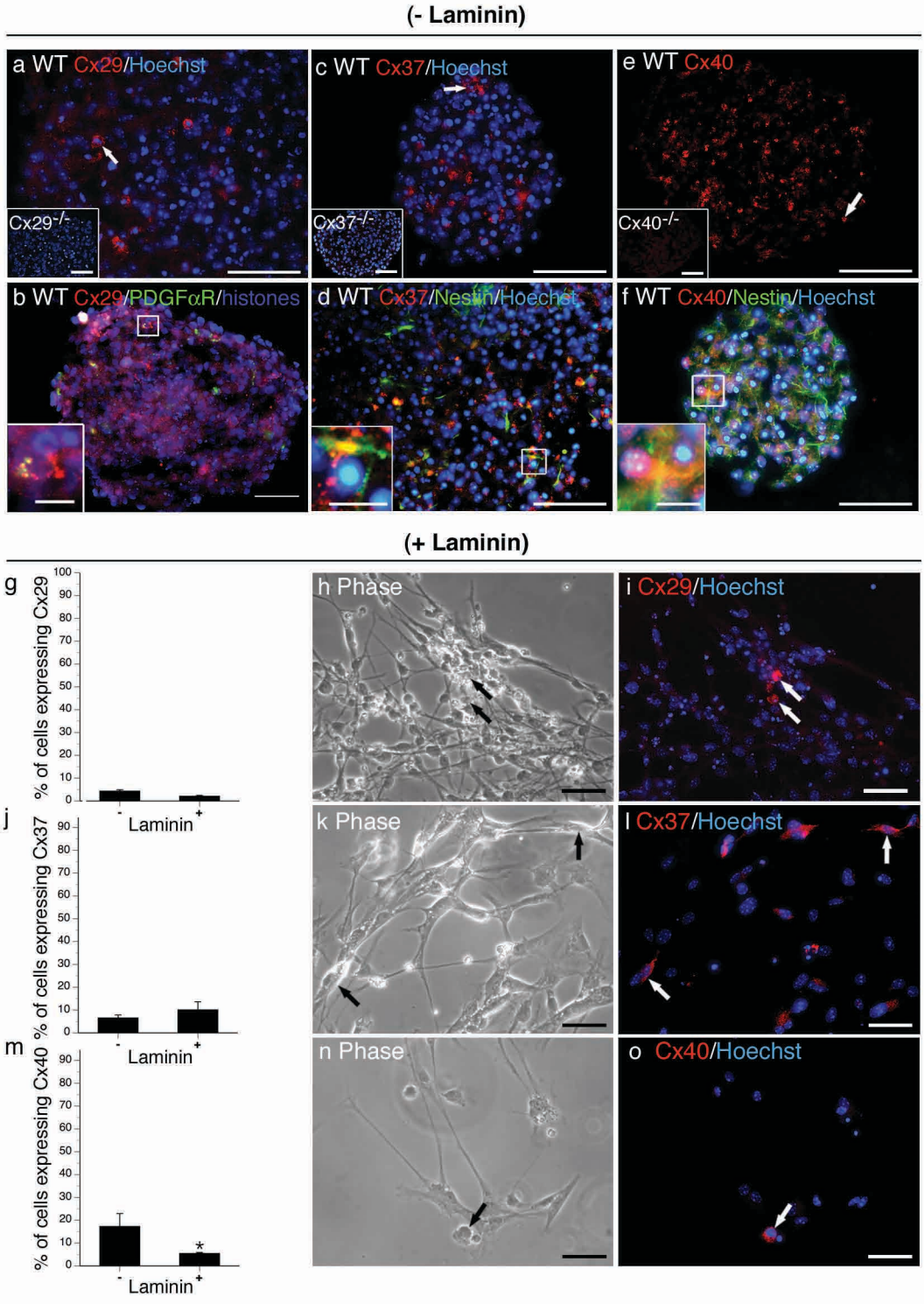
(- Laminin)
a WT Cx29/Hoechst
Cx29-/-
c WT Cx37/Hoechst
Cx37-/-
e WT Cx40
Cx40-/-
b WT Cx29/PDGFαR/histones
d WT Cx37/Nestin/Hoechst
f WT Cx40/Nestin/Hoechst
(+ Laminin)
g
% of cells expressing Cx29
Laminin
h Phase
i Cx29/Hoechst
j
% of cells expressing Cx37
Laminin
k Phase
l Cx37/Hoechst
m
% of cells expressing Cx40
Laminin
n Phase
o Cx40/Hoechst

Figure 2.8

Figure 2.8 - Cx29, Cx37, and Cx40 are expressed by discrete cell populations with only Cx40 responsive to laminin.

Cx29 (a, arrow), Cx37 (c, arrow) and Cx40 (e, arrow) were found in a small number of cells within neurospheres. Insets depict immunostaining performed on null-mutant controls demonstrating antibody specificity. Cx29 was expressed by PDGFαR$^+$ OPCs (b). Cx37 and Cx40 expression were detected in nestin$^+$ Type 2a cells (d, f). Laminin did not alter the frequency of Cx29$^+$ (g-l, arrows) or Cx37$^+$ (j-l, arrows) cells but decreased the number of Cx40$^+$ cells detected in culture (m-o, arrows). Data represent mean of 5-15 sections (- laminin) or field (+ laminin) counted over n=5-10 cultures/condition $\pm$ SEM (g,j.m). Scale bars, 50 μm. Inset in b, 10 μm. Inset in d,f, 25 μm. * $p<0.05$ Student's *t*-test.

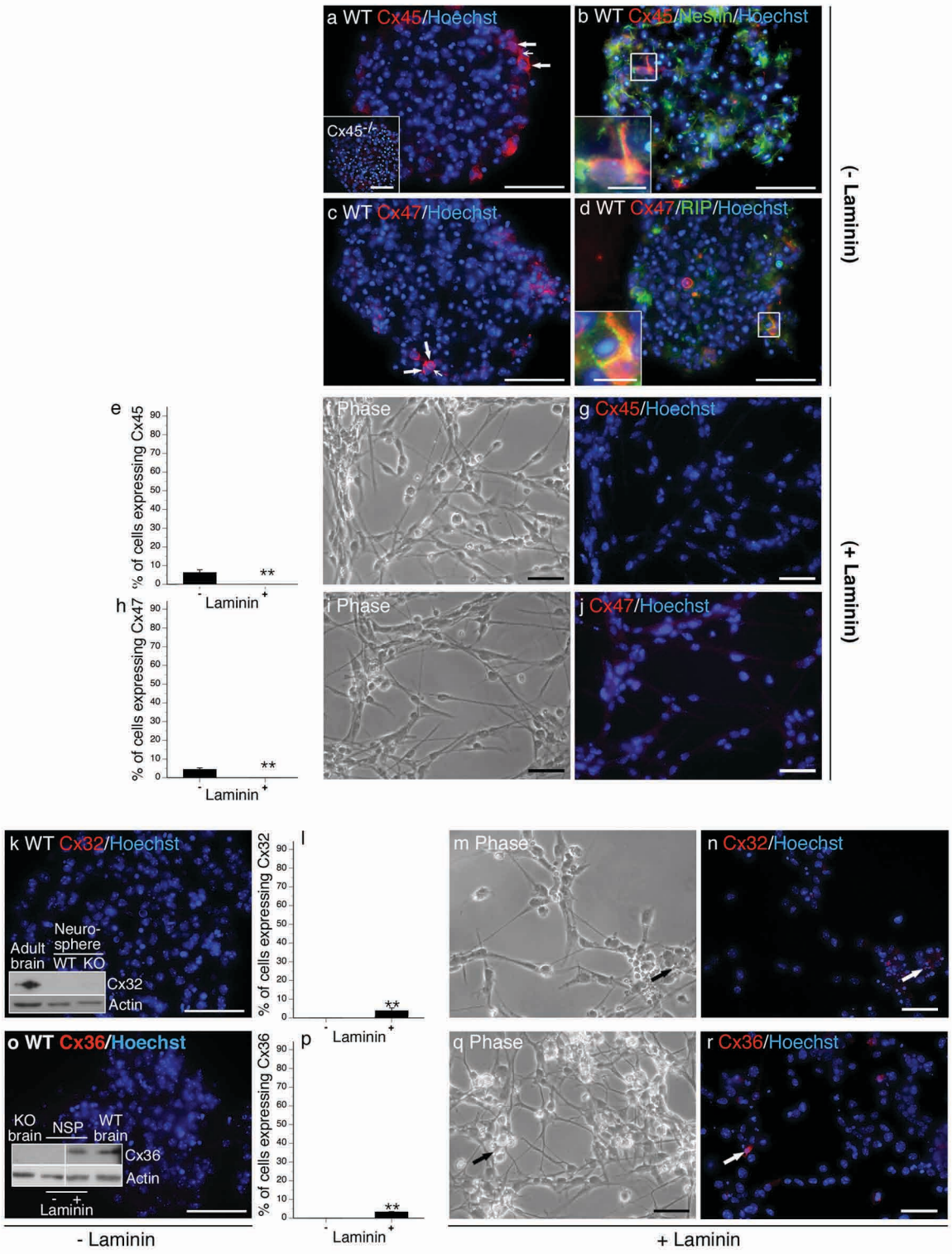
a WT Cx45/Hoechst
Cx45-/-
b WT Cx45/Nestin/Hoechst
c WT Cx47/Hoechst
d WT Cx47/RIP/Hoechst
(- Laminin)
e
% of cells expressing Cx45
Laminin
f Phase
g Cx45/Hoechst
h
% of cells expressing Cx47
i Phase
j Cx47/Hoechst
(+ Laminin)
k WT Cx32/Hoechst
Adult brain
Neuro-sphere
WT KO
Cx32
Actin
l
% of cells expressing Cx32
m Phase
n Cx32/Hoechst
o WT Cx36/Hoechst
KO brain
NSP
WT brain
Cx36
Actin
Laminin
p
% of cells expressing Cx36
q Phase
r Cx36/Hoechst
- Laminin
+ Laminin

Figure 2.9

Figure 2.9 - Cx32, Cx36, Cx45, and Cx47 are expressed by rare cells and are laminin-responsive.

Cx45 (a, arrows) and Cx47 (c, arrows) were expressed in rare populations of cells. Cx45 was detected in Type 2a nestin^{+} cells (b, inset). Cx47 was restricted to RIP^{+} oligodendrocytes (d, inset). Both connexins were downregulated when plated on a laminin substrate (Cx45 e-g, Cx47 h-j). Cx32 (k) and Cx36 (o) protein was not detected in the absence of laminin but was induced following adherence to substrate (Cx32 (l-n) and Cx36 (p-r)). To confirm the lack of Cx32 protein in suspension cultures, western analysis was performed using adult WT brain as a positive control (k, inset). Similarly, western analysis was carried out to confirm Cx36 expression in the + laminin condition and to verify antibody specificity (p, inset). Adult WT and null-mutant (KO) brain served as positive and negative controls. Actin immunoblotting was performed as a loading control. Scale bars, 50 μm, Insets, 25 μm. ** $p<0.01$ Student's *t*-test.

cells (<10%) restricted primarily to the periphery of neurospheres cultured in the absence of laminin. These connexins were often expressed in adjoining cells (Figure 2.9a, c, large arrows) where contiguous labeling was detected at the plasma membrane (Figure 2.9a, c, small arrows). Cx45 localized to nestin$^+$ Type 2a NPCs (Figure 2.9b, inset) and was not detected in GFAP$^+$ cells (data not shown). Cx47 expression was restricted to RIP$^+$ oligodendrocytes (Figure 2.9d, inset).

(IV) Both Cx32 and Cx36 were detected at the mRNA level (Fig 2d,e) but no immunodetection of protein was observed (Figure 2.9k, o). Antibody efficacy was confirmed by Western analysis (Figure 2.9k, o, insets).

2.7.4 Exposure to exogenous laminin substrate alters NPC expression of most connexins

To assess the effect of ECM on connexin expression, NPCs were expanded for 8 DIV in suspension before plating on laminin-coated glass coverslips (Figure 2.1). Cultures exposed to laminin rapidly adhered to the ECM substrate. Cells at the periphery of the neurosphere migrated from the core as adherent monolayers over 6 DIV. Fewer Cx43$^+$ cells (Figure 2.4j-l) and Cx40$^+$ cells (Figure 2.8m-o) and no Cx45$^+$ (Figure 2.9e-g) or Cx47$^+$ (Figure 2.9h-j) cells were observed when NPCs were cultured on laminin substrate. Laminin increased the number of Cx26$^+$ cells (Figure 2.7d-f) and induced Cx32 (Figure 2.9l-n) and Cx36 (Figure 2.9p-r) protein. Cx32$^+$ cells exhibited small nuclei (Figure 2.9m,n, arrow) and were immunopositive for the OPC marker NG2 (data

not shown). Cx36 was expressed in refractile cells with neuritic extensions (Figure 2.9r,s, arrow) and were immunoreactive for the neuronal marker Tuj1-bIII tubulin (data not shown). Although exposure to the ECM substrate did not alter the percentage of cells expressing Cx30 (Figure 2.4g), a change in subcellular localization was observed. Immunoreactivity was markedly less punctate (compare Figure 2.4a and i) with protein detected throughout the cytosol of laminin-treated cultures (Figure 2.4h,i, arrows). Contact with laminin did not change the number of cells expressing Cx29 (Figure 2.8g-i), Cx30 (Figure 2.4g-i), or Cx37 (Figure 2.8j-l).

An alternative explanation for these changes lies in the mechanical impact of NPC plating to an adhesive substrate (Stout et al., 2002; Suadicani et al., 2006). To test this, we plated neurospheres on poly-L-lysine, laminin, or a Matrigel mixture containing the following ECM components (56% laminin, 31% collagen IV, 8% entactin) and assessed Cx43 expression (Figure 2.10). The same reduction in Cx43-expressing cells was observed following engagement with Matrigel as plating on laminin alone. Plating on poly-L-lysine had no effect on Cx43 expression. Taken together, these data provide converging evidence for a laminin-specific regulation of connexin expression as compared to a physico-mechanical influence of culture condition.

2.7.5 Impact of laminin-induced changes in connexin expression on cell-cell and hemichannel communication

Transmembrane flux of the low molecular mass fluorescent dye, LY, was used to assess hemichannel activity in neurosphere cultures. RD was used to

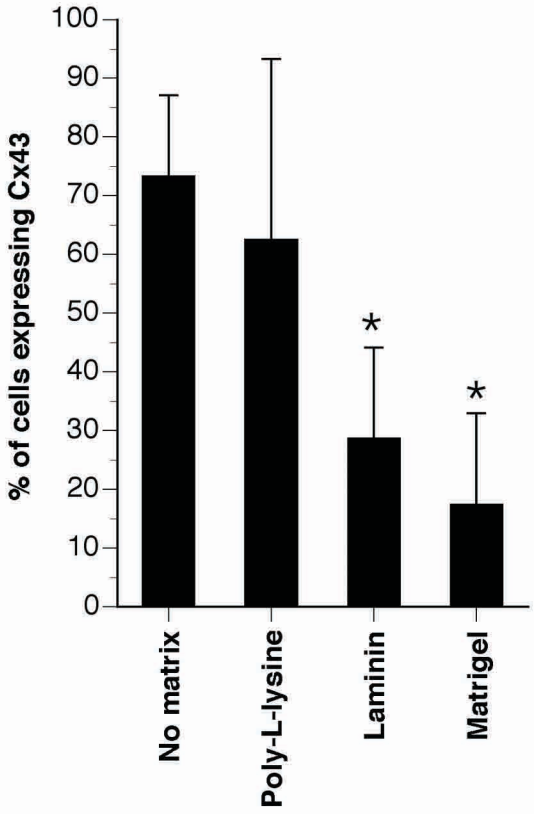
% of cells expressing Cx43
100
90
80
70
60
50
40
30
20
10
0
*
*
No matrix
Poly-L-lysine
Laminin
Matrigel

Figure 2.10

Figure 2.10 - Laminin and laminin-ECM mixtures but not poly-L-lysine alter the expression of Cx43.

The number of Cx43 expressing cells was reduced when NPCs were plated on laminin or a Matrigel matrix composed of laminin/collagen/entactin but not when cultures were plated on poly-L-lysine. * $p<0.05$, ANOVA, *post-hoc* Dunnett's *t*-test compared to neurospheres cultured in the absence of matrix.

control for uptake resulting from plasma membrane damage. Hemichannel opening was induced by mechanical stimulation with glass microbeads as previously described (Lampe and Lau, 2000). Cultures were expanded in suspension for 8 DIV, then plated and analyzed immediately after adherence (Figure 2.11a, DIV 1) prior to any detectable change in connexin protein expression (data not shown) or after 6 DIV in contact with laminin (Figure 2.11a, DIV 6) following the observed changes in connexin expression (Figure 2.4-2.8). Mechanical stimulation elicited a significant increase in dye uptake on DIV 1 (Figure 2.11a, DIV 1) that was largely inhibited by the dual-specificity chloride channel and connexin/pannexin-channel blocker FFA (Elias et al., 2007) but not the chloride channel inhibitor DIDS (Figure 2.11a, DIV 1). Hemichannel activity was lost when NPCs were cultured on laminin for 6 DIV (Figure 2.11a, DIV 6). We cannot, however, rule out that these changes are due to an effect of ECM on pannexin channel formation as we have determined that NPCs cultured in suspension express both pannexin 1 and 2 mRNA (data not shown) yet we have not investigated impact of laminin on this expression at the protein level.

Biochemical coupling indicative of GJIC was assessed by scrape loading. Little to no dye transfer was observed on DIV 1 or DIV 6 (Figure 2.11b, Proliferative conditions, DIV 1 and DIV 6). These data were surprising given the ubiquitous Cx30 and Cx43 expression detected in the majority of cells prior to laminin engagement. As a positive control, we differentiated NPCs to a predominantly nestin^{-}/GFAP^{+} astrocytic lineage (Figure 2.12). Robust GJIC was detected in these cultures and was inhibited by gap junction channel blocker

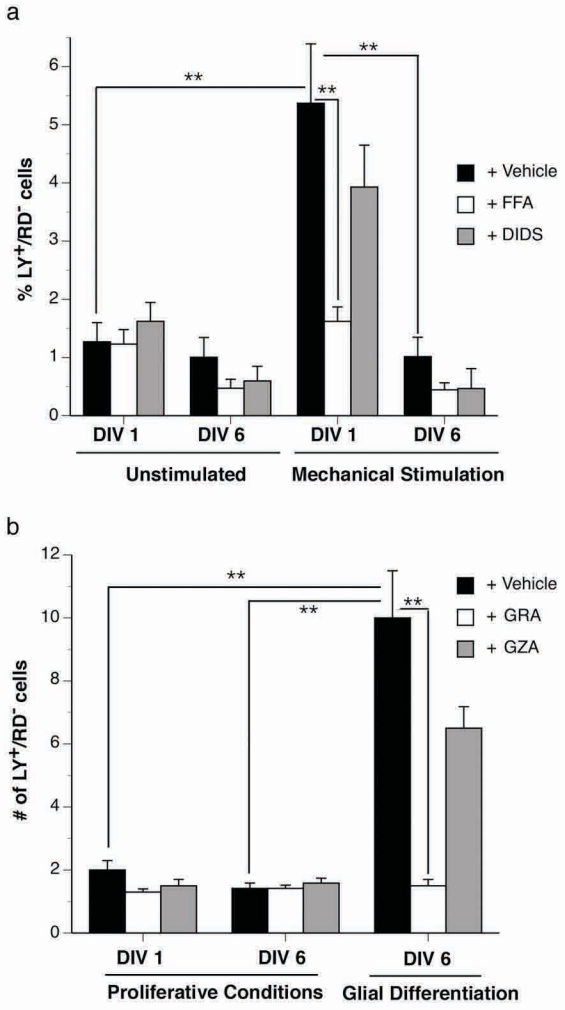
a
% LY+/RD- cells
0
1
2
3
4
5
6
**
**
**
+ Vehicle
+ FFA
+ DIDS
DIV 1
DIV 6
DIV 1
DIV 6
Unstimulated
Mechanical Stimulation
b
# of LY+/RD- cells
0
2
4
6
8
10
12
**
**
**
+ Vehicle
+ GRA
+ GZA
DIV 1
DIV 6
DIV 6
Proliferative Conditions
Glial Differentiation

Figure 2.11

Figure 2.11 - Laminin engagement alters functional hemichannel but not GJIC activity.

Functional hemichannel activity was assessed by anionic LY dye uptake (a) of cultures exposed to laminin for 1 DIV (prior to any changes in connexin expression) or 6 DIV (following changes in connexin expression). Spontaneous LY uptake was observed at low levels after 1 and 6 DIV. Open hemichannel activity could be induced by mechanical stimulation with glass microbeads within 1 DIV but not 6 DIV. Dye uptake was inhibited by the hemichannel/chloride channel inhibitor FFA but not the chloride channel blocker DIDS. LY^+/RD^- cells is expressed as a percentage of the total number of cells per microscopic field ± standard error of the mean (SEM) counted in n=5 fields per experiment conducted in triplicate experiments. GJIC was assayed using the scrape-loading method (b). Significant LY^+/RD^- dye transfer was not observed after 1 or 6 DIV when NPCs were cultured in the presence of mitogens (proliferative conditions). As a positive control, we performed the same experiment in a condition known to promote glial cell differentiation and obtained robust GJIC which was significantly inhibited by the gap junction blocker GRA but not the inactive analog GZA (glial differentiation). In GJIC assays, the number of LY^+/RD^- cells along the scrape line was established in serial photographs taken along the entire length of the scrape (9–14 photos/coverslip) over triplicate cultures. Two coverslips were assessed per culture for a total of n=54–84 measurements per condition. Data are expressed as the mean number of LY^+/RD^- cells $\pm$ SEM. *p<0.05, **p<0.01, ANOVA, *post-hoc* Tukey test.

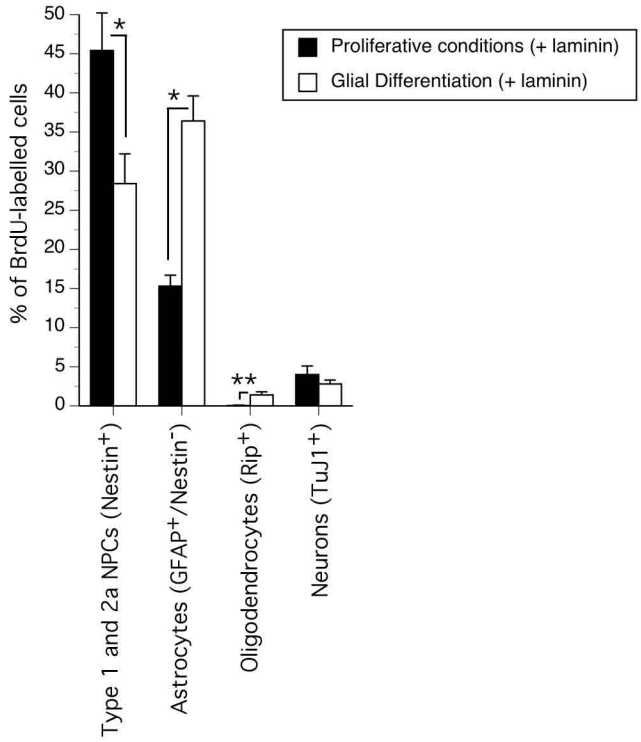
% of BrdU-labelled cells
50
45
40
35
30
25
20
15
10
5
0
*
*
**
Proliferative conditions (+ laminin)
Glial Differentiation (+ laminin)
Type 1 and 2a NPCs (Nestin+)
Astrocytes (GFAP+/Nestin-)
Oligodendrocytes (Rip+)
Neurons (TuJ1+)

Figure 2.12

Figure 2.12 - Culture composition following mitogen or glial differentiating conditions.

Cultures were pulsed with BrdU 24 h prior to plating on a laminin substrate in the presence of EGF and FGF-2 (proliferative conditions) or RA and FBS (glial differentiation). Data are expressed as the percentage of cells actively proliferating at the time of plating that retained a Type1/2a NPC identity or specified to astrocytes, oligodendrocytes, or neurons. *$p<0.05$, **$p<0.01$, ANOVA, *post-hoc* Tukey test.

GRA but not its inactive analog GZA (Figure 2.11b). To provide mechanistic insight into the lack of LY transfer in NPC cultures, we assessed Cx43 phosphorylation status and found, in neurospheres cultured without laminin, that the P2, P3, and hyperphosphorylated forms of Cx43 predominated (Figure 2.13). Previous studies have indicated that the degree of LY dye coupling is inversely correlated with phosphorylation state (Worsdorfer et al., 2008) suggesting that post-translation modification of Cx43 in NPC cultures may account for this lack of dye transfer. Taken together, these data indicate that postnatal NPC cultures do not establish gap junctions permeable to LY, but do exhibit hemichannel activity that is suppressed upon exposure to laminin.

2.8 Discussion

Our data indicate hippocampal-derived postnatal progenitor cells express a broad array of connexin genes and that connexin expression, intracellular distribution, and channel activity are regulated by ECM-cell interactions. When cultured as neurospheres in the absence of exogenous ECM, postnatal NPCs express Cx26, Cx29, Cx30, Cx37, Cx40, Cx43, Cx45, and/or Cx47 mRNA and protein as well as Cx32 and/or Cx36 mRNA. Exposure to a laminin substrate markedly increases the number of $Cx26^+$ cells present in culture and elicits the appearance of Cx32 and Cx36 protein. Conversely, laminin treatment decreases the frequency of $Cx40^+$, $Cx43^+$, and $Cx45^+$ cells without impacting on the number of $Cx29^+$, $Cx30^+$, or $Cx37^+$ NPCs. Surprisingly, we found that, while postnatal NPC cultures exhibit hemichannel activity, they do not transfer LY, a well-

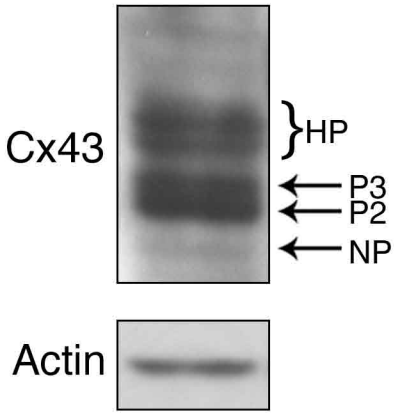

Figure 2.13

Figure 2.13 - Phosphorylation status of Cx43 in NPCs cultured as neurospheres.

Protein lysates were prepared from neurosphere cultures on DIV 14. Two predominant phosphoisoforms were detected corresponding to the P2 and P3 phosphovariants as well as hyperphosphorylated forms. Very low levels of the faster migrating unphosphorylated (NP) and little to no of the P1 phosphoisoform was detected. Data are representative of two independent cultures.

established hallmark of junctional communication, until differentiated to a mature astroglial lineage. The lack of dye transfer is likely the result of post-translational modification of the connexin proteins, specifically Cx43. Further, hemichannel activity is suppressed by plating on laminin. Together, these findings suggest a new role for ECM-cell interaction, specifically laminin, in the regulation of intrinsic connexin expression and function in postnatal NPC cultures. This regulation may predispose postnatal NPCs toward channel-independent connexin communication as recently demonstrated in the developing CNS wherein channel-independent connexin adhesion influences the migration of newly born neurons along radial glia (Bittman and LoTurco, 1999; Nadarajah et al., 1997).

2.8.1 Localization of connexins to discrete subsets of postnatal hippocampal progenitor cells

The repertoire of connexins identified here is much larger than that reported by Wörsdöfer *et al*., who demonstrated expression of seven connexin transcripts but protein for only Cx31, Cx43, and Cx45 (Rochefort et al., 2005; Worsdorfer et al., 2008). However, these studies used ECM components and not suspension-grown neurospheres. Furthermore, they used embryonic stem (ES) cells rather than postnatal NPCs. Thus, the differences in connexin expression patterns likely reflect the unique connexin signature of the different stem and progenitor cell populations present in these cultures as well as the impact of ECM adhesion on connexin expression. Here, we show that Type 1 and Type 2a multipotential NPCs express Cx26, Cx30, and Cx43. Cx26 and Cx43 have been previously observed in embryonic NPC populations (Menichella

et al., 2003) but this is the first demonstration that Cx30, exclusively expressed postnatally (Worsdorfer et al., 2008), is also expressed by multipotential Type 1 NPCs. We found that progression to a Type 2a lineage is associated with *de novo* Cx37, Cx40, and Cx45 protein expression. Further commitment to an oligodendrocyte lineage is accompanied by the loss of all of the connexins identified in multipotential NPCs and *de novo* expression of connexins associated with mature oligodendrocytes (Cx29, Cx32, Cx47) (Flaim et al., 2005; Goetz et al., 2006; Philp et al., 2005). Surprisingly, Cx32 transcript but not protein was detected in OPCs and oligodendroglia in the absence of laminin. The presence of Cx32 transcript but not protein is also observed in ES cells (Guo et al., 2001; Isakson et al., 2006; Lampe et al., 1998) with unknown consequence on cell fate. Taken together, these data provide evidence of intrinsic connexin signatures expressed by progenitor cell subsets over the course of commitment *in vitro*.

2.8.2 Effect of laminin on connexin expression and function

Exposure of NPCs to a laminin substrate altered connexin protein expression and/or stability, intracellular distribution, and functional channel activity. The simplest explanation for these changes is that this profile mirrors the effect of laminin on the differentiation of NPCs. Plating of NPCs on ECM is known to alter the rate of spontaneous specification and survival of embryonic stem cell cultures (Elias et al., 2007). Indeed, we found more cells retained a multipotential Type 1/2 phenotype in laminin-treated cultures than those expanded in the absence of laminin. An alternative explanation is a direct effect

of laminin/integrin signaling on the regulation of connexin protein expression or stability in NPCs. Certainly, laminin has been shown to alter connexin expression and function in other cell types (Melanson-Drapeau et al., 2003). The consequences of these changes have only begun to be appreciated. In CNS, the recent finding that gap junctional plaques formed by Cx26 or Cx43 are required for migration of neuronal progenitors along radial glial cells during cortical development in the mouse (Cina et al., 2009; Elias et al., 2007) suggests that ECM regulation of connexin expression is involved in cortical lamination and thus is likely to influence postnatal NPC migration. This hypothesis represents an unexplored role for ECM-connexin interactions *in vivo.*

The changes in connexin profile detected in the presence of laminin are not restricted to NPCs. OPC and neuronal profiles are also altered. Cx32 and Cx36 protein, not observed in the absence of exogenous ECM, are present following laminin engagement. Cx32 null-mutation has previously been shown to delay terminal differentiation of $NG2^+$ OPCs in adult hippocampus (Kleopa et al., 2004; Orthmann-Murphy et al., 2008). Here, we find that the induction of Cx32 protein in $NG2^+$ OPCs by laminin is associated with a moderate increase in the frequency of RIP^+ oligodendrocytes suggesting enhanced oligodendrogenesis. Surprisingly, these new oligodendrocytes did not express Cx47 in the presence of laminin suggesting that perhaps different functional subset of cells are generated. Recent studies indicate that Cx32 and Cx47 do not oligomerize and do not form the same heterotypic intercellular channels taken as evidence for functional differences between these connexins and perhaps between subsets of

oligodendrocytes (Bittman et al., 1997; Kandler, 1997; Lo Turco and Kriegstein, 1991). Finally, the induction of Cx36 protein in $TuJ1^+$ neurons in the face of reduced neurogenesis was equally surprising. This observation may recapitulate the changes observed between early and late phases of embryonic neurogenesis. Cx36 mRNA is initially detected in multipotential neural stem cells during early but not late neurogenesis where expression is restricted to their neuronal progeny (Duval et al., 2002). It may be that the functional changes in channel activity observed following engagement of neurosphere cultures with laminin regulate NPC and OPC commitment.

At no point did we detect cell-cell coupling with respect to the passage of the anionic dye LY. While surprising given that embryonic progenitor populations are coupled during early neurogenesis (Pampaloni et al., 2007) our data agree with the findings of Duval *et al.* who demonstrated GJIC in astrocytes but not in other NPC-derived cells . Here, we show passage of LY only when NPC cultures are induced to differentiate towards a primarily astroglial lineage and likely corresponds with a shift in the phosphorylation status of Cx43. Conversely, robust transmembrane dye flux indicative of functional hemichannel activity is evident prior to ECM-induced changes in connexin protein profiles. Laminin engagement suppresses this activity. It is tempting to speculate that connexin (or pannexin)-mediated hemichannel activity promotes specification whereas channel-independent adhesion supports the migration through ECM-defined domains in postnatal hippocampus. However, care must be taken in directly extrapolating our results to the *in vivo* situation given the complex three

dimensional character of the developing hippocampus where many different matrices and cellular scaffolds are present given evidence of different cell phenotypes within 2D and 3D culture systems. Clearly, it will be important to establish the impact of ECM control of connexin protein expression on postnatal NPC proliferation, migration, and specification *in vivo*.

2.9 Conclusions

We have identified the repertoire of connexins expressed by postnatal hippocampal NPCs over the course of commitment *in vivo*. We have demonstrated that ECM engagement, more specifically laminin, can alter not only the fate of cultured postnatal hippocampal NPCs, but also their connexin expression profile and related channel function. Taken together, these data suggest that ECM control of connexin expression and signaling may play a role in the direction of multipotential NPCs towards a neuronal or glial lineage.

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Chapter 3 – Connexin 45 and neurogenesis: its expression and role in adult hippocampal neural progenitor cell proliferation, specification and survival*

3.1 Objectives of this study

1) Localize Cx45 mRNA and protein expression within the adult hippocampal dentate gyrus
2) Determine the role of Cx45 in adult hippocampal NPC proliferation, specification and survival using a loss of function approach

3.2 Statement of author contributions

AP and SALB conceived of and designed the experiments. AP performed all the experiments, data analysis, and generated the figures. AP and SALB wrote the manuscript. The manuscript has been reformatted to meet the University of Ottawa's thesis requirements.

* A version of this chapter has been submitted to the Journal of Neuroscience

3.3 Connexin 45 regulates adult hippocampal neural stem cell proliferation

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Conflict of Interest: The authors report no conflicts of interest.

3.4 Summary

Neural stem and progenitor cells depend upon connexin-mediated communication for cell-cell communication during central nervous system development. It is not clear whether this communication is required for adult neurogenesis or whether loss of connexin function impairs activation of adult neural progenitor cells. Here, we localized connexin45 mRNA to both mature neurons and adult neural progenitor cells within the subgranular zone and granule cell layer of the adult hippocampal dentate gyrus. Using Cx45eGFP BAC transgenic mice, Cx45 fusion protein localized to all adult neural progenitor cells subtypes (type 1, type 2a, type 2b) with the exception of type 3 doublecortin-positive neuroblasts. To assess its role in neurogenesis, we used brain-specific null mutants to delete connexin45 from all cells of the neural lineage. Conditional Cx45 knockout mice displayed a significant reduction in type 1 neural stem cell-like proliferation but not in the activation and expansion of other downstream neurogenic lineages. These data support the continued requirement for connexin-mediated communication in the adult neurogenesis process and specifically in the proliferation of the early progenitor populations.

3.5 Introduction

The connexins are a protein family consisting of 21 and 20 members in humans and mice, respectively (Evans and Martin, 2002; Sohl et al., 2005; Sohl and Willecke, 2003). Six connexin subunits hexamerize to form a connexon or single membrane channel in non-junctional membranes which can allow for communication with the extracellular environment. When two connexons are elaborated at adjacent membranes, they can dock forming an intercellular channel allowing for the passage of ions, small metabolites and second messengers of up to 1.6 kDa in mass and less than 16 Å in size between coupled cells (Evans and Martin, 2002). Collections of intercellular channels form the morphologically defined gap junction. Individual connexins exhibit temporally and spatially distinct patterns of expression over development and their composite channels display different permeabilities supporting the hypothesis that each family member has a distinct functional role in cell-cell communication (Bedner et al., 2011; Kanaporis et al., 2011). Gap junctional intercellular communication (GJIC) is one of the primary means by which neural progenitor cells (NPCs) can communicate with both adjacent cells and their extracellular environment during development for the coordination of NPC activity, maintenance of ion homeostasis, neurotransmitter uptake, proliferation, differentiation, neuronal migration and myelination of axons (Duval et al., 2002; Kunze et al., 2009; Sutor and Hagerty, 2005; Weissman et al., 2004).

Neurogenesis, which is defined as the generation of new functionally integrated neurons, is ongoing within two regions of the adult mammalian brain:

the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Zhao et al., 2008). The production of these new neurons occurs through a stepwise process involving the proliferation, specification, and maturation of increasingly committed NPCs. As cell mature they are either apoptotically deleted or functionally integrated as new post-mitotic neurons. While the individual steps in adult neurogenesis have been well characterized, underlying regulatory events have only begun to be elucidated (Doetsch, 2003a; Kempermann et al., 2004). A comprehensive understand of these regulatory processes is required if the therapeutic potential of these cells is to be realized.

The role of juxtacrine communication, and more specifically connexin-mediated communication, in adult neurogenesis has only begun to be assessed. Within the adult brain, two connexin family members are expressed by neurons, Cx36 and Cx45 (Sohl et al., 2005). Cx45 has been reported in mature NeuN-positive neurons of the cerebellar cortex, thalamus and CA3 region of the hippocampus (Maxeiner et al., 2003; von Maltzahn et al., 2009). Cx45 is likely expressed over the course of adult neurogenesis as protein expression has been noted in the transit amplifying NPCs and neuroblasts of the SVZ; transcript has also been reported in a subset of oligodendrocyte precursor populations (Doetsch, 2003b; Maxeiner et al., 2003; von Maltzahn et al., 2009). We have previously localized Cx45 to type 2a postnatal hippocampal NPCs *in vitro* (Imbeault et al., 2009); however, the functional implications of this expression on adult neurogenesis are not known. To address this question, we show here that

Cx45 is dynamically expressed over the course of adult hippocampal neurogenesis and regulates the proliferation of primordial (type 1) stem-like hippocampal progenitors.

3.6 Experimental Procedures

3.6.1 Animals

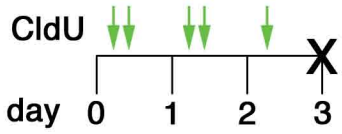
Cx45flx/flx breeding pairs on a mixed C57Bl/6 X 129/SvJ background (Nishii et al., 2003) were the generous gift of Dr. Kiyomasa Nishii (Kuyshu University, Japan). To produce brain-specific conditional knockouts, we crossed these mice with nestin-specific Cre-recombinase transgenics on a FVB/N genetic background (Berube et al., 2005) kindly provided by Dr. Ruth Slack (University of Ottawa, Canada). Two lines with identical hybrid genetic background were established by F2 matings: 1) a C57Bl/6 X 129/SvJ X FVB/N NesCre-:Cx45+/+ colony generating wildtype (WT) control mice and 2) a NesCre+:Cx45flx/flx X NesCre-:Cx45flx/flx colony generating both brain-specific null-mutants and littermates with the floxed Cx45 allele lacking Cre. To address potential impact of the Cx45 floxed allele, NesCre+:Cx45flx/flx animals were compared to both NesCre-:Cx45+/+ and NesCre-:Cx45flx/flx mice where indicated. Animals were kept on a 12 h light-dark cycle (light phase 0600 h -1800 h) with access to food and water *ad libitum*. All experiments performed on conditional knockouts were initiated using eight week old male mice. To localize Cx45, one year old female Cx45eGFP BAC mice (von Maltzahn et al., 2009), generously provided by Dr.

Klaus Willecke (University of Bonn, Germany), were used. All experimental protocols were approved by the Animal Care Committee (ACC) of the University of Ottawa conforming to the strict guidelines set forth by the Canadian Council on Animal Care for ethical treatment of experimental animals.

3.6.2 In vivo labeling of proliferating cells

Two labeling protocols were used in this study (Figure 3.1). The first cohort of NesCre-:Cx45+/+ (n=4), NesCre-:Cx45flx/flx (n=2) and NesCre+:Cx45flx/flx (n=4) mice received five injections over three days of 5-chloro-2'-deoxyuridine (CldU; 42.5 mg/kg) (prepared to 17 mg/ml in 0.625 M Tris base in sterile water; Sigma, catalog #C6891, Saint Louis, MO) intraperitoneally (i.p.) at eight weeks of age and were sacrificed 24 hours later to determine baseline proliferation for survival analysis. A second cohort of NesCre-:Cx45+/+ (n=3), NesCre-:Cx45flx/flx (n=2) and NesCre+:Cx45flx/flx (n=5) mice received five injections over three days of CldU (42.5 mg/kg) at eight weeks of age and 22 days later five injections of 5-iodo-2'-deoxyuridine (IdU; 57.5 mg/kg) (prepared to 23 mg/ml in 0.895 M Tris base in sterile water; MP Biomedicals, catalog #100357, Solon, OH) i.p. Mice were sacrificed 24 hours later to assess the 28 day survival and fate of CldU positive cells along with the proliferation and identity of the IdU labeled cells at 12 weeks of age. A third cohort of NesCre-:Cx45+/+ (n=14) and NesCre+:Cx45flx/flx (n=14) received the same IdU and CldU labeling as cohort

A - Proliferation at 8 weeks of age



B - 28 day survival and proliferation at 12 weeks of age

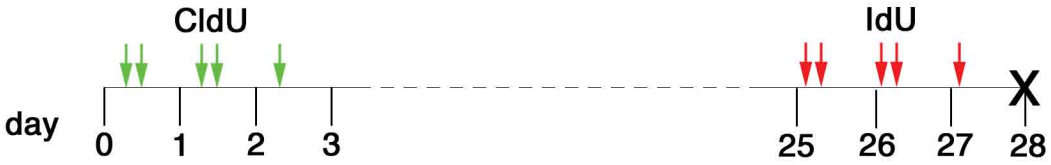


Figure 3.1

Figure 3.1 - Illustration of the mitotically active cell labeling paradigms.

IdU injections (57.5 mg/kg, i.p.) and CldU injections (42.5 mk/kg, i.p.) are indicated by red and green arrows respectively. A black X denotes the time of sacrifice.

two but were used for lineage and fate mapping analysis of the proliferating and surviving NPCs. IdU and CldU concentrations were as described in Vega and Peterson (2005) with the exception that Tris base was used to enhance solubility in place of sterile saline and 5 N NaOH as we have previously published (Pettit et al., 2012). The final pH of the solutions was pH 8.5 as recommended by Vega and Peterson (Vega and Peterson, 2005). Previous studies have confirmed equal labeling efficiencies when equimolar delivery is used (Vega and Peterson, 2005).

3.6.3 Tissue Preparation

Mice were euthanized at the time points indicate above and depicted in figure 1 with euthanyl (Bimeda-MTC Animal Health Ins, Cambridge ON) prepared in sterile water to a final concentration of 65 mg/ml and transcardial perfusion with 10 mM phosphate buffered saline (PBS; 10mM sodium phosphate and154mM NaCl) followed by 3.7% paraformaldehyde in 10 mM PBS. Brains were removed, post-fixed for 24 h and cryoprotected in 20% sucrose. For the eight week old cohort and the first 8-12 week cohort, serial 30 µm coronal cryosections (Leica Microsystems, Wetzlar, Germany) through the entire hippocampus (bregma -0.82 mm to -4.30 mm) were collected in a 1-in-9 series and stored in 0.1M PBS + 0.1% sodium azide (NaN_3) at 4°C until processed for immunofluorescence. For the second 8-12 week cohort and Cx45eGFP tissue, serial 10 µm coronal cryosections were collected through the hippocampus and slide mounted immediately (Superfrost/Plus, Fisher, catalog # 12-550-15).

3.6.4 Cx45 mRNA detection

For *in situ* hybridization, the following DIG labeled oligoprobes for Cx45 were designed using Clone Manager (SciEd Softwear, Cary, NC, USA) and generated by Integrated DNA Technologies (Coralville, IA, USA): 3' labeled probe – gattgctaggtccaagcgttcctgagccattctgatctcc-DIG and 5' labeled probe – DIG-gctccgagctgccttcttgtctgcctcgccatgctccatt. 10 µm sections were used. The hybridization step was done with 100 pg/ul of a 1:1 mixture of the two probes and was carried out in a humid chamber containing 50% formamide (EMD chemicals, Darmstadt, Germany) in a 1X salt solution (20 mM NaCl, 9 mM Tris-HCL, 1 mM Tris base, 5 mM dihydrous sodium phosphate, 5 mM disodium phosphate and 5mM EDTA) at 65ºC overnight. The hybridization solution contained 50% deionized formamide, 20% of a 50% dextran sulfate solution, 0.1 mg/ml yeast rRNA, 1X Denhardt's solution (2% bovine serum albumin, 2% Ficoll 400, 2% polyvinyl pyrolidone) in a 1X salt solution. Hybridization was detected using a sheep anti-DIG – alkaline phosphatase conjugated (Roche Diagnostics, Mannheim, Germany) antibody, diluted 1:1500 in blocking solution (20% sheep serum, 2% blocking reagent (Roche Diagnostics) in 1X maleic acid buffer with 0.1% Tween-20). Alkaline phosphatase was developed using staining buffer (100mM NaCl, 100mM Tris-Cl pH9.5, 50mM $McCl_2$, 0.1% Tween-20) with 4.5 ul/mL 4-nitro-blue-tetrazolium chloride and 3.5ul/mL 5-bromo-4-chloro-3-indoyl-phosphate (Roche Diagnostics). Unless otherwise noted all chemicals were obtained from Sigma (Sigma, Saint Louis, MO).

Cx45 mRNA expression was also assessed by immunofluorescence for β-galactosidase, the protein product of the nuclear localized LacZ reporter construct placed downstream of the Cx45 promoter in the Cx45flx/flx mice (Nishii et al., 2003). 10 µm sections were incubated with primary rabbit anti-β-galactosidase (1:1500; Millipore, catalog#AB1211, Temecula, CA) overnight at 4˚C, washed with 10 mM PBS, incubated with donkey anti-rabbit Cy3 (1:600, Jackson Immunoresearch, catalog #711-166-152) for 1 h at room temperature, and washed with PBS prior to being coverslipped in ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, P-36931). All antibodies were diluted in antibody buffer (3% bovine serum albumin, 0.3% Triton X-100 in 10 mM PBS).

3.6.5 Cx45 protein localization

Endogenous Cx45 protein was labeled using rabbit anti-Cx45 (1:1000; Millipore, catalog #AB1745), mouse anti-human Cx45, (1:400; Millipore, catalog #MAB3101), or a rabbit anti-dog Cx45 generated by T.H.Steinberg (Civitelli et al., 1993) (1:1000). Cx45eGFP fusion protein expression was identified using rabbit anti-GFP (1:2000, Abcam, catalog #ab290, Cambridge, MA) and localized to specific cell types by double and triple immunofluorescence for specific lineage markers. The primary antibodies used for lineage analysis were mouse anti-nestin (1:50; Millipore, catalog #MAB353), rat anti-GFAP (1:20, Invitrogen, catalog #130300, Eugene, Oregon, USA), guinea pig anti-doublecortin (1:400; Millipore, catalog #AB2253), mouse anti-Tuj1 (1:250, Fitzgerald International Inc.,

catalog #10R-T136a), goat anti-calbindin D28K (1:50, Santa Cruz Biotechnology, catalog #sc-7691, Santa Cruz, CA). Secondary antibodies were as above or DyLight 649 or Cy3 conjugated anti-mouse IgG (1:100, 1:800; Jackson Immunoresearch, catalog #715-495-151 and 715-165-150 respectively), FITC conjugated anti-rabbit IgG (1:100; Jackson Immunoresearch, catalog #711-096-152), Cy3 conjugated anti-goat IgG (1:800, Jackson Immunoresearch, catalog #705-165-147) and Cy3 conjugated anti-guinea pig IgG (1:800; Jackson Immunoresearch, catalog #706-165-147). Sections were incubated with primary antibodies overnight at 4˚C, washed with 10 mM PBS, incubated with secondary antibodies for 1 h at room temperature, and washed with PBS prior to being coverslipped in ProLong Gold antifade reagent (Invitrogen, catalog #P-36930). All antibodies were diluted in antibody buffer (3% bovine serum albumin, 0.3% Triton X-100 in 10 mM PBS).

3.6.6 Proliferation and Survival Analyses

An entire series of hippocampal sections were mounted, rostral to caudal, on glass slides (Superfrost/Plus, Fisher) and air-dried. Sections underwent antigen retrieval in 0.01 M citric acid, pH 6.0 at 95°C for 15 min, DNA denaturation in 2 N HCl for 30 min at RT and neutralization in 0.1 M borate buffer, pH 8.5. IdU and CldU incorporation was detected using mouse anti-BrdU clone 44 (1:500; Becton Dickinson Immunocytochemistry Systems, catalog #347580, San Jose, CA) and rat anti-BrdU (1:100, AbD Serotec, catalog #OBT0030, Raleigh, NC) respectively. Secondary antibodies were as above in

addition to Cy3 or Cy5 anti-rat IgG (1:300, 1:100; Jackson Immunoresearch, catalog # 712-166-150 and 112-15-167 respectively). Sections were incubated with primary antibodies overnight at 4˚C, washed with 10 mM PBS, incubated with secondary antibodies for 1 h at room temperature, and washed with PBS prior to being coverslipped in ProLong Gold antifade reagent (Invitrogen, catalog #P-36930). All antibodies were diluted in antibody buffer (3% bovine serum albumin, 0.3% Triton X-100 in 10 mM PBS).

3.6.7 Lineage/fate mapping and cell cycle analyses

Using two 10 µm serial sections, bregma matched between mice, double and triple immunofluorescence for IdU or CldU in addition to antigenic lineage markers or Ki67 was used to determine (a) the identity of actively proliferating (IdU-labeled) cells, (b) fate of cells surviving 28 days after labeling (CldU-labeled) and (c) the cell cycle stage at the time of sacrifice of proliferating cell (IdU labeled). Sections were incubated in 2 N HCl for 30 min at 37˚C and neutralized in 0.1 M borate buffer (pH 8.5). The primary antibodies employed were as above in addition to rabbit anti-GFAP (1:100; Sigma, catalog #G9269), rabbit anti-nestin (1:1000; Covance, catalog #PRB-315C, Emeryville, CA), rabbit anti-NG2 (1:100; Millipore, catalog #AB5320), mouse anti-NeuN (1:100; Millipore, catalog #MAB377) and rabbit anti-Ki67 (1:100; Abcam, catalog #ab66155). Secondary antibodies were as above plus FITC conjugated anti-mouse IgG (1:100; Jackson Immunoresearch, catalog #715-095-150). Sections were incubated with primary antibodies overnight at 4˚C, washed with 10 mM PBS, incubated with secondary

antibodies for 1 hr at room temperature, and washed with PBS prior to being coverslipped in ProLong Gold antifade reagent (Invitrogen, catalog). All antibodies were diluted in antibody buffer (3% bovine serum albumin, 0.3% Triton X-100 in 10 mM PBS).

3.6.8 Cell Death

Dying cells were labeled by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL, catalog #11684795910, Montreal Canada) in bregma matched 10 µm serial sections as we have previously described (Melanson-Drapeau et al., 2003).

3.6.9 Microscopic Analysis and Quantification

i) Cx45 localization – *In situ* hybridization for Cx45 mRNA and β-galactosidase immunostaining was visualized using light and epifluorescent microscopy, respectively, on a DMRXA2 microscope (Leica Microsystems). Confocal laser scanning microscopy, Leica TCS Sp5 (Leica Microsystems), was used to identify Cx45eGFP positive cells, co-labeled with antigenic lineage markers within the hippocampal dentate gyrus. Cx45eGFP-positive cells were identified and then assessed for the presence of the lineage marker of interest in z-stacks captured with Leica LAS AF software (version 2.41) using an HCX PL APO CS 63X 1.4 oil objective with a pinhole size of 1 Airy unit (or equal optical slices for multiple fluorophores). We considered antigenic lineage markers colocalized with

Cx45eGFP-labeled cells only if all labels extended from top to bottom of the z-stack.

ii) NPC proliferation and Survival - Immunofluorescence was assessed using a DMXRA2 epifluorescent microscope (Leica Microsystems). Quantification of the total number of IdU and CldU-labeled cells has been previously described (Lagace et al., 2010). The areas of interest assessed were of (a) the SGZ, defined as the region bordering the granule cell layer and the hilus (3 cell widths into each), (b) the hilus (c) the GCL of the DG and (d) the CA1 region of the hippocampus. All IdU or CldU positive cells within an area of interest, in both the left and right hippocampi of a complete series of sections through the rostro-caudal extent of the hippocampus were counted by two independent observers blinded as to the identity of the sections using the 40x objective while scanning in the z plane. These values were averaged to yield a single number per region, section, and animal. Because labeled cells were not evenly distributed and are relatively rare, we did not use defined counting frames but rather counted all labeled cells within these defined areas in accordance with previous studies (Guo et al., 2010; Thallmair et al., 2006). In proliferation studies, IdU-labeled cells as well as the number of IdU positive cell clusters, defined as 3 or more IdU positive cells in contact were counted. The total number of labeled cells in a given region per animal was calculated as the sum of the cell counts in all sampled sections multiplied by 9 to account for the inter-section interval. Means and standard errors of the mean (SEM) are reported for each experimental group.

iii) NPC lineage and fate mapping – Total IdU or CldU-positive nuclei in each

area of interest were counted and then assessed for the presence of the lineage marker of interest in merged epifluorescent images taken with a 40X objective. To provide further confirmation, confocal images were captured with Leica LAS AF software (version 2.41) using an HCX PL APO CS 63X 1.4 oil objective with a pinhole size of 1 Airy unit (or equal optical slices for multiple fluorophores) through *z*-stacks. We considered antigenic lineage markers colocalized with IdU or CldU-labeled nuclei only if the lineage label extended from top to bottom of the z-stack. An average of 130 IdU-positive cells per mouse were analyzed to identify the lineage of proliferating cells and 50 CldU-positive cells per mouse were analyzed to establish fate specification. Data are presented as the percentage of a given cell type in the total IdU or CldU population.

iv) Cell cycle and cell death - TUNEL counts were completed in the GCL, SGZ, and hilus, while Ki67/IdU counts were restricted to the SGZ and GCL, of both hemispheres using a 10X objective in two serial sections bregma matched between mice (n = 4 counts per mouse). Details are as we have previously published (Melanson-Drapeau et al., 2003). Briefly, counts were performed by two independent investigators blinded as to the identity of the sections. The area of the GCL, SGZ, and hilus or SGZ and hilus was established for each hemisphere/section using the Advanced Measurement Module of OpenLab 5.0.2 (Improvision) software. Area measurements allowed us to confirm anatomical bregma matching and to express final data as the number of positive cells per $0.1mm^2$ in the areas of interest. For Ki67/IdU analysis these values were then used to calculate the percentage of cells in G_0 (Ki67-/IdU+), S (Ki67+/IdU+) or

G_2+M (Ki67+/IdU-) phase for final data presentation.

3.6.10 Statistical Analysis

The data are presented as mean ± SEM and were analyzed by Student's t-test or ANOVA (one-way or two-way) followed by *post-hoc* Bonferroni or Tukey's post-hoc tests or planned comparisons as indicated. Statistics were performed using Prism v5.0c or SPSS v19.

3.7 Results

3.7.1 Cx45 localizes to type 1, 2a, and 2b NPCs but not type 3 neuroblasts during adult hippocampal neurogenesis

We used *in situ* hybridization to assess Cx45 mRNA expression in the neurogenic hippocampal niche of adult wildtype (WT: NesCre-:Cx45+/+ or NesCre-:Cx45flx/flx) mice. Robust Cx45 mRNA labeling was detected throughout the granule cell layer (GCL), SGZ, and hilus of the dentate gyrus (Figure 3.2A). Brain-specific null-mutants served as negative controls to confirm probe specificity (Figure 3.2B). NesCre+ transgenics (Berube et al., 2005) were crossed with Cx45flx/flx mice (Nishii et al., 2003) to generate conditional null-mutants (Cx45 cKO: NesCre+:Cx45flx/flx). Mice were viable, exhibited comparable weight gain over the course of development, and showed no overt CNS phenotype.

To confirm this expression profile, we used anti-β-galactosidase

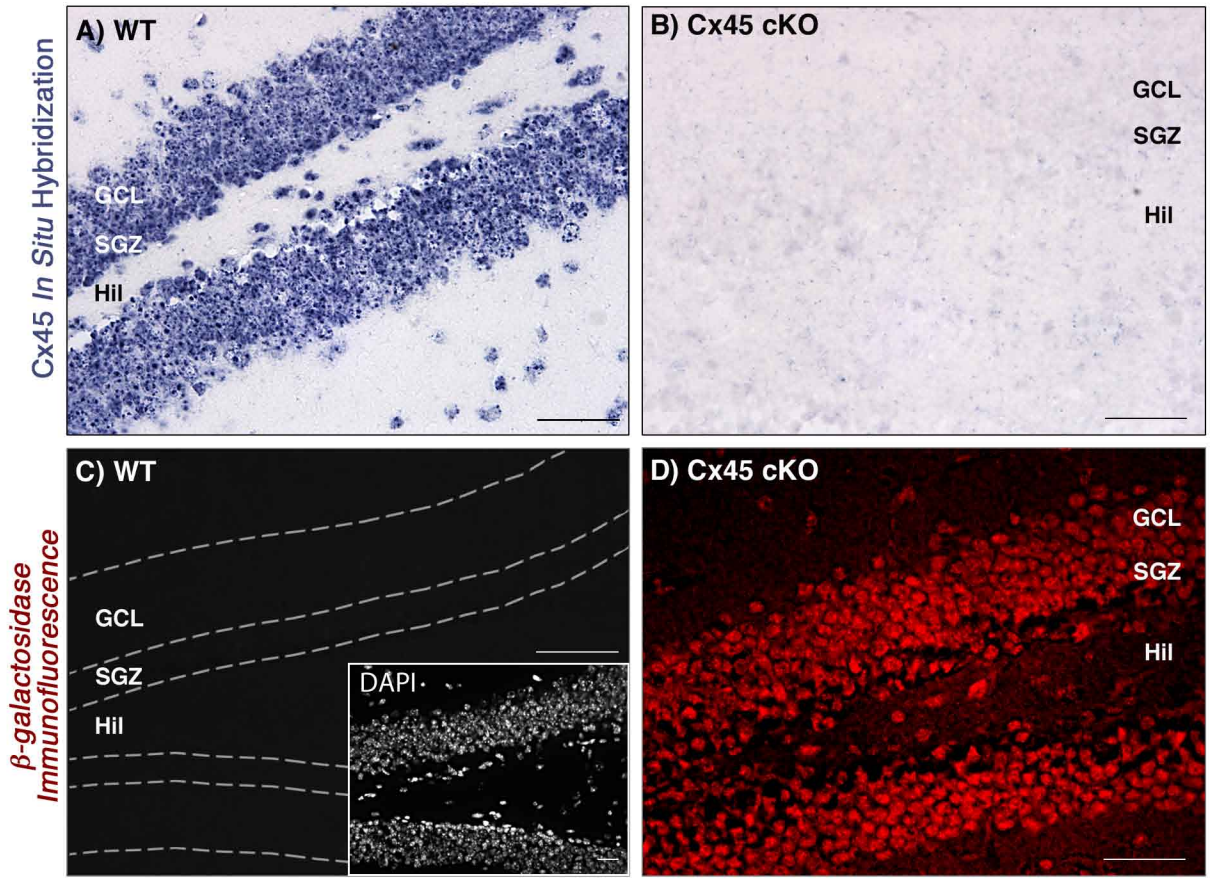

Cx45 In Situ Hybridization
β-galactosidase Immunofluorescence
A) WT
GCL
SGZ
Hil
B) Cx45 cKO
GCL
SGZ
Hil
C) WT
GCL
SGZ
Hil
DAPI
D) Cx45 cKO
GCL
SGZ
Hil

Figure 3.2

Figure 3.2 - Cx45 transcript is expressed throughout the majority of cells in the subgranular zone of the adult hippocampal dentate gyrus.

GCL, granule cell layer of the dentate gyrus; SGZ, subgranular zone; Hil, hilus. *In situ* hybridization for Cx45 mRNA in WT (A) and Cx45 cKO (B) adult murine hippocampal dentate gyrus at 40x magnification. Immunofluorescence for β-galactosidase, the lacZ gene product, under the control of the Cx45 promoter in Cx45 cKO in WT (C) and Cx45 cKO (D) adult hippocampal dentate gyrus at 40x magnification. Inset presents the DAPI channel on the WT section devoid of marker expression for orientation. All scale bars are 50 μm.

immunoreactivity to assess reporter gene product expression in our conditional null-mutants. Cx45 cKO mice express a lacZ reporter gene with nuclear localization signal placed under control of the Cx45 promoter following Cre-mediated excision of the floxed coding region (Nishii et al., 2003). As observed for Cx45 *in situ* hybridization in WT mice (Figure 3.2A,B), robust β-galactosidase immunoreactivity was detected in cells throughout the GCL, SGZ and hilus of Cx45 cKO mice (Figure 3.2C,D).

To determine when Cx45 protein is first expressed over the course of adult neurogenesis, type 1, 2a, 2b, and 3 NPCs, immature neurons, and mature neurons were identified using the panel of antigenic markers listed in Figure 3.4A and three different anti-Cx45 antibodies. AB1745 (Millipore) failed to detect any signal in WT or Cx45 cKO hippocampus whereas both MAB3101 (Millipore) and a proprietary Cx45 antibody (Civitelli et al., 1993) labeled glial-like cells in both WT and Cx45 cKO hippocampus (Figure 3.3). Lacking a Cx45 antibody with the required specificity, we applied a genetic approach to track Cx45-fusion protein over the course of adult neurogenesis. Cx45eGFP BAC mice express a C-terminal eGFP-tagged Cx45 fusion protein under the control of the Cx45 promoter (von Maltzahn et al., 2009). Although the BAC transgene cannot rescue the embryonic lethality of a Cx45-deficient background resulting from cardiac malformations, suggesting that the formation of functional channels is impaired, the distribution of the Cx45 fusion protein product has been reported to accurately recapitulate that of endogenous Cx45 in all tissues studied to date (Chapman et al., 2012; Kumai et al., 2000; von Maltzahn et al., 2009). Using

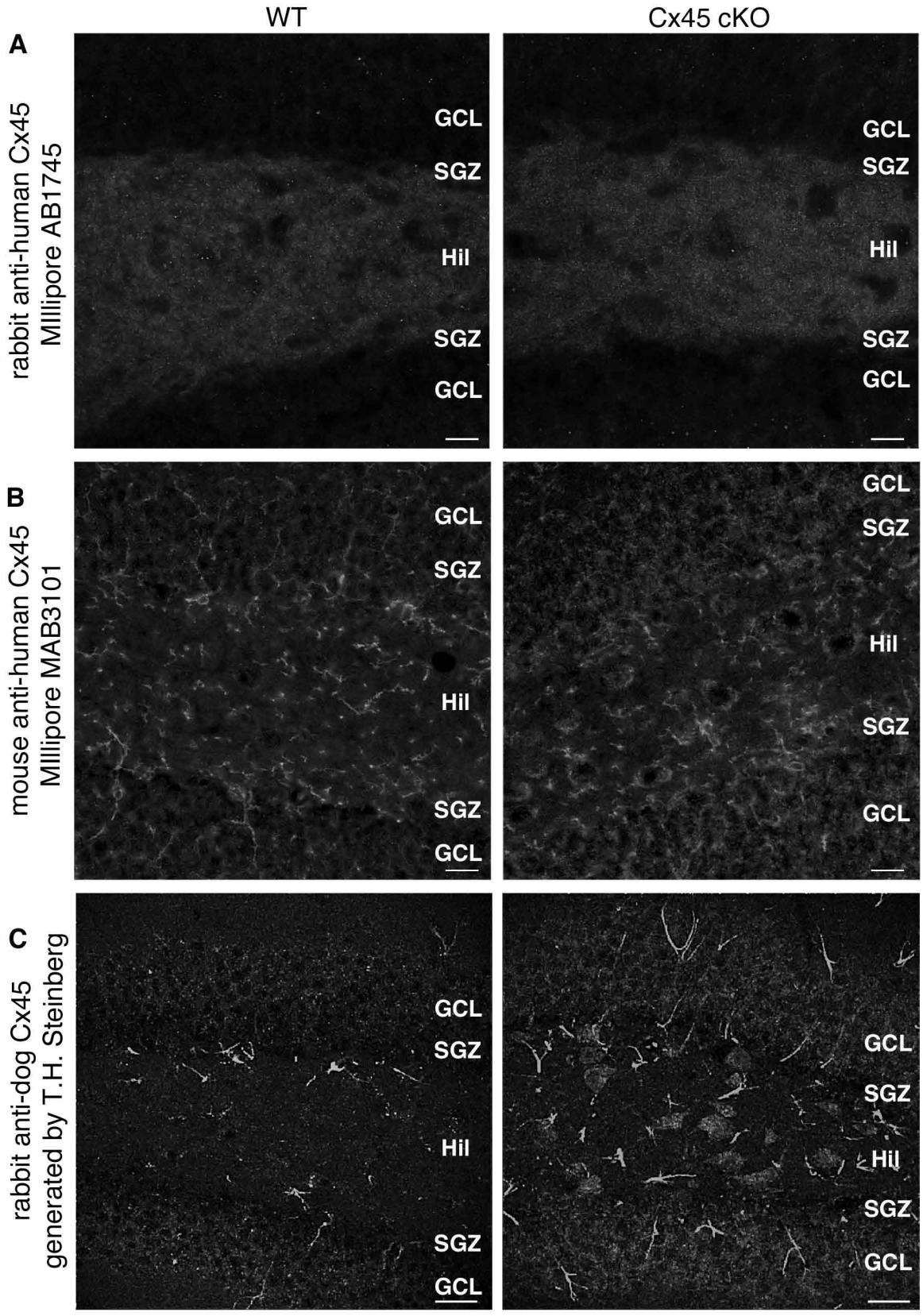

WT
Cx45 cKO
A
rabbit anti-human Cx45
MIllipore AB1745
GCL
SGZ
Hil
SGZ
GCL
B
mouse anti-human Cx45
MIllipore MAB3101
C
rabbit anti-dog Cx45
generated by T.H. Steinberg

Figure 3.3

Figure 3.3 - Existing Cx45 antibodies lack specificity in the brain.

Immunofluorescence for Cx45 in WT and Cx45 cKO adult hippocampal dentate gyrus using (A) rabbit anti-human Cx45, Millipore AB1745, (B) mouse anti-human Cx45, Millipore MAB3101 and (C) rabbit anti-dog Cx45 generated by T.H.Steinberg (Civitelli et al., 1993).

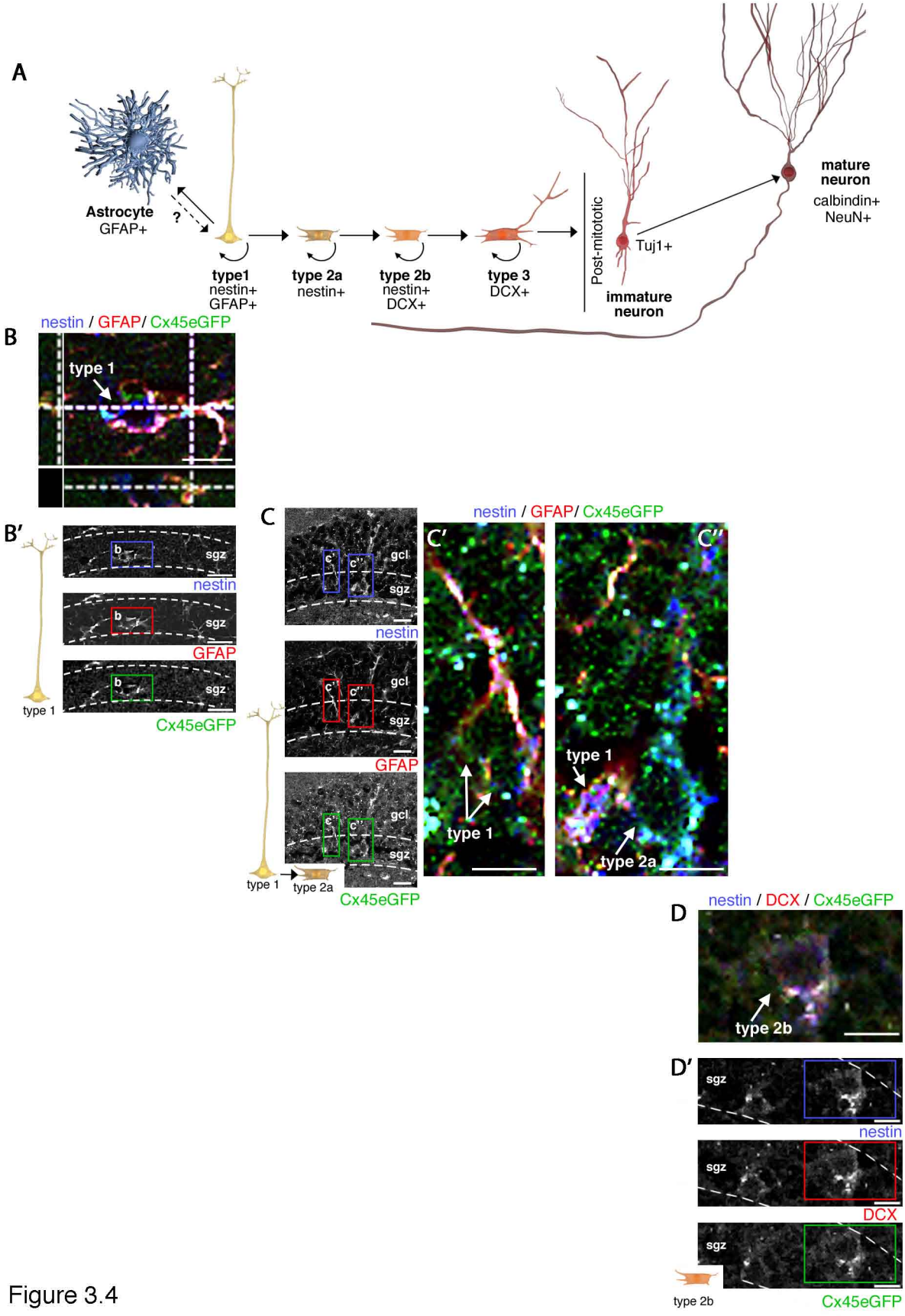

A
Astrocyte
GFAP+
?
type1
nestin+
GFAP+
type 2a
nestin+
type 2b
nestin+
DCX+
type 3
DCX+
Post-mitototic
Tuj1+
immature
neuron
mature
neuron
calbindin+
NeuN+
B
nestin / GFAP/ Cx45eGFP
type 1
B'
b
sgz
nestin
GFAP
Cx45eGFP
type 1
C
c'
c''
gcl
sgz
nestin
GFAP
Cx45eGFP
type 1
type 2a
C'
C''
nestin / GFAP/ Cx45eGFP
type 1
type 2a
D
nestin / DCX / Cx45eGFP
type 2b
D'
sgz
nestin
DCX
Cx45eGFP
type 2b

Figure 3.4

Figure 3.4 - Cx45eGFP fusion protein localizes to type 1, 2a and 2b NPCs in the adult hippocampal dentate gyrus.

Acronyms are as defined in figure 3.2. (A) Diagram of postnatal hippocampal neurogenesis including protein markers used for lineage analysis of proliferating and postmitotic cell types. (B-D) Confocal microscopy images of: Cx45eGFP colocalizing to nestin and GFAP-positive cells in type 1 NPCs (panel B, C, C' and C'') or to cells positive for nestin alone in type 2a NPCs (panel C and C''). Cx45eGFP is also detected in nestin and DCX-positive type 2b NPCs (panel D and D'). Scale bars are 10 μm (panel B, C', C'', D and D'), or 20 μm (panel C and B').

these mice, we found that Cx45-eGFP localized to nestin-positive/GFAP-positive cells in the dentate gyrus (Figure 3.4B, C, C' and C''). These cells were further defined as type 1 stem-like NPCs by their distinctive triangular soma in the SGZ and single process projecting through the GCL (Figure 3.4B, C, C' and C''). Fusion protein was also evident in the nestin-positive/GFAP-negative type 2a progeny of type 1 NPCs (Figure 3.4C and C'') as well as a majority of nestin-positive /DCX-positive type 2b NPCs (Figure 3.4D and D''). At no point, did we see Cx45-eGFP in nestin-negative/DCX-positive type 3 neuroblasts (Figure 3.5A). However, following terminal commitment, Cx45eGFP was again detected in both post-mitotic immature Tuj1-positive (Figure 3.5B) and mature calbindin-positive neurons of the SGZ and GCL (Figure 3.5C).

3.7.2 Proliferation of type 1 NPCs is decreased by the loss of Cx45

The production of new neurons in the adult hippocampus occurs through the stepwise proliferation, specification, migration, and terminal differentiation of type 1, 2a, 2b, and 3 NPCs. To examine whether Cx45 is required for the proliferation of these populations, we quantified the number of proliferating cells in the SGZ of WT and Cx45 cKO mice at eight and 12 weeks of age (Figure 3.6A,B). Actively proliferating cells were labeled with the antigenically distinct thymidine analogs, CldU and IdU (Vega and Peterson, 2005). Delivery schedules (Figure 3.1) were optimized to label both slowly dividing type 1 and 2a populations and rapidly dividing type 2b and 3 NPCs in sufficient numbers to

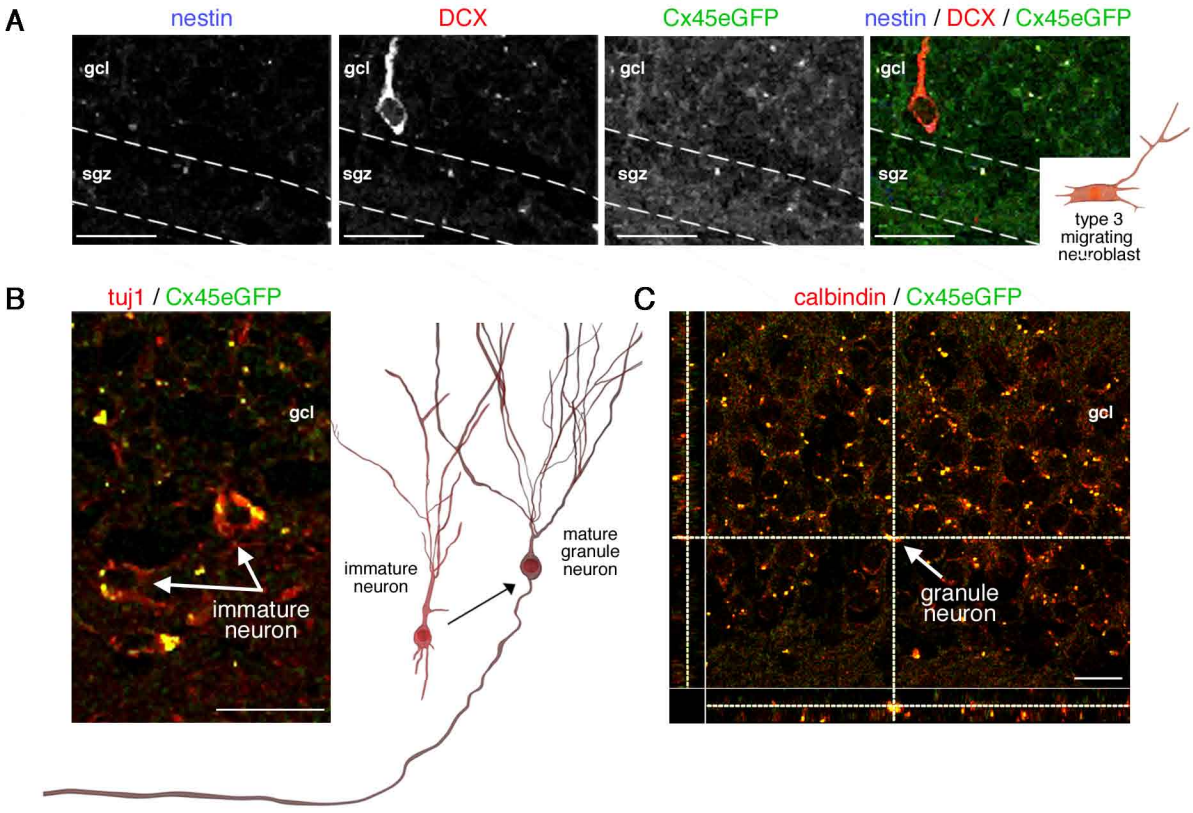

A
nestin
DCX
Cx45eGFP
nestin / DCX / Cx45eGFP
gcl
sgz
type 3
migrating
neuroblast
B
tuj1 / Cx45eGFP
immature
neuron
mature
granule
neuron
C
calbindin / Cx45eGFP
granule
neuron

Figure 3.5

Figure 3.5 - Cx45eGFP protein is expressed by post-mitotic immature and mature neurons but not type 3 DCX positive neuroblasts in the hippocampal dentate gyrus.

Acronyms are as defined in figure 2. Confocal microscopy images demonstrating the absence of Cx45eGFP in DCX-positive type 3 cells (panel A, scale bar 20 μm). Cx45eGFP co-localizes with tuj1 in immature neurons (panel B, scale bar 25 μm) and calbindin-positive mature neurons (panel C, scale bar 12.5 μm).

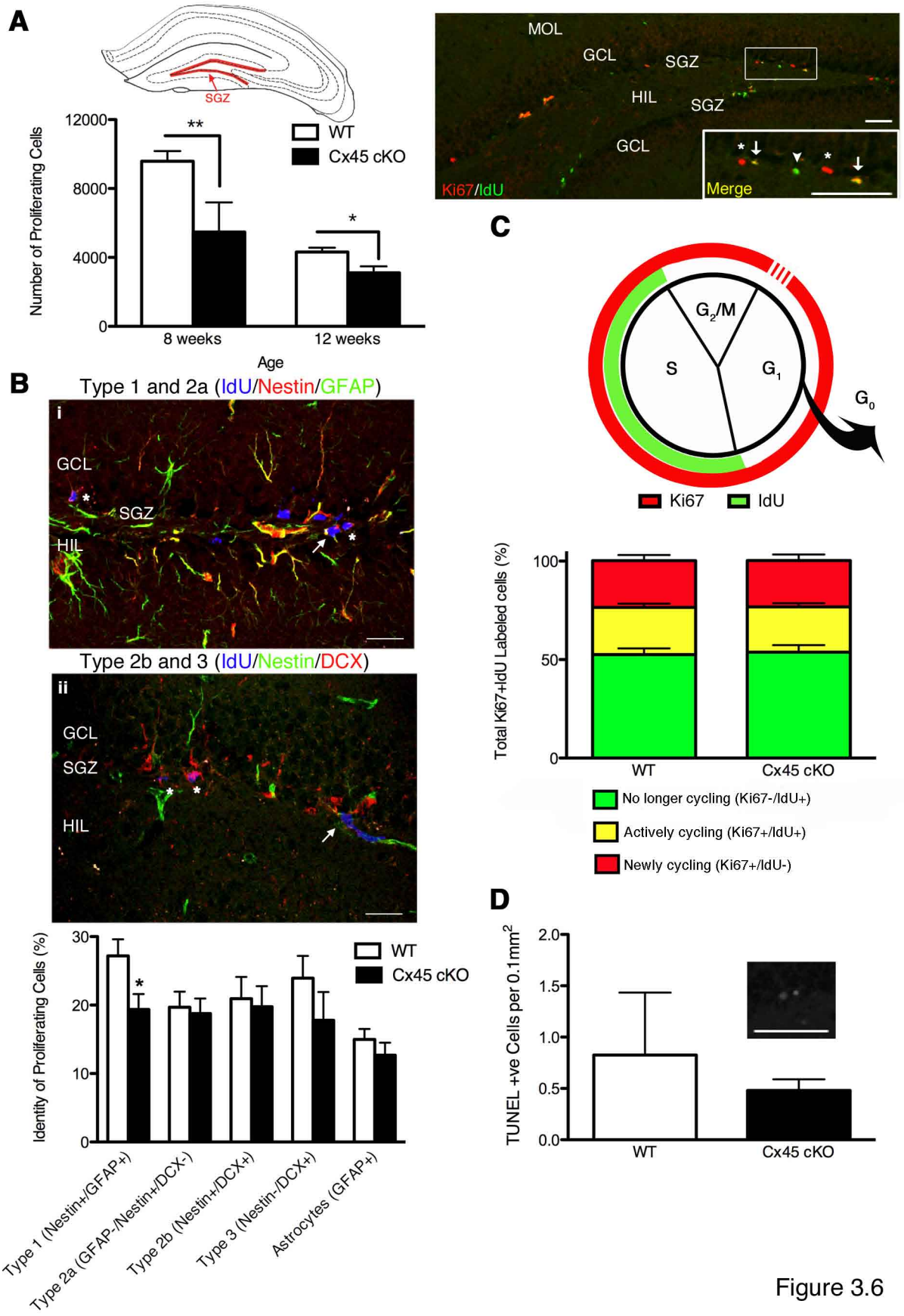

A
SGZ
Number of Proliferating Cells
12000
8000
4000
0
**
*
WT
Cx45 cKO
8 weeks
12 weeks
Age
MOL
GCL
SGZ
HIL
SGZ
GCL
Ki67/IdU
Merge
B
Type 1 and 2a (IdU/Nestin/GFAP)
i
GCL
SGZ
HIL
Type 2b and 3 (IdU/Nestin/DCX)
ii
GCL
SGZ
HIL
Identity of Proliferating Cells (%)
30
20
10
0
*
WT
Cx45 cKO
Type 1 (Nestin+/GFAP+)
Type 2a (GFAP-/Nestin+/DCX-)
Type 2b (Nestin+/DCX+)
Type 3 (Nestin-/DCX+)
Astrocytes (GFAP+)
C
G2/M
S
G1
G0
Ki67
IdU
Total Ki67+IdU Labeled cells (%)
100
50
0
WT
Cx45 cKO
No longer cycling (Ki67-/IdU+)
Actively cycling (Ki67+/IdU+)
Newly cycling (Ki67+/IdU-)
D
TUNEL +ve Cells per 0.1mm2
2.0
1.5
1.0
0.5
0.0
WT
Cx45 cKO

Figure 3.6

Figure 3.6 - Type 1 NPC proliferation is decreased in the SGZ of the hippocampal dentate gyrus of mice lacking Cx45.

MOL, molecular layer of the dentate gyrus, all other acronyms are as define in figure 2. (A) Quantification of the proliferating cells in the SGZ of the dentate gyrus of 8 and 12 week old WT and Cx45 cKO mice. * $p<0.05$, ** $p<0.01$, two-way ANOVA, post-hoc Tukey's test. (B) Representative confocal micrographs used for lineage analysis of proliferating NPCs. (i) IdU/Nestin/GFAP (type 1 cell, closed arrow and type 2a cells, asterisk), (ii) IdU/Nestin/DCX (type 2b cell, closed arrow and type 3 cells, asterisk). Scale bars, 25 μm. Double and triple positive cells in the SGZ were quantified at 12 weeks of age in WT (white bars) and Cx45 cKO (black bars) mice. * $p<0.05$ by Student's *t*-test for each lineage. (C) Double immunofluorescence for IdU (green) and Ki67 (red) in the DG at 10x magnification to assess for cell cycle defects. Inset depicts cells that are no longer cycling (green, arrow head) positive for IdU alone, cells that continue to cycle phase (yellow, arrow) positive for both IdU and Ki67 and cells that have recently entered the cell cycle (red, asterisk) positive for Ki67 alone. Scale bar, 50 μm. Schematic of the expression of Ki67 and IdU labeling across the cell cycle. Single and double-labeled cells were quantified and the percentage in each cell cycle stage is not different between WT and Cx45 cKO mice at 12 weeks of age. $p>0.05$ by two-way ANOVA. (D) The number of TUNEL positive cells per $0.1mm^2$ in the DG of WT and Cx45 cKO mice is not different at 12 weeks of age. Inset, representative image of TUNEL positive cells. Scale bar, 50 μm.

detect an increase or decrease in proliferative index (Nowakowski et al., 1989; Pettit et al., 2012; Taupin, 2007). Proliferating cell number decreased with age in both genotypes with Cx45 cKO mice exhibiting fewer actively proliferating cells compared to WT (Figure 3.6A two-way ANOVA, main effects of age and genotype, * $p<0.05$, ** $p<0.01$ relative to WT by post-hoc Tukey's test). There was no difference in the number of labeled cells in the CA1 field of the hippocampal formation indicating that the bioavailability was comparable between genotypes and thymidine analogs (data not shown).

To identify the NPC populations affected by the loss of Cx45, double and triple immunofluorescence for IdU and the antigenic lineage markers presented in Figure 3.4A were assessed in WT and Cx45 cKO mice at 12 weeks of age (Figure 3.6B). Double and triple labeled cells were quantified and expressed as a percentage of the total proliferating (IdU-positive) population. Cx45 cKO mice displayed a significant reduction in proliferating type 1 nestin-positive/GFAP-positive NPCs (Figure 3.6B, ** $p<0.01$ student t-test). The numbers of all other activated NPC populations were comparable between genotypes (Figure 3.5B, $p>0.05$, student t-test).

The reduction in proliferating type 1 cells in Cx45-deficient mice could be due a change in cell cycle kinetics or an increase in cell death. A decrease in cell cycle rate, which occurs most commonly due to a lengthening of G_1 phase, could result in fewer NPCs being labeled in Cx45cKO mice. The possibility of a disrupted cell cycle is particularly intriguing due to the slow turnover over of type 1 hippocampal NPCs and the requirement for GJIC, at least in embryonic

progenitors, to enter S phase (Bittman et al., 1997; Filippov et al., 2003; Kempermann et al., 2004). To address whether cell cycle kinetics are altered by the loss of Cx45, cells were labeled with IdU, an S phase marker and immunostained for Ki67, an endogenous cell cycle protein expressed during all stages except G_0 and early G_1 (Figure 3.6C). The percentage of cells entering the cell cycle, proliferating or having recently exited from the cell cycle did not vary between genotypes (Figure 3.6C, $p>0.05$, two-way ANOVA) suggesting that there is no gross cell cycle defect as a result of the loss of Cx45. Moreover, no difference in apoptotic-like deletion of cells was detected by TUNEL (Figure 3.6D, $p>0.05$, student t-test). Taken together, these findings suggest that proliferation is reduced specifically in type 1 NPCs in the absence of Cx45.

3.7.3 Loss of Cx45 does not alter the specification or survival of NPC progeny

To complete the assessment of the neurogenic potential of the Cx45 cKO mice, the long-term (28 day) survival of CldU labeled progeny was assessed. Mice were labeled with five injections of CldU over three days at eight weeks of age and were sacrificed 28 days later (Figure 3.1B). Quantification of the total number of surviving cells in the GCL and SGZ of WT and Cx45 cKO mice revealed no significant differences (Figure 3.7A; $p>0.05$, student t-test). There were no differences in the distribution of the CldU positive cells between genotypes (Figure 3.7B). Using double immunofluorescence for a mature neuronal or glial marker, NeuN and GFAP respectively, and CldU we identified

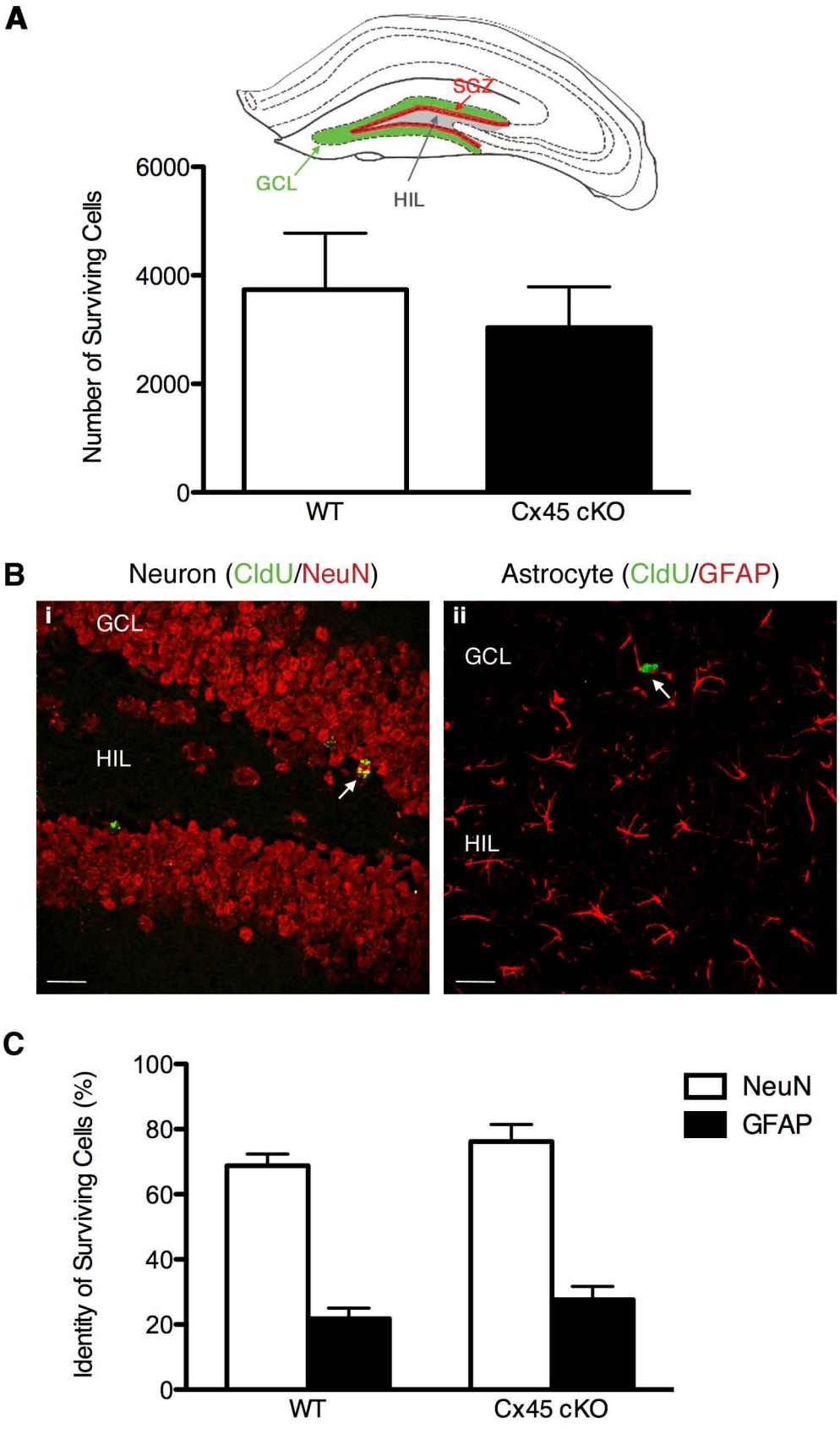

A
SGZ
GCL
HIL
Number of Surviving Cells
6000
4000
2000
0
WT
Cx45 cKO
B
Neuron (CldU/NeuN)
Astrocyte (CldU/GFAP)
i
GCL
HIL
ii
GCL
HIL
C
Identity of Surviving Cells (%)
100
80
60
40
20
0
NeuN
GFAP
WT
Cx45 cKO

Figure 3.7

Figure 3.7 - Loss of Cx45 does not alter NPC survival or fate.

(A) Number of surviving cells 28 days after labeling in the GCL and SGZ of the hippocampal dentate gyrus of WT and Cx45 cKO mice. $p>0.05$, Student's *t*-test. (B) Representative confocal microscope micrographs used for fate mapping analysis of surviving NPC progeny. (i) Neurons (CldU/NeuN), (ii) astrocytes (CldU/GFAP), double positive cells are indicated by white arrows. Scale bars 25 μm. (C) The percentage of total CldU-positive cells which are double positive for the lineage markers NeuN (white) or GFAP (black) by immunofluorescence in the GCL and SGZ of the hippocampal dentate gyrus of WT and Cx45 cKO mice is not altered. $p>0.05$, two-way ANOVA.

the fate of surviving progenitor-derived cells (Figure 3.7B). Double positive cells were expressed as a percentage of the total surviving population (Figure 3.6C). No significant difference in the proportion of surviving neurons or glia was seen between genotypes (Figure 3.7C, $p>0.05$, two way-ANOVA). Taken together this data suggests that the loss of Cx45 does not affect hippocampal progenitor cell survival or fate specification.

3.8 Discussion

Here, we report that the loss of Cx45 restricts the expansion of type 1 stem-like progenitors in the uninjured adult hippocampus. We show that protein is dynamically expressed over the course of adult hippocampal neurogenesis localizing to type 1, 2a, and 2b NPCs and re-expressed by mature neurons following terminal specification. In Cx45-deficient mice, brain-specific deletion results in a reduction in the number of slowly dividing type 1 NPCs in the adult hippocampus without affecting the survival or specification of their progeny.

This is the first report to describe the expression and localization of Cx45 within the neurogenic niche of the adult hippocampal dentate gyrus. Cx45 has been previously reported in the CA3 region of the hippocampus (Maxeiner et al., 2003; von Maltzahn et al., 2009; Zlomuzica et al., 2010). Here, by both *in situ* hybridization in WT mice and ß-galactosidase immunofluorescence in our conditional knockout mouse, we show robust Cx45 expression throughout the GCL and SGZ. Using the Cx45eGFP BAC transgenic we similarly observed a strong Cx45eGFP signal throughout the GCL and SGZ it is detected throughout

the early stages (type1, 2a and 2b) of adult neurogenesis. Interestingly, type 3 NPCs appear to be devoid of Cx45 protein, yet immature and mature neurons once again express Cx45 protein. We posit that this could be due to the intense level of selection which occurs between the type 3 and immature neuron stage which results in some receptor subtypes being down regulated to allow for this selection to occur undisturbed (Garcia et al., 2004).

While this is the first report that Cx45 regulates type 1 progenitor cell proliferation, our results are consistent with previous reports of decreased NPC proliferation in Cx30/43 double knockout mice (dKO) and several *in vitro* reports showing a requirement for GJIC in precursor proliferation (Cheng et al., 2004; Duval et al., 2002; Kunze et al., 2009). Kunze *et al.* also reported a decrease in the number of radial glial like cells, which are comparable with type 1 cells in our study, in their Cx30/43 dKO mice. This supports the idea that the primordial type 1 cells retain a strong requirement for connexin-mediated communication, likely a conserved role from the embryonic neurogenic program (Bittman et al., 1997; Filippov et al., 2003; Tozuka et al., 2005; Wang et al., 2005). Type 1 cells have a very slow proliferative rate and, and with the exception of seizures which increases their proliferation, are generally thought to be otherwise unresponsive to neurogenic stimuli beyond basal activation (Kempermann et al., 2004; Kronenberg et al., 2003; Steiner et al., 2008). Similar to the Cx30/43 dKO mice phenotype, we also failed to detect any changes in the number or specification of NPC progeny despite the overall reduction in proliferating type 1 cells (Kunze et al., 2009). Ongoing adult neurogenesis is required for normal cognitive

functioning and learning, possibly by modulating temporal encoding and/or long term consolidation of memories (Aimone et al., 2010; Aimone et al., 2006; Deng et al., 2010). Cx45 cKO display normal novel environment habituation and object place recognition but impaired novel object recognition, a known hippocampal dependent task (Zlomuzica et al., 2010). Electrophysiological studies of these mice also revealed reduced amplitude of kainite-induced γ-oscillations in the CA3 and a larger full width maximum in the CA1, which is suggestive of a slight desynchronization of neurons in these mice which is known to disrupt long term memory formation (Axmacher et al., 2006). Our data suggest that these deficits may also be mediated by the impairment in type 1 NPC proliferation.

There are several possible underlying molecular mechanisms that could account for the proliferative changes seen in brain-specific Cx45 conditional null mutants. Radial glial like cells in the embryonic ventricular zone express Cx26 and 43 where they are responsible for forming specific proliferative units and their expression is dynamically regulated in relation to the cell cycle (Bittman et al., 1997; Bittman and LoTurco, 1999; Nadarajah et al., 1997). Cell cycle analysis within the proliferating population of our Cx45 cKO mice did not reveal any gross defects suggesting that this role is not conserved in the adult brain. Cx43 and 26 also play an important role within the developing ventricular zone where they permit progenitor cell migration along their radial glial processes thanks to the adhesive properties of gap junctions (Elias et al., 2007). Given that both the overall architecture of the Cx45-deficient brain appears normal and that the distribution of both the labeled proliferating and surviving cells is not different

from their WT counterparts, it seems unlikely that defects in migration could underlie our results. Activation of the cAMP signal transduction cascade resulting in the phosphorylation of the cAMP response element binding (CREB) is known to increase the proliferation of adult hippocampal NPCs and conversely the presence of a dominant negative CREB results in decreased NPC proliferation *in vivo* (Nakagawa et al., 2002). This cascade is not activated within the proliferating NPCs themselves but rather the surrounding cells in the SGZ and GCL of the hippocampus suggesting that activation of this cascade results in the secretion of pro-proliferative signals that then acts upon the NPCs (Nakagawa et al., 2002). Interestingly, *in vitro* studies have demonstrated that Cx45-containing gap junctions have a relatively high permeability to cAMP when compared with other connexin family members (Bedner et al., 2006). Taken together, it is tempting to speculate that the loss of Cx45 disrupts instructive cAMP signaling between mature neurons within the SGZ, thereby underlying a decrease in the production of pro-proliferative growth factors. Furthermore the loss of Cx45 within the progenitor cell populations themselves could render them unable to receive pro-proliferative signals further reducing NPC proliferation. While an intriguing possibility, this pathway will require further study.

In summary, we report, for the first time, functional regulation of adult type 1 NPCs in the hippocampus by Cx45. Our data indicate that Cx45 is required for the normal proliferation of type 1 NPCs but not for their survival or specification. These data support the emerging hypothesis that connexin-mediated

communication is required for adult hippocampal neurogenesis, and specifically the expansion and activation of early progenitor populations.

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Chapter 4 – Exogenous regulation of adult hippocampal neurogenesis by opiate analgesia*

4.1 Objective of this study

1) Determine the impact of opiate analgesia adult hippocampal progenitor cell proliferation, specification and survival

4.2 Statement of author contributions

AP and SALB conceived of and designed the experiments. AP performed all the experiments, data analysis, and generated the figures. RD completed experimental double counting for these experiments. AP and SALB wrote the manuscript. The manuscript has been reformatted to meet the University of Ottawa's thesis requirements.

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4.3 The opiate analgesic buprenorphine decreases proliferation of adult hippocampal neuroblasts and increases survival of their progeny

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Conflict of Interest: The authors report no conflicts of interest.

4.4 Summary

Although opiate drugs of abuse have been shown to decrease adult hippocampal neurogenesis, the impact of opiate analgesics has not been tested. North American regulatory boards governing the ethical treatment of experimental animals require the administration of analgesics, such as buprenorphine, following minor surgical interventions. Here, we show that two commonly used post-operative buprenorphine dosing regimes significantly inhibit the proliferation of doublecortin-positive neuroblasts but not other hippocampal stem and progenitor populations in adult mice. Buprenorphine, administered in schedules of three 0.05 mg/kg subcutaneous injections over a single day or seven 0.05 mg/kg injections over a three day period decreased the number of actively proliferating 5-iodo-2'-deoxyuridine-labelled doublecortin-positive cells for up to six days after opiate withdrawal. The minimal (three injection), but not standard (seven injection), analgesic paradigm also reduced basal indices of hippocampal progenitor cell apoptosis and enhanced survival of newly born cells for up to 28 days. Taken together, these data provide the first evidence that the routine of administration of opiate analgesics has transient but long-lasting effects on neurogenesis and further emphasize that analgesic dosage and schedule should be reported and considered when interpreting the magnitude of neural stem and progenitor cell activation in response to *in vivo* intervention.

4.5 Introduction

Adult neurogenesis is primarily restricted to two regions of the adult mammalian brain: the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles (Gage, 2000). The production of new neurons in these regions occurs through the stepwise expansion, specification, migration, and terminal differentiation of increasingly committed neural stem and progenitor cell populations. In the SGZ, resident neural stem cells (NSCs) identified, in part, by their expression of both nestin and glial fibrillary acidic protein (GFAP), can self-renew or give rise to nestin-positive transiently amplifying progenitor cells (TAPs). TAPs, in turn, can generate doublecortin-positive (DCX) neuroblasts capable of terminal neuronal differentiation (Ables et al., 2010; Berube et al., 2005; Doetsch, 2003; Garcia et al., 2004; Kempermann et al., 2004). Their post-mitotic neuronal progeny can either mature and integrate into adult hippocampal circuitry or be deleted by apoptosis (Deng et al., 2010). Each step of this process is discretely regulated and is postulated to affect hippocampal function in the uninjured brain (Zhao et al., 2008).

Here, we asked whether the routine prescription of opiate analgesics alters hippocampal neurogenesis. Although chronic administration of opiate drugs of abuse (i.e., morphine and heroin) is known to inhibit proliferation of neural progenitor cell populations in the SGZ (Arguello et al., 2009; Arguello et al., 2008; Eisch et al., 2000; Kahn et al., 2005), the effects of opiate analgesics have yet to be assessed. We tested buprenorphine, a μ opioid receptor (MOR)

and opioid like receptor-1 (ORL-1) agonist with mixed κ opioid receptor (KOR) agonist and antagonist activities. Clinically buprenorphine is routinely prescribed for pain management and is increasingly administered “off-label” to treat addiction and mood disorders (Davis, 2007; Howland, 2010; Likar et al., 2007). Buprenorphine is also recommended by both American and Canadian regulatory boards as an analgesic following painful experimental interventions such as the minor surgeries commonly used to manipulate neurogenesis in laboratory animals (1993; 1996; 2009a).

Certainly, the use of appropriate analgesia is one of the predominant refinements associated with the 3R principles of replacement, reduction, and refinement practiced to ensure the ethical use of experimental animals (Russell, 1959; Smythe, 1978). To obtain consistent plasma levels, reduce handling stress and target drug delivery, test compounds are frequently administered via osmotic minipumps. Basic subcutaneous (s.c.) implantation of these pumps involves a minor skin incision. North American regulatory boards mandate the administration of analgesia as a standard post-operative procedure in such cases (1993; 1996; 2009a). While best practice protocols vary between institutions, 0.05 mg/kg s.c. buprenorphine administered at 3 - 12 hour intervals is considered the ‘gold-standard’ generating long-lasting analgesia with minimal side-effects (Curtin et al., 2009; Dobromylskyi et al., 2000; Hawk et al., 2005; Martin et al., 2001). Injections are commonly performed as a standard post-operative procedure by veterinary staff often without the full awareness of the primary research team. Consequently, details of exposure, dosage, specific schedule,

and type of opiate analgesic are rarely reported (Stokes et al., 2009). Such variations in best-practice protocols are also characteristic of clinical practice (Likar et al., 2007).

Here, we assessed the effects of two common dosing schedules of buprenorphine on adult hippocampal progenitor cell proliferation, specification, and survival. Our data indicate that buprenorphine inhibits the proliferation of DCX-positive neuroblasts, regardless of the schedule of administration, and enhances the survival of their progeny born at the time of analgesic treatment. Taken together, these data provide the first evidence that the routine administration of opiate analgesics has transient but long-lasting effects on neurogenesis and further emphasize that analgesic dosage and schedule should be both reported and considered when interpreting the magnitude of neural stem and progenitor cell activation in response to pharmacological or molecular intervention *in vivo.*

4.6 Experimental Procedures

4.6.1 Animals

Male C57Bl/6 x FVB/N x 129/SV hybrid mice were eight weeks of age at time of treatment. All experimental animals were compared to age matched drug-naïve controls. This strain of mice was selected to assess impact of buprenorphine on one of the common genetic backgrounds used in neurogenic studies (2009b; Yoshiki and Moriwaki, 2006). Specifically, the C57Bl/6 x FVB/N x 129/SV strain is representative of the hybrid crosses of nestin-specific cre-

recombinase FVB/N mice mated to floxed lines generated on a C57BL/6 X 129/SV background (Ackermann et al., 2011; Berube et al., 2005; Dubois et al., 2006; Schultz et al., 2011). Animals were kept on a 12 h light-dark cycle (light phase 0600 h-1800 h) with access to food and water *ad libitum*. Five to ten mice were used per experimental group (Figure 4.1A-C). All protocols were approved by the Animal Care Committee of the University of Ottawa according to guidelines set forth by the Canadian Council on Animal Care. Modifications to institutional standard operating procedures for the purposes of testing the effect of the analgesic schedule are indicated below and were approved by the University of Ottawa's Animal Care Committee.

4.6.2 Opiate Analgesia

Buprenorphine (Chiron Compounding Pharmacy Inc., Guelph, ON) was prepared in sterile water to a final concentration of 0.03 mg/ml. Delivery schedules, defined as either as "Standard", representing the routine practice of veterinary staff at the University of Ottawa, or "Minimal", representing a reduced number of opiate analgesic injections that still provided effective pain management according to the Canadian Council for Animal Care guidelines, are depicted in Figure 4.1A-C. Under the standard protocol, mice received three injections (0.05 mg/kg, s.c.) of analgesic every six h on the day of surgery and two injections (0.05 mg/kg, s.c.) separated by eight h for two subsequent days for a total of seven injections over a 72 h period. Under the minimal protocol,

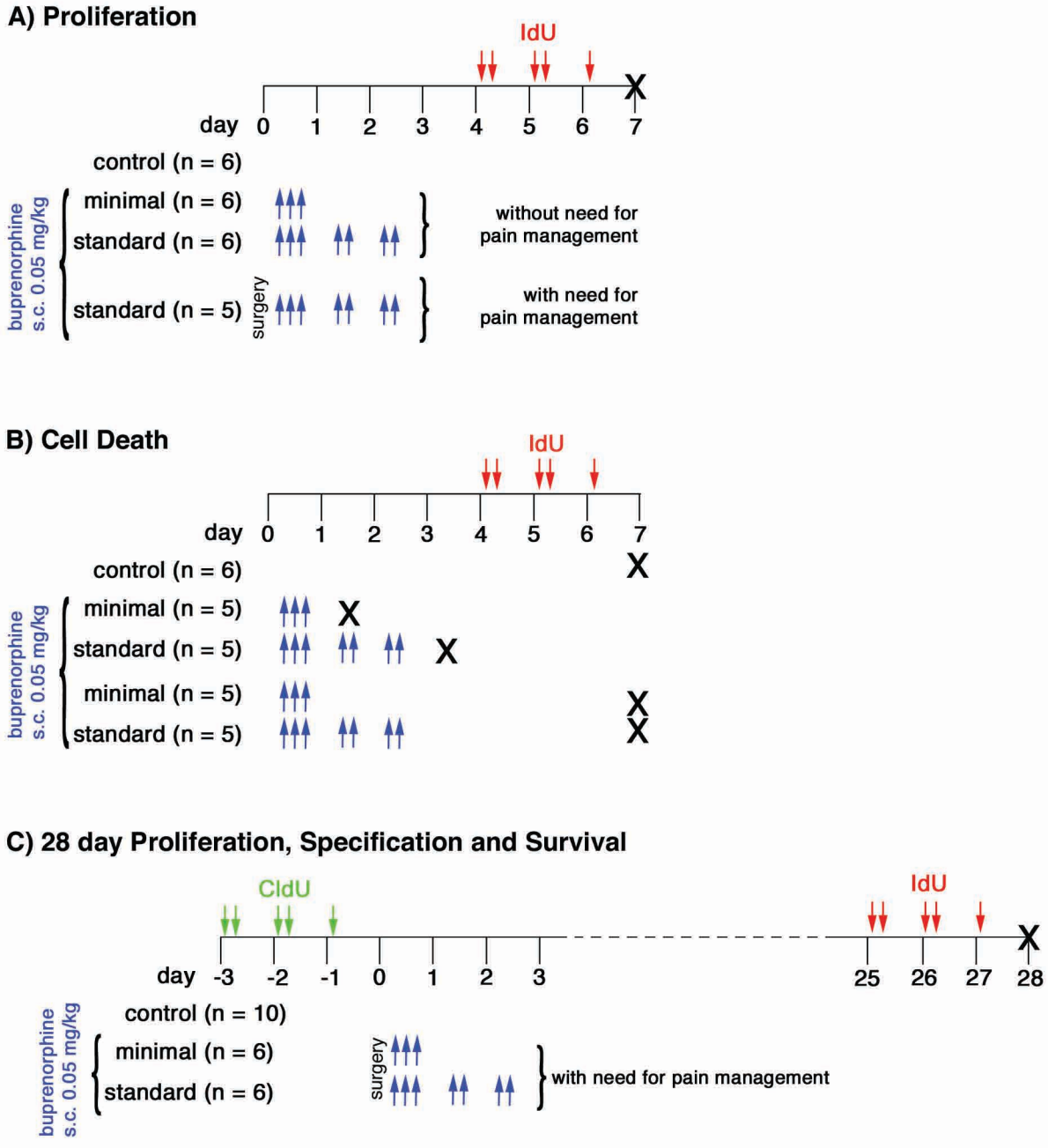

A) Proliferation
IdU
day 0 1 2 3 4 5 6 7
control (n = 6)
minimal (n = 6)
standard (n = 6)
standard (n = 5)
buprenorphine s.c. 0.05 mg/kg
surgery
without need for pain management
with need for pain management
B) Cell Death
IdU
day 0 1 2 3 4 5 6 7
control (n = 6)
minimal (n = 5)
standard (n = 5)
minimal (n = 5)
standard (n = 5)
buprenorphine s.c. 0.05 mg/kg
C) 28 day Proliferation, Specification and Survival
CldU
IdU
day -3 -2 -1 0 1 2 3 25 26 27 28
control (n = 10)
minimal (n = 6)
standard (n = 6)
buprenorphine s.c. 0.05 mg/kg
surgery
with need for pain management

Figure 4.1

Figure 4.1 – Treatment paradigms.

Buprenorphine injections (0.05 mg/kg, s.c.) are indicated by blue arrows; IdU injections (57.5 mg/kg, i.p.) are indicated by red arrows; CldU injections (42.5 mg/kg, i.p.) are indicated by green arrows. Mice subjected to surgical intervention were implanted with osmotic minipumps subcutaneously on day 0, delivering 20% 2-hydroxypropyl)-β-cyclodextrin, as indicated by "surgery". Time of sacrifice is indicated by a black X. (A) Assessment of cell proliferation following minimal or standard schedules of analgesia with or without the need for pain management. Proliferating cells were labeled on days 4, 5, and 6 after the initiation of opiate analgesia. Animals were sacrificed 1 week after the onset of analgesic treatment. (B) Apoptotic-like cell death was assessed 24 h, 72 h, and 6 days after the final buprenorphine injection for both the minimal and standard analgesia regimen. (C) To establish whether acute exposure to buprenorphine under either the minimal or standard schedule permanently alters proliferative capacity, IdU was administered on days 25, 26 and 27 following surgery and IdU-positive proliferating cell number was assessed 28 days after the onset of opiate analgesia. To assess specification and survival of stem and progenitor cells following surgery and buprenorphine treatment, proliferating cells were labeled with CldU over the 3 days prior to surgery. Survival and fate specification were assessed 28 days later.

mice received three injections (0.05 mg/kg, s.c.) separated by six h over a 24 h period. Delivery schedules were designed to model variations in pain management strategies observed across institutions (Curtin et al., 2009; Dobromylskyi et al., 2000; Hawk et al., 2005; Martin et al., 2001). All subjects were compared to age-matched, drug-naïve, control mice.

4.6.3 Surgery

To assess the effects of opiate analgesia with and without the need for pain management, uninjured mice were compared to mice that underwent minor surgery. The surgical group was anesthetized with an inhalant mixture of oxygen and isoflurane. Alzet osmotic minipumps (model 2001 (seven day implantation) or model 2004 (28 day implantation), DURECT Corporation, Cupertino, CA) pre-filled with 20% (2-hydroxypropyl)-β-cyclodextrin (catalog #H107, Sigma, Saint Louis, MO) in sterile normal saline (0.9% NaCl) were subcutaneously implanted on the back of the mouse. Both standard and minimal analgesic schedules were employed as pain management strategies (Figure 4.1A,C).

4.6.4 *In vivo* labeling of proliferating cells

Mice received five injections over three days of 5-chloro-2'-deoxyuridine CldU (42.5 mg/kg) (prepared to 17 mg/ml in 0.625 M Tris base in sterile water; Sigma, catalog #C6891, Saint Louis, MO) and/or 5-iodo-2'-deoxyuridine IdU (57.5 mg/kg) (prepared to 23 mg/ml in 0.895 M Tris base in sterile water; MP Biomedicals, catalog #100357, Solon, OH) intraperitoneally (i.p.). Concentrations

were as described in Vega and Peterson (2005) with the exception that Tris base was used to enhance solubility in place of sterile saline and 5 N NaOH. The final pH of the solutions was pH 8.5 as recommended by Vega and Peterson (Vega and Peterson, 2005). Delivery schedules for CldU and IdU (Figure 4.1A-C) for each experiment were optimized to label not only more rapidly dividing TAPS but also the slowly dividing NSC population (Nowakowski et al., 1989; Taupin, 2007).

4.6.5 Tissue Preparation

Mice were euthanized at the time points outlined in Figure 4.1A-C by injection with euthanyl (Bimeda-MTC Animal Health Ins, Cambridge ON) prepared in sterile water to a final concentration of 65 mg/ml and transcardiall perfusion with 10 mM phosphate buffered saline (PBS; 10 mM sodium phosphate and 154 mM NaCl) followed by 3.7% paraformaldehyde in 10 mM PBS. Brains were removed, post-fixed for 24 hrs, and cryoprotected in 20% sucrose. Serial 10 µm coronal cryosections were collected using a Leica CM1900 cryostat (Leica Microsystems, Wetzlar, Germany), throughout the dorsal domain of hippocampus from bregma -1.0 to -3.4 using the coordinates of Hof et al. (Hof et al., 2001).

4.6.6 Immunofluorescence

Sections were incubated in 2 N HCl for 30 min at 37˚C and neutralized in 0.1 M borate buffer (pH 8.5). IdU and CldU incorporation was detected by immunofluorescence using mouse anti-BrdU clone 44 (1:500; Becton Dickinson Immunocytochemistry Systems, catalog #347580, San Jose, CA) and rat anti-

BrdU-FITC (1:100, AbD Serotec, catalog #OBT0030F, Raleigh, NC) respectively. We used double and triple immunofluorescence for antigenic lineage markers to determine (a) the identity of actively proliferating (IdU-labeled) cells and (b) fate of cells actively proliferating at the time of opiate analgesic exposure (CldU-labeled). The primary antibodies employed were rabbit anti-GFAP (1:100; Sigma, catalog #G9269, Saint Louis, MO), rabbit anti-nestin (1:1000; Covance, catalog #PRB-315C, Emeryville, CA), guinea pig anti-doublecortin (1:400; Millipore, catalog #AB5910, Temecula, CA), rabbit anti-NG2 (1:100; Millipore, catalog #AB5320), and Alexa488 conjugated and unconjugated mouse anti-NeuN (1:100; Millipore, catalog #MAB377X and MAB377 respectively). Secondary antibodies were FITC or Cy3 conjugated anti-mouse IgG (1:100, 1:800; Jackson Immunoresearch, catalog #715-095-150 and 715-165-150 respectively), Cy3 conjugated anti-rabbit IgG (1:600; Jackson Immunoresearch, catalog #711-166-152), and Cy3 conjugated anti-guinea pig IgG (1:800; Jackson Immunoresearch, catalog #706-165-147). Sections were incubated with primary antibodies overnight at 4˚C, washed with 10 mM PBS, incubated with secondary antibodies for 1 h at room temperature, and washed with PBS prior to being coverslipped in ProLong Gold antifade reagent (Invitrogen, catalog #P-36930, Eugene, Oregon, USA). All antibodies were diluted in antibody buffer (3% bovine serum albumin, 0.3% Triton X-100 in 10 mM PBS).

4.6.7 Cell Death

Dying cells were labeled by terminal deoxynucleotidyl transferase-

mediated dUTP nick end labeling (TUNEL, catalog #11684795910, Montreal Canada) as we have previously described (Melanson-Drapeau et al., 2003).

4.6.8 Microscopic Analysis and Quantification

Immunofluorescence was assessed using a DMXRA2 epifluorescent microscope (Leica Microsystems). Additional confocal imaging was performed on a Leica TCS SP5 (Leica Microsystems). Quantification of the total number of IdU and CldU-labeled cells was as described (Guo et al., 2010; Thallmair et al., 2006) with the following minor modification: counts were restricted to the dorsal domain of the hippocampus as defined by (Fanselow and Dong, 2010). This focus facilitated a clear anatomical distinction between the dorsal zone of the hippocampus and the intermediate/ventral zones that would otherwise require the use of marker genes to distinguish boundaries in more posterior sections (Dong et al., 2009). An average of 10 bregma matched sections serially sampled along the entire anterior-posterior axis of the dorsal hippocampus, bregma -1.0 and -3.4 were analyzed per animal. Five to ten animals were counted per condition (Figure 4.1). The area of (a) the SGZ, defined as the region bordering the granule cell layer and the hilus (3 cell widths into each), (b) the hilus, and (c) the entire GCL was established for each section using the Advanced Measurement Module of OpenLab 5.0.2 (Improvision) software. The number of labeled cells was assessed in both the left and right hippocampi by two independent observers blinded as to the identity of the sections. These values were averaged to yield a single number per region, section, and animal. Because

labeled cells were not evenly distributed and are relatively rare, we did not use defined counting frames but rather counted all labeled cells within these defined areas in accordance with previous studies (Guo et al., 2010; Thallmair et al., 2006). In proliferation studies, IdU-labeled cells were counted in the SGZ and hilus using a 10x objective. In survival studies, CldU-labeled cells were counted throughout both the GCL and hilus as new cells, born in the SGZ, will migrate into the granule cell layer of the DG. The hilus was included in all analyses to include $NG2^+$ gliogenic progenitor cells as well as to acknowledge the possibility of ectopic localization of SGZ progenitor progeny following treatment. The total number of labeled cells in a given region per animal was calculated as the sum of the cell counts in all sampled sections multiplied by the total number of serial sections collected between bregma -1.0 and -3.4 and divided by the number of sections sampled (Guo et al., 2010; Thallmair et al., 2006). Means and standard errors of the mean (SEM) are reported for each experimental group.

Identification of IdU or CldU-positive cells, co-labeled with antigenic lineage markers, were completed using a 40X objective for epifluorescence and confirmed by confocal laser scanning microscopy using a 63X objective. Total IdU or CldU-positive nuclei in each area of interest were counted and then assessed for the presence of the lineage marker of interest in merged epifluorescent images. To provide further confirmation, confocal images were captured with Leica LAS AF software (version 2.41) using an HCX PL APO CS 63X 1.4 oil objective with a pinhole size of 1 Airy unit (or equal optical slices for multiple fluorophores) through *z*-stacks. We considered antigenic lineage

markers colocalized with IdU or CldU-labeled nuclei only if the lineage label extended from top to bottom of the z-stack. An average of 350 IdU-positive cells per mouse were analyzed to identify proliferating cells and 45 CldU-positive cells per mouse were analyzed to establish fate specification. The total number of cells positive for each proliferative phenotype was calculated by multiplying the total number of IdU-positive cells by the proportion positive for the appropriate lineage makers yielding a single value per mouse. Five to ten mice were assessed per condition as indicated (Figure 4.1A-C). Data are presented as mean and SEM of all animals counted.

TUNEL counts were completed in the GCL, SGZ, and hilus of both hemispheres using a 10X objective in two serial sections between bregma -1.7 and -2.7 matched between mice (n = 4 counts per mouse). Details are as we have previously published (Melanson-Drapeau et al., 2003). Briefly, counts were performed by two independent investigators blinded as to the identity of the sections. The area of the GCL, SGZ, and hilus was established for each hemisphere/section using the Advanced Measurement Module of OpenLab 5.0.2 (Improvision) software. Area measurements allowed us to confirm anatomical bregma matching and to express final data as the number of positive cells per $0.1mm^2$ in the combined areas of interest (GCL, SGZ, and hilus).

4.6.9 Statistical Analysis

Data were analyzed by one-way ANOVA followed by *post-hoc* Tukey's test or Bonferroni's test as indicated.

4.7 Results

4.7.1 Buprenorphine reduces proliferating cell number in the adult hippocampus

The standard post-operative analgesic protocol following minor surgery (i.e., s.c. implantation of osmotic minipumps) at the University of Ottawa is seven buprenorphine injections (0.05 mg/kg/s.c) administered over three days (Figure 4.1A). We found that this schedule, in the absence of any other intervention, reduced the number of actively proliferating IdU-positive cells in the SGZ and hilus of the hippocampal dentate gyrus assessed one week after analgesic treatment (Figure 4.2A,B; ANOVA, *post hoc* Tukey's test, ** $p < 0.01$ relative to control). The effect of buprenorphine on hippocampal cell proliferation was not dependent on the schedule of opiate administration. Mice that received an abbreviated (minimal) experimental protocol of three injections over the course of a single day (Figure 4.1A) also showed a significant reduction in proliferating IdU-positive cell number (Figure 4.2A,B; ANOVA, *post hoc* Tukey's test, * $p<0.05$ relative to control). There was no difference in IdU incorporation in the CA1 region of the hippocampus between any of the treatment groups indicating that IdU bioavailability was equivalent across experimental manipulations.

To test whether buprenorphine inhibits hippocampal cell proliferation in the context in which it is routinely used (i.e., as a pain management strategy), mice were subcutaneously implanted with osmotic minipumps containing 20% 2-hydroxypropyl)-β-cyclodextrin, a common drug delivery vehicle

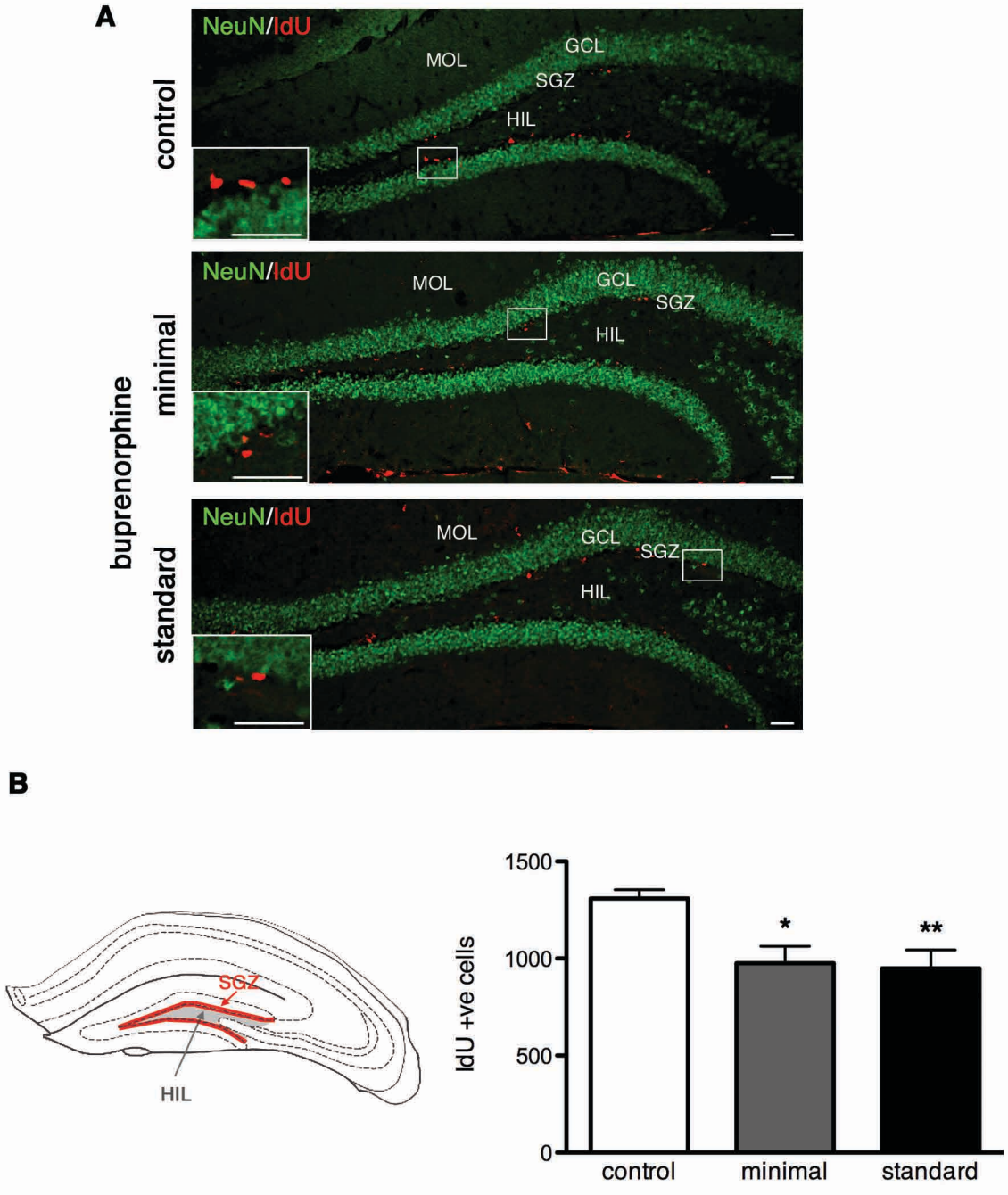

A
NeuN/IdU
MOL
GCL
SGZ
HIL
control
buprenorphine
minimal
standard
B
SGZ
HIL
IdU +ve cells
1500
1000
500
0
*
**
control
minimal
standard

Figure 4.2

Figure 4.2 – Proliferating cell number is decreased in the SGZ and hilus of the hippocampal dentate gyrus seven days after the initiation of either standard or minimal buprenorphine treatment schedules.

Abbreviations: GCL, granule cell layer of the dentate gyrus; HIL, hilus; MOL, molecular layer of the dentate gyrus; SGZ, subgranular zone. (A) Double immunofluorescence for NeuN (green) and IdU (red) in the dentate gyrus of mice who did not receive buprenorphine injections (control) or following minimal or standard buprenorphine injection paradigms. Insets depict IdU labeling at higher magnification. Scale bar 50 μm. (B) Schematic of the hippocampal dentate gyrus highlighting the SGZ (red) and hilus (grey), the areas used for quantification of IdU-positive cells. Quantification of IdU immunoreactive cells in the SGZ and hilus of controls (n=6) and of mice receiving a minimal (n=6) or standard (n=6) buprenorphine treatment regimen. Statistics were ANOVA followed by *post-hoc* Tukey test. * $p<0.05$, ** $p<0.01$ relative to control

(Loftsson et al., 2005), and treated under the standard schedule of buprenorphine administration (Figure 4.1A). The number of IdU-positive cells in the SGZ and hilus of the adult hippocampal dentate gyrus was significantly reduced seven days following the initiation of treatment both with and without need for pain management compared to drug-naïve uninjured animals. (Figure 4.3; ANOVA, *post hoc* Tukey's test, *p<0.05 and **p<0.01 vs. control). Moreover, proliferating cell number was found to be further suppressed in mice that had undergone minor surgery compared to pain-free animals (Figure 4.3; ANOVA, *post hoc* Tukey's test, ## p<0.01 without vs. with the need for pain management). As it is required by the Canadian Council for Animal Care that mice receive opiate analgesia after minor surgery, we could not ethically include a surgical control group that did not receive buprenorphine to assess impact of pain alone on hippocampal cell proliferation. An isoflurane anesthesia alone control group also was not included as recent reports indicate that isoflurane does not affect the proliferation or survival of adult hippocampal progenitors (Zhu et al., 2010).

4.7.2 The buprenorphine-mediated reduction in hippocampal cell proliferation is transient following surgery

To assess the duration of buprenorphine's effects on proliferation, we quantified the number of IdU-positive cells in the SGZ and hilus 28 days following standard or minimal buprenorphine treatment and minor surgery (Figure 4.1C). Proliferating cell number was not permanently changed by opiate analgesia (Figure 4.4; ANOVA, *post hoc* Tukey's test, p>0.05). We did, however, detect a

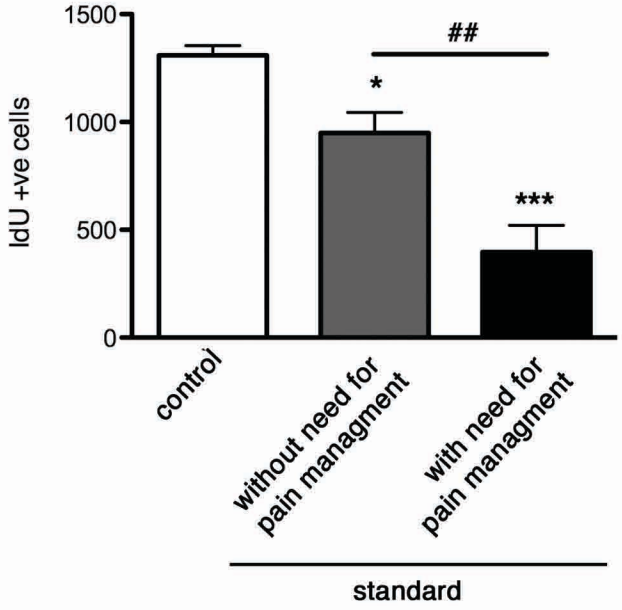

1500
1000
500
0
IdU +ve cells
##
*
***
control
without need for pain managment
with need for pain managment
standard

Figure 4.3

Figure 4.3 – Proliferating cell number is further suppressed in SGZ and hilus of the hippocampal dentate gyrus seven days following surgery after application of the standard pain management strategy.

Quantification of IdU-positive cells in the SGZ and hilus of drug-naïve littermates (control, n=6) and uninjured mice (n=6, without need for pain management) or mice implanted with osmotic mini-pumps (n=5, with need for pain management) administered the standard schedule of buprenorphine. Proliferating cell number was assessed 1 week after the initiation of treatment. Statistics were ANOVA, *post-hoc* Tukey test. * $p<0.05$, ** $p<0.01$ relative to control, ## $p<0.01$ without vs. with the need for pain management.

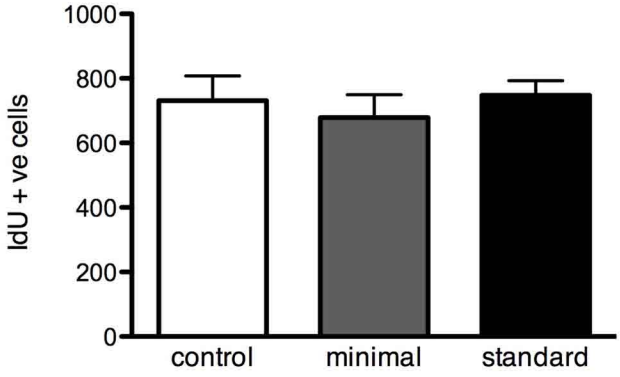


Figure 4.4

Figure 4.4 – Proliferating cell number is comparable to control levels 28 days after buprenorphine treatment.

Quantification of IdU-positive cells in the SGZ and hilus of controls (n = 10) and mice implanted with osmotic mini-pumps delivering 20% 2-hydroxypropyl)-β-cyclodextrin and administered either a minimal (n = 6) or standard (n = 6) schedule of buprenorphine. The control group represents uninjured drug-naïve littermates. Proliferating cell number was assessed 28 days after the initiation of treatment. Statistics were ANOVA, *post-hoc* Tukey test. $p > 0.05$.

difference in proliferating cell number between control animals aged 8 weeks of age (Figure 4.3) and 12 weeks of age (Figure 4.4) consistent with previously published data (Ben Abdallah et al., 2010).

4.7.3 Buprenorphine inhibits proliferation of DCX-positive neuroblasts

Hippocampal stem and progenitor cells exhibit differential sensitivity to various pharmacological manipulations including opiate drugs of abuse (Kronenberg et al., 2003; Sargeant et al., 2008). To identify the proliferating cell populations affected by buprenorphine, cells were labeled *in vivo* with IdU and identified 24 hrs after the last IdU injection using a panel of antigenic lineage markers and morphological characteristics (Figure 4.5A). NSCs in the SGZ were identified by their co-expression of IdU and the glial antigen GFAP along with a distinctive triangular soma and single process projecting through the granule cell layer (GCL) (Figure 4.5B - i, open arrow). All GFAP-positive cells with ovoid cell bodies lacking a single projection into the GCL were classified as astrocytes (Figure 4.5B - i, filled arrow). Confocal imaging of IdU/Nestin/GFAP triple-labeled sections confirmed these results (Figure 4.5B-ii). Proliferating TAPs were IdU-positive / nestin-positive but did not express GFAP (Figure 4.5B - iii). Proliferating neuroblasts were identified by their DCX immunoreactivity (Figure 4.5B - iv). Proliferating early oligodendrocyte precursor cells (OPCs) were identified by their NG2 immunoreactivity (Figure 4.5B – v) and were almost exclusively found in the hilus. All IdU positive cells that did not co-label with the lineage markers described above were classified as “other,” and are most likely immature neurons

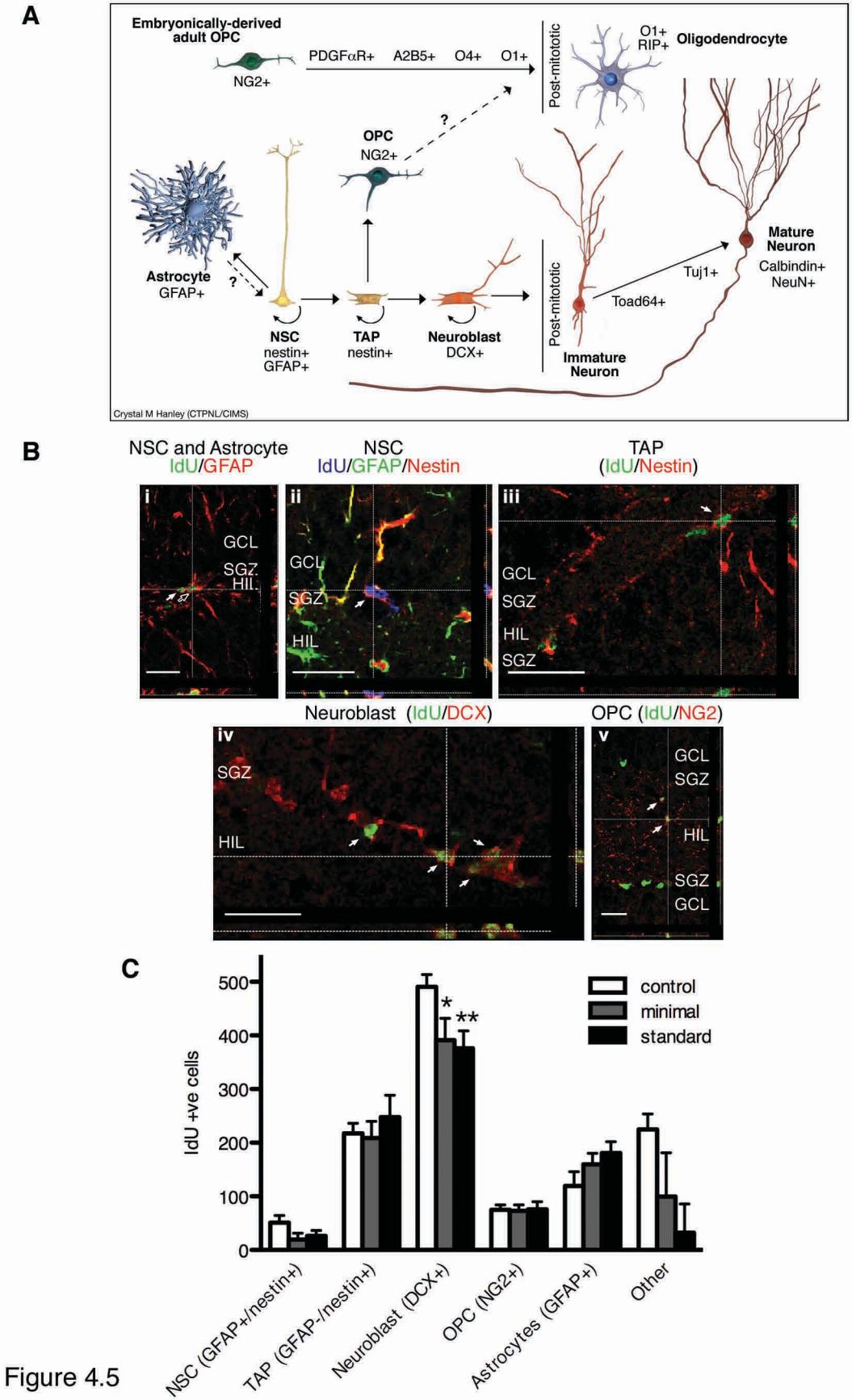

A
Embryonically-derived adult OPC
NG2+
PDGFαR+
A2B5+
O4+
O1+
Post-mitototic
O1+ RIP+
Oligodendrocyte
OPC NG2+
?
Astrocyte GFAP+
NSC nestin+ GFAP+
TAP nestin+
Neuroblast DCX+
Immature Neuron
Toad64+
Tuj1+
Mature Neuron
Calbindin+ NeuN+
Crystal M Hanley (CTPNL/CIMS)
B
NSC and Astrocyte IdU/GFAP
NSC IdU/GFAP/Nestin
TAP (IdU/Nestin)
i
ii
iii
GCL
SGZ
HIL
Neuroblast (IdU/DCX)
OPC (IdU/NG2)
iv
v
C
IdU +ve cells
500
400
300
200
100
0
control
minimal
standard
*
**
NSC (GFAP+/nestin+)
TAP (GFAP-/nestin+)
Neuroblast (DCX+)
OPC (NG2+)
Astrocytes (GFAP+)
Other

Figure 4.5

Figure 4.5 – Buprenorphine inhibits the proliferation of DCX-positive neuroblasts.

Abbreviations are as defined in Figure 4.2. (A) Schematic of the process of adult hippocampal neurogenesis depicting the morphological characteristics of the individual cell types and the antigenic lineage markers used to follow the process. (B) Representative confocal micrographs used for lineage analysis of proliferating stem and progenitor cells. (i) IdU/GFAP (NSC, open arrow and astrocyte, filled arrow), (ii) IdU/Nestin/GFAP (NSC, filled arrow), (iii) IdU/Nestin (TAP, filled arrow), (iv) IdU/DCX (neuroblast, filled arrows) and (v) IdU/NG2 (OPC, filled arrows) Scale bars 25 μm. (C) Quantification of IdU-labeled NSCs, TAPS, neuroblasts, OPCs, and astrocytes in drug naïve mice (control, n=9) or following minimal (n=6) or standard (n=6) buprenorphine injection paradigms without requirement for pain management. The number if IdU-positive/DCX-positive neuroblasts were significantly reduced following treatment with both the minimal and standard schedule of buprenorphine treatment. Statistics for each lineage marker were ANOVA followed by *post-hoc* Bonferroni's test. * $p<0.05$, ** $p<0.01$ relative to control.

or possibly more committed gliogenic progenitor cells (Figure 4.5A). Buprenorphine reduced the number of both IdU-labeled NSCs and neuroblasts, but not IdU-labeled TAPS, OPCs, astrocytes, or other cell types following either minimal or standard treatment protocols; however, only the reduction in IdU-labeled neuroblasts reached statistical significance (Figure 4.5C; ANOVA, *post hoc* Bonferroni's test, *p<0.05, **p<0.01).

4.7.4 Buprenorphine enhances cell survival in adult hippocampus

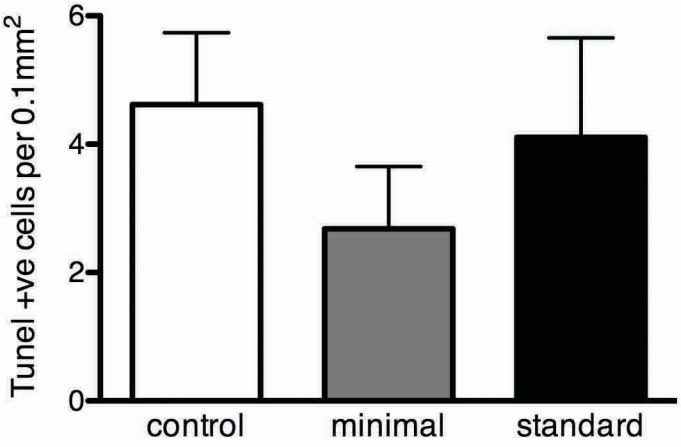
The transient reduction in DCX-positive neuroblast cell proliferation following buprenorphine treatment could be due to three possibilities: a decrease in cell proliferation, as has been observed following chronic exposure to opiate drugs of abuse (Eisch et al., 2000), an increase in the apoptotic index of actively dividing cells, as has been observed in NG108-15 neuroblastoma/glioma cells treated *in vitro* with buprenorphine (Kugawa et al., 2000), and/or an increase in the maturation rate of DCX-positive neuroblasts. Because the percentage of IdU-positive cells that did not co-label with the lineage markers we tested following buprenorphine treatment (i.e., neuroblasts that had stepped out of the cell cycle over the course of labeling and developed into DCX-negative immature neurons) was lower than that of controls (Figure 4.5C), it is unlikely that an increase in the rate of neuroblast maturation underlies the decrease in IdU labeling. To distinguish between the two remaining hypotheses, dying cells were labeled by TUNEL. Because cell loss in adult hippocampus attributed to opiate drugs of abuse is restricted to cells in S-phase and newly born immature neurons

(Arguello et al., 2008), we assessed cell death in both actively proliferating cells, 24 hrs after IdU labeling, and immature neurons that had matured seven days following the initiation of analgesic treatment (Figure 4.1B). No significant difference in the total number of TUNEL-positive cells was observed 24 hrs after the last buprenorphine injection, suggesting that analgesic treatment does not compromise the viability of actively proliferating cells in the hippocampus whether administered through a standard or minimal schedule (Figure 4.6A). A reduction in the hippocampal apoptotic index was evident following both treatment paradigms seven days after initiating buprenorphine treatment. Two-fold less TUNEL-positive cells were detected in the GCL, SGZ, and hilus compared to controls under both administration paradigms, however this decrease only reached statistical significance in the group receiving the minimal analgesic schedule (Figure 4.6B; ANOVA, *post hoc* Tukey test, *$p<0.05$).

4.7.5 An abbreviated schedule of buprenorphine analgesia enhances the survival of newly born cells

To directly assess the impact of buprenorphine on hippocampal stem and progenitor cell survival, we tracked the number and fate of cells labeled with CldU prior to manipulation 28 days after minor surgery and analgesic treatment (Figure 4.1C). Consistent with the decrease in cell death observed 7 days post minimal buprenorphine treatment in mice without need for pain management (Figure 4.6B), we found that the survival of CldU-labeled cells in the hippocampal dentate gyrus of mice that received a minimal buprenorphine treatment regimen following surgery significantly increased relative to both control and standard

A - 24 hrs following the final buprenorphine injection



B - 7 days following the initiation of buprenoprhine treatment

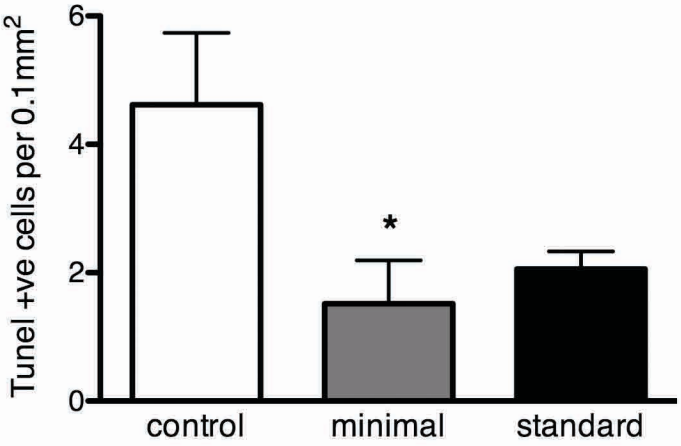


Figure 4.6

Figure 4.6 – Apoptotic cell death is decreased in the hippocampal dentate gyrus 7 days following the initiation of buprenorphine treatment.

Quantification of TUNEL-positive cells per 0.1 mm^2 of control, minimal and standard buprenorphine treated mice without need for pain management. (A) 24 hrs after the final buprenorphine injection (control n=6, minimal n=5, standard n=5) or (B) 7 days following the first buprenorphine injection (control n=6, minimal n=5, standard n=5). Statistics were ANOVA followed by *post-hoc* Tukey test. * $p<0.05$ relative to control.

treatment (Figure 4.7A; ANOVA, *post hoc* Tukey's test, *p<0.05 relative to control, ## p<0.01 relative to standard treatment). We next identified these CldU-positive cells using markers for mature neurons (NeuN) or astrocytes (GFAP) (Figure 4.7B,C). Double-positive cells were quantified and expressed as a percentage of the total number of CldU-positive cells. No difference in the percentage of terminally differentiated cells was detected between groups (Figure 4.7D), indicating that while the minimal protocol of opiate analgesic intervention enhanced the survival of newly born cells, it did not alter their specification.

4.8 Discussion

Here, we report that buprenorphine, a routinely administered opiate analgesic, transiently inhibits the proliferation of DCX-positive neuroblasts and, under an acute schedule of administration, enhances survival of newly born cells. Using two different administration paradigms (standard; seven injections over 72 hrs, and minimal; three injections over 24 hours), we show that buprenorphine decreases the number of proliferating DCX-positive cells in the adult hippocampus for up to one week after the last buprenorphine injection. These effects are further exacerbated by post-operative pain with animals subjected to both surgery and buprenorphine showing larger reductions in proliferating cell number than pain-free animals administered buprenorphine alone. Surprisingly, our data also indicate that acute exposure to opiate analgesics enhances the survival of newly born cells for up to 28 days. Following a minimal schedule of

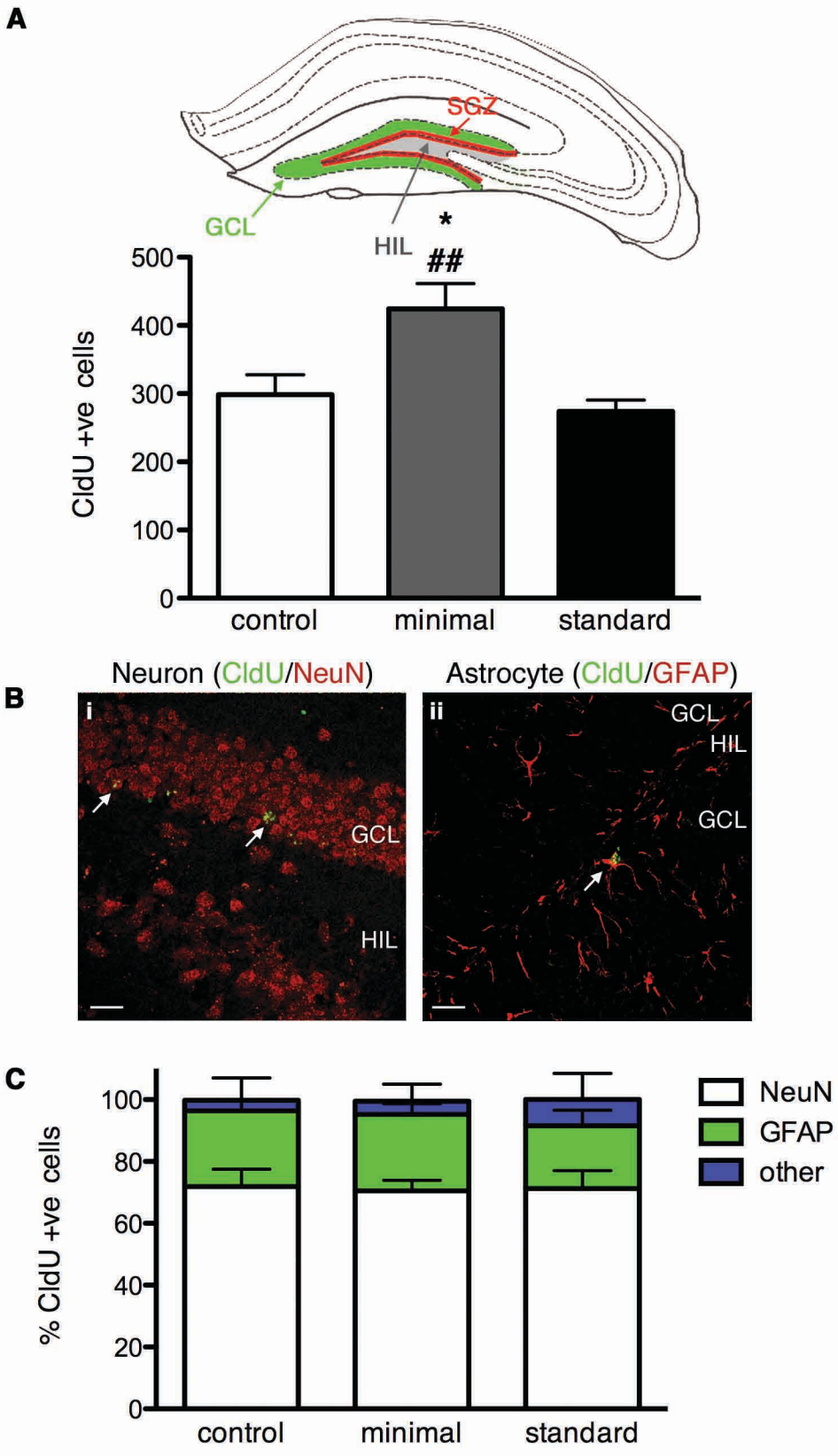

A
SGZ
GCL
HIL
*
##
500
400
300
200
100
0
CldU +ve cells
control
minimal
standard
B
Neuron (CldU/NeuN)
Astrocyte (CldU/GFAP)
i
ii
GCL
HIL
GCL
HIL
GCL
C
100
80
60
40
20
0
% CldU +ve cells
control
minimal
standard
NeuN
GFAP
other

Figure 4.7

Figure 4.7– The survival of newly born cells is increased with minimal buprenorphine treatment following surgery.

Abbreviations are as defined in Figure 4.2. (A) Schematic of the hippocampal dentate gyrus highlighting the GCL (green), SGZ (red) and hilus (grey), the areas used for quantification of CldU-positive cells and quantification of surviving CldU-positive cells in the dentate gyrus of controls (n=10) and mice implanted with osmotic mini-pumps administered a minimal (n=6) or standard (n=6) schedule of buprenorphine 28 days following surgery. Statistics were ANOVA followed by *post-hoc* Tukey test. * $p<0.05$ relative to control, ## $p<0.01$ relative to standard buprenorphine treatment. (B) Representative confocal micrographs of double immunofluorescence for (i) CldU/NeuN and (ii) CldU/GFAP used to assess fate specification in the dentate gyrus. Double positive cells are indicated by white arrows. Scale bars 25 μm. (C) Percentage of total CldU-positive cells double-positive for the lineage markers NeuN (white), GFAP (green) or other (blue) in the dentate gyrus. Note there is no difference in the percentage of terminally differentiated neurons or glia generated by NSC and progenitor cells in the presence or absence of opiate analgesia and surgery.

buprenorphine administration, basal apoptotic indices are reduced and long-term survival of newly born cells is enhanced.

While this is the first study to demonstrate that opiate analgesics alter hippocampal neurogenesis, our data are also consistent with the well-documented negative impact of opiate drugs of abuse on the proliferation of hippocampal neural progenitor cells and specifically DCX-positive/nestin-negative TAP populations (Arguello et al., 2009; Eisch et al., 2000; Kahn et al., 2005). We did not test whether the return to basal proliferative indices observed in this study is gradual or involves a rebound increase in proliferating cell number as seen after withdrawal from chronic morphine treatment (Kahn et al., 2005). The acute nature, however, of the buprenorphine-mediated phenotype is novel. Measurable decreases in hippocampal progenitor cell proliferation depend upon chronic exposure to opiate drugs of abuse whether through s.c. pellet implantation (morphine) or intravenous self-administration (heroin) (Eisch et al., 2000). A single intraperitoneal injection of morphine does not alter proliferating hippocampal progenitor cell number (Eisch et al., 2000). The threshold of opiate exposure required to elicit these effects has yet to be determined and it may be that our acute injection paradigm of three s.c. injections in a single day represents the minimal threshold of opiate exposure necessary to inhibit neuroblast proliferation.

Perhaps even more intriguingly, the viability and maturation of hippocampal progenitor cells are known to be impaired by chronic exposure to opiate drugs of abuse (Arguello et al., 2009; Eisch et al., 2000) whereas here we

show here that the shortest buprenorphine analgesic paradigm improves survival of newly born cells exposed to opiate analgesia. Again, this may be a compensatory effect of analgesic withdrawal as extended exposure to buprenorphine *in vitro* has been shown to elicit apoptosis (Kugawa et al., 2000) thus limiting the duration of exposure to an apoptogenic stimuli *in vivo* may be sufficient to trigger compensatory anti-apoptotic pathways that enhance cell survival in the absence of cell death. Alternatively, the differential effects of buprenorphine and opiate drugs of abuse on cell survival may reflect different mechanisms of action. Certainly stress and opiate treatment can activate the hypothalamic-pituitary-adrenal (HPA) axis causing an increase in the production and secretion of stress hormones, notably corticosterone, that negatively regulate neurogenesis (Bryant et al., 1991; Coe et al., 2003; Olfe et al., 2010; Urquhart et al., 1984). Buprenorphine is unique among opiates, as it does not activate the HPA axis (Gomez-Flores and Weber, 2000). We cannot rule out the possibility that the behavioral impact of our s.c. injection paradigm, independent of the drug administered, activated the HPA axis. However, while restraint stress is associated with activation of the HPA axis and impaired neurogenesis, this effect is variable and dependent of numerous factors including age, sex, duration and frequency of restraint and habituation to handling (Barha et al., 2010; Hanson et al., 2011). Moreover, both of our treatment paradigms fall within the mild end of the stress spectrum suggesting that the reduced proliferation and enhanced survival observed is primarily directly attributable to buprenorphine.

Buprenorphine targets the MOR, KOR, and ORL-1 opiate receptors, all of which are expressed by hippocampal neurons (Mollereau et al., 1994; Skyers et al., 2003; Tallent et al., 2001; Yasuda et al., 1993). NSC and progenitor populations have been shown to express MORs and respond to MOR ligands but appear not to express KOR or respond to KOR-specific ligands, suggesting a direct role for MOR signaling in the phenotypes observed in this study (Berglund et al., 2004; Gutstein et al., 1997; Hauser et al., 1996; Learish et al., 2000; Persson et al., 2003; Zheng et al., 2010). While not extensively studied, buprenorphine has also been reported to be neuroprotective following retinal optic nerve transection *in vivo* (Ozden and Isenmann, 2004) and this protection is thought to be mediated by its direct action on MORs (Faden, 1996). It is not yet known whether hippocampal neuroblasts express ORL-1, but given its robust expression by granule neurons of the dentate gyrus (Tallent et al., 2001), it may be that buprenorphine modulates the “instructive” signaling associated with neuron-progenitor cell interactions influencing their survival and specification (Song et al., 2002). *In vivo*, ORL-1 expression increases in neurons that survive traumatic brain injury; thus, suggesting that activation of these receptors could be implicated in the increased survival of newly born cells observed in this study (Witta et al., 2003). Clearly, the expression of ORL-1 by hippocampal stem and progenitor cells must be determined before these hypotheses can be further explored mechanistically.

Taken together, this study identifies a hitherto unreported source of variability in neurogenic studies and a novel potential mechanism that could

impact upon clinical efficacy. Here, we demonstrated the impact of buprenorphine on neurogenesis in the dorsal hippocampus. Adult hippocampal neurogenesis has been implicated in learning and memory, with newborn neurons capable of being functionally integrated into the existing hippocampal circuitry and activated by specific memory tasks, most often spatial in nature (Kee et al., 2007; Winocur et al., 2006; Zhao et al., 2008). Accordingly, we and others have focused our studies of neurogenesis on the dorsal domain of the hippocampus which is responsible for these cognitive functions (Fanselow and Dong, 2010). There is, however, an increasing awareness of the functional heterogeneity of the hippocampus along its longitudinal axis and a growing body of research investigating the role of neurogenesis within the ventral hippocampus with respect to the regulation of emotion and the outcome of motivated behaviors (Fanselow and Dong, 2010; Kheirbek and Hen, 2011). This is particularly interesting in the context of opiate treatment in general, and buprenorphine more specifically, as even acute treatment may lead to cognitive and psychomotor impairment as well as modulate the magnitude of emotional outcomes in healthy subjects (Dolcos et al., 1998; Jensen et al., 2008; Panksepp et al., 1978). While such potential functional implications are important in the context of experimental design when examining neurogenesis and/or hippocampal dependent behaviors, they are of even greater importance in the clinic where the acute effect of buprenorphine on neurogenesis may negatively impact the already heightened negative emotions and cognitive impairments seen in patients with chronic pain, anxiety and/or depression, whereas timely removal of opiate treatment following

amelioration of pain could facilitate recovery through neurogenic impact not only in dorsal but also ventral hippocampus (Beaudreau and O'Hara, 2008; Hart et al., 2000; Hart et al., 2003; McDermott and Ebmeier, 2009; Wade et al., 1990). Future studies dissecting the impact of various opiates on neurogenesis in the dorsal versus ventral hippocampal domains and separating the functional impact on each region using appropriate behavioral paradigms will be of critical importance, notably when exploring the off-label use of buprenorphine to treat addiction and mood disorders (Davis, 2007; Howland, 2010; Likar et al., 2007).

In no way do our data support curtailing the use of analgesics in neurogenic studies. Rather, our findings are presented to highlight an important source of variability affecting the interpretation of changes in acute cell proliferation following neurogenic intervention strategies. It is widely accepted that surgery, even minor surgery, is stressful, both physically and psychologically to both humans and experimental animals, and that opiate analgesics can attenuate many of the immune modulatory events underlying the stress response in addition to pain management, thereby improving post-operative recovery (reviewed in (Shavit et al., 2006)). Here, we show that the inhibition of cell proliferation in animals treated with buprenorphine is significantly greater in animals experiencing post-operative pain. While ethical considerations precluded the direct examination of the effect of surgery alone on neurogenesis in our study, these data are evidence of a profound modulatory role of pain on hippocampal neurogenesis. Given the need to minimize the impact of pain in studies designed to test neurogenic interventions that require surgical

manipulation of experimental animals, we encourage researchers to carefully consider the potential impact of analgesic treatments on experimental outcomes and to include these details in reported methodologies as a potential explanation for a transient decrease in neuroblast proliferation following surgical intervention. Only 20% of current peer-reviewed publications detail the post-operative care of animals undergoing minor experimental surgery (Coulter et al., 2011; Piersma et al., 1999; Stokes et al., 2009). Failure to report analgesic use is not surprising; these procedures are commonly performed as standard operating procedures by veterinary staff monitoring the post-operative recovery of experimental animals, often without the involvement (or direct knowledge) of the primary research team. Given these necessary but “silent” interventions, our data suggest that differences in practice between institutions likely affect the acute assessment of hippocampal progenitor cell proliferation following surgical interventions and, potentially, the long-term survival of progenitor cell progeny without research teams being fully aware of these critical procedural differences.

4.9 References

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Chapter 5 – Exogenous regulation of adult hippocampal neurogenesis by selective serotonin reuptake inhibitor, antidepressant therapy

5.1 Objectives of this study

1) Determine if chronic SSRI treatment alters adult hippocampal neurogenesis

2) Determine if the neurogenic outcomes are drug specific

3) Determine if SSRI-mediated neurogenic effects are required for their effective therapeutic outcomes

5.2 Statement of author contributions

AP and SALB conceived of and designed the experiments. AP performed all the experiments, data analysis and generated the figures. AP and SALB wrote the manuscript.

5.3 A subset of selective serotonin reuptake inhibitors increase neural progenitor cell proliferation

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5.4 Summary

Amelioration of a depressive phenotype by chronic antidepressant (AD) treatment correlates with an increase in neural progenitor cell (NPC) proliferation and maturation in experimental models that match the kinetics of clinical AD efficacy. Here, we asked whether the effect of selective serotonin reuptake inhibitors on NPC proliferation is drug-specific. Escitalopram and citalopram, its racemate, are two of the most widely prescribed SSRIs on the market, presumably with identical mechanisms of action, yet few studies have examined their impact on NPC biology in the neurogenic subgranular zone of the dentate gyrus of the hippocampus. Using halogenated thymidine analogues, to label specific progenitor cell populations and behavioural analysis of depression-like behavior, we assessed the impact of vehicle (20% (2-Hydroxypropyl)-β-cyclodextrin), 10 mg/kg escitalopram and 10 or 20 mg/kg citalopram treatment for 7,14 and/or 28 days on NPC proliferation, survival and behavioural outcomes. Fluoxetine (10 mg/kg) was used as a positive control. Chronic fluoxetine and escitalopram, but not citalopram treatment significantly increased NPC proliferation but not survival. Furthermore, we show that environmental changes (drinking water and bedding) and standard elements of animal care (post-operative analgesia) will significantly modulate these effects and should be given greater consideration in the interpretation of experimental results. These results support the conclusion that neurogenesis associated with effective antidepressant treatment segregates to a subsets of SSRIs and is not a common molecular mechanism of action.

5.5 Introduction

The World Health Organization estimates that depression affects up to 20% of the global population (Wong and Licinio, 2001). Although staggering, this statistic likely remains a gross underestimation as it does not include diagnoses of depression co-morbid to another condition (Sudhir Khandelwal, 2001). Depression is characterized by the persistence of depressed mood, altered appetite and sleep habits, feelings of hopelessness, apathy, anhedonia, and suicidal ideations. While the underlying pathophysiology is poorly understood, this large range of symptoms is likely indicative of a multifactorial etiology involving many brain regions. The hippocampus is best known as being critical for the consolidation of learning and memory; however, its role in emotional regulation as part of the limbic circuit has lead to an increased focus on hippocampal dysfunction in many neuropsychiatric conditions, particularly depression (Dupret et al., 2008; Eisch et al., 2008; Papez, 1995).

The hippocampus, specifically the subgranular zone (SGZ) of the dentate gyrus, is also unique for being one of two sites in adult brain capable of significant neurogenesis throughout life (Zhao et al., 2008). A decrease in hippocampal volume, correlating with the length of a depressive episode, has been observed in both animal models of depression and human disease. (Bergouignan et al., 2008; MacQueen et al., 2003; Sheline, 1996; Sheline et al., 1999; Videbech and Ravnkilde, 2004). Hippocampal atrophy in these conditions is often accompanied by a decrease in adult neurogenesis, generally attributed to the molecular impact of stress, albeit with some controversy (Alonso et al., 2004;

Boldrini et al., 2009; Coe et al., 2003; Jaako-Movits et al., 2006; Mineur et al., 2007). Conversely, multiple antidepressant therapies increase neural progenitor cell (NPC) proliferation (Duman et al., 1999; Madsen et al., 2000; Malberg and Duman, 2003; Malberg et al., 2000; Perera et al., 2007; Santarelli et al., 2003). These data, and in particular the marked correlation between the 28-day time-frame required for adult neuronal specification and the three to four week lag in the appearance of appreciable clinical improvements following the onset of pharmacological antidepressant therapy, underlie 'the neurogenesis hypothesis of depression' (Frazer and Benmansour, 2002; Kempermann et al., 2004). Here, altered neurogenesis is explored as a causal factor for depression and/or a potential therapeutically relevant "off-target" effect of antidepressant treatment that has yet to be fully understood. Compelling evidence for the potential of this line of investigation has also been provided by loss of function studies wherein pharmacological antidepressant therapy ceased to improve behavioral indices of depressions in animal models following the elimination of adult neurogenesis (Perera et al., 2011; Santarelli et al., 2003).

It is not yet clear whether neurogenesis is required for antidepressant efficacy or represents an "off-target" effect of particular treatments. Few studies have compared comprehensively the effect of common selective serotonin reuptake inhibitors (SSRI), the leading treatment prescribed for the treatment of depression, on the complete neurogenic process. The majority of research has focused on Prozac (fluoxetine) and it remains to be determined whether the observed effects are drug-specific (Alahmed and Herbert, 2008; Encinas et al.,

2006; Hodes et al., 2010; Huang et al., 2008; Klempin et al., 2010; Malberg and Duman, 2003; Malberg et al., 2000; Pechnick et al., 2011; Perera et al., 2011; Santarelli et al., 2003; Wang et al., 2008). While there is an emerging consensus that stress and/or antidepressants reciprocally impact upon NPC proliferation (Petrik et al., 2011; Samuels and Hen, 2011; Snyder et al., 2011), little is known about NPC specification beyond a paucity of evidence that, in some cases, antidepressants enhance maturation and increase survival of NPC progeny (Duman et al., 1999; Madsen et al., 2000; Malberg and Duman, 2003; Malberg et al., 2000; Perera et al., 2007; Santarelli et al., 2003; van Praag et al., 1999, 2000; Wang et al., 2008). Perhaps, most damaging to the overarching hypothesis, some antidepressant effects are not reproducible between research groups or appear to be specific to the rodent strain, depression model, neurogenesis ablation protocol, or behavioral test used to assess treatment efficacy in a given experiment, and, in a few cases, have even been shown to reduce NPC proliferation (Alahmed and Herbert, 2008; Bessa et al., 2009; Hodes et al., 2010; Huang et al., 2008; Kornack and Rakic, 1999; Petersen et al., 2008; Snyder et al., 2011; Surget et al., 2008). Finally, relatively little is known about the molecular mechanisms by which antidepressants alter the neurogenic process in NPC populations that lack the functional chemical synapses characteristic of mature neurons canonically targeted by SSRIs. To address whether increasing NPC proliferative indices is a common mechanism of action among SSRIs, we compared the actions of citalopram and its active isomer, escitalopram, with fluoxetine on adult hippocampal NPC proliferation and survival. We show here

that fluoxetine and escitalopram, but not citalopram, increase adult hippocampal NPC proliferative indices without an effect on survival and moreover that these actions are modulated significantly by environmental changes and drug interactions with commonly prescribed analgesics.

5.6 Materials and Methods

5.6.1 Animals

Eight week old, male, C57Bl/6 x FVB/N x SV-129 mice were used. Animals were kept on a 12 h light-dark cycle (light phase 0600 h-1800 h) with access to food and water *ad libitum*. Mice were individually housed starting at seven weeks of age. Mice undergoing environmental enrichment were housed in standard cages with Harlan Laboratories (USA),¼ inch Teklad corn cob sanitized bedding in place of the standard PWI industries (Montreal, QC), mixed hardwood bedding. All experimental protocols were approved by the Animal Care Committee (ACC) of the University of Ottawa according to guidelines set forth by the Canadian Council on Animal Care.

5.6.2 Labeling mitotically active cells

Mice received five intraperitoneal (i.p.) injections of 5-choloro-2'-deoxyuridine (CldU; 42.5 mg/kg) (prepared to 17 mg/ml in 0.625 M Tris base in sterile water; Sigma, catalog #C6891, Saint Louis, MO) over three days prior to osmotic minipump implantation and five i.p. injections of 5-iodo-2'-deoxyuridine (IdU; 57.5 mg/kg) (prepared to 23 mg/ml in 0.895 M Tris base in sterile water; MP Biomedicals, catalog #100357, Solon, OH) over the three final days of SSRI

treatment. IdU and CldU concentrations were as described by Vega and Peterson (2005) with the exception that Tris base was used to enhance solubility in place of sterile saline and 5 N NaOH. The final pH of the solutions was pH 8.5 as recommended (Vega and Peterson, 2005). Delivery schedules for CldU and IdU for each experiment were optimized to label not only more rapidly dividing type 2 and 3 NPCs but also the slowly dividing type 1 population, see figure 5.1 for a schematic of the delivery schedules (Nowakowski et al., 1989; Taupin, 2007).

5.6.3 SSRI treatment

All antidepressant drug treatments and control compounds were administered via osmotic minipump. Alzet osmotic minipumps (model 2001, 2002 and 2004, DURECT Corporation, Cupertino, CA) were prefilled with vehicle, 20% (2-Hydroxypropyl)-β-cyclodextrin (Sigma, catalog #H107, Saint Louis, MO) in sterile normal saline (0.9% NaCl), fluoxetine-HCl (10 mg/kg; Enzo life sciences, catalog #BML-NS140, Plymouth meeting, PA), citalopram-HBr (10 or 20 mg/kg; Lundbeck, Copenhagen, Denmark) or escitalopram-oxalate (10 mg/kg; Lundbeck), all dosages were established based on published standards. Twenty eight day pumps (model 2004) were pre-incubated individually in 5 ml normal saline at 37°C for 36 h prior to implantation as per manufacturers instructions. Seven and 14 day pumps were implanted immediately after being filled. For mini-pump implantation, mice were anesthetized with an inhalant mixture of oxygen and isofluorane, a small skin incision followed by a

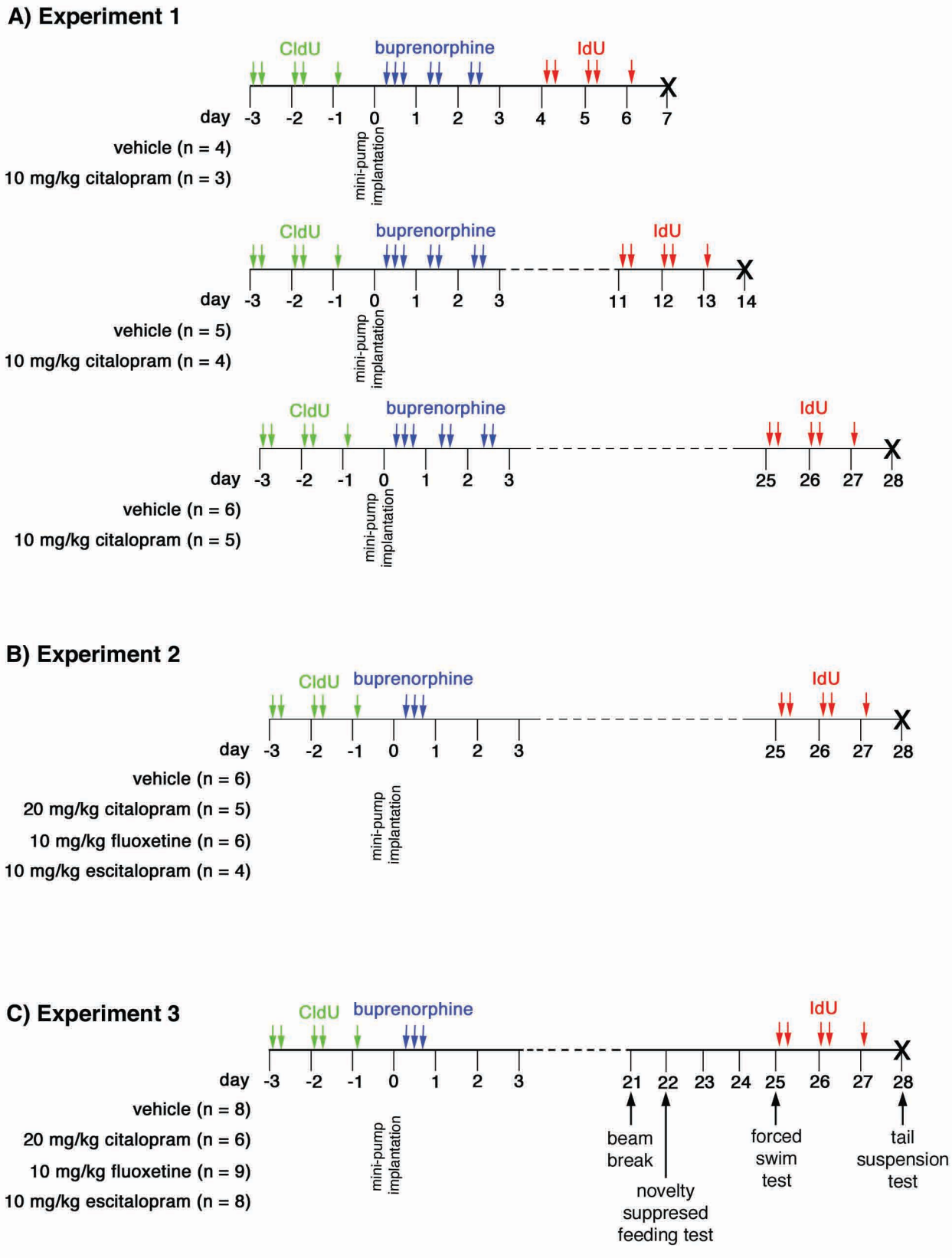

A) Experiment 1
CldU
buprenorphine
IdU
day -3 -2 -1 0 1 2 3 4 5 6 7
vehicle (n = 4)
10 mg/kg citalopram (n = 3)
mini-pump implantation
CldU
buprenorphine
IdU
day -3 -2 -1 0 1 2 3 11 12 13 14
vehicle (n = 5)
10 mg/kg citalopram (n = 4)
mini-pump implantation
CldU
buprenorphine
IdU
day -3 -2 -1 0 1 2 3 25 26 27 28
vehicle (n = 6)
10 mg/kg citalopram (n = 5)
mini-pump implantation
B) Experiment 2
CldU
buprenorphine
IdU
day -3 -2 -1 0 1 2 3 25 26 27 28
vehicle (n = 6)
20 mg/kg citalopram (n = 5)
10 mg/kg fluoxetine (n = 6)
10 mg/kg escitalopram (n = 4)
mini-pump implantation
C) Experiment 3
CldU
buprenorphine
IdU
day -3 -2 -1 0 1 2 3 21 22 23 24 25 26 27 28
vehicle (n = 8)
20 mg/kg citalopram (n = 6)
10 mg/kg fluoxetine (n = 9)
10 mg/kg escitalopram (n = 8)
mini-pump implantation
beam break
novelty suppresed feeding test
forced swim test
tail suspension test

Figure 5.1

Figure 5.1 – **Treatment paradigms**.

Buprenorphine injections (0.05 mg/kg, s.c.) are indicated by blue arrows; IdU injections (57.5 mg/kg, i.p.) are indicated by red arrows; CldU injections (42.5 mg/kg, i.p.) are indicated by green arrows. Time of sacrifice is indicated by a black X. The vehicle for all SSRIs was 20% (2-hydroxypropyl)-β-cyclodextrin. (A) Assessment of cell proliferation and survival of NPCs following a seven, 14 or 28 day treatment with vehicle or 10 mg/kg citalopram with seven 0.05 mg/kg s.c. injections of buprenorphine following surgery. CldU was administered over the three days prior to mini-pump implantation and proliferating cells were labeled with IdU over the three final days of treatment. Animals were sacrificed 24 h after the last IdU injection. (B) To establish SSRI specificity, 20 mg/kg citalopram, 10 mg/kg fluoxetine or 10 mg/kg escitalopram were compared. IdU was administered on days 25, 26 and 27 following surgery and IdU-positive proliferating cell number was assessed 28 days after the onset of SSRI treatment. To assess specification and survival of stem and progenitor cells following SSRI treatment, proliferating cells were labeled with CldU over the three days prior to the imitation of treatment. Survival and fate specification were assessed 28 days later. To assess drug interactions, a reduced schedule of three 0.05 mg/kg s.c. injections of buprenorphine analgesia was used following mini-pump implantation. (C) To determine the baseline therapeutic outcomes of chronic SSRI treatment in non-depressed mice, SSRI treatments were carried out as in B in addition to a battery of depression behavioural tests in an additional cohort of mice. Mice, underwent beam break locomotion assessment, novelty suppressed feeding testing, forced swim testing and tail suspension testing on treatment day 21, 22, 25 and 28 respectively.

subcutaneous pocket were made on the back of the mouse, mini-pumps were placed in the pocket with the dispensing end away from the incision and finally the incision was closed with one stitch and a single wound clip. As an analgesic measure following surgery buprenorphine (Chiron Compounding Pharmacy Inc., Guelph, ON) was prepared in sterile water to a final concentration of 0.03 mg/ml and either seven 0.05 mg/kg subcutaneous injections were administered over the day of surgery and the two following days (experiment 1) or three 0.05 mg/kg subcutaneous injections were administered on the day of surgery (experiment 2 and 3). In experiment 1, mice received either vehicle or 10 mg/kg citalopram for seven, 14 or 18 days. In experiment 2 and 3 mice received vehicle, 10 mg/kg fluoxetine, 20 mg/kg citalopram or 10 mg/kg escitalopram for 28 days. All three cohorts were used for the analysis of neurogenesis and cohort three was also used for behavioural studies. Following sacrifice residual pump volumes were measured to confirm appropriate dispensing of the contents.

5.6.4 Behavioral Testing

For all behavior tests mice were transferred to the testing room, with the white noise generator playing at 70 dB, 60 min prior to beginning the test to allow for acclimation.

i. beam break locomotion testing – Locomotor activity of the mice was measured using infrared beam break boxes on day 21 of treatment (AccuScan Instruments, Columbus, OH). Mice were monitored for 2 h between 1200 and 1600 h. Data was collected and analyzed using the Micromax Software (AccuScan Instruments).

ii. Novelty suppressed feeding test – On treatment day 21, 18 h prior to testing all food was removed for the home cage. The testing room was lit to 45 lux in the center of the testing apparatus, a 45x45x45 cm white plastic box without bedding. One quarter of a standard mouse chow pellet was placed in the center of the box and the mouse introduced into the corner of the box. Latency to eat was measured by a single observer for all mice up to a maximum of five min. Immediately following the test, mice were returned to their home cage where the amount of food consumed over an additional five-minute period was measured to control for differences in appetite.

iii. Forced swim test – The testing room was lit with red light. Mice were placed in a cylinder 22 cm in diameter and 37 cm high filled with 23-25°C water to a depth exceeding the distance their tail could reach preventing them from touching the bottom. Mice were left in the cylinder for 6 min. Ethovision software version 7 (Noldus, Waganingen, Netherlands) was used to video record and analyze mouse movement for total immobility time.

iv. Tail suspension test – The testing room was lit to 45 lux at the center of the apparatus. Mice were suspended from a metal bar by tape, 2 mm from the base of the tail, attached to a strain gauge (Med Associates inc., St Albans, VT, USA) for 6 min. The duration of immobility was measured by tail suspension software (Med Associates inc.).

5.6.5 Tissue preparation

Mice were sacrificed 24 h after the final IdU injection with euthanyl (Bimeda-MTC Animal Health Ins, Cambridge ON) prepared in sterile water to a

final concentration of 65 mg/ml and transcardial perfusion with 10 mM phosphate buffered saline (PBS; 10 mM sodium phosphate and154 mM NaCl) followed by 3.7% paraformaldehyde in 10 mM PBS. Brains were removed, post-fixed for 24 h, and cryoprotected in 20% sucrose. Serial 10 µm coronal cryosections were collected using a Leica CM1900 cryostat (Leica Microsystems, Wetzlar, Germany), throughout the dorsal domain of hippocampus from bregma -1.0 to -3.4 using the coordinates of Hof et al. (Hof et al., 2001).

5.6.6 Immunofluorescence

Serial coronal sections were incubated in 2 N HCl for 45 min at 37˚C and neutralized in 0.1 M borate buffer (pH 8.5). IdU and CldU incorporation was detected by immunofluorescence using mouse anti-BrdU clone 44 (1:500; Becton Dickinson Immunocytochemistry Systems, catalog #347580, San Jose, CA) and rat anti-BrdU-FITC (1:100, AbD Serotec, catalog #OBT0030F, Raleigh, NC) respectively. Cy3 conjugated anti-mouse IgG (1:800; Jackson Immunoresearch, catalog #715-165-150) was the secondary antibody used. Sections were incubated with primary antibodies overnight at 4˚C, washed with 10 mM PBS, incubated with secondary antibodies for 1 h at room temperature, and washed with PBS prior to being coverslipped in ProLong Gold antifade reagent (Invitrogen, catalog #P-36930, Eugene, Oregon, USA). All antibodies were diluted in antibody buffer (3% bovine serum albumin, 0.3% Triton X-100 in 10 mM PBS).

5.6.7 Microscopic Analysis and Quantification

Immunofluorescence was assessed on a DMXRA2 epifluorescent microscope (Leica Microsystems) using OpenLab 5.0.2 (Improvision) software. Quantification was completed as we have previously described (Melanson-Drapeau et al., 2003). Briefly, the area of (a) the SGZ, defined as the region bordering the granule cell layer and the hilus (3 cell widths into each), (b) the hilus, and (c) the entire GCL was established for each section using the Advanced Measurement Module of OpenLab 5.0.2 (Improvision) software. The number of labeled cells was assessed in both the left and right hippocampi by two independent observers blinded as to the identity of the sections. These values were averaged to yield a single number per region, section, and animal. IdU-labeled cells were counted in the SGZ and hilus using a 10x objective. In survival studies, CldU-labeled cells were counted throughout both the GCL and hilus as new cells, born in the SGZ, will migrate into the granule cell layer of the DG. The hilus was included to assess the possibility of ectopic localization of SGZ progenitor progeny following treatment. The total number of labeled cells in a given region per animal was calculated as the number of IdU or CldU positive cells per 0.1 mm^2 using the appropriate area measurements for the regions counted.

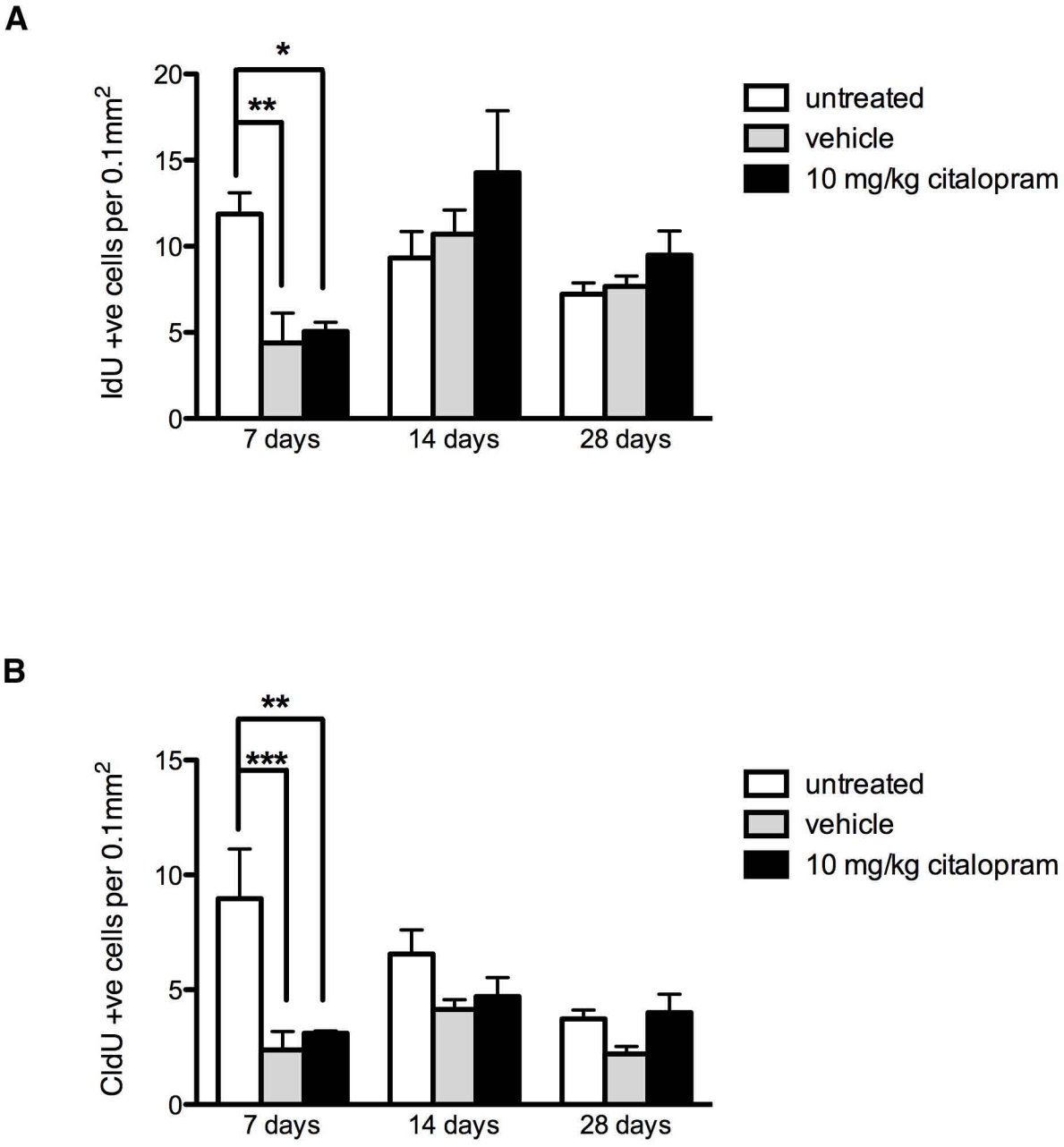
5.6.8 Statistical Analysis

Data are presented as mean ± standard error of measurement (SEM). Data were analyzed by one-way or two-way ANOVA followed by *post-hoc* Tukey or Bonferroni test or Student's t-test where appropriate.

5.7 Results

5.7.1 Experiment 1

To determine the effect of acute (seven day), sub-chronic (14 day) and chronic (28 day) 10 mg/kg citalopram treatment on neurogenesis, we first compared its effects on NPC proliferation in the SGZ and hilus of the dentate gyrus of the hippocampus. Osmotic minipumps were used for administration to more easily maintain steady state levels of the drugs given the increased metabolic rate of rodents relative to humans and to eliminate the restraint stress associated with daily injections that could, in and of itself, elicit a depressed state and affect NPC proliferation (Barha et al., 2010; Demetrius, 2005). To minimize immediate surgical discomfort and assess potential for pharmacological cross talk, mice received seven 0.05 mg/kg subcutaneous injections over the first 3 days following surgery, a schedule that we have previously shown acutely suppresses NPC proliferation without affecting survival of their progeny (Pettit et al., 2012). Antidepressants were solubilized in 20% (2-hydroxypropyl)-β-cyclodextrin, a benign, routinely used vehicle that increases the solubility of hydrophobic molecules and is compatible with osmotic minipump administration (Loftsson et al., 2005; Yaksh et al., 1991). Control mice were implanted with pumps containing 20% (2-hydroxypropyl)-β-cyclodextrin only. Using this


A
IdU +ve cells per 0.1mm2
20
15
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*
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7 days
14 days
28 days
untreated
vehicle
10 mg/kg citalopram
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CldU +ve cells per 0.1mm2
15
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0
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***
7 days
14 days
28 days
untreated
vehicle
10 mg/kg citalopram

Figure 5.2

Figure 5.2 - Effect of 10 mg/kg citalopram on hippocampal progenitor cell proliferation and survival.

Following a seven, 14, or 28-day treatment with vehicle (20% (2-hydroxypropyl)-beta-cyclodextrin) or 10 mg/kg citalopram via subcutaneous osmotic minipump. (A) Proliferation of IdU positive cells per $0.1mm^2$ in the SGZ and hilus of the dentate gyrus of the hippocampus was reduced with seven-day vehicle or citalopram treatment. (B) The number of surviving CldU positive cells per $0.1mm^2$ in the hilus, SGZ and GCL of the hippocampus was reduced with 7 day vehicle or citalopram treatment. Data are presented as mean ± SEM. * $p<0.01$, ** $p<0.01$ *** $p<0.001$ (two-way ANOVA, *post-hoc* Bonferroni test).

treatment paradigm (Figure 5.1A) we show that a seven-day treatment with both the vehicle and 10 mg/kg citalopram significantly reduced the proliferation of IdU positive NPCs (Figure 5.2A, * $p<0.05$, ** $p<0.01$ compared to vehicle by two-way ANOVA followed by post-hoc Bonferroni test). This suppression was only observed acutely and partially overlapped with the period of time mice received both antidepressant or vehicle (seven days) and opiate analgesia (three days). Longer 14 and 28 day treatments with vehicle or citalopram, following withdrawal of buprenorphine, had no significant effect on NPC proliferation in adult hippocampus (Figure 5.2A, $p>0.05$ compared to vehicle by two-way ANOVA followed by post-hoc Bonferroni test).

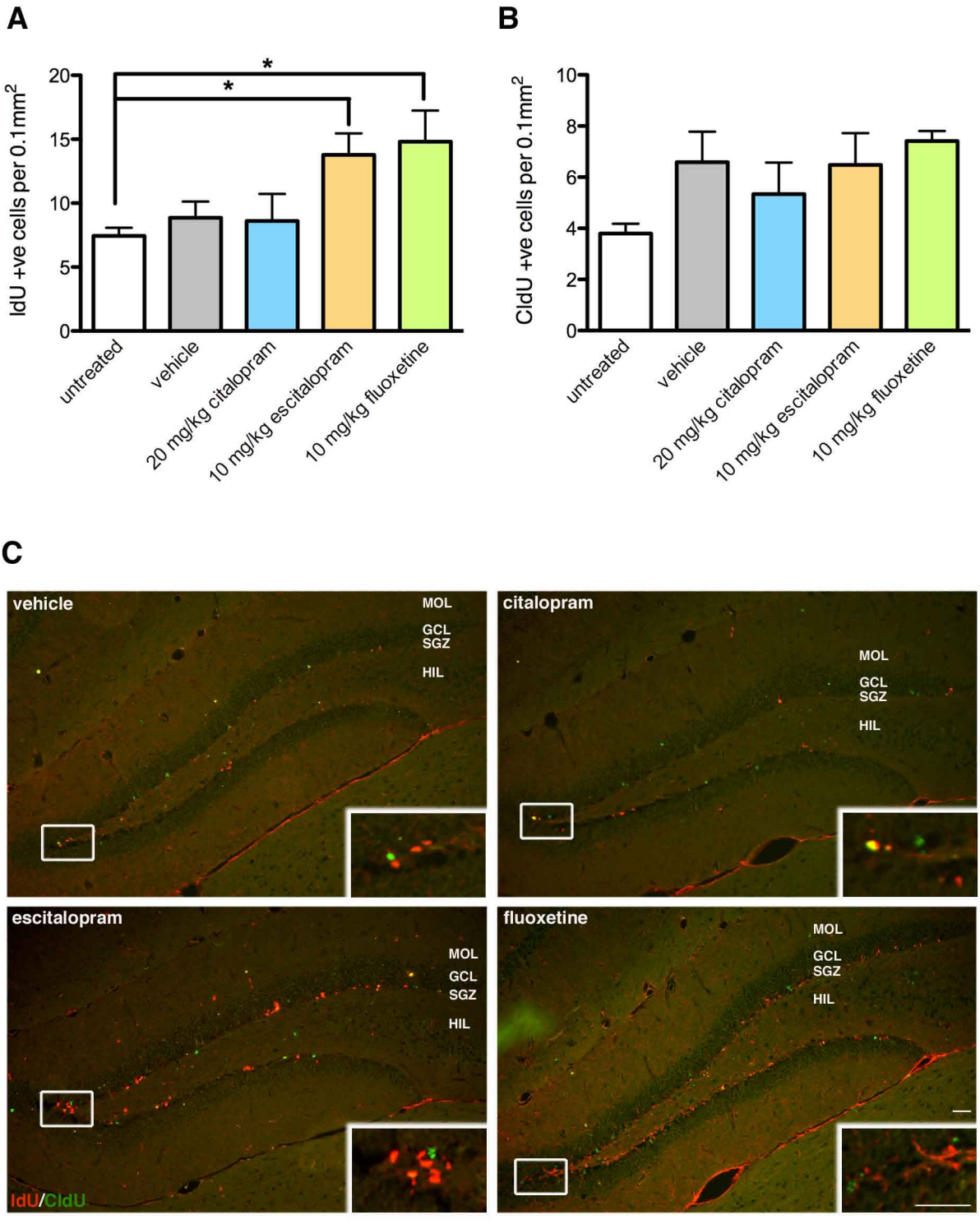
Survival of NPC progeny was also assessed. In order to avoid the unknown effect of SSRI withdrawal on neurogenesis, proliferating cells were labeled with CldU over the three days prior to osmotic minipump implantation and those surviving after seven, 14 or 28 days of treatment were quantified (Figure 5.1A). As with proliferation, we observed a significant decrease in survival following seven days of vehicle or citalopram treatment with concomitant opiate analgesia over the first three test days (Figure 5.2B, ** $p<0.01$, *** $p<0.001$ compared to vehicle by two-way ANOVA followed by post-hoc Bonferroni test) but no additional effect with 14 or 28 day treatments and withdrawal of opiate analgesia (Figure 5.2B, $p>0.05$ compared to vehicle by two-way ANOVA followed by post-hoc Bonferroni test). There was no difference in IdU or CldU incorporation in the CA1 region of the hippocampus between any of the

treatment groups at any time point. This indicates that IdU and CldU bioavailability was equivalent across experimental manipulations.

5.7.2 Experiment 2

To determine if altered neurogenesis is a common mechanism of action for SSRIs, the effects of chronic SSRI treatment with two structural analogs (escitalopram and citalopram) on NPC proliferation were compared to fluoxetine. To minimize pharmacological cross talk on proliferative indices with the opiate analgesics required immediately after surgery, buprenorphine was administered using a schedule we have previously shown to have no effect on NPC proliferation but modestly enhance survival of their progeny (Pettit et al., 2012). Fluoxetine, which has been most consistently shown to increase hippocampal NPC proliferation, was administered at 10 mg/kg. Escitalopram, a newer formulation of citalopram consisting only of the active S enantiomer was administered at 10 mg/kg (Hyttel et al., 1992). Citalopram, a racemic mixture, was administered at 20 mg/kg to match the amount of the active S enantiomer delivered in the escitalopram alone.

Both escitalopram and fluoxetine effectively doubled the number of proliferating IdU labeled NPCs in the adult hippocampal dentate gyrus compared to untreated mice (Figure 5.3A and C, * $p<0.05$ compared to untreated, one way ANOVA followed by *post-hoc* Tukey's test). Vehicle and 20 mg/kg citalopram treatment had no effect on NPC proliferation (Figure 5.3A and C, $p>0.05$, one way ANOVA followed by *post-hoc* Tukey's test). None of the treatments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20 mg/kg citalopram
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10 mg/kg fluoxetine
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IdU/CldU
fluoxetine
MOL
GCL
SGZ
HIL

Figure 5.3

Figure 5.3 - Effect of chronic citalopram, escitalopram or fluoxetine treatment on hippocampal progenitor cell proliferation and survival.

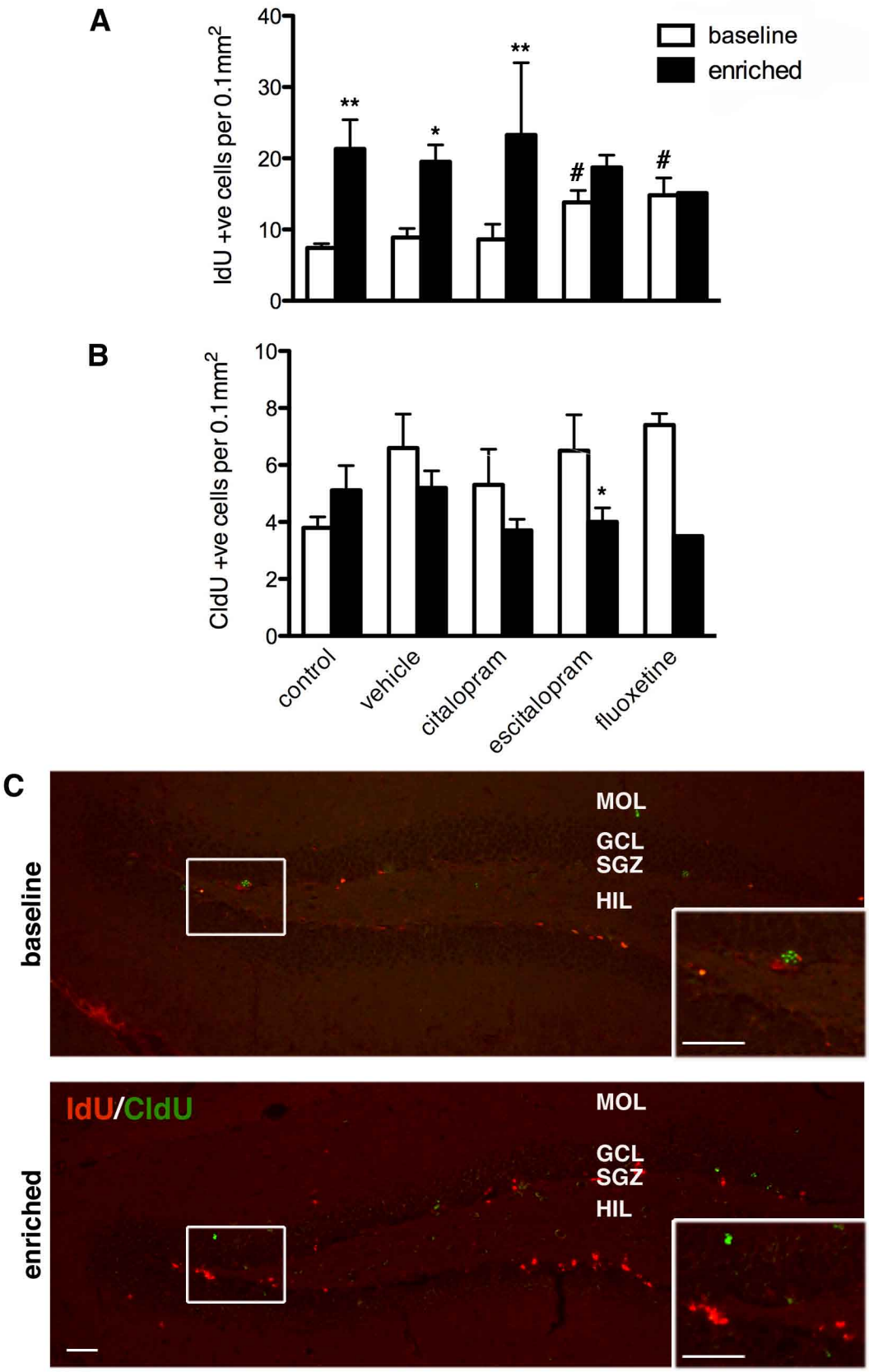
Following a 28-day treatment with vehicle (20% (2-hydroxypropyl)-beta-cyclodextrin), 20 mg/kg citalopram, 10 mg/kg escitalopram or fluoxetine via subcutaneous osmotic minipump. (A) Proliferation of IdU positive cells per $0.1mm^2$ in the SGZ and hilus of the dentate gyrus of the hippocampus was increased with escitalopram and fluoxetine treatment. (B) The number of surviving CldU positive cells per $0.1mm^2$ in the hilus, SGZ and GCL of the hippocampus was unchanged following treatment. Data are presented as mean ± SEM. * $p<0.05$ (one-way ANOVA, *post-hoc* Tukey's test). (C) Representative 10x micrographs of double immunofluorescence for IdU (red) and CldU (green) following treatment. Scale bars 50 μm. Abbreviations: GCL, granule cell layer of the dentate gyrus; HIL, hilus; MOL, molecular layer of the dentate gyrus; SGZ, subgranular zone.

significantly altered the survival of NPC progeny labeled immediately prior to treatment (Figure 5.3B and C, $p>0.05$, one way ANOVA). There was no difference in the IdU or CldU incorporation in the CA1 region (data not shown) indicating that their bioavailability remains unchanged following treatment.

5.7.3 Experiment 3

To address whether SSRI-mediated increases in hippocampal NPC proliferation are required for their effective therapeutic outcomes we attempted an environmental behavioural gain of function study. Rather, than ablate proliferating NPCs and assess impact on SSRI therapeutic efficacy, we sought to non-invasively enhance NPC proliferative rates and establish whether this increase would obviate

requirement for SSRIs in behavioural tests of depressive phenotype. To minimize environmental manipulations, we chose a single, mild, enrichment intervention. Homecage bedding for animals in experiment 3 was changed from a woodchip type bedding to a corncob, gravel-like bedding. The later bedding is routinely recommended for marble burying studies as it motivates digging behavior in rodents (Deacon, 2006). Given that this bedding is known to be stimulating, encouraging increased activity in rodents and that we know that environmental enrichment increases neurogenesis with the exercise component affecting NPC proliferation specifically (Deacon, 2006; van Praag et al., 1999, 2000), we sought first to determine if this mild intervention would abrogate the impact of SSRIs on NPC proliferation by elevating basal indices. Animals were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fluoxetine
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baseline
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SGZ
HIL
enriched
IdU/CldU
MOL
GCL
SGZ
HIL

Figure 5.4

Figure 5.4 - Effect of environmental enrichment on chronic citalopram, escitalopram or fluoxetine treatment on hippocampal progenitor cell proliferation and survival.

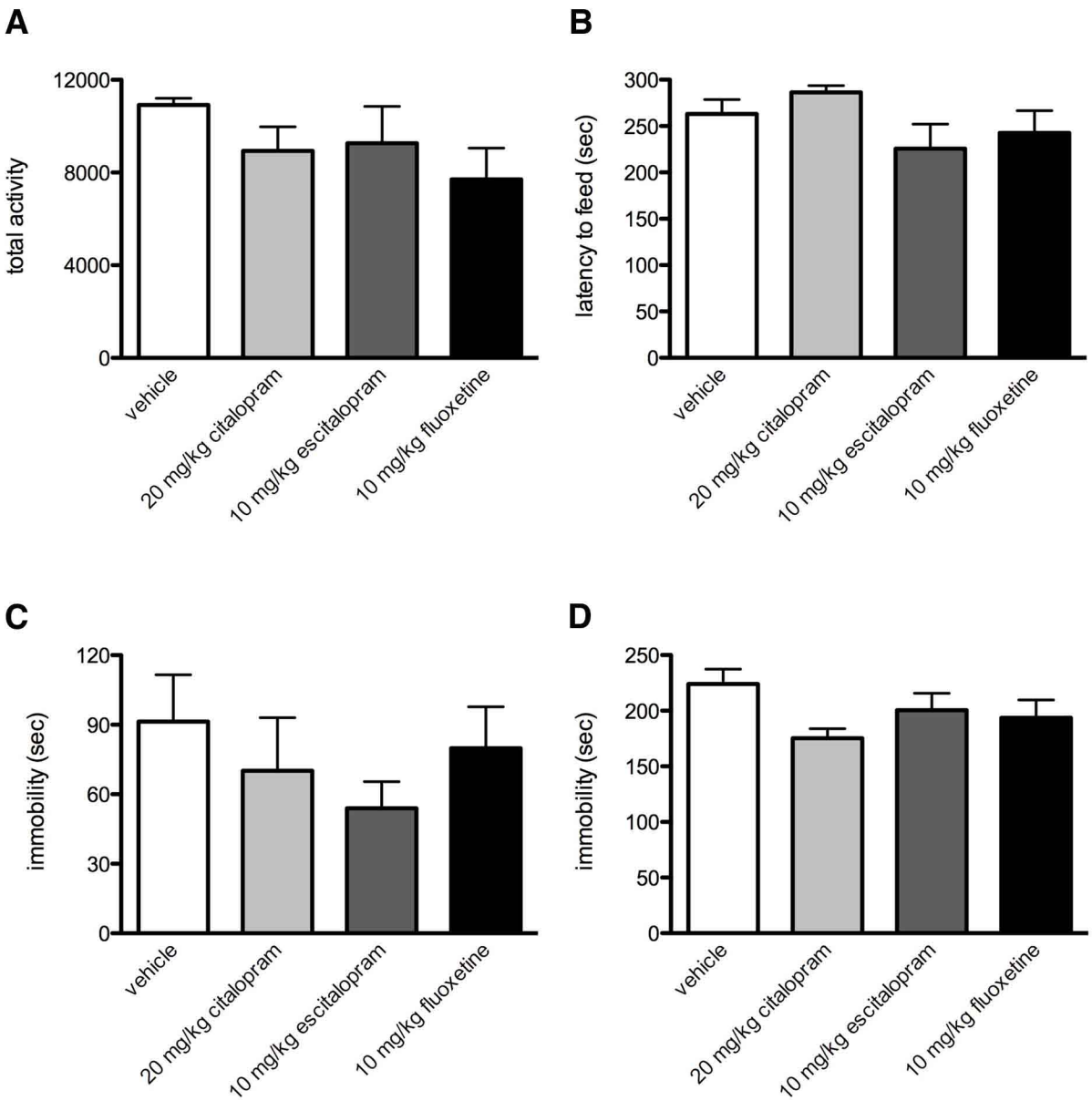
Following a 28-day treatment with vehicle (20% (2-hydroxypropyl)-beta-cyclodextrin), 20 mg/kg citalopram, 10 mg/kg escitalopram or fluoxetine via subcutaneous osmotic minipump and housing in baseline (while bars) or enriched, corn cob gravel bedding, (black bars) conditions. (A) Proliferation of IdU positive cells per $0.1mm^2$ in the SGZ and hilus of the dentate gyrus of the hippocampus is increased by environmental enrichment compared to baseline conditions and when combined with vehicle or fluoxetine treatment. (B) The number of surviving CldU positive cells per $0.1mm^2$ in the hilus, SGZ and GCL of the hippocampus was reduced following escitalopram and fluoxetine treatment in the presence of enrichment. Data are presented as mean ± SEM. Baseline comparisons by one-way ANOVA, post-hoc Tukey's test, #$p<0.05$ relative to control, paired comparisons by two-way ANOVA, *post-hoc* Tukey's test, * $p<0.05$, ** $p<0.01$ relative to baseline. (C) Representative 10x micrographs of double immunofluorescence for IdU (red) and CldU (green) in control animals under baseline housing conditions and following housing in enriched conditions. Abbreviations are as in previous figures. Scale bars 50 μm.

implanted for 28 days with osmotic minipumps containing vehicle (20% % (2-hydroxypropyl)-β-cyclodextrin), citalopram (20 mg/kg), escitalopram (10 mg/kg) or fluoxetine (10 mg/kg) and compared to control (untreated mice). Post-operative opiate analgesia was identical to that described for experiment 2. A significant increase in the basal rate of NPC proliferation was evident in the "enriched" control group relative to animals housed with standard wood-chip bedding (Fig 5.4A and C, ** $p<0.01$ relative to baseline by two-way ANOVA and post-hoc Tukey's test). Moreover, this simple environmental change enhanced IdU-positive NPC proliferation of both the vehicle-treated control and citalopram-treated groups to that observed following chronic escitalopram- or fluoxetine-treated animals (Figure 5.4A, *$p<0.05$, **$p<0.01$ relative to baseline by two-way ANOVA and post-hoc Tukey's test). No further increases in these two groups were seen when compared to untreated animals (Figure 5.4A and B, $p>0.05$ by two-way ANOVA). Furthermore the number of surviving NPC progeny were reduced by enrichment when combined with escitalopram and also approached significance with fluoxetine treatment (Figure 5.4B, *$p<0.05$ relative to baseline by two-way ANOVA and post-hoc Tukey's test).

Prior to sacrifice, the effect of 28 day SSRI treatment on gold standard behavioural measures of depression in mice were then assessed in the "enriched" cohort in the final week of treatment (Figure 5.1C). Although a depressive phenotype was not induced prior to testing, both the forced swim test (FST) and tail suspension test (TST) have been used previously to screen for antidepressant compounds following acute treatment (Cryan et al., 2005; Porsolt

et al., 2001) and assess the impact of chronic treatment (Santarelli et al., 2003). In both the FST and TST, mice receiving antidepressant therapy display a reduced immobility time demonstrating an increased struggle associate with an increased 'will to live' or desired to escape from what is perceived to be an unpleasant or dangerous situation. The novelty suppressed feeding test (NSF) is uniquely sensitive to chronic antidepressant treatment with therapeutically relevant pharmacological activity associated with a reduced latency to feed in the novel environment (Alahmed and Herbert, 2008; Huang et al., 2008; Santarelli et al., 2003). Home cage beam break testing is routinely used to assess overall motor activity as a control to account for any baseline changes to movement, this may occur in these studies due to serotonin overstimulation known as serotonin syndrome following SSRI treatment (Martin, 1996).

Examining first the gross locomotor effects of SSRI treatment in our mice by home cage beam break analysis, we saw no effect with any of the SSRI treatments (Figure 5.5A, $p>0.05$ by one way ANOVA). The NSF test revealed no change in the latency to feed in a novel environment following any of SSRI treatments compared to vehicle (Figure 5.5B, $p>0.05$ by one way ANOVA). This was not due to differences in appetite as home cage food consumption over the 5 min post testing was not different between groups (data not shown). Furthermore, neither the FST nor TST showed a significant change in immobility time following SSRI treatment (Figure 5.5C and D, $p>0.05$ by one way ANOVA). It should be noted that tests were not carried out on the cohort that did not receive behavioural enrichment.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10 mg/kg fluoxetine
B
latency to feed (sec)
300
250
200
150
100
50
0
vehicle
20 mg/kg citalopram
10 mg/kg escitalopram
10 mg/kg fluoxetine
C
immobility (sec)
120
90
60
30
0
vehicle
20 mg/kg citalopram
10 mg/kg escitalopram
10 mg/kg fluoxetine
D
immobility (sec)
250
200
150
100
50
0
vehicle
20 mg/kg citalopram
10 mg/kg escitalopram
10 mg/kg fluoxetine

Figure 5.5

Figure 5.5 - Effect of chronic citalopram, escitalopram or fluoxetine treatment on depressive-like behavior.

Over the final week of a 28-day treatment with vehicle (20% (2-hydroxypropyl)-beta-cyclodextrin), 20 mg/kg citalopram, 10 mg/kg escitalopram or fluoxetine mice were tested for depression-like behaviors. Overall ambulatory activity (A), latency to feed in a novel environment (B), immobility in both the forced swim (C) and tail suspension (D) tests was unchanged following treatment (one-way ANOVA). Data are presented as mean ± SEM.

5.8 Discussion

We show here that chronic treatment with a subset of SSRI antidepressants is able to increase the proliferation of adult hippocampal NPCs but does not alter survival of their progeny and that these proliferative effects are exquisitely sensitive to environmental manipulation. We show that both fluoxetine and escitalopram, when administered chronically, increase NPC proliferation but that citalopram does not despite equimolar administration of the active isomer. Further, we show that NPC proliferation, whether animals are treated with vehicle or citalopram, can be transiently inhibited by 72 h co-treatment with opiate analgesics. Finally, our data suggests that manipulations that elevate NPC proliferation masks the phenotypic effects of antidepressants on behavioural indices of depression although this latter finding will require further corroboration in mice wherein basal indices of NPC proliferation are not already elevated.

These data, while still in an early state, raise some important considerations for the design of experiments targeting the pharmacoefficacy of neurogenic manipulation strategies. Certainly, it has been recognized in the literature that certain factors, rodent strain most prominently, may alter the neurogenic outcomes in response to antidepressant therapy (Alahmed and Herbert, 2008; Bergstrom et al., 2007; Cryan et al., 2005; Huang et al., 2008; Petit-Demouliere et al., 2005). Variability between seemingly identical cohorts has not, however, been reported, in part due to the current culture of publication favoring positive results in lieu of experiments that identify factors that contribute to “failure”.

Personal communication with several independent laboratories has however, revealed that this is surprisingly common with respect to antidepressant effects on neurogenesis. Understanding such inconsistencies and variability is crucial to our ability to reduce wasted time, effort, animals, and taxpayers operating support in future studies.

As we have previously published, one important consideration when trying to tease apart these events is the care and handling of research animals. Given that a technical staff rather than researchers themselves manage the day-to-day aspects of animal care in most institutions, it is often overlooked in experimental analysis. One such example is post-operative analgesia, which while mandated by the national animal care governing bodies, varies significantly between institutions. Here, we show that in addition to direct effects on NPC proliferation (Pettit et al., 2012), drug interactions between buprenorphine and citalopram are sufficient to reduce acute NPC proliferative indices 7 days after treatment with citalopram is initiated. Taken together, we suggest that off-target drug interactions will impact significantly the neurogenic process, potentially altered by SSRIs.

We also show here that relatively “insignificant” differences in housing will also impact upon the ability of antidepressants to modulate neurogenesis. Here, we manipulated bedding demonstrating a robust induction of NPC proliferation in all groups that may have abrogated, or alternatively masked, theneed for antidepressant efficacy in our behavioural assessment. It is likely that due to this already heightened basal proliferative rate we were unable to see a further

increase in response to SSRI treatment in experiment 3 animals due to a ceiling effect. Such a ceiling effect has been previously suggested where the application of positive neurogenic stimuli in combination are not additive with respect to neurogenic outcomes as they are for some behavioural measures, this however remains a difficult concept test (Klaus et al., 2009). Interestingly, enrichment combined with escitalopram and fluoxetine treatment resulted in decreased survival. While the mechanism remains unclear this is consistent a prior report demonstrating SSRI mediates decreases in neurogenesis in a model of depression wherein there is increased basal neurogenesis, and further supports the conclusion that select SSRIs specifically target neurogenesis as a part of their mechanism of action (Petersen et al., 2008).

It should be noted that, in addition to the bedding manipulation, one other change was implemented during the experiment 3. The treatment process applied to the drinking water given to the mice changed significantly. Our first two cohorts of animals were given *ad libitum* access to regular city of Ottawa tap water while the latter were given HCl acidified, UV filtered, reverse osmosis treated water with a pH of 2.5-3. This is considered an industry standard, originally developed and implemented due to its bacteriostatic properties (Fox, 2007). Such a standard was adopted and applied despite early studies demonstrating that mice drank less and had slower weight gain, along with impacts on immune response, when given this water (Hall et al., 1980; Hermann et al., 1982). These findings suggest that it is not an innocuous manipulation although its effects on neurogenesis specifically remain unknown. For this

reason, we have elected to repeat experiment 2 and 3 with animals receiving the same acidified water in addition to bedding change before pursuing the hypothesis that an increase in neurogenesis is sufficient to elicit mild antidepressant activity.

Another important point of consideration which arises from our data lies in the disconnect between citalopram and escitalopram. Consistent with prior reports we demonstrated that chronic fluoxetine treatment increases hippocampal NPC proliferation in a non-depressed mouse (Santarelli et al., 2003). While published literature is minimal for both citalopram and escitalopram, our finding of no change and increased proliferation respectively are consistent prior reports in rats (Jaako-Movits et al., 2006; Jayatissa et al., 2006). The most likely explanation for the different effects of citalopram and escitaloparm on neurogenesis lies in subtle differences in the receptor and transporter-binding profiles for each drug. The primary target to which escitalopram, fluoxetine and citalopram bind with the greatest affinity is the serotonin reuptake transporter (SERT). Escitalopram, fluoxetine and citalopram, listed in decreasing order of affinity for the SERT, are the top three most selective SERT inhibitors on the market today (Owens et al., 2001). The primary difference between them lies with citalopram, which is a racemic mixture of the R and S enantiomer of the drug (Hyttel et al., 1992). It is the S enantiomer to which all positive activity is attributed with the R enantiomer having what is now known to be an inhibitory effect on the activity of the active isomer (Hyttel et al., 1992; Sanchez, 2006; Sanchez et al., 2004). The SERT has at least 2 citalopram binding sites, the

primary high affinity site and a second low affinity allosteric site. Binding of the S enantiomer to the primary site induces a conformational change reducing the reuptake of serotonin from the synaptic cleft (Sanchez, 2006). Binding of the R enantiomer to the allosteric site reduces the occupancy time of the S enantiomer at the primary site, while binding of a second S enantiomer increases occupancy at the primary site potentiating the reuptake inhibition (Sanchez, 2006). This difference is known to delay the onset of all of the molecular, electrophysiological and clinical outcomes of citalopram therapy when compared to escitalopram examined thus far (Dremencov et al., 2009; El Mansari et al., 2005; Sanchez, 2006; Sanchez et al., 2004). Thus, it is conceivable that the R-enantiomer might also delay the effects of the S enantiomer on neurogenic parameters measured in this study.

Each of these SSRIs also have what are considered secondary targets where they also display antagonist properties, albeit at very low levels, which are thought to contribute more to their side effect profile than their antidepressant action (Carrasco and Sandner, 2005; Palvimaki et al., 1999). Citalopram, and to a much lesser extent fluoxetine and escitalopram, have a greater affinity for the histamine H_1 receptor relative to other SSRIs. This activity contributes to the mild anti-histamine and sedating properties of these drugs; however, its potential impact on NPC proliferation is unclear (Carrasco and Sandner, 2005). Fluoxetine is unique among the SSRIs having an affinity for the $5HT_{2C}$ receptor (Carrasco and Sandner, 2005; Klempin et al., 2010; Millan et al., 1998; Palvimaki et al., 1999). Traditionally thought to contribute to the norepinephrine and

dopamine modulation that lead to activation and weight loss in response to fluoxetine treatment, recent evidence suggests that this may also contribute to its neurogenic effects (Klempin et al., 2010; Millan et al., 1998; Santarelli et al., 2003). Klempin demonstrated that it is the acute activation of the $5HT_{1A}$ receptors, seen immediately following SSRI treatment, that leads to increased proliferation in the SGZ but that acute inhibition of $5HT_2$ receptors will also enhance proliferative indices, likely of the type 2a NPC subpopulation. Acute activation of the $5HT_{2C}$ receptors specifically tempers this response by suppressing proliferation (Klempin et al., 2010). We therefore hypothesize that the combined pro-proliferative effects of acute $5HT_{1A}$ activation and $5HT_2$ antagonism unique to fluoxetine underlies the robust and reproducible activation of NPC proliferation seen with this drug, even in the absence of depression (Santarelli et al., 2003).

The effects of SSRIs on NPC survival are less well studied and more controversial. When researchers have reported the impact of SSRI treatment on the survival of NPC progeny it has been almost exclusively positive (Hodes et al., 2010; Klempin et al., 2010; Malberg et al., 2000; Marlatt et al., 2010; Santarelli et al., 2003). Both fluoxetine and citalopram have previously been reported to increase survival under specific conditions; escitalopram has not been examined (Jaako-Movits et al., 2006; Malberg et al., 2000). Most often survival analysis was completed using BrdU administration on the final days of SSRI treatment with animals then being sacrificed after 14-30 days of SSRI withdrawal. Our experimental design avoided the withdrawal aspect of these survival studies by

labeling the proliferating NPCs upon the initiation of treatment and assessing the number of surviving progeny 28 days later. Using this strategy we saw no difference in survival following SSRI treatment. To our knowledge, only one other group has used this approach demonstrating increased survival following chronic 10 mg/kg fluoxetine treatment (Klempin et al., 2010). An important difference between this study and ours is that they used female mice that were housed 5 to a cage generating both a very different hormonal and social experimental context which can significantly alter neurogenic effects independent of drug treatment (Dranovsky et al., 2011; Galea, 2008). We cannot however rule out that NPC survival is only affected in NPCs chronically exposed to SSRIs and not to NPCs specifying over the course of SSRI treatment. Our labeling paradigm does not address the necessity for prolonged activation of the $5HT_{2C}$ receptor, which occurs with chronic SSRI treatment previously shown to increase the number of late stage progenitor and neurons (Klempin et al., 2010). Labeling cells to assess survival following two weeks of SSRI treatment would clarify this. Alternatively, it is well known that stimuli that promote NPC proliferation, namely voluntary exercise, do not necessarily confer an overall increase in neurogenesis (Fabel et al., 2009; van Praag et al., 1999). Rather increased proliferation is thought to prime the system, increasing the number of progenitors available for selection, which leads to increases in neurogenesis only when there is a need for these cells (i.e., following injury) or a when a second survival-promoting stimuli is present. It is therefore possible that SSRIs act only at the level necessary to increase proliferation, priming the hippocampal environment for the possibility of

the increased production of new neurons, whereas it is the effects of depression itself which lead to a requirement for new neurons to replace those lost to the disease and therefore provides the second survival promoting stimuli needed to increase overall neurogenesis. Thus in the conditions studied here, where the mice have normal baseline neurogenesis in their non-depressed state (and in the absence of environmental enrichment), SSRIs are able to increase proliferation but there is no requirement for increased survival of their progeny, whereas in a model of depression that suppresses baseline proliferation, such as chronic mild stress, we might expect to see SSRI treatment increasing survival. Results with citalopram support this model where treated rats show significantly increased survival following olfactory bulbectomy, a model of depression, but not sham surgeries (Jaako-Movits et al., 2006).

In summary we show here that fluoxetine and escitalopram increase proliferation of adult hippocampal NPCs and that survival, at least of those progenitors proliferating upon the initiation of treatment in a healthy mouse, are unaffected. Furthermore, we highlight the exquisite sensitivity of the adult neurogenic program to both drug interactions and mild environmental changes suggesting that such elements should be paid further attention with regard to experimental outcomes.

5.9 - References

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Chapter 6 – Ablating Adult Hippocampal Progenitor Cells

6.1 Objective of the study

1) Optimize the experimental protocol to maximize ablation of adult hippocampal progenitor cells

6.2 Statement of author contributions

AP and SALB conceived of and designed the experiments. AP performed all the experiments with the exception of Figure 6.4 and 6.6, which were done by Mark Akins. AP performed all data analysis and generated the figures. This chapter describes research performed to inducibly ablate type 1 and 2 neural progenitor cells (NPC) or oligodendrocyte progenitor cells (OPC). This model was to be used in both Chapter 2 and Chapter 5. Because further optimization is required, it is presented separately as a final proof of principle study. The paper format of the chapter has been preserved for consistency; however, this work does not represent a manuscript in preparation.

6.3 Inducible toxigenic ablation of adult hippocampal neural and oligodendrocyte progenitor cells

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6.4 Summary

The hippocampus has long been recognized to play a critical role in learning and memory and more recent studies have also linked it to the regulation of emotions. The discovery of ongoing adult neurogenesis within this region was therefore hypothesized to be functionally correlated with these outcomes. While many researchers have used correlative findings to link these, proof of a true causal relationship requires that neurogenesis be eliminated and the corresponding behavioural outcome to be disrupted. Recently scientist have begun to use techniques which take advantage to the proliferative nature of NPCs within the otherwise postmitotic environment that is the brain and kill these cells using primarily gamma irradiation or cytotoxic agents. These techniques are however rife with concerns of potential off target effects leading us and others to investigate inducible toxigenic approaches to ablate neurogenesis in a more selective manner. Here we investigate the potential of the Cre-inducible expression of the simian diphtheria toxin receptor (iDTR mice) under to control of an inducible NestinCreER^{T2} or NG2CreER^{TM} followed by the systemic administration of diphtheria toxin to ablate NPCs or OPCs respectively.

6.5 Introduction

Ongoing neurogenesis in the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles of the adult mammalian brain has garnered great scientific interest (Ming and Song, 2011; Suh et al., 2009). This excitement stems not only from the potential to exploit this endogenous repair mechanism following injury or illness but also its apparent importance in the learning and memory processes which are impaired during the normal process of aging leading to a significant societal and medical burden. Until relatively recently most functions attributed to adult neurogenesis have been based on correlative findings (Gould et al., 1992; Gould et al., 1998; Kempermann and Gage, 1999; van Praag et al., 1999). Definitive attribution of specific aspects of brain function or outcomes of other experimental manipulations on the process of adult neurogenesis requires a method of suppressing neurogenesis without any other manipulation, which until the development of genetic toxigenic approaches had been lacking.

Several strategies to reduce or eliminate specific cells in the central nervous system (CNS) have been developed to date, many of which have been applied to the study of neurogenesis with varying degrees of success. The earliest of these focused on physical lesioning the desired brain region subsequently refined using laser-guided ablation (Huang and Moody, 1992; Kay et al., 2005). This approach lacks the specificity needed for the study of neurogenesis destroying both target cells and neighboring instructive cells with significant invasive manipulation. Chemical susceptibility of specific cell types,

such as the use to 6-hyroxydopamine to target cells expressing the catecholamine transporters necessary for its uptake, as well as antibodies conjugated to toxins, are yet another set of approaches that have been implemented for specific cellular ablation in the CNS (Palmiter, 2001). Cell specific ablation has also been achieved using transgenic animals to drive expression of a toxin or a pro-apoptotic gene, under the expression of a specific promoter, however this leads to ablation starting from embryogenesis and lacks the temporal specificity necessary to answer many functional questions (Breitman et al., 1987; Palmiter et al., 1987). By far the most commonly used approaches to ablate neurogenesis are anti-mitotic agents and gamma-irradiation (Bessa et al., 2009; Doetsch et al., 1999; Kim et al., 2011; Kokoeva et al., 2005). Both of these approaches preferentially affect actively proliferating cells, which in the brain are predominantly activated glia and the NPC populations. Both techniques can be applied at a desired developmental or experimental time point. Despite assurances that the treated mice remain healthy (and the inclusion of many control experiments suggesting that there are no significant off-target effects due to these manipulations), it cannot be overlooked that there is a significant potential to induce an inflammatory response and negatively affect other proliferative cell types within the body such as those in the immune system and the GI tract, along with the neurovasculature itself (Bessa et al., 2009; Santarelli et al., 2003; Singer et al., 2009). Furthermore, personal communication with researchers suggests that these off-target effects are far more common than is apparent in the literature.

Nevertheless, these methods, irradiation in particular, are the most robust and reproducible means of ablating neurogenesis available today and, as such, are often used as a second measure to confirm results even when more recent genetic approaches to neurogenesis ablation have also been used (Burghardt et al., 2012; Sahay et al., 2011; Saxe et al., 2007).

The need for temporal control of neurogenic manipulations, to eliminate the developmental element of constitutive transgenic mice, in addition to the quest to eliminate the larger systemic effects of anti-mitotic approaches has lead to the development of inducible toxigenic methods to ablate neurogenesis. These approaches fall into two main categories. First are those, which use cell-specific promoters to drive the expression of transgenes that encode enzymes such as thymidine kinase (TK) or nitroreductase, capable of catalyzing the conversion of a prodrug to a cytotoxic product (Borrelli et al., 1988; Clark et al., 1997; Singer et al., 2009). Ectopic expression renders the target neural cell sensitive to an otherwise “benign” prodrug. This approach benefits from being reversible upon cessation of drug treatment; however, these prodrugs are systemically toxic, necessitating direct intracerebroventricular (ICV) administration, furthermore long treatment periods are generally required to get significant ablation reducing temporal specificity. Moreover, in our hands and others, these mice often suffer from transgene silencing requiring a large number of breeders to obtain the necessary animals for experiments and the TK gene is not transmitted through the male germline due to TK expression in the testis

which kills spermatids (Behringer et al., 1988; Bush et al., 1998; Palmiter et al., 1987; Rhim et al., 1994).

A second approach has taken advantage of the resistance of mice to diphtheria toxin (DT) in combination with the Cre/loxP system. Mice have been engineered to express either the DTA subunit or the simian DT receptor (DTR, simian *Hbegf,* iDTR mouse line*)* following excision of a loxP flanked upstream stop cassette (Brockschnieder et al., 2006; Buch et al., 2005). Expression is then targeted to a specific population of cells using a cell type specific Cre recombinase and may be further temporally regulated using an inducible version of these Cre mouse lines or through the application of a constitutive cell type specific Cre recombinase following viral infection. The iDTR mouse line has the added advantage of two levels of temporal regulation; first induction of the expression of the DTR results in the tagging of a desired cell population and the timing of the administration of the DT allows for temporal specificity of the ablation step also, this is particularly useful in research examining the role of NPCs once them have matured (Arruda-Carvalho et al., 2011). Systemic administration of the DT results in its binding to the DTR, internalization into endosomes where the A chain then dissociates and crosses into the cytoplasm where it inactivates the elongation factor 2 through ADP-ribosylation arresting protein synthesis and resulting in apoptotic cell death (Palmiter, 2001). Apoptotic cell death is desirable in that it should not induce the inflammatory response elicited with other ablation techniques. The DT dependent cell death is highly sensitive, reportedly requiring only a very low dose of DT, as a single active

molecule of the DT-A subunit (DTA) is sufficient to kill a cell, yet high dosages remain non-toxic to mice and sustained administration does not lead to a significant immune response (Palmiter, 2001; Yamaizumi et al., 1978). These mouse lines have been successfully used to ablate several cell types, with no adverse effects, including four different cell populations in the CNS, demonstrating that the efficacy of this model and providing proof of principle of the blood brain barrier permeability for systemically administered DT (Arruda-Carvalho et al., 2011; Ghosh et al., 2011; Han et al., 2009; Hatori et al., 2008; Luque et al., 2011; Okuyama et al., 2010; Wrobel et al., 1990).

We have selected this later approach to investigate with respect to its ability to ablate adult hippocampal progenitor cells, for use in future functional studies, using the inducible NestinCreER^{T2} and NG2CreER^{TM} mouse lines mated with iDTR mice (Buch et al., 2005; Lagace et al., 2007; Zhu et al., 2011). Some lines were crossed to a mT/mG ROSA reporter background wherein all cells express a membrane bound tandem-dimer Tomato gene prior to Cre-mediated excision and a membrane-targeted green fluorescent protein (GFP) after excision or a yellow fluorescent protein (YFP)-ROSA background (Muzumdar et al., 2007; Srinivas et al., 2001). We have studied the ablation of NPCs and OPCs respectively in the adult hippocampus using these mouse lines.

6.6 Methods

6.6.1 Animals

iDTR mice – The iDTR mice were generated and described by the Waisman laboratory (Buch et al., 2005) and have been obtained from the Jackson laboratory (Maine, USA; stock #007900). These mice express the simian DTR (heparin-binding epidermal growth factor-like growth factor, *Hbegf*) under the control of the ubiquitous ROSA26 promoter, its expression is however dependent on the Cre mediated-removal of an upstream loxP flanked STOP cassette.

NesCreER^{T2} mice - The NesCreER^{T2} mice were generated and described by the Eisch laboratory (Lagace et al., 2007) and were a kind gift from Dr. Diane Lagace (University of Ottawa, Canada). These mice express a Cre recombinase/mutant estrogen receptor fusion protein (Indra et al., 1999) under the control of the nestin promoter and exons 1-3 of the nestin gene. They express the CreER^{T2} fusion protein which is cytoplasmically localized until tamoxifen treatment upon which it undergoes nuclear translocation.

NG2CreER^{TM} mice - The NG2CreER^{TM} mice were generated, described and generously donated by the Nishiyama laboratory (Zhu et al., 2011). These mice express a Cre recombinase/mutant estrogen receptor fusion protein (Indra et al., 1999) under the control of the NG2 promoter. They express the CreER^{TM} fusion protein which is cytoplasmically localized until tamoxifen treatment upon which it undergoes nuclear translocation.

ROSA-reporter mice – The ROSA-YFP and ROSA-mT/mG reporter mice lines have been previously described (Muzumdar et al., 2007; Srinivas et al., 2001) and were obtained from the Jackson laboratory (stock # 006148 and 007676 respectively). The ROSA-YFP mouse expresses an enhanced yellow fluorescent protein under the control of the ubiquitous ROSA26 promoter, its expression is however dependent on the Cre mediated-removal of an upstream loxP flanked STOP sequence. The Rosa-mT/mG mouse expresses a tandem-dimer red fluorescent tomato protein whose coding sequence is flanked by loxP sites, following Cre mediated excision of this tomato sequence the downstream GFP is expressed.

Mouse models – In our laboratory, breeding was completed to generate the following lines: NesCreER^{T2}xiDTR (Figure 6.1), NesCreER^{T2} x ROSA-YFP (Figure 6.2), NG2CreER^{TM}xROSA-mT/mG (Figure 6.3) and NG2CreER^{TM}xROSA- mT/mG xiDTR (Figure 6.4). Genotyping was performed upon weaning on genomic DNA as previously described (Buch et al., 2005; Lagace et al., 2007; Muzumdar et al., 2007; Zhu et al., 2011). Animals were kept on a 12 h light-dark cycle (light phase 0600 h -1800 h) with access to food and water *ad libitum*. Mice were individually housed starting at seven weeks of age, with both male and female mice being used experimentally. All experimental protocols were approved by the Animal Care Committee (ACC) of the University

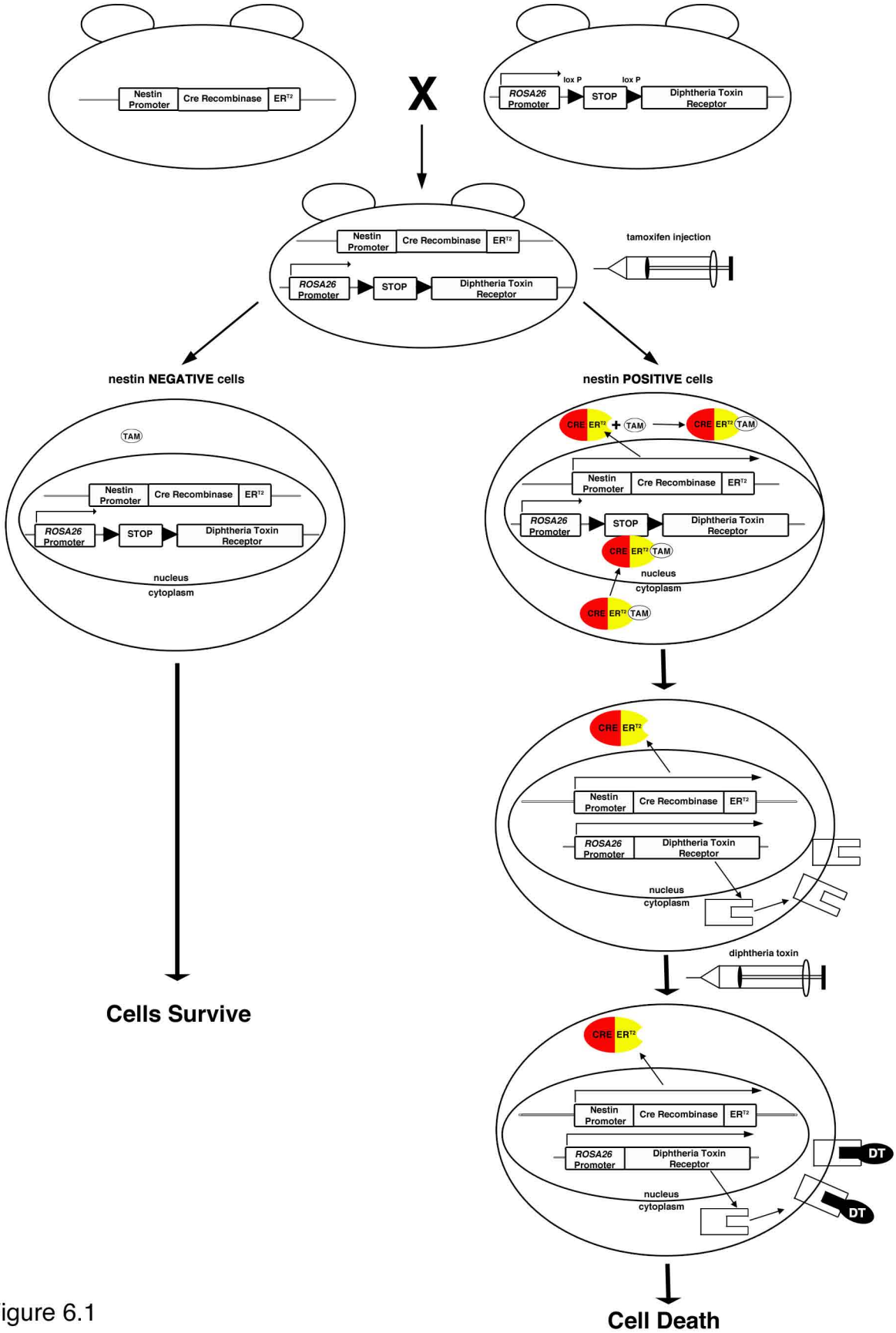

Nestin Promoter
Cre Recombinase
ER^{T2}
X
lox P
lox P
ROSA26 Promoter
STOP
Diphtheria Toxin Receptor
tamoxifen injection
nestin NEGATIVE cells
nestin POSITIVE cells
TAM
CRE
nucleus
cytoplasm
Cells Survive
diphtheria toxin
DT
Cell Death

Figure 6.1

Figure 6.1 - Schematic of the generation of the NesCreERT2 x iDTR mouse line and cellular ablation using it.

Briefly, following the mating of these two mouse lines cells which do not express nestin will not express the Cre recombinase and will be unaffected by subsequent manipulations. Cells that express nestin will express the $CreER^{T2}$ fusion protein. Following tamoxifen administration, the Cre fusion protein will translocate to the nucleus where it excises the STOP cassette upstream of the diphtheria toxin receptor (DTR) coding sequence, enabling expression. Once administered, the diphtheria toxin (DT) binds to its receptor now present on nestin-positive cells, and their progeny, leading to apoptotic death of these cells.

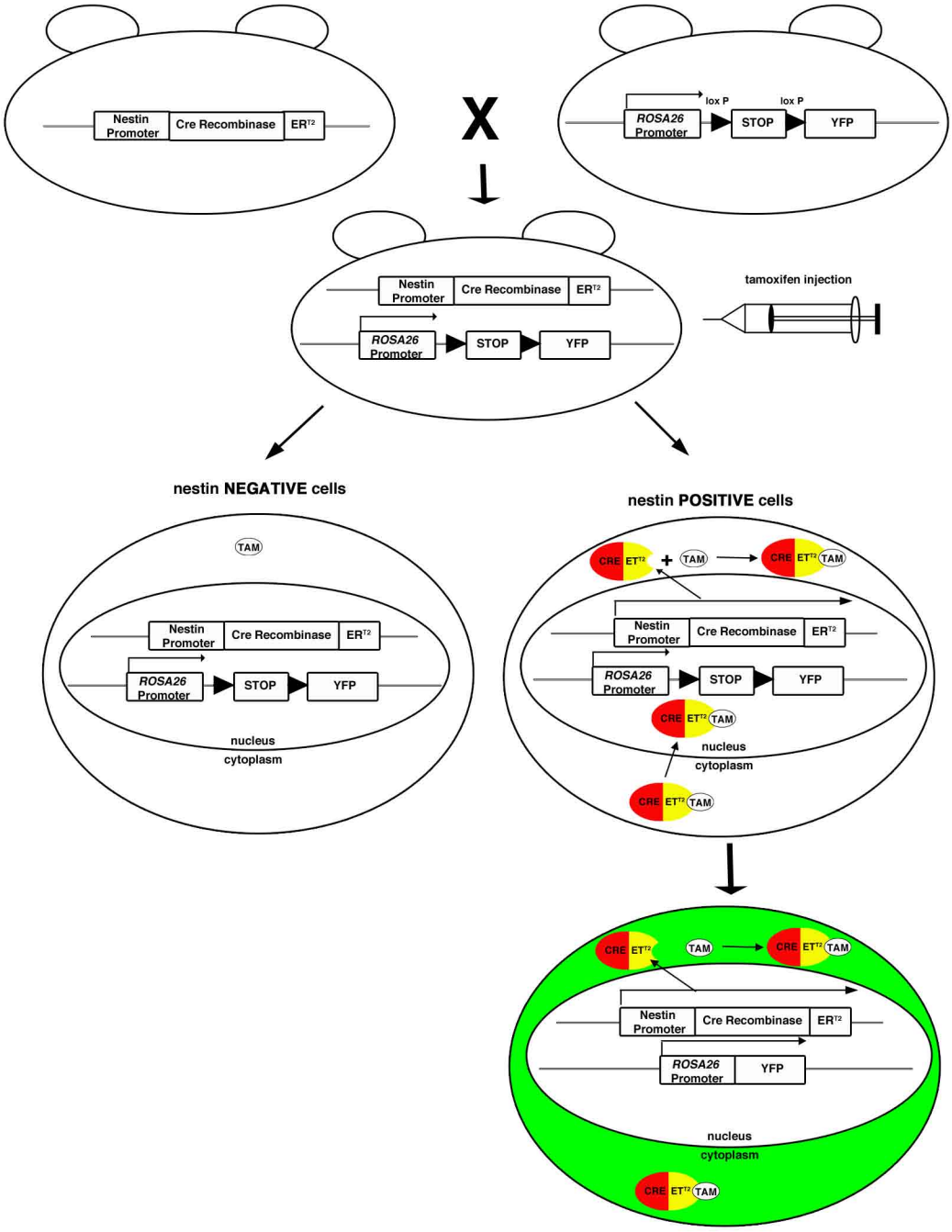

Nestin Promoter
Cre Recombinase
ER^{T2}
X
lox P
lox P
ROSA26 Promoter
STOP
YFP
tamoxifen injection
nestin NEGATIVE cells
nestin POSITIVE cells
TAM
CRE
ET^{T2}
nucleus
cytoplasm

Figure 6.2

Figure 6.2 - Schematic of the generation of the NesCreER^{T2} x ROSA-YFP mouse line.

Briefly, following the mating of these two mouse lines, cells which express nestin will express the CreER^{T2} fusion protein, following tamoxifen administration, the Cre will translocate to the nucleus where it will excise the loxP STOP cassette resulting in the expression of the downstream YFP.

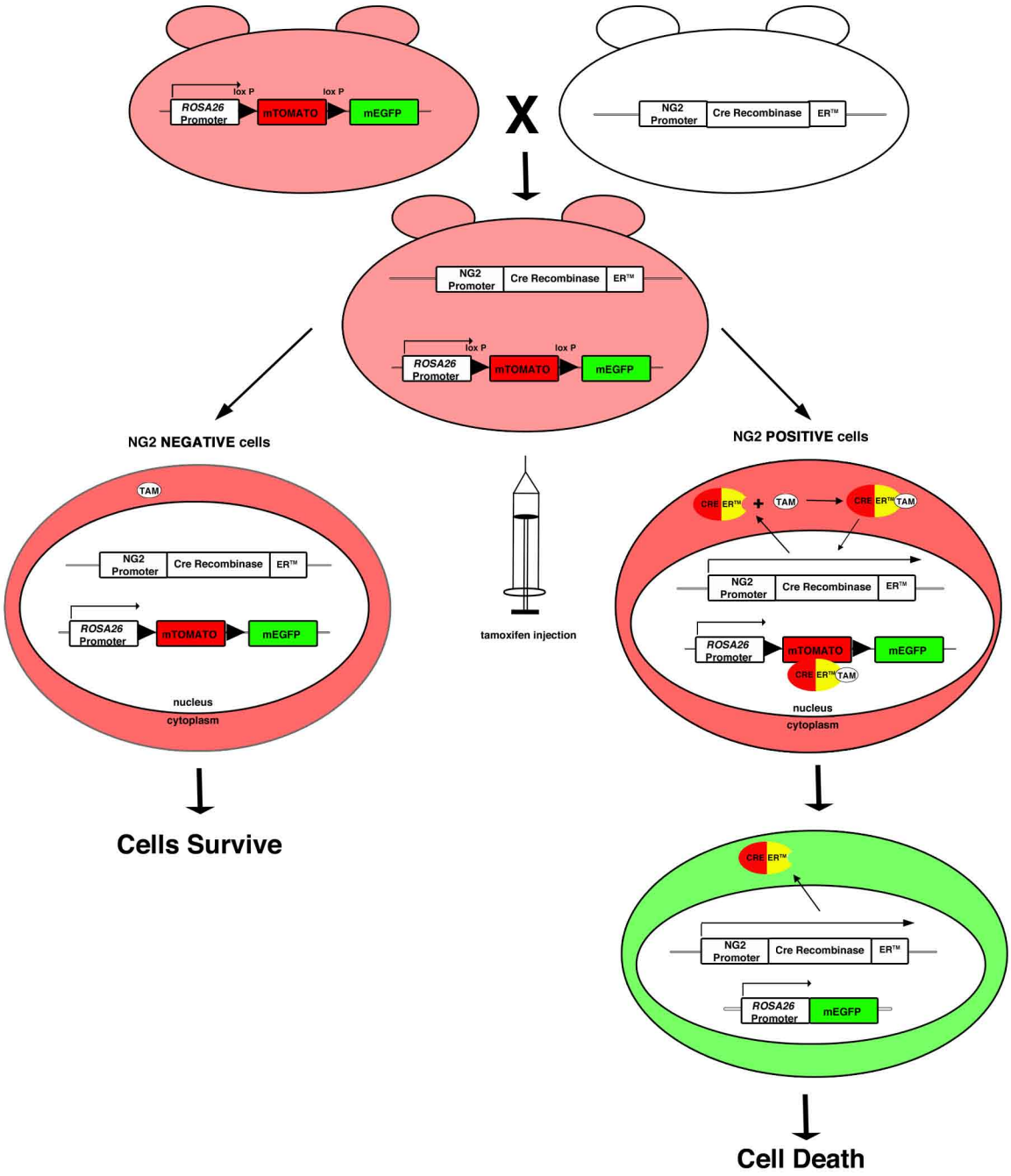

lox P
lox P
ROSA26 Promoter
mTOMATO
mEGFP
X
NG2 Promoter
Cre Recombinase
ER™
NG2 NEGATIVE cells
NG2 POSITIVE cells
TAM
CRE ER™
CRE ER™ TAM
nucleus
cytoplasm
tamoxifen injection
Cells Survive
Cell Death

Figure 6.3

Figure 6.3 – Schematic of the generation of NG2^{TM}xROSA-mT/mG mouse line.

Briefly, following the mating of these two mouse lines, cells which do not express NG2, will express the tomato protein and will not express the Cre recombinase. Cells that express NG2 will express the CreER^{TM} fusion protein. Following tamoxifen administration, the Cre will translocate to the nucleus where it excises the loxP flanking the tomato protein resulting in the expression of the downstream GFP.

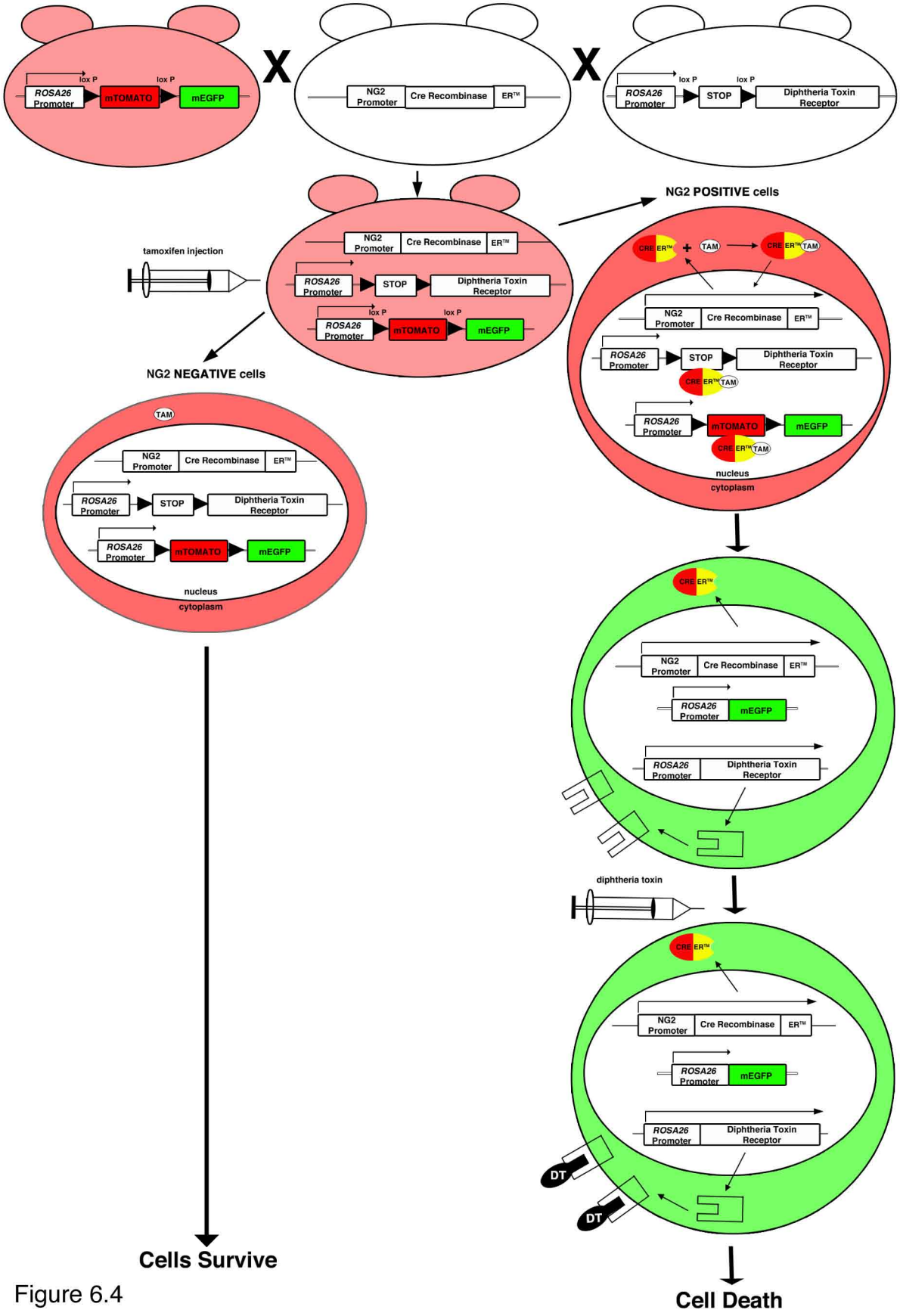

lox P
ROSA26 Promoter
mTOMATO
mEGFP
X
NG2 Promoter
Cre Recombinase
ER™
X
lox P
ROSA26 Promoter
STOP
Diphtheria Toxin Receptor
NG2 POSITIVE cells
tamoxifen injection
NG2 NEGATIVE cells
TAM
CRE ER™
nucleus
cytoplasm
diphtheria toxin
DT
Cells Survive
Cell Death

Figure 6.4

Figure 6.4 - Schematic of the generation of the NG2CreER^{TM} x ROSA-mT/mG x iDTR mouse line and cellular ablation using it.

Briefly, following the mating of these three mouse lines, cells which do not express NG2, will express the tomato protein and will not express the Cre recombinase and will be unaffected by subsequent manipulations. Cells that express NG2 will express the CreERT2 fusion protein. Following tamoxifen administration, the Cre will translocate to the nucleus where it excises the STOP cassette upstream of the diphtheria toxin receptor (DTR) coding sequence, leading to its expression. The Cre also excises the loxP flanked tomato protein resulting in the expression of the downstream GFP. Once administered, the diphtheria toxin (DT) binds to its receptor and leads to the apoptotic death of these cells.

of Ottawa according to guidelines set forth by the Canadian Council on Animal Care.

6.6.2 Drugs

Thymidine analogues - Mice received a single intraperitoneal (i.p.) injection of 5-choloro-2'-deoxyuridine (CldU; 42.5 mg/kg) (prepared to 17 mg/ml in 0.625 M Tris base in sterile water; Sigma, catalog #C6891, Saint Louis, MO) the day prior to diphtheria toxin administration and a single i.p. injection of 5-iodo-2'-deoxyuridine (IdU; 57.5 mg/kg) (prepared to 23 mg/ml in 0.895 M Tris base in sterile water; MP Biomedicals, catalog #100357, Solon, OH) 24 h prior to sacrifice. IdU and CldU concentrations were as described by Vega and Peterson (2005) with the exception that Tris base was used to enhance solubility in place of sterile saline and 5 N NaOH. The final pH of the solutions was pH 8.5 as recommended (Vega and Peterson, 2005). See figure 6.5 for a schematic of the delivery schedules (Nowakowski et al., 1989; Taupin, 2007).

Tamoxifen – Tamoxifen (Sigma, Saint Louis, MO, catalog # T5648) was dissolved in minimal ethanol and sunflower seed oil to a final concentration of 30 mg/ml as previously described (Lagace et al., 2007). Mice received either received a single 180 mg/kg i.p. injection for five days (900 mg/kg total, Figure 6.5, 6.6 and 6.7) or a single 120 mg/kg i.p. injection followed by two days with twice daily 90 mg/kg injections followed by a final single 120 mg/kg injection on a fourth day (600 mg/kg total, Figure 6.4).

Diphtheria toxin – DT (Sigma, catalog #D0564) was dissolved in phosphate buffered saline (PBS; 10 mM sodium phosphate and154 mM NaCl) to a final concentration of 5 µg/ml. Mice received a single i.p. injection of 16 µg/kg following the protocol of (Arruda-Carvalho et al., 2011; Han et al., 2009).

6.6.3 Tissue Preparation

Mice were sacrificed with euthanyl (Bimeda-MTC Animal Health Ins, Cambridge ON) prepared in sterile water to a final concentration of 65 mg/ml and transcardial perfusion with 10 mM PBS followed by 3.7% paraformaldehyde in 10 mM PBS. Brains were removed, post-fixed for 24 h, and cryoprotected in 20% sucrose. The right hemisphere of NesCreER^{T2}xiDTR brains were serially sectioned in the coronal plane at 30 µm on a freezing microtome (Lecia). Sections were collected in a 1 in 9 series, through the entire hippocampus (bregma -0.82mm to -4.30mm) and stored in 0.1M PBS + 0.1% sodium azide (NaN_3) at 4°C until processed for immunofluorescence. For all other brains, serial 10µm coronal cryosections were collected through the hippocampus and slide mounted immediately (Superfrost/Plus, Fisher, catalog # 12-550-15).

6.6.4 Immunofluorescence

i) Proliferation and Survival Analysis – An entire series of 30 µm hippocampal sections were mounted, rostral to caudal, on glass slides (Superfrost/Plus, Fisher) and air-dried. Sections underwent antigen retrieval in 0.01M citric acid,

pH 6.0 at 95°C for 15min, DNA denaturation in 2N HCl for 30 min at RT and neutralization in 0.1 M borate buffer, pH 8.5. IdU and CldU incorporation was detected using mouse anti-BrdU clone 44 (1:500; Becton Dickinson Immunocytochemistry Systems, catalog #347580, San Jose, CA), FITC conjugated rat anti-BrdU (1:100, AbD Serotec, catalog #OBT0030F, Raleigh, NC) respectively and Cy3 conjugated anti-mouse IgG (1:800; Jackson Immunoresearch, catalog #715-165-150).

ii) YFP and GFP Analysis – YFP and GFP were visualized in 10 µm serial sections using rabbit anti-GFP (1:2000, Abcam, Cambridge, MA, catalog #ab290) and FITC conjugated anti-rabbit IgG (1:100; Jackson Immunoresearch, catalog #711-096-152).

In all cases sections were incubated with primary antibodies overnight at 4˚C, washed with 10 mM PBS, incubated with secondary antibodies for 1 h at room temperature, and washed with PBS prior to being coverslipped in ProLong Gold or ProLong Gold with DAPI antifade reagent (Invitrogen, catalog #P-36930 and P-36935 respectively, Eugene, Oregon, USA). All antibodies were diluted in antibody buffer (3% bovine serum albumin, 0.3% Triton X-100 in 10 mM PBS).

6.6.5 Microscopic Analysis

i) NPC proliferation and Survival - Immunofluorescence was assessed using a DMXRA2 epifluorescent microscope (Leica Microsystems). Quantification of the

total number of IdU and CldU-labeled cells has been previously described (Lagace et al., 2010). The areas of interest assessed were of (a) the SGZ, defined as the region bordering the granule cell layer and the hilus (3 cell widths into each), (b) the hilus (c) the GCL of the DG and (d) the CA1 region of the hippocampus. All IdU or CldU positive cells within an area of interest, in right hippocampi of a complete series of sections through the rostro-caudal extent of the hippocampus were counted using the 40x objective while scanning in the z plane. Because labeled cells were not evenly distributed and are relatively rare, we did not use defined counting frames but rather counted all labeled cells within these defined areas (Guo et al., 2010; Thallmair et al., 2006). The total number of labeled cells in a given region per animal was calculated as the sum of the cell counts in all sampled sections multiplied by two to account for the two hemispheres and nine to account for the inter-section interval. Means and standard errors of the mean (SEM) are reported for each experimental group.

ii) YFP and GFP Expression – YFP and GFP immunofluorescence following antibody enhancement were visualized using a DMXRA2 epifluorescent microscope (Leica Microsystems).

6.7 – Results

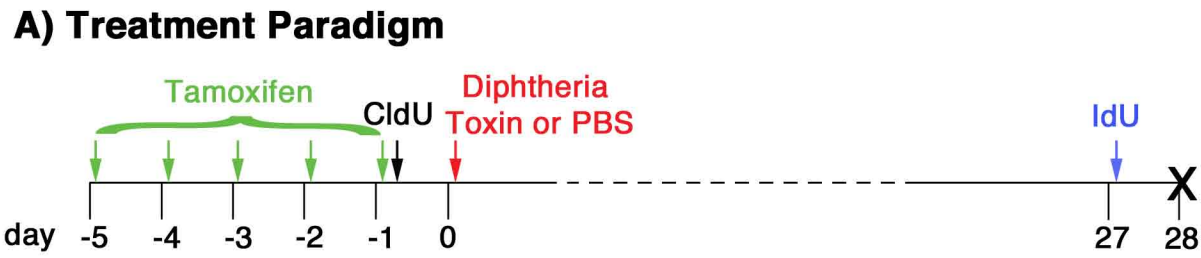
6.7.1 Adult hippocampal NPC ablation in NesCreER^{T2} x iDTR mice

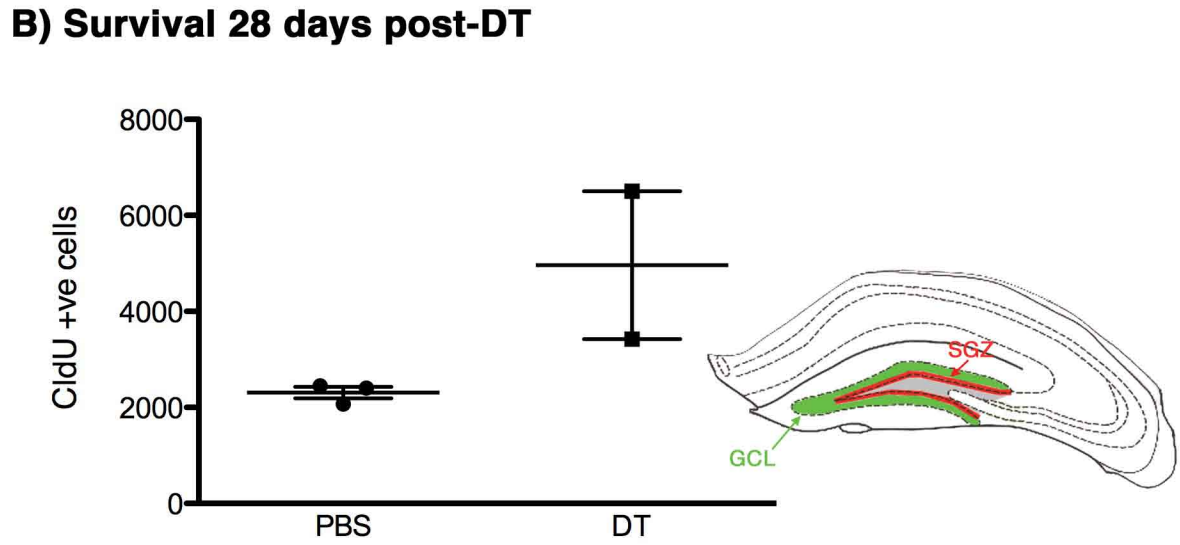
To investigate the ability of the iDTR mouse line to effectively ablate adult hippocampal NPCS we crossed the iDTR mouse line the NesCreER^{T2} mouse

line in which the expression of Cre recombinase is under the control of an NPC specific nestin promoter (Buch et al., 2005; Lagace et al., 2010). The resultant CreER^{T2} fusion protein is cytoplasmically localized until the administration of tamoxifen, which allow for its nuclear translocation and therefore excision of the STOP cassette upstream of the DTR coding sequence. All recombined nestin positive cells and their progeny from this time forward express the DTR and following DT administration should be ablated (Figure 6.1).

To test the efficacy of this system we administered tamoxifen using the protocol, previously optimized for the NesCreER^{T2} mice to achieve maximal recombination (Lagace et al., 2010). Prior to DT administration, proliferating cells were labeled with CldU. Mice were then administered DT or its vehicle, PBS. Residual proliferating cells were IdU labeled 24 h before sacrifice and mice were scarified 28 days following DT administration (Figure 6.5A). A full stereological analysis of the surviving CldU positive (Figure 6.5B) and proliferating IdU positive (Figure 6.5C) populations in the hippocampal dentate gyrus revealed no difference between PBS and DT treated animals. This suggested that no appreciable ablation had occurred.

Lack of ablation could be explained by an insufficient concentration of DT or poor recombination and therefore DTR expression. Given that a single active DTA molecule is sufficient to kill a DTR positive cell, poor recombination seemed more likely (Yamaizumi et al., 1978). To assess the recombination efficiency of this tamoxifen administration in the NesCreER^{T2} mice, they were crossed onto a reporter line in which YFP is expressed under the control of the ubiquitous


A) Treatment Paradigm
Tamoxifen
CldU
Diphtheria
Toxin or PBS
IdU
day
-5
-4
-3
-2
-1
0
27
28


B) Survival 28 days post-DT
CldU +ve cells
8000
6000
4000
2000
0
PBS
DT
SGZ
GCL

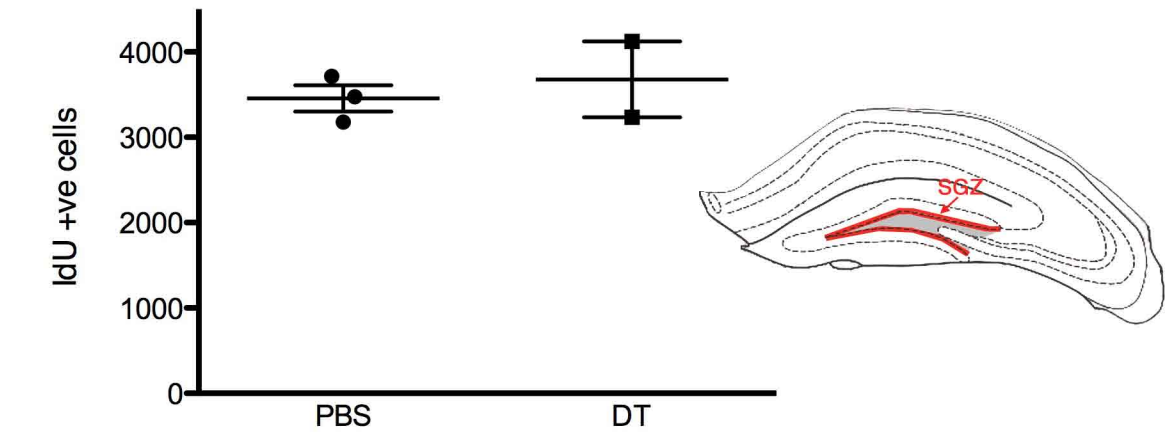

C) Proliferation 28 days post-DT
IdU +ve cells
4000
3000
2000
1000
0
PBS
DT
SGZ

Figure 6.5

Figure 6.5 - Optimizing ablation of neurogenesis using the NesCreER^{T2} x iDTR mice.

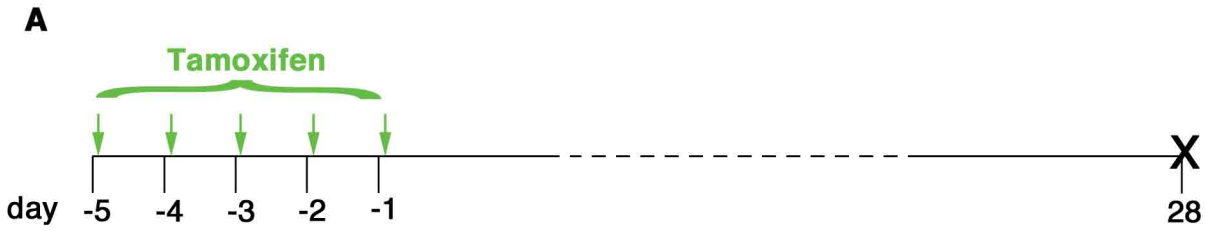
(A) Schematic of the treatment protocol used. Tamoxifen injections (180 mg/kg/d) are indicated by green arrows, CldU (42.5 mg/kg) by black arrows, IdU (57.5 mg/kg) blue arrows, diphtheria toxin (16 ug/kg) or vehicle (10mM PBS) red arrows and the time of sacrifice by the black X. Surviving (B) and proliferating (C) cell number was unaffected by DT treatment. Quantification of surviving CldU labeled cells was completed in the granule cell layer (GCL) and subgranular zone (SGZ) of the dentate gyrus and proliferating cells in the SGZ only as shown in the schematics of the hippocampus.

ROSA26 promoter, following the Cre mediated recombination of an upstream STOP sequence (Figure 6.2). Mice were sacrificed 28 days following tamoxifen administration (Figure 6.6A) and immunofluorescence revealed very few YFP positive recombined cells (Figure 6.6B).

6.7.2 Adult hippocampal OPC ablation in NG2CreER^{TM} x ROSA-mT/mG x iDTR mice

In order to examine the ablation of OPCs we used the NG2CreER^{TM} mouse line (Zhu et al., 2011). First to characterize the tamoxifen induced NG2CreER^{TM} mediated recombination, these mice were crossed onto a tomato/GFP reporter mouse line (Figure 6.4). The ROSA-mT/mG mouse expresses a red fluorescent tomato protein whose coding sequence is flanked by loxP sites, following Cre mediated excision of this tomato sequence the downstream GFP is expressed. Using a reduced tamoxifen administration protocol (600 mg/kg vs. 900 mg/kg total, Figure 6.7A) and sacrifice 24 hours post-tamoxifen treatment, a large number of GFP positive cells were visible (Figure 6.7B) indicative of good recombination efficiency.

In order to test the ability of the iDTR mouse line to effectively ablate adult OPCs we crossed the NG2CreER^{TM} x ROSA-mT/mG mice with the iDTR mouse line. In these mice treatment with tamoxifen allows Cre mediated recombination in NG2 positive cells which results to the expression of both the DTR and a switch from the ubiquitously expressed tomato protein to GFP (Figure 6.4). Following the standard 900 mg/kg total tamoxifen administration, mice were either treated with DT or PBS and sacrificed 28 days later (Figure 6.8A).



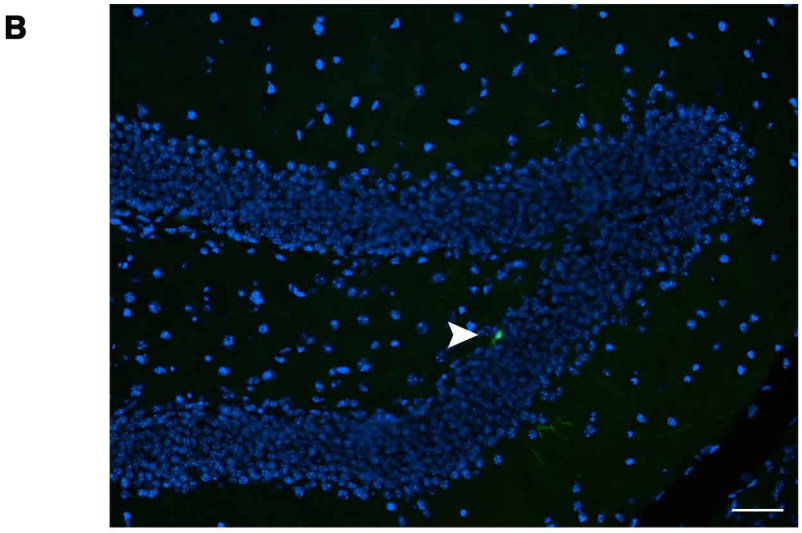
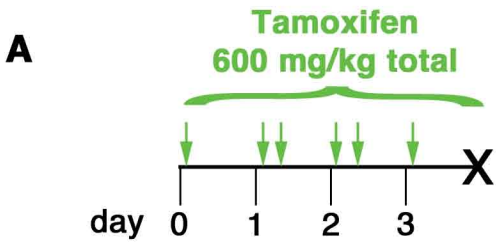


Figure 6.6

Figure 6.6 - NesCreER^{T2} x ROSA-YFP mice have poor recombination efficiency following tamoxifen treatment.

(A) Schematic of the treatment protocol used. Tamoxifen injections (180mg/kg/d) are indicated by green arrows and the time of sacrifice by the black X. (B) Micrograph taken at 20x magnification of immunofluorescence for YFP and DAPI in the dentate gyrus. A single YFP positive recombined cell (arrow head) can be seen. Scale bar 50 μm.



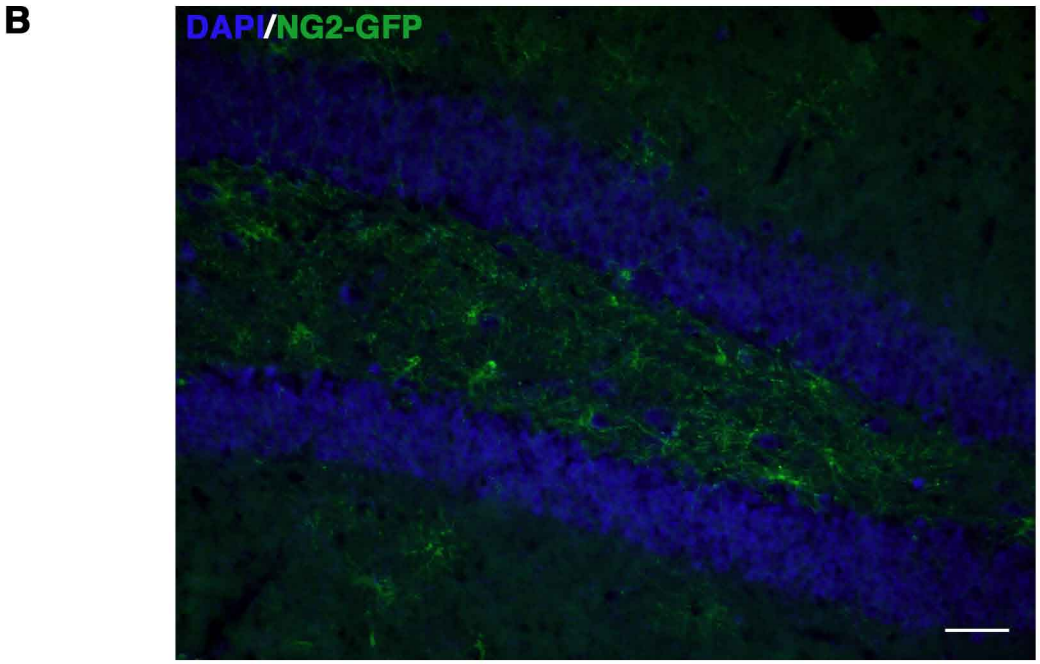


Figure 6.7

Figure 6.7 - NG2CreER^{TM} x ROSA- mT/mG mice have excellent recombination efficiency following tamoxifen treatment.

(A) Schematic of the treatment protocol used. Tamoxifen injections are indicated by green arrows and the time of sacrifice by the black X. (B) Micrograph taken at 20x magnification of immunofluorescence for GFP and DAPI in the dentate gyrus. Multiple GFP positive recombined cells can be seen. Scale bar 50 μm.

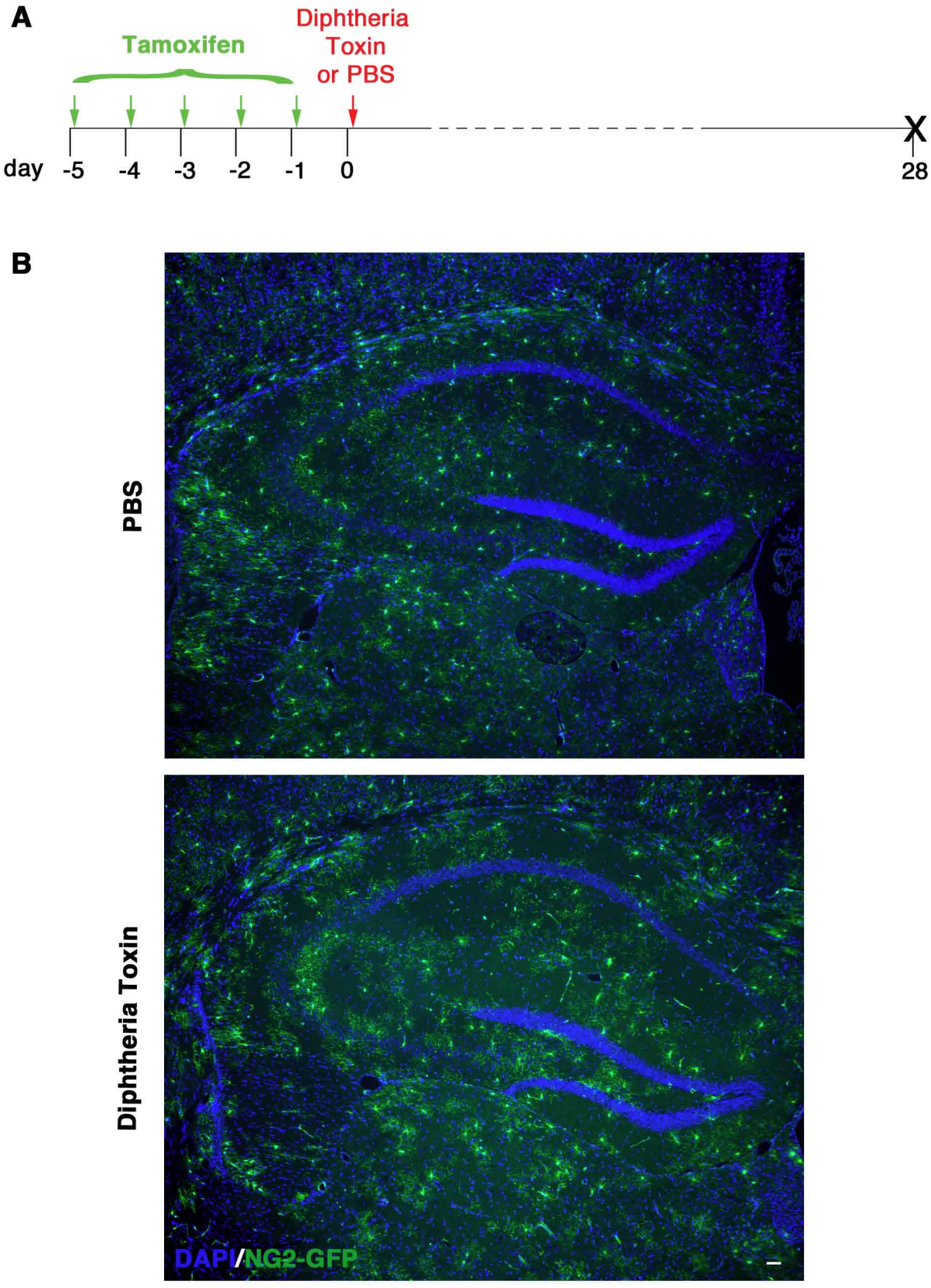

A
Tamoxifen
Diphtheria
Toxin
or PBS
day
-5
-4
-3
-2
-1
0
X
28
B
PBS
Diphtheria Toxin
DAPI/NG2-GFP

Figure 6.8

Figure 6.8 - Optimizing cellular ablation in the NG2CreER^{TM} x ROSA-mT/mG x iDTR mice.

(A) Schematic of the treatment protocol used. Tamoxifen injections (180 mg/kg/d) are indicated by green arrows, diphtheria toxin (16 ug/kg) or vehicle (10mM PBS) by red arrows and the time of sacrifice by the black X. (B) Micrograph taken at 5x magnification of immunofluorescence for GFP and DAPI of the hippocampus. Many GFP positive recombined cells can be seen in both the vehicle and DT treated sections indicating that despite excellent recombination there has been no appreciable cellular ablation. Scale bar 50μm.

Immunofluorescence for GFP was used to again confirm recombination efficiency in the PBS treated mice and compared with the persistence of GFP positive cells in the DT treated animals to assess ablation. GFP positive cells were not quantified but qualitative assessment reveals significant recombination having occurred but no appreciable cellular ablation following DT treatment (Figure 6.8B). Taken together, these results highlight problems with both the NesCreER^{T2} line and the schedule of DT administration employed that must be addressed before this approach can be successfully used. Our lab is closely collaborating with that of Dr Diane Lagace to address and eliminate these problems. To this end, we have obtained another iDTR breeding pair from Jackson and placed them on our NG2CreER^{TM}x ROSA-mT/mG mice. The experiments described above were repeated without any improvement in DTR-mediated ablation. These data highlight the need to focus on the schedule of DT administration.

6.8 - Discussion

We report the successful breeding of the NesCreER^{T2}xiDTR and NG2CreER^{TM}x ROSA-mT/mG xiDTR mouse line. Using the previously optimized tamoxifen administration protocol our results suggest that there is very little recombination occurring in the NesCreER^{T2}xiDTR mice and that this contributes to the lack of ablation observed following DT administration. While we observed very good recombination in the NG2ER^{TM}x ROSA-mT/mG xiDTR mice, we were unable to detect cell loss following DT administration.

Low level ablation using a genetic ablation strategy is not surprising. The field has almost universally struggled to achieve the of degree ablation seen with irradiation and cytotoxic agents. Irradiation generally boasts a 90% or greater ablation of the BrdU positive proliferating cells in the SGZ (Kim et al., 2011; Santarelli et al., 2003). Of the genetic approaches in use today, those which use cell type specific promoters to drive the expression of transgenes that encode enzymes such as thymidine kinase (TK), allowing for the conversion of a prodrug to a cytotoxic product, seemingly have the greatest success in ablating NPCs (Borrelli et al., 1988; Clark et al., 1997; Singer et al., 2009). On average these mouse lines are able to generate a 90% ablation of late doublecortin positive progenitors, this however take 3-6 weeks of drug administration and even when a nestin specific promoter is used to drive the TK there is very poor ablation of the early nestin positive progenitors which is what allows for the NPC population to return to normal following cessation of drug treatment (Blaiss et al., 2011; Yu et al., 2008). Inducible approaches thus far have had even poorer results. Imayoshi's NesCreER^{T2} line and the Lagace NesCreER^{T2} line crossed onto separate DTA mouse lines have both resulted in less that 30% ablation following tamoxifen administration (Imayoshi et al., 2008).

Our poor ablation with the NesCreER^{T2}xiDTR line appears to be a function of the lack of recombination and therefore lack of DTR expression that is occurring. This was unexpected as the mouse line was obtained from its founder and recombination was induced using the previously established protocol that was highly effective (Lagace et al., 2007). This is not however unique to our

laboratory, all groups within the University of Ottawa using this mouse line since its move here from the University of Texas Southwestern, including Dr.Lagace's lab have noted the same results but have been unable to identify the root cause (D.Lagace, S.Bennett, R.Slack, personal communication). In an effort to circumvent this problem, the Lagace and Bennett laboratories have obtained the Imayoshi NesCreER^{T2} mouse line for future experiments.

Using a different mouse line will not necessarily be an easy solution. When these mice were mated to an inducible NSE-DTA line, using the optimized tamoxifen administration protocol they were only able to generate a 15% ablation of BrdU+NeuN+ neurons 12 weeks post tamoxifen treatment (Imayoshi et al., 2008). This mouse line has also been mated to the iDTR mouse line by another research group to study the effect of ablating mature neurons following behavioural training, this lab however had to induce the DTR expression over the course of 3 months to have sufficient numbers of labeled progeny to effect a change following ablation (Arruda-Carvalho et al., 2011). The difficulties with inducing recombination in these cells likely relates to two factors: first the poor blood brain barrier permeability to tamoxifen and second, the rapid rate at which proliferating progenitors pass through the nestin positive stage (Kempermann et al., 2004; Mongiat and Schinder, 2011). Clearly there are technical difficulties that need to be overcome with regards to the induction of recombination within nestin positive progenitor cells if there approaches are to be successful.

Recombination within NG2 positive cells does not however appear to be the limiting step in ablating OPCs in the NG2ER^{TM}x ROSA-mT/mG xiDTR mouse

line. However, despite using a previously published does of DT we did not see any notable ablation (Han et al., 2009). We recognize that, despite prior successes with this dosage of DT, and the need for only a single DTA molecule to kill a cell, it is the lowest published dosage used in the iDTR mice (Buch et al., 2005; Hatori et al., 2008; Yamaizumi et al., 1978). Therefore we cannot rule out the possibility that a higher dosage or more sustained treatment with DT is necessary to kill this population of cells. To address this problem, we are currently pursuing chronic administration via the osmotic minipump paradigm described in chapter 3. In keeping, with the theme of this thesis, it was decided to include these negative results with the intent of perhaps assisting other researchers struggling with similar problems (i.e., reporting negative data is also a valuable exercise in scientific endeavor).

6.9 – References

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Chapter 7 – General Discussion

The existence of ongoing adult hippocampal neurogenesis is now a well-accepted phenomenon. While researchers continue to debate the properties of individual stem and progenitor cell populations, their regulation, and how they contribute to functional outcomes it has become clear that this process does, in fact, contribute functionally to hippocampal-dependent learning, memory, and mood control (Bonaguidi et al., 2012; Deng et al., 2009; Sahay et al., 2011; Samuels and Hen, 2011; Snyder et al., 2011). It is therefore of great interest to identify methods of manipulating these endogenous properties for therapeutic purposes (Petrik et al., 2012). Neurogenesis is a dynamically regulated process where the proper progression through distinct developmental stages is required for the generation of terminally differentiated and functionally integrated mature neurons. The neurogenic niche, and communication within it, are recognized as key players in this regulatory process, furthermore numerous exogenous pharmacological and environmental cues have now been identified that alter hippocampal neurogenesis (Fabel et al., 2009; Kodama et al., 2004; Miller and Gauthier-Fisher, 2009; Petrik et al., 2012; Santarelli et al., 2003). This thesis focused on identifying and understanding the contribution of novel endogenous and exogenous, yet clinically relevant, regulators of adult hippocampal neurogenesis. In this general discussion, I seek to place the relevance of these findings within the larger context of the literature and prevailing issues related to the reporting and replication of neurogenic studies.

7.1 Endogenous Regulation by Connexin Mediated Communication and Niche Cues

As the principal requirement for ongoing adult neurogenesis under basal conditions, the neurogenic niche is a central focus when investigating the regulation of this process. This thesis adds to the existing body of knowledge pertaining to the regulation of adult neurogenesis by demonstrating how instructive cells communicate with the immature NPCs located in the hippocampus, how the surrounding extracellular matrix contributes to the regulation of adult hippocampal neurogenesis, and how the interplay between these two events is modulated over the course of specification (both *in vitro* and *in vivo*) (Figure 7.1).

Until recently, the role of connexin-mediated communication in the regulation of adult neurogenesis remained unknown despite robust evidence for its importance in brain development (Bruzzone and Dermietzel, 2006; Sutor and Hagerty, 2005). In chapter 2 I demonstrate that connexin mRNA and protein, specifically Cx43 and Cx45, are expressed by cultured postnatal hippocampal neural progenitor cells. Furthermore, I show that spontaneous differentiation results in a restricted pattern of connexin expression including a downregulation of Cx43 protein levels and complete loss of Cx45 (Figure 7.1A). This capacity for connexin-mediated communication is fine-tuned by cross-talk with other juxtacrine communication systems including the integrin-ECM system as I observed downregulation only when cultures are allowed to spontaneously

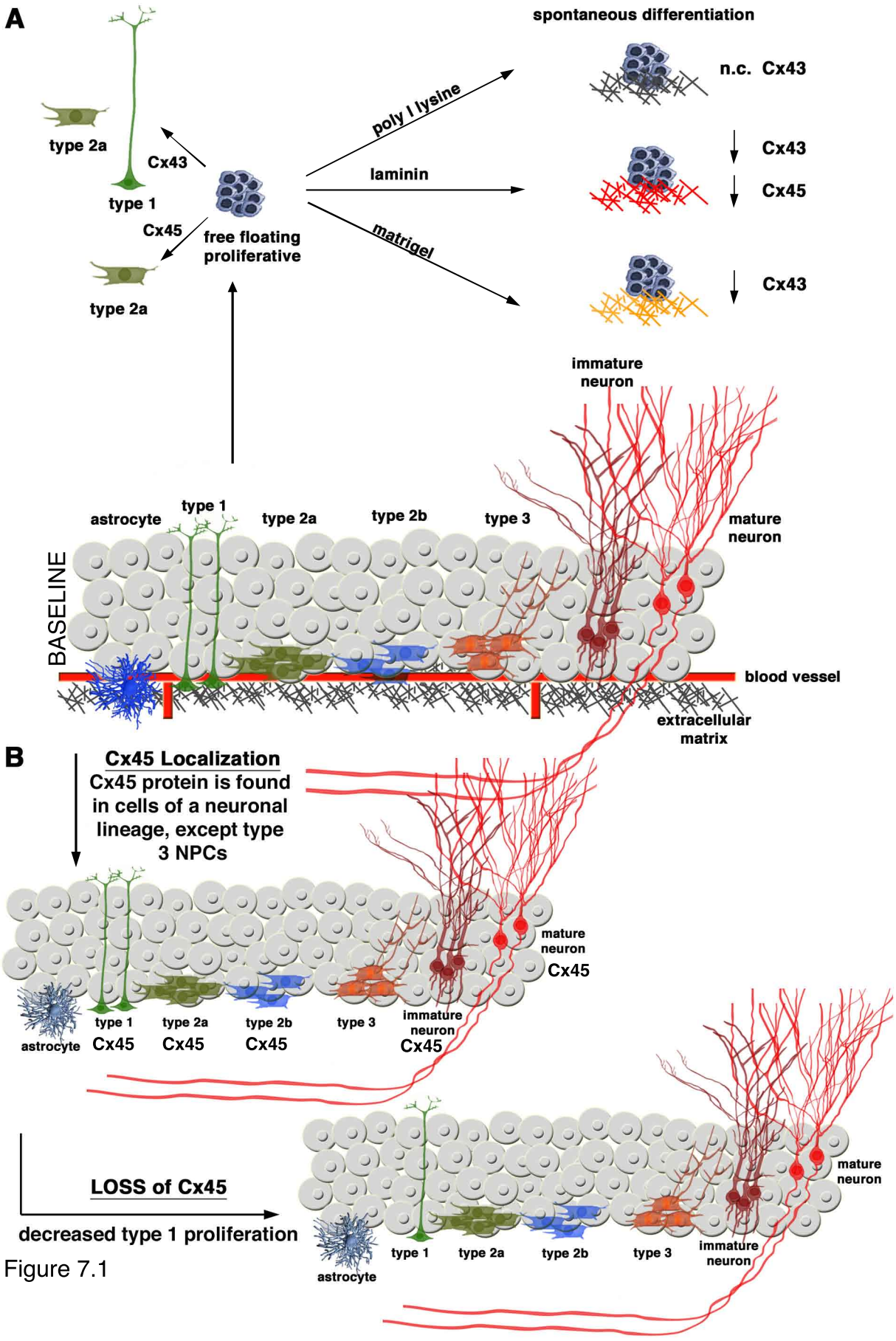

A
spontaneous differentiation
n.c. Cx43
Cx43
Cx45
Cx43
poly l lysine
laminin
matrigel
type 2a
Cx43
type 1
Cx45
free floating
proliferative
type 2a
immature
neuron
astrocyte
type 1
type 2a
type 2b
type 3
mature
neuron
BASELINE
blood vessel
extracellular
matrix
B
Cx45 Localization
Cx45 protein is found
in cells of a neuronal
lineage, except type
3 NPCs
mature
neuron
Cx45
astrocyte
type 1
Cx45
type 2a
Cx45
type 2b
Cx45
type 3
immature
neuron
Cx45
LOSS of Cx45
decreased type 1 proliferation
mature
neuron
astrocyte
type 1
type 2a
type 2b
type 3
immature
neuron

Figure 7.1

Figure 7.1 - Summary of the endogenous regulators of adult hippocampal neurogenesis studied in this thesis.

A) Free floating proliferative post-natal neurospheres express Cx43 in type 1 and 2a cells along with Cx45 in the type 2a cells. Plating neurospheres on extracellular matrix components induces spontaneous differentiation interaction. Plating on laminin containing ECM specifically reduces the protein level of both Cx43 and 45. B) Cx45 protein is localized to all cells of a neuronal lineage with the exception of type 3, doublecortin positive cells, in the adult hippocampal dentate gyrus. Loss of Cx45 in vivo decreases proliferation of the type 1 NPCs but does not affect fate specification or survival.

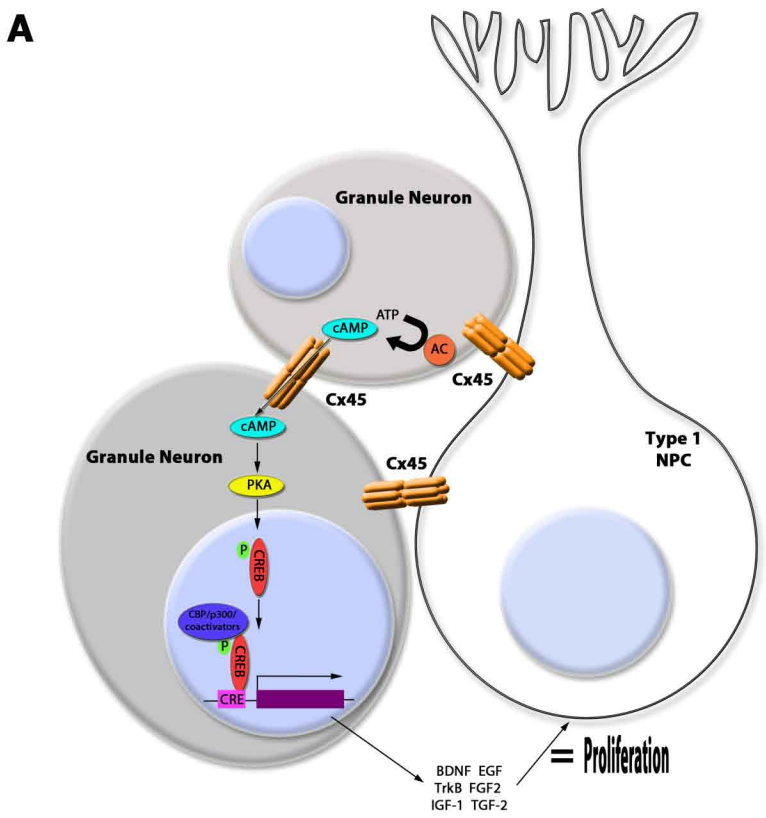
specify on a laminin and not on poly-l-lysine. I suggest that this alteration in the structural subunits of gap junction channels expressed by the progenitor cells represents one means by which the extracellular environment within the neurogenic niche can regulate the neurogenic process. Specifically it directs how NPCs can synchronize communication with each other and/or neighboring instructive cells, that until now had not been appreciated in the adult neurogenic process.

Chapter 3 sought to extend our *in vitro* findings with early postnatal NPCs to the intact adult brain examining the role of Cx45 in regulating neurogenesis within the adult hippocampus. Until this thesis, the localization of Cx45 in the adult hippocampus had only been described in the CA fields (Maxeiner et al., 2003; von Maltzahn et al., 2009; Zlomuzica et al., 2010). I demonstrate here robust expression of Cx45 mRNA throughout the granule cell layer (GCL) and subgranular zone (SGZ) in addition to cells of a neuronal morphology within the hilus and molecular layer of the hippocampus. Similarly, Cx45 protein was identified in these areas and was further found to be dynamically expressed over the course of adult neurogenesis localizing to both primordial NSCs and their immediate NPC progeny, including type 2a and type 2b cells but not type 3 neuroblasts (Figure 7.1B).

The lack of Cx45 protein within type 3 cells was unexpected but not unprecedented. We have previously shown that the neuronal channel forming protein pannexin 2 (Panx 2) is similarly found in early NPCs, lost in more committed progenitors and re-expressed in post-mitotic mature neuronal progeny

(Swayne et al., 2010). The reason for this pattern of expression remains unclear but I suggest that regulation of Cx45 (and possibly Panx2) may be similar to that of the glucocorticoid receptors which are thought to be downregulated at this developmental stage to allow for undisturbed selection of those NPCs which are to progress to the post-mitotic stage of maturation (Garcia et al., 2004).

With respect to the neurogenic process, I also show in chapter 3 that Cx45 is necessary for normal basal proliferation of adult hippocampal NPCs and specifically the type 1 nestin/glial fibrillary acidic protein (GFAP) double positive population. Proliferation is reduced but not abrogated in type 1 progenitors in the absence of Cx45. Despite the significant loss of proliferating progenitors, these mice develop a reduced but normal number of neurons and glia relative to the proliferating population suggesting that Cx45 does not play a significant role in the specification and survival of the NPCs and their progeny (Figure 7.1B). I hypothesize that the observed effects in our loss of function system stem from the requirement for Cx45 channels to allow for normal cAMP signaling. cAMP is preferentially passed through Cx45 containing gap junctions, and is known to be required for the expression of pro-proliferative growth factors within granule neurons and therefore the normal proliferation of hippocampal progenitor cells (Figure 7.2) (Bedner et al., 2006; Nakagawa et al., 2002). However, we cannot, at this time, rule out other direct signaling by second messengers passed between neurons and Cx45 expressing progenitor or between Cx45 expressing progenitors themselves. These are significant findings as they represent the first description of Cx45 expression within the hippocampal DG, implication of a


A
Granule Neuron
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Type 1
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CBP/p300/
coactivators
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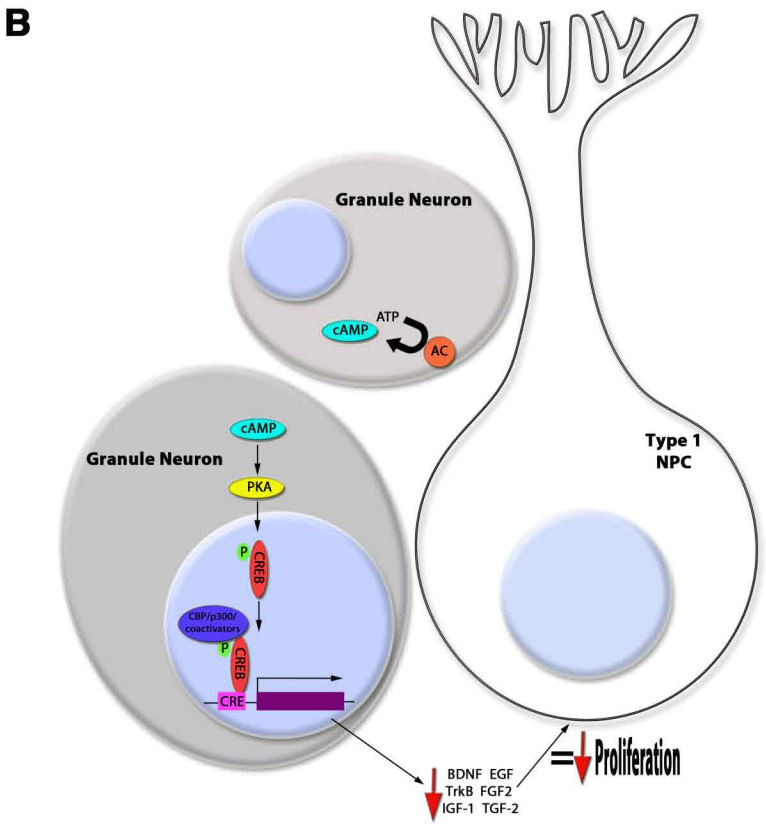
CREB
CRE
BDNF EGF
TrkB FGF2
IGF-1 TGF-2
= Proliferation


B
Granule Neuron
ATP
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AC
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Granule Neuron
Type 1
NPC
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CREB
CBP/p300/
coactivators
P
CREB
CRE
BDNF EGF
TrkB FGF2
IGF-1 TGF-2
= Proliferation

Figure 7.2

Figure 7.2 - Proposed model for the role of Cx45 in adult hippocampal neurogenesis.

It is known that cAMP dependent phosphorylation of CREB within the granule cell layer (GCL) of the hippocampus is required for the production of pro-proliferative growth factors and their receptors which are in turn necessary for normal NPC proliferation. We hypothesize that under normal conditions (A) cAMP levels are mediated, at least in part, by homotypic Cx45 gap junctions between granule neurons and that in the absence of Cx45 (B) this cAMP signaling is disrupted, reducing the production of the pro-proliferative molecules leading to a reduction in the proliferation of NPCs in the adult hippocampus.

neuronal connexin in the adult neurogenic program, and identification of novel modulator of type 1 NPC proliferation.

When placed in the context of the existing literature, my findings build upon work of Bittman, Elias and Kriegman, among others, wherein a role for altering the repertoire of connexins has been shown to regulate proliferation and migration of NPCs in developing brain. These researchers have established a role for gap junctional coupling between radial glial cells and neuronal precursors, but not immature neurons, for the synchronization and regulation of proliferation, as well as the interaction of radial glial cells and immature neurons through gap junction plaques to direct migration via their adhesive properties in the developing ventricular zone and neocortex (Bittman et al., 1997; Bittman and LoTurco, 1999; Elias et al., 2007). Kunze and colleagues have recently demonstrated through dye coupling that radial glia in the adult hippocampus are also coupled through gap junctions and while the distance is greatly reduced, immature neurons in the adult hippocampus migrate from the SGZ to the inner third to half of the GCL suggesting that both roles for GJIC could also be conserved in the adult context (Kunze et al., 2009; Ming and Song, 2011).

In this thesis, I have demonstrated a pattern of connexin expression, which is dynamically regulated across the adult neurogenic process both *in vitro* and *in vivo*. I argue that this occurs to allow for the dynamic coupling and uncoupling between specific NPCs and their surrounding instructive cells within the neurogenic niche necessary for both these proliferation and migration events to occur in the adult brain (Imbeault et al., 2009). Within the adult brain,

connexin expression is restricted relative to the embryo, particularly with respect to the neuronal connexins where Cx45 is the only neuronal connexin expressed *in vivo* in the adult hippocampal dentate gyrus and therefore has the potential to play a crucial role in the development of new neurons (Condorelli et al., 2000*)*. I suggest that the two distinct periods of expression I have described here for Cx45 expression allow for the segregation of the proliferative aspects from the migratory and/or instructive role of Cx45 in the neurogenic process. Within the developing brain dynamic coupling and uncoupling through Cx26 and 43, independently of one another, is necessary for proliferation and more specifically S phase entry (Bittman et al., 1997). Within the adult hippocampus I have shown that type 1, 2a and 2b, proliferative NPC types, express Cx45 and that the loss of Cx45 results in reduced proliferation. Bittman and colleagues have shown that as NPCs in the developing brain progress to the immature neuron stage they are no longer dye coupled to these same connexin coupled proliferative units (Bittman et al., 1997). As I have shown here type 3 DCX positive NPCs are devoid of Cx45 and suggest this marks the end of the association of these neuroblasts with their less mature stem and progenitor cell counterparts, allowing for their cell cycle exit and progress into the subsequent immature neuronal phase.

A requirement for uncoupling at this stage could also explain the lack of fate specification and survival defects observed in the Cx45cKO mice. While freeze fracture and dye coupling studies have only detected coupling of mature neurons to other mature neurons or microglia, it is possible that immature

neurons are also coupled, as the *de novo* expression of Cx45 in the immature Tuj1 positive neurons would suggest (Dobrenis et al., 2005; Elias et al., 2007; Rash et al., 2000; Rash et al., 2001). These coupling studies were focused on coupling between mature cell types and may therefore have missed this relatively minor but important contribution to cell-cell coupling or similarly to the developing brain, open channels may not be necessary and Cx45 could instead contribute to the migration of these cells to their mature position within GCL through their adhesive properties. I posit that this would likely occur through Cx43/Cx45 (radial glia or astrocyte and immature neuron) heterotypic channels which are known to be compatible, at least in the heart (Desplantez et al., 2004). While there are no gross neuronal migration defects apparent in the Cx45cKO mice studied here, a more detailed analysis is necessary to rule out the possibility of subtle defects. Interestingly, is it at this developmental time point that immature neurons transition from being tonically activated by GABA to receiving mature synaptic GABA inputs which result in depolarization through a CREB dependent mechanism (Jagasia et al., 2009; Ming and Song, 2011). This CREB dependent mechanism is what I have also hypothesized to be necessary for the transition to an instructive role wherein Cx45-expressing granule neurons produce the growth factors required to regulate basal proliferation of neighboring NPCs via paracrine release (Figure 7.2). While further work is necessary to confirm these speculative 'bigger picture' roles of connexin mediated communication (and Cx45 specifically), in the adult brain, the data I have presented in chapters 2 and 3 add

significantly to the frame-work within which we understand the role of connexin mediated communication in the adult hippocampal neurogenic niche.

7.2 Exogenous Regulators of Adult Hippocampal Neurogenesis

The desire to manipulate the neurogenic process for therapeutic purposes has lead to the investigation of the effects of numerous existing behavioural and pharmacological paradigms on adult neurogenesis. These studies have uncovered both positive and negative regulators of the process although relatively little is known about the mechanistic underpinnings of these effects as was highlighted in chapter 1. Presented in this thesis are data demonstrating the positive impact of a subset of selective serotonin reuptake inhibitors (SSRIs) and the negative impact of the opiate analgesic buprenorphine on adult hippocampal neurogenesis (Figure 7.3). The significance of these findings lies within the routine clinical use of these drugs without consideration of their neurogenic effects.

7.2.1 Selective Serotonin Reuptake Inhibitor Treatment

SSRIs, amongst other antidepressant therapies, were first identified as positive regulators of adult hippocampal neurogenesis in the late 1990's (Duman et al., 1999; Malberg et al., 2000). Despite continued research since that time, there remains significant controversy with regards to many key findings in this field (Petrik et al., 2011). Data presented in chapter 5 demonstrate that both fluoxetine and escitalopram increase hippocampal NPC proliferation with no

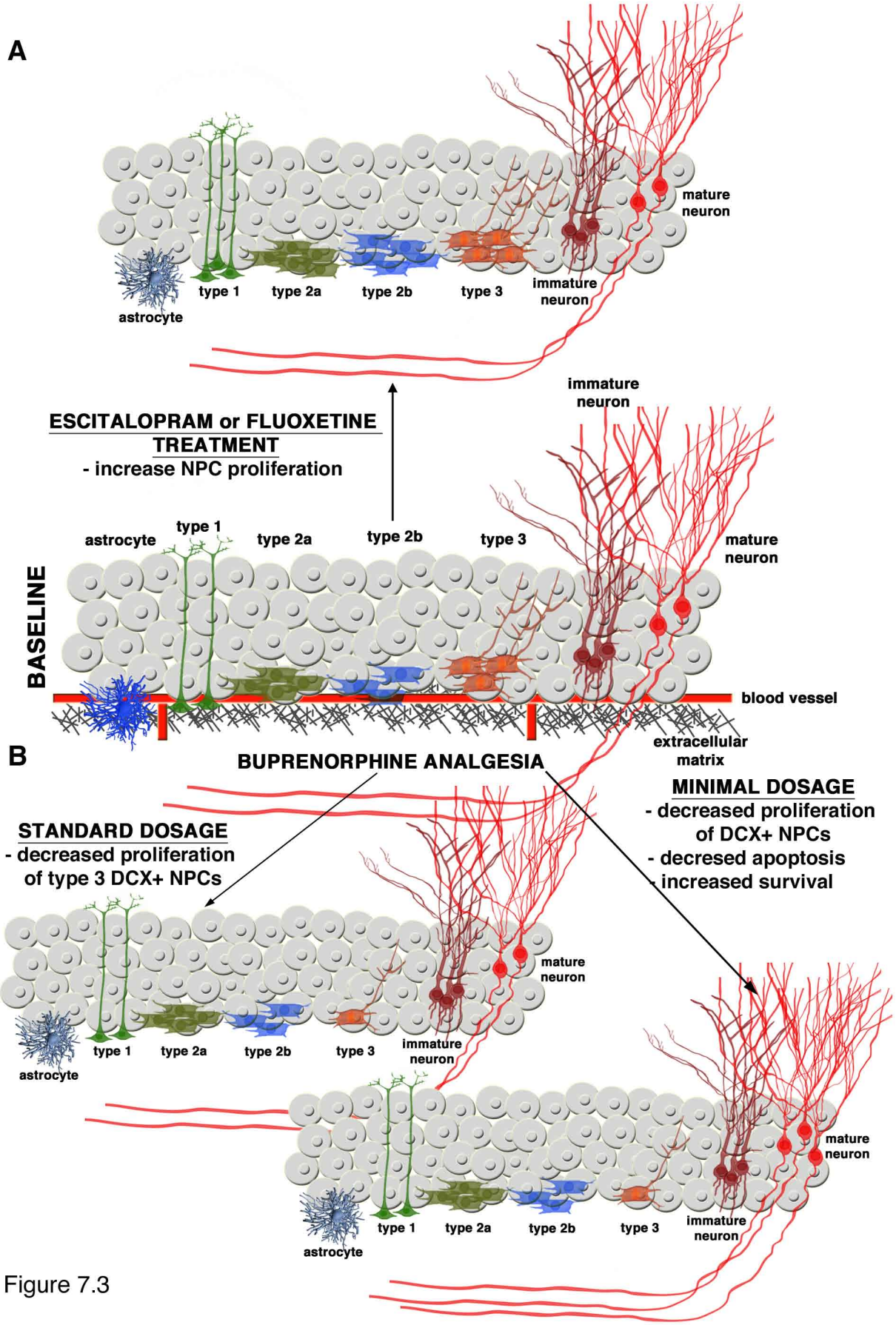
A
astrocyte
type 1
type 2a
type 2b
type 3
immature neuron
mature neuron
ESCITALOPRAM or FLUOXETINE TREATMENT
- increase NPC proliferation
BASELINE
blood vessel
extracellular matrix
B
BUPRENORPHINE ANALGESIA
STANDARD DOSAGE
- decreased proliferation of type 3 DCX+ NPCs
MINIMAL DOSAGE
- decreased proliferation of DCX+ NPCs
- decresed apoptosis
- increased survival

Figure 7.3

Figure 7.3 - Summary of the exogenous regulators of adult hippocampal neurogenesis studied in this thesis.

A) A subset of SSRIs, escitalopram and fluoxetine, increase proliferation of hippocampal NPCs but do not affect their survival. B) Post-operative analgesia reduces proliferation of DCX+ cells in the adult hippocampus for up to 7 days after administration and at a minimal dosage also increases survival of their progeny.

effect on survival or fate specification (Figure 7.3A). These findings did not extend to a third family member, citalopram, suggesting that neurogenic effects are not a universal mechanism of action of SSRI family of antidepressants and/or that the “inactive” racemate is, in fact, a modulator of NPC response. The finding that fluoxetine did not alter the survival of NPC progeny is contradictory to several other published reports, although it should be noted that we did use a different labeling paradigm (Encinas et al., 2006; Wang et al., 2008). While we cannot rule out that our treatment did impact upon progeny survival as cells were not exposed to SSRI at the moment of their “birthdating” but rather over the course of specification, I hypothesize that antidepressant therapies are instead targeting the proliferative aspect of neurogenesis readying a larger pool of progenitors for selection and maturation when needed due to the stress-associated aspects of depression. This would not be at all unexpected given the more robust effect of antidepressant therapies when tested in conjunction with a model of depression (Jaako-Movits et al., 2006; Santarelli et al., 2003).

While these findings are generally regarded as being relevant in the context of understanding the multifactorial mechanisms of action of antidepressant therapies, earlier generalizations that the neurogenic benefits of antidepressant therapy were seen with all classes of antidepressant drug and behavioural therapies, in addition to a lack of understanding of the functional/behavioural outcomes of this pro-neurogenic effect, has hampered the application of such bench findings to clinical therapeutic application. SSRIs represent the current front line therapy for depression and yet the response rate

to initial therapy is still poor and the rationale for selecting one family member over another is often unclear, owing most notably to a lack of studies directly comparing the effects of multiple antidepressants in a single test group (Gartlehner et al., 2007). Certainly, given our growing understanding of the association between the severity/duration of depressive episodes with hippocampal atrophy (and therefore the need for regeneration), combined with the functional interplay between the deficits in cognition and emotional regulation seen in depression with hippocampal neurogenesis, it is plausible that one day the selection of an antidepressant for a specific subtype of depression (i.e. - situational depression vs. persistent major depression) could include a consideration of the neurogenic profile of the available drugs (Austin et al., 2001; Samuels and Hen, 2011; Sheline, 1996; Sheline et al., 1999). For this to be possible, data such as I have presented in chapter 5, where a direct comparison of SSRI family members demonstrated that fluoxetine and escitalopram promote NPC proliferation whereas citalopram does not change the neurogenic profile are essential first steps.

A further goal of this thesis, as it was originally conceived, was to assess the requirement for neurogenesis in effective SSRI mediated-antidepressant therapy. To that end, I bred mice to generate a transgenic mouse model in which it would be possible to ablate adult neurogenesis by administering diphtheria toxin (DT) following the tamoxifen mediated induction of expression of the diphtheria toxin receptor (DTR) in nestin positive stem and progenitor cells, the NesCreER^{T2}:iDTR mouse line. Significant ablation has not yet been achieved

due to both the poor tamoxifen-induced recombination in the NesCreER^{T2} line tested and the ineffectiveness of the dosage of DT used. While inducible toxigenic ablation continues to be pursued by the field as a whole, it still requires significant refinement prior to standing on its own and therefore continues to be used in parallel with traditional irradiation methods for confirmation at this current time (Burghardt et al., 2012; Denny et al., 2012; Snyder et al., 2011). Ongoing work in our laboratory seeks to optimize the protocols necessary to obtain significant ablation using this mouse model, as it is a valuable experimental tool necessary to tease out the causal relationships between neurogenesis and its functional outcomes. More specifically use of these approaches to elucidate the impact of the loss of the neurogenic effects of specific antidepressant therapies in models of depression will be critical to helping inform physicians in their selection of appropriate therapeutics in specific depression subtypes as outlined earlier.

7.2.2 Unexpected Regulators of Neurogenesis – Post-Operative Analgesia and Beyond

In addition to the SSRIs targeted in this thesis, other environmental regulators became apparent over the course of my studies. While only the effect of buprenorphine on adult hippocampal neurogenesis was studied in detail, these manipulations will be discussed here as they highlight the importance of a well designed experiment, the need to examine the impact of unexpected variables on your results, and the need for a suitable platform to discuss such findings in the scientific literature.

When we examined the initial cohort of animals for the SSRI studies, it was noted that both vehicle and citalopram treated mice showed a significant reduction in proliferation relative to untreated animals. This prompted the investigation of the impact of post-operative buprenorphine analgesia, a treatment that all the mice received following the implantation of the osmotic mini-pumps used to deliver the test compounds, on neurogenesis. Despite it having been established that opiates reduce neurogenesis in the context of abuse, there had been no study of their impact in this far more prevalent usage context, analgesia (Eisch and Harburg, 2006). Chapter 4 of this thesis demonstrates that two different, yet commonly used, treatment schedules significantly reduce proliferation of DCX-positive NPCs, with the lower dosage also significantly reducing cell death and increasing survival of their progeny (Figure 7.3B). As shown in chapter 5, this effect can persist in the presence of other drug treatments and possibly override their effects.

It is tempting to speculate that these neurogenic changes may underlie the cognitive, psychomotor and emotional reactions to buprenorphine that have been previously demonstrated in both animal models and human patients (Dolcos et al., 1998; Jensen et al., 2008; Panksepp et al., 1978). Impaired cognition has been noted as soon as 105 min after a single infusion of buprenorphine and cognitive deficits have long been recognized in opiate abuse (Cipolli and Galliani, 1987; Guerra et al., 1987; Jensen et al., 2008; Spain and Newsom, 1991). The strong association between dorsal hippocampal neurogenesis and learning and memory, as outlined in section 1.5.1, supports the notion that the negative effects

demonstrated in chapter 3 could contribute to these functional deficits (Fanselow and Dong, 2010). Similarly, both short term and chronic exposure to opiates lead to altered emotional regulation. Thus, it is also possible that the observed effects on neurogenesis could contribute to this outcome through modulation of ventral neurogenesis, now increasingly being associated with emotional regulation, as outlined in section 1.5.2 (Dolcos et al., 1998; Nieto et al., 2002; Panksepp et al., 1978). As we have demonstrated here, even very minimal exposure has the potential to alter neurogenesis in an otherwise healthy individual. While this could certainly impact the average patient using buprenorphine for short-term analgesic purposes, it will certainly have a more substantial impact upon patients using a sustained course of buprenorphine, be it in the context of abuse, treatment of opiate dependence, or off-label antidepressant purposes. These potential neurogenic "side effects" will therefore need to be considered when being selected for therapeutic applications. In fact a recent study demonstrated that methadone, a related synthetic opioid drug used both in the treatment of opiate dependence and for analgesia, resulted in increased co-morbid depression in heroin users, grey matter volume reductions, increased anxiety and likelihood of relapse even after having been free from illicit drug use for a minimum of 12 months suggesting that this therapy could, at least in regards to neurogenesis and its functional outcomes, be impairing recovery (Lin et al., 2012).

As highlighted in chapter 3, in the contest of basic science, post operative analgesia represents a very important potential source of variability in neurogenic

research that most often goes unreported in the literature with fewer than 20% of publications detailing the analgesia and anesthesia employed in their experimental paradigm (Piersma et al., 1999; Stokes et al., 2009). Furthermore I show in chapter 5 that a very minor environmental manipulation, a change to corn husk gravel-like bedding, can profoundly impact basal neurogenic parameters. While the regulatory bodies governing animal care in a research setting, such as the Canadian Council on Animal Care (CCAC), caution researchers to factor housing conditions and routine procedures into their experimental analysis, it is very easy and therefore common to overlook these factors which we as experimenters do not control ourselves or often do not even see occurring (Lipman and Perkins, 2002). These factors can, at least in some instances, easily account for the variability of results between research labs even when repeating identical experiments or explain differences between cohorts in the same researchers hands. With greater then 60% of “negative data” estimated to never reach publication, there are surely many more examples lost in these ‘failed’ or ‘unexpected/uninterpretable’ results. While there is a growing awareness that environment, rather than experimental protocol, may dictate the differences seen in behavioural testing results between labs, this is not yet a widely held belief (Wahlsten et al., 2003). The neurogenesis field has only recently begun to acknowledge concerns about the lack of skepticism with which correlative findings between behavioural outcomes and neurogenic profiles are accepted without consideration for many potential relevant factors and yet they continue to overlook these same factors with regards to their impact on the

neurogenic program at the cellular level in the first place (Lazic, 2010). Unfortunately it is difficult to publish (and therefore openly discuss the impact of) such unexpected confounding variables due to our prevailing culture of publication, which openly supports the publication of top tier, cutting edge "positive" data wherever possible. However, in an era when we are trying to do more with fewer funding dollars there is a greater need than ever to avoid needlessly repeating or refining research protocols that are limited or otherwise influenced by these unexpected confounding variables. I therefore argue that researchers must take greater care to truly control for and recognize the effects that these seemingly routine or innocuous changes/procedures may be having on their experimental readouts. Furthermore, we would benefit from the development of a better platform on which these variables can be openly discussed and therefore raise the awareness of a greater number of researchers.

7.3 Mechanistic Insight

While seemingly very different, each of the modulators of neurogenesis investigated in this thesis likely has a related mechanism of action. Over the last ten years there has been an increasing awareness that many of the broader signaling pathways that have been identified as regulating adult hippocampal neurogenesis ultimately have an impact at the level of the individual transcription factors (TFs) that govern this process (Faigle and Song, 2012; Hsieh, 2012). Figure 7.4 provides an overview of TFs with known roles in the adult neurogenic process along with the broader pathways and signaling molecules regulating

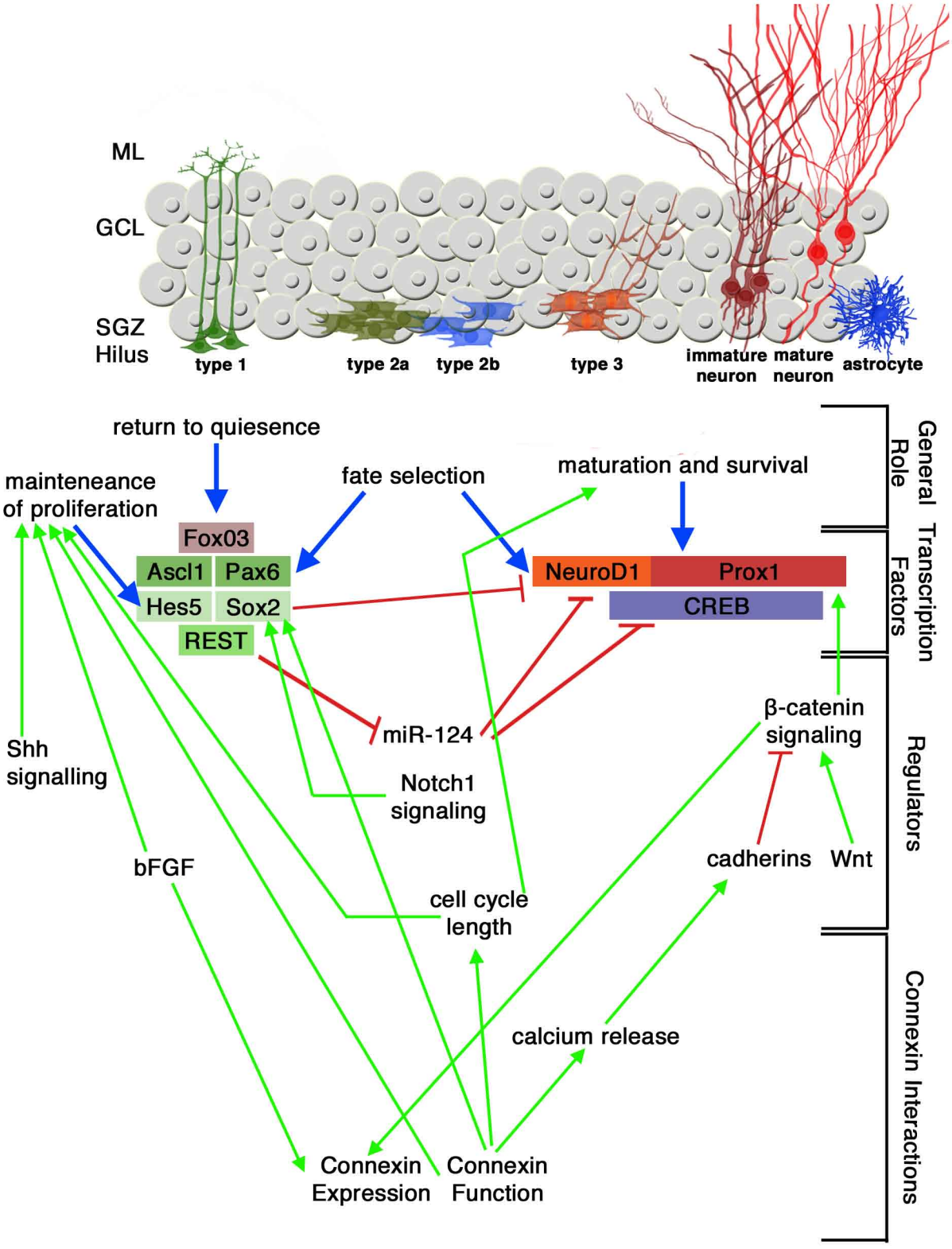
ML
GCL
SGZ
Hilus
type 1
type 2a
type 2b
type 3
immature neuron
mature neuron
astrocyte
return to quiesence
maintenance of proliferation
fate selection
maturation and survival
Fox03
Ascl1
Pax6
Hes5
Sox2
REST
NeuroD1
Prox1
CREB
miR-124
Shh signalling
Notch1 signaling
β-catenin signaling
cadherins
Wnt
bFGF
cell cycle length
calcium release
Connexin Expression
Connexin Function
General Role
Transcription Factors
Regulators
Connexin Interactions

Figure 7.4

Figure 7.4 – Transcriptional Regulation of Neurogenesis

Schematic outlining transcription factors known to regulate adult hippocampal neurogenesis, the general role they play, known regulatory pathways and/or molecules and the interactions these each of these have with connexin expression and/or function.

their expression and/or function, many of which are conserved from the developmental process.

The regulation of neurogenesis, is often investigated starting from the binding of a molecule of interest to its specific receptor at the plasma membrane and proceeding through its effects on the known intracellular signaling pathways. While this is a valid approach, particularly when examining pathways previously characterized in a developmental context, it is important to remember that NPCs do not yet express a full complement of functional receptors and as such how the impact of many manipulations, particularly exogenous pharmacologically relevant molecules, on the neurogenic process actually reach the NPCs is unclear. Juxtacrine and paracrine communication from within the neurogenic niche, occuring at least in part through connexin mediated communication, is posited to play a significant role (Bruzzone and Dermietzel, 2006; Song et al., 2002). We and others have now demonstrated the importance of connexin mediated communication in the basal regulation of adult hippocampal neurogenesis and continue to identify how numerous exogenous stimuli may impact upon the expression and/or function of these individual proteins and their composite channels thereby altering the neurogenic process (Imbeault et al., 2009; Kunze et al., 2009; Melanson-Drapeau et al., 2003). Figure 7.4 therefore also highlights how the connexin family of proteins, regulates and is regulated by the same pathways affecting the TFs important for neurogenesis. This is a key finding, the impact of which has yet to be fully appreciated.

As highlighted by Figure 7.4 the regulation of important TFs for neurogenesis is complex and a single neurogenic modulator has the potential to impact upon multiple signaling pathways. In the interest of simplicity I have focused on how each of the neurogenic modulators investigated within thesis could impact upon a single pathway. As outlined earlier, cAMP mediated activation of PKA results in the phosphorylation of CREB (Figure 7.2), which is required for the expression of growth factors necessary for the normal proliferation of NPCs (Cameron et al., 1998; Nakagawa et al., 2002). As one of the gap junction channels known to be most permissive to cAMP passage and the more prominently expressed neuronal connexin in the hippocampal dentate gyrus I have hypothesized that Cx45 plays a critical role in the maintenance of cAMP homeostasis within the GCL and SGZ which ultimately regulates the expression and secretion of the growth factors necessary for the proliferation of the type 1 NPC population (Figure 7.2). Future studies will seek to test this hypothesis by confirming functional GJIC coupling and/or hemichannel activity of Cx45 in the dentate gyrus using dye transfer studies, compare CREB phosphorylation and expression of its target genes in WT and Cx45cKO mice and finally investigate the ability of a phosphodiesterase-4 inhibitor (rolipram) to rescue the type 1 NPC proliferation defect in the Cx45 cKO mouse by increasing cAMP levels.

As I demonstrated in this thesis buprenorphine treatment also decreases hippocampal progenitor cell proliferation (Pettit et al., 2012). Buprenorphine treatment has been shown to decrease cAMP levels, as I hypothesize with the

loss of Cx45, this could therefore reduce the downstream CREB phosphorylation and ultimately growth factor production required for NPC proliferation (Liu et al., 1999). This could occur via the direct effect of buprenorphine binding the μ opioid receptor (MOR receptors) on granule neurons and NPCs, preventing the activation of the adenylate cyclase (AC) responsible for the production of cAMP (Gutstein et al., 1997; Hauser et al., 1996; Sargeant et al., 2007). However, we must also consider the possibility of indirect effects due action at buprenorphine's other targets, the κ opioid receptor and opioid like receptor-1 (ORL-1), which are not expressed or have not been characterized in NPCs, respectively, yet are both found in the dentate gyrus (Gutstein et al., 1997; Hauser et al., 1996; Mollereau et al., 1994; Tallent et al., 2001; Yasuda et al., 1993). Interestingly, connexin mediated communication is required for the antinociceptive properties of morphine, suggesting that buprenorphine could also indirectly affect NPC proliferation through connexin mediated communication (Suzuki et al., 2006). Future studies will further explore these possibilities by investigating the expression pattern of ORL-1 in NPCs using immunofluorescence, assessing whether buprenorphine acts directly on the MOR to elicit changes in cAMP signaling using MOR knockout mice and finally the contribution of GJIC to these neurogenic outcomes by examining changes in connexin expression and functional requirement using pharmacological inhibitors of hemichannels and/or gap junctions.

Finally, I have demonstrated that chronic treatment by a subset of SSRIs increases proliferation of NPCs in the SGZ (chapter 5). Chronic treatment with

multiple antidepressants have previously been shown to increase CREB expression, activation and the expression of downstream growth factors in the rodent hippocampus suggesting that it may be a key pathway contributing to the changes in the neurogenic parameters seen in my studies (Nibuya et al., 1996). As with buprenorphine it remains to be seen if these changes are occurring through direct or indirect mechanisms. NPCs in the SGZ are reported to express the $5HT_{1A}$, $5HT_{2A}$ and $5HT_{2C}$ receptors at a minimum, supporting the possibility of the direct activation of AC (Klempin et al., 2010). The possibility of indirect effects via SSRI effects on neighboring instructive cells has also been suggested as the expression of Cx43 is upregulated in response to chronic antidepressant treatment and appears to be required for their proneurogenic effects which are lost with reduced Cx43 expression (Fatemi et al., 2008; Kong et al., 2009). Future investigations will focus on the requirement for connexin mediated communication in the effective therapeutic outcomes of antidepressant treatment in addition to seeking to clarify the contribution of connexin mediated communication vs the direct activation of 5HT receptors in NPCs with regards to CREB activation using both *in vitro* pharmacological manipulations and *in vivo* loss of function approaches.

7.4 Summary

In summary, this thesis has demonstrated that connexins are expressed by adult hippocampal NPCs as seen both *in vitro* with Cx43 and 45 and *in vivo* with Cx45. Not only are they expressed; their patterns of expression are altered by interactions with laminin containing extracellular matrix components, which

results in spontaneous differentiation. Thus we have implicated this niche factor in the intrinsic regulation of adult hippocampal neurogenesis through the modulation of the NPCs capacity to communicate: this is a significant addition to the framework generated from the study of the developing brain. This thesis also adds to the growing number of environmental and pharmacological manipulations that affect neurogenesis. I have demonstrated that a subset of SSRIs increase hippocampal NPC proliferation, which is required for effective therapeutic outcomes, but not survival of their progeny. This work also brought attention to the overall sensitivity of neurogenesis to what are considered by most to be relatively minor or at times even irrelevant changes in experimental environment and/or treatment protocol. I demonstrate that a simple switch to gravel-like bedding, in which mice actively seek to dig, is able to increase basal NPC proliferation rates. Perhaps even more important was the discovery that post-operative analgesia with buprenorphine, a nationally mandated standard operating procedure in experimental animal research, reduces proliferation of DCX positive neuroblasts and depending on the dosing regimen increases survival of their progeny. The findings based on these pharmacological and environmental manipulations have potential far-reaching and profound impacts upon our considerations for experimental design as well as the clinical therapeutic applications of these drugs. Clearly the regulation of neurogenesis is a complex and dynamic event that changes in response to both endogenous and exogenous cues. This plasticity (and its environmental sensitivity) is notably relevant to pursuing cell replacement strategies for mental health disorders.

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