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Role of "neuronal" gap junction proteins in regulating post-natal hippocampal neurodegeneration and neurogenesis *in vitro* and *in vivo*

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# Role of "neuronal" gap junction proteins in regulating post-natal hippocampal neurodegeneration and neurogenesis *in vitro* and *in vivo*

Catherine D. Sorbara

A thesis submitted to the Faculty of Graduate and
Postdoctoral studies in partial fulfillment of the
requirements for the degree of Masters of Science.

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## Abstract

Adult neural stem and progenitor cells (NPCs) are located within specific neurogenic niches, such as the subgranular zone of the dentate gyrus. Cell-cell communication through gap junction channels is believed to play an important role in embryonic neurogenesis. Connexins, the protein subunits of gap junctions, are expressed in NPCs and connexin36 (Cx36) is considered the prototypical neuronal connexin although a role in neurogenesis had yet to be assessed. Here, I examined the influence of Cx36 on NPC proliferation, specification, and survival in the hippocampus *in vitro* and *in vivo* in both wild type and $Cx36^{-/-}$ mice. Using cultured neurospheres from P0-P3 mice pup hippocampi, I show that Cx36 protein is first expressed upon engagement to a laminin matrix. Moreover, a Cx36 null mutation enhances neurogenesis under certain microenvironment conditions apparently by accelerating differentiation. Using thymidine analogs to label actively proliferating cells in uninjured mice and following kainic acid-induced seizures, I show that Cx36 deletion does not alter cell proliferation in the SGZ. However, the loss of Cx36 is neuroprotective following hippocampal injury without affecting neuroregenerative capacity. Finally, I demonstrate that the gap junction-like protein, pannexin2 (Panx2), is also expressed within mature neurons of the central nervous system (CNS) although functional channel activity and a role in neurogenesis have not elucidated. Here, I characterized the localization and morphology of Panx2 within hippocampal derived neurospheres *in vitro* and discovered that Panx2 exhibits a unique rod-like morphology with Type I and Type IIa NPCs. Together, these data implicate Cx36 in regulating postnatal NPC fate in the postnatal hippocampus and suggest a distinctive, regulatory role for Panx2 in neurogenesis.

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I would like to thank my supervisor Dr. Steffany Bennett for all her support and encouragement during my time in Ottawa. She has instilled in me the confidence to continue to pursue a career in the scientific field and her unbridled enthusiasm for science and making a difference in society is unmatched by anyone I have met. I would also like to thank Dr. Paul from Harvard Medical School and his students for providing me with the Cx36 mouse colony and allowing me to visit Boston to study under his supervision. To all the Bennett Lab Members both past and present; it has been my sincere pleasure to work with everyone. The environment created by your presence made coming into work everyday fun and enjoyable. Specifically, I need to thank Leigh Anne Swayne who opened the door to the wonderful world of pannexins and has provided me with invaluable advice and assistance. Stephanie Fowler; you have become my very best friend and words cannot express how grateful I am we have met. Finally, I need to extend my deepest and most sincere thanks to my family and friends for their support and open hearts.

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## List of Abbreviations

**aCSF**: Artificial cerebrospinal fluid
**Aß:** Amyloid-ß
**AD:** Alzheimer's Disease
**BrdU:** Bromodeoxyuridine
**CA1, 2, 3, Py:** Pyramidal cell fields of the hippocampus (*Cornu ammonis*)
**CldU:** Chlorodeoxyuridine
**CNS:** Central nervous system
**Cx:** Connexin
**Cy3:** Cyanine-3
**Cy5:** Cyanine-5
**DCX:** Doublecortin
**DG:** Dentate gyrus
**DIV:** Days *in vitro*
**DMEM/F12**: Dulbecco's modified Eagle's medium F-12
**E:** Embryonic day
**ECM:** Extracellular matrix
**EGF:** Epidermal growth factor
**ER:** Endoplasmic reticulum
**FGF-2:** Fibroblast growth factor
**FITC:** Flurescein isothiocyanate
**GABA:** Gamma-aminobutyric acid
**GFAP:** Glial fibrillary acidic protein
**GJIC:** Gap junction intercellular communication
**GrDG:** Granule cell layer of the dentate gyrus
**H&E:** Hematoxylin and eosin
**hESC:** Human embryonic stem cell
**HI:** Hypoxia-ischemia
**HD:** Huntington's Disease
**hEGF:** Human epidermal growth factor
**IdU:** Iododeoxyuridine
**KA:** Kainic acid
**LV:** Lateral ventricle
**MCAO:** Middle Cerebral Artery Occlusion
**NPC:** Neural progenitor cells
**NSC:** Neural stem cells
**OB:** Olfactory bulb
**OPC:** Oligodendrocyte precursor cell
**P:** Postnatal day
**Panx:** Pannexin
**PBS:** Phosphate buffered saline
**PD:** Parkinson's Disease
**PCR:** Polymerase chain reaction
**PDGFRαR:** Platelet-derived growth factor receptor alpha

**PLAP:** Placental alkaline phosphatase
**PVE:** Pseudostratified ventricular epithelium
**RMS:** Rostral migratory stream
**RT-PCR:** Reverse transcription-polymerase chain reaction
**SGZ:** Subgranular zone
**SE:** Status epilepticus
**SVZ:** Subventricular zone
**TdT:** Terminal deoxynucleotidyl transferase
**tPA:** tissue plasminogen activator
**TUNEL:** TdT-mediated dUTP nick-end labeling
**WT:** Wildtype

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# Chapter 1: Introduction

## 1.1 Therapeutic cell replacement strategies for neurodegenerative disease

The frequency of neurodegenerative disease increases with age and the elderly currently represent the fastest growing population in Western society (Elbaz and Moisan, 2008; Salloway, 2008). These diseases markedly impact upon the lives of millions, posing significant public health challenges. A range of genetic determinants and environmental toxins have been implicated in the etiology of neurodegenerative disease, however, the mechanisms of neuronal pathologies are still poorly understood (reviewed in (Coppede et al., 2006)). These diseases include Alzheimer's Disease (AD), Parkinson's Disease (PD) and Huntington's Disease (HD) along with related dementias and motor-type disorders. Each disorder is defined by a characteristic loss of neurons in distinct regions of the central nervous system (CNS). Initial synaptic dysfunction leads to a decline in cognitive and/or motor function. Progressive neuronal loss leads to an irreversible impairment in cognitive and/or motor function that greatly decreases the quality of life and patient life expectancy (for recent reviews see (Pillay et al., 2004; Imarisio et al., 2008; Toulouse and Sullivan, 2008)).

Current treatment for neurodegenerative disease remains inadequate. The lack of therapeutic options has been called "the single biggest unmet medical need in neurology" (Citron, 2002). In the case of PD, primary symptoms are a result of reduced synthesis and action of dopamine from dopaminergic neurons, leading to reduced stimulation of the motor cortex by the basal ganglia (see review (Toulouse and Sullivan, 2008)). Therefore, the primary method of treatment is the administration of a dopamine replacement provided in the form of a dopamine precursor (L-DOPA) capable of crossing the blood

brain barrier. While able to reduce some of the motor symptoms and delay morbidity, dopamine replacement therapy is associated with its own motor complications including involuntary chorea, is unable to halt disease progression, and does not ameliorate all symptoms of the disease, including later-stage dementia (Olanow, 2004). As we gain a greater understanding of the molecular basis of neurodegeneration, the ultimate goal is to expand our arsenal of neuroprotective-based therapies. These therapies must be capable of ensuring neuronal survival following acute injury or over the course of degenerative insult, preserving neuronal function, and increasing long-term patient survival with appropriate quality of life. A second therapeutic focus lies in enhancing or directing the latent neuroregenerative capacity of adult brain whether through mobilizing endogenous neural precursor populations or through transplantation strategies.

Currently, there are no pharmaceutical interventions capable of halting or delaying progression of AD. Treatment aims at reducing the severity and frequency of the behavioral symptoms associated with the progressive neuronal dysfunction and death (Lukiw, 2008). The 'amyloid cascade hypothesis' is currently the most widely accepted explanation of AD progression and centers around the idea that the plaques and neurofibrillary tangles that are the pathological hallmark in AD patients are composed of amyloid-ß (Aß) peptide (plaques) leading to a cascade of tau hyperphosphorylation and aggregation (tangles) (reviewed in (Sorrentino and Bonavita, 2007)). Current research goals have concentrated on the generation of Aß peptide antibodies for immunotherapy, however, this promise has yet to be validated after treatment in clinical trials led to encephalitis requiring alternant approaches to the vaccination protocol and antigenic source (reviewed in (Schenk, 2002)). Thus, the most common therapeutic obstacles to

treatment of multiple neurodegenerative disorders is the inability to reduce the rate of neuronal loss or reverse the progressive reduction in neuronal number once diagnosis and prophylactic treatments have been initiated.

Recent progress in the field has resulted in the discovery of a renewable source of neurons in the adult mammalian brain, an environment once believed to be incapable of generating new neurons (Reynolds and Weiss, 1992; Eriksson et al., 1998; Emsley et al., 2005; Ming and Song, 2005; Colucci-D'Amato and di Porzio, 2008). Neural precursor cells represent a new potential therapeutic target. Mobilization of these populations to replace dead or damaged neurons raises new hope for cell replacement strategies as a treatment of neurodegenerative disease. By understanding the basic cell biology underlying the activation, migration, survival, and specification of these cells, it may become possible to isolate and perpetuate precursor cell populations in sufficient quantities, to generate "type-specific" neurons for transplantation, or to stimulate endogenous populations by use of proliferation and differentiation-promoting factors. Clearly, validation will require rigorous evaluation of whether appropriate cell lineages generated from these precursors can be effectively integrated into degenerating tissue.

### 1.2 Neural stem and progenitor cells

The term neural precursor cell encompasses two overarching populations: neural stem cells (NSCs) and neural progenitor cells (NPCs). NSCs are characterized by their capacity for unlimited self-renewal and their ability to differentiate into all cells of the CNS lineage: astrocytes, oligodendrocytes or neurons. NPCs are often more committed toward a specific lineage, and have a decreased capacity for self-renewal compared to primordial NSCs.

During CNS development, specification of NSCs and their NPC progeny is temporally regulated. Neurogenesis precedes gliogenesis. The majority of neurons are born embryonically while gliogenesis is completed postnatally (Altman, 1966; LeVine and Goldman, 1988). Subsets of NSCs and NPCs are multipotential with the capacity to specify into both neurons and glia under the control of extrinsic and intrinsic cues specific to particular microenvironments in the brain (Qian et al., 2000; Sauvageot and Stiles, 2002). Briefly, cortical neurons are born in the proliferative pseudostratified ventricular epithelium (PVE), a lining of the ventricular cavities of the developing cerebral wall. Developmental neurogenesis occurs in two waves between embryonic day 10.5 (E10.5) and E15.5 and again between E16.5 and E18.5. Gliogenesis also takes place here but over a broader temporal window and over a larger area of the subcortical cerebral wall (Caviness et al., 1995; Takahashi et al., 1995). New glia are generated by embryonically derived $NG2^+$ early oligodendrocyte precursor cells (OPCs) that migrate to their final destination while retaining their capacity to self-renew and produce new oligodendrocytes in the adult brain. These OPCs are generated in two waves, first in the developing medial ganglionic eminence and the entopeduncular beginning at embryonic day 9.5 (E9.5) and then in the embryonic subventricular zone at E18.5 (Ivanova et al., 2003). The different types of neurons and glia derived from this embryonic ventricular niche are vast in number and distinct in their properties and characteristics. They must migrate from their place of birth to their final destination where regionally specific extrinsic cues, in part, enable them to survive and adopt a lineage appropriate to their final function and site of integration.

Multipotential NSCs and NPCs are retained in adult brain albeit in more restricted locations and, possibly, with finite regenerative potential with respect to the cell types that are generated. Two regions are enriched in cells capable of generating new neurons. These neurogenic niches are found within the subventricular zone (SVZ) of the lateral ventricle and subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Figure 1.1). Neurons born in the SVZ migrate along the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into inhibitory neurons, either granule or periglomerular cells (Lois and Alvarez-Buylla, 1994; Doetsch et al., 1999). Meanwhile, neurons born in the SGZ migrate only a short distance to the granule cell layer of the dentate gyrus where surviving progeny adopt a granule cell lineage. These new neurons extend dendritic extensions to the molecular layer of the dentate gyrus and axonal projections to the cornu ammonis (CA) 3 region of the hippocampus through the mossy fibre pathway (Markakis and Gage, 1999; Kempermann et al., 2003).

In adult tissue, NSC and NPC specification to a specific neuronal lineage involves several cell intermediates, each exhibiting increasingly restricted lineage commitment and variable ability to self-replicate (reviewed in (Emsley et al., 2005)). These intermediates are identified by their morphology and expression of specific antigenic cell markers. There has been great debate as to whether each of these niches contains true NSCs (capable of unlimited self-renewal) or populations of NPCs each with finite capacity for self-renewal but different neurogenic and gliogenic potential (Rakic, 2002; Seaberg and van der Kooy, 2002). In this thesis, I will define each sub-population by its antigenic identity and thus refer to these regenerative cells in the adult brain as "neural

**Figure 1.1 Neurogenic niches in the adult mammalian brain.**

This diagram depicts the two regions with signficant neurogenic capacity in the adult brain. Representative coronal (A,B) and sagittal sections (C) of an N3 C57Bl/6 X 129SV/J adult hybrid mouse stained with cresyl violet are depicted. NPCs are found in (A) the SVZ of the LV between bregma 1.10 mm and 0.5 mm as well as (B) the SGZ of the DG in the hippocampal formation between bregma -1.68 mm and -2.08 mm. (C) Sagittal orientation depicts the path of migration of a NPC from the SVZ along the RMS to the OB where it terminally differentiates. NPCs born in the SGZ migrate to the granule layer of the DG and send axonal projections to the CA3 region of the hippocampus. Abbreviations: NPC, neural progenitor cell; SVZ, subventricular zone; LV, lateral ventricle; SGZ, subgranular zone; DG, dentate gryus; RMS, rostral migratory stream; OB, olfactory bulb; CA3, cornu ammonis 3.

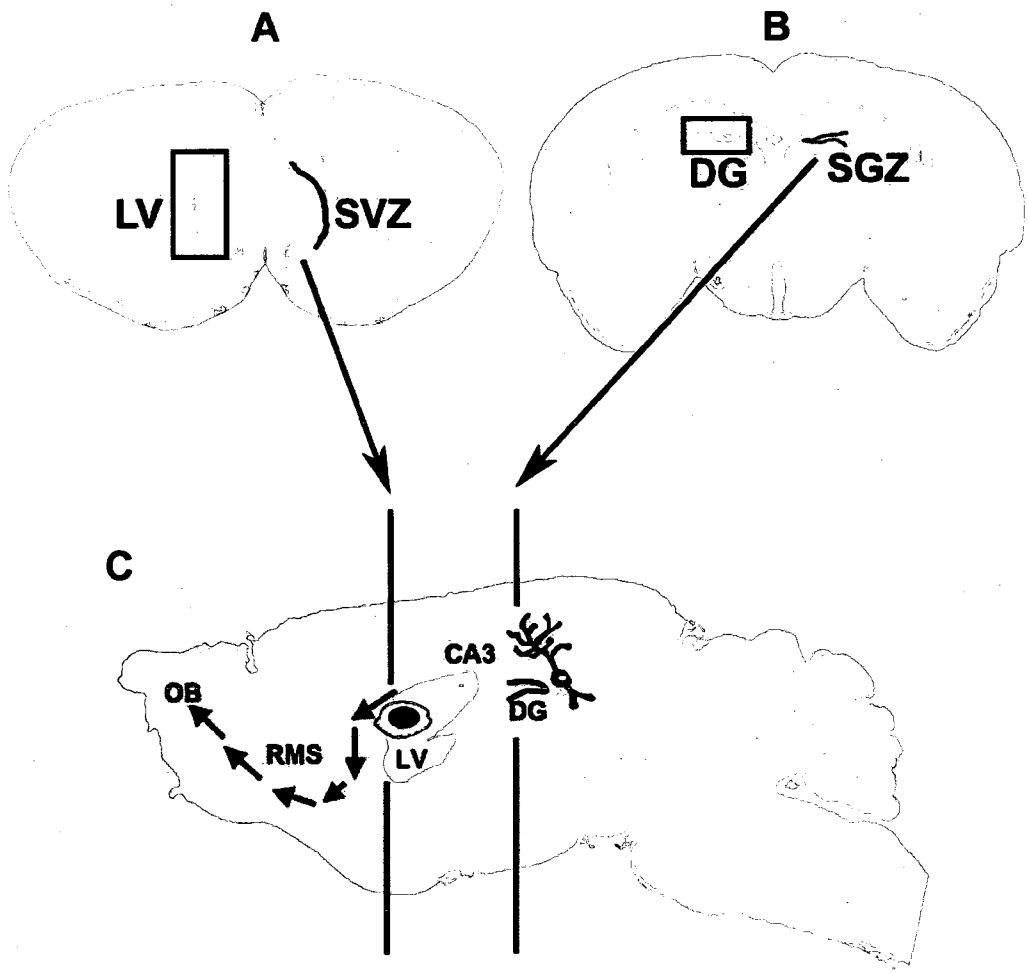


**Figure 1.1**

progenitor cells (NPCs)" as I will not address their absolute capacity for self-renewal and thus cannot definitively distinguish primordial NSCs from their NPC progeny in my experiments.

### 1.3 Assessment of post-natal neurogenesis

The first evidence of post-natal neurogenesis was determined using [3H]-thymidine autoradiography to label proliferating cells (Altman and Das, 1965; Altman, 1969). Now, *in vivo* studies regarding birth-dating and proliferation are carried out for the most part using the halogenated thymidine analog, bromodeoxyuridine (BrdU) (Taupin, 2007). BrdU and related analogs can be incorporated into cells undergoing DNA synthesis with the bromine group detected *in situ* by immunolabelling. In concert with cell markers for specific NPC lineages, NPCs can thereby be identified *in situ* by (a) their capacity for proliferation (BrdU-labelling) and (b) their antigenic properties (described below).

Two different nomenclatures are commonly used to describe neurogenesis in the SVZ and SGZ (Figures 1.2, 1.3). According to the SVZ neurogenic lineage model, Type B cells likely represent the self-renewing, primordial NSCs that can produce transit-amplifying Type C NPCs. Type C cells then form Type A neuroblasts that migrate to the OB and differentiate to interneurons (Doetsch, 2003; Alvarez-Buylla and Lim, 2004). Type B cells express astrocytic marker glial fibrillary acidic protein (GFAP) as well as nestin. Type C express solely nestin while Type C cells express doublecortin (DCX) (Figure 1.2). Commitment to an oligodendrocyte lineage is more complex in that it involves several more cell intermediates. Within the SVZ, Type I cells can generate $NG2^+$ glial progenitors that can give rise to platelet derived growth factor alpha receptor

**Figure 1.2 Model of SVZ neurogenesis in the adult mammalian brain.**

One of the two adult neurogenic niches in located in the SVZ of the LV, shown in a representative coronal section in (A). Here, Type B cells likely represent NSCs and express GFAP and nestin (B). They are capable of self-renewal or commitment to transit-amplifying Type C cells that express solely nestin. These cells have limited self-renewal capability and differentiate to Type A $DCX^+$ neuroblasts. Type A cells migrate from the SVZ along the RMS to the olfactory bulb where they fully differentiate to $NeuN^+$ neurons. Abbreviations are as described in Fig 1.1 including: GFAP, glial fibrillary acidic protein; DCX, doublecortin.

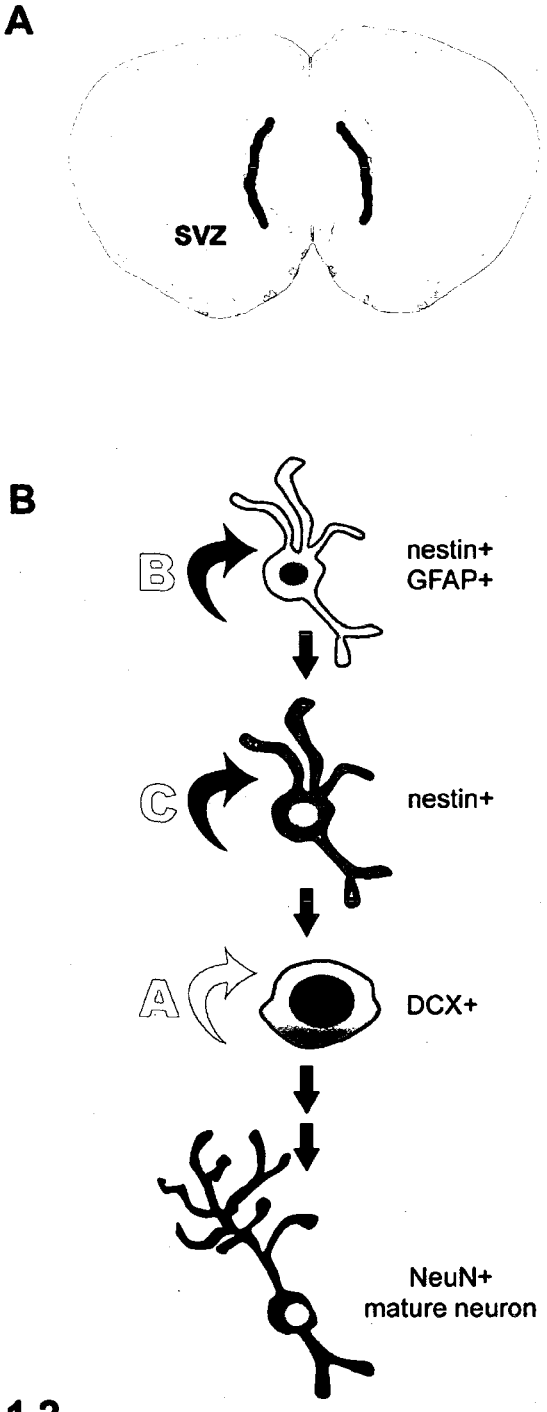


**Figure 1.2**

**Figure 1.3 Model of SGZ neurogenesis in the adult mammalian brain.**

The SGZ of the dentate gyrus in the hippocampal formation also carries out neurogenesis into adulthood, shown in a representative coronal section in (A). Here the Type I nestin/GFAP$^{+}$ cell may represent the putative NSC with capacity for self-renewal. Each daughter cell thereafter has increasingly limited self-renewal ability combined with an increasing commitment to a mature neuron (B). Type III DCX$^{+}$ neuroblasts then exit the cell cycle and become post-mitotic immature TuJ1/Toad64$^{+}$ neurons. These neurons, once fully mature, send axonal projections to the CA3 region and migrate to the granule cell layer of the dentate gyrus. Abbreviations are as Fig 1.1 and 1.2 including CA3, cornu ammonis 3.

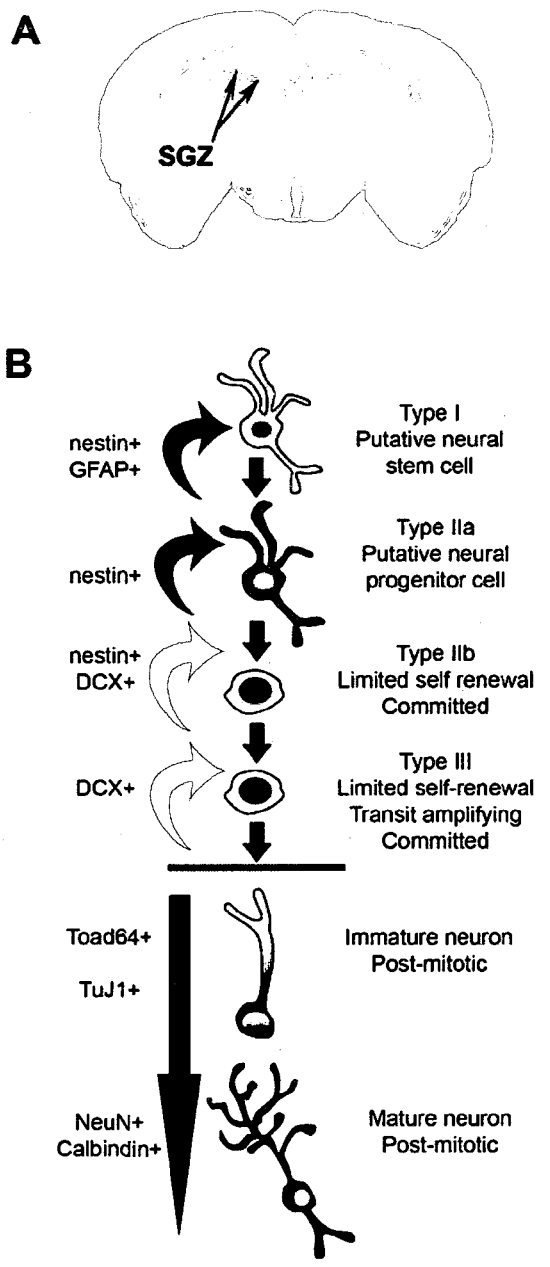
A
SGZ
B
nestin+
GFAP+
Type I
Putative neural
stem cell
nestin+
Type IIa
Putative neural
progenitor cell
nestin+
DCX+
Type IIb
Limited self renewal
Committed
DCX+
Type III
Limited self-renewal
Transit amplifying
Committed
Toad64+
TuJ1+
Immature neuron
Post-mitotic
NeuN+
Calbindin+
Mature neuron
Post-mitotic

**Figure 1.3**

(PDGFαR) oligodendrocytes progenitors and become mature oligodendrocytes (Gritti et al., 2002). It has also been shown that $NG2^+$ cells can migrate along the RMS and contribute to both neurogenesis and gliogenesis postnatally in the SVZ as well as exhibit Type C-like properties in the SGZ and generate interneurons in the hippocampus postnatally (Aguirre and Gallo, 2004; Aguirre et al., 2004). Kempermann *et al* first outlined the second type of nomenclature used to describe hippocampal neurogenesis in 2002 (Figure 1.3). Here, Type I cells are likely NSCs and express nestin/GFAP. Cells then lose their expression of GFAP and become Type IIa cells followed by a gain of expression of DCX, thereby producing Type IIb cells. These putative progenitor cells are increasingly committed to a neuronal lineage and have decreased self-renewal potential compared to a Type I cell. Finally, the Type IIb cell loses expression of nestin and becomes a Type III neuroblast. Within one or two cell divisions, migrating neuroblasts exit the cell cycle and begin to terminally differentiate becoming immature granule neurons. During this process synaptic connections with neighbouring cells are made, migration to the granule cell layer of the DG occurs, and axonal projections are sent to the CA3. Once complete, these cells will express the mature neuronal marker, NeuN and adopt a unique granule neuronal identity appropriate to their location in the granule cell layer of the DG (Kempermann et al., 2004).

NPCs are assessed *in vitro* by isolating and culturing the cells from either the SVZ or SGZ in fetal or adult brain (reviewed in (Jensen and Parmar, 2006)). These cells are disaggregated and plated in media containing mitogens, epidermal growth factor (EGF) and fibroblast growth factor (FGF-2). NPCs, but not terminally differentiated astrocytes or post-mitotic neurons and oligodendrocytes, will then proliferate in suspension to form

free-floating spherical clusters termed neurospheres with which subsequent studies can be performed (Reynolds and Weiss, 1992; Bez et al., 2003).

### 1.4 Neurogenesis in injured adult tissue

Cell replacement may be different in injured and uninjured tissue. Certainly, the process of adult neurogenesis is altered during neurodegenerative disease (see review (Abdipranoto et al., 2008)). Recently, using an AD-like mouse model of neurodegeneration, a functional correlation was shown between neurogenesis in the hippocampus and severity of neuronal loss in that neurogenesis decreased as the stage of neuronal loss worsened (Chen et al., 2008). Deposition of Aß-peptide alone has also been shown to affect hippocampal neurogenesis via impairment of proliferation, survival and neuronal differentiation (Haughey et al., 2002; Zhang et al., 2007). Indeed neurogenesis has even been shown to decrease with aging further reducing neuroregenerative potential (Bondolfi et al., 2004; Rao et al., 2006).

It remains to be determined whether new neurons formed subsequent to disease or injury can migrate to the site of damage and repair or replace neurons or whether regeneration in diseased tissues results in the integration of inappropriate cell types detrimental to brain function. Arvidsson *et al.* suggested a possible answer to this question in 2002. The authors measured the rate of proliferation in the SVZ and the amount of neuronal replacement following ischemic injury induced by two hours of middle cerebral artery occlusion (MCAO), a model of acute stroke. Remarkably, there was a 31-fold increase in the number proliferating neurons in the ipsilateral striatum four weeks following injury. Yet, the fraction of dead neurons replaced six weeks following injury was estimated to be only 0.2% (Arvidsson et al., 2002). Further, in seizure models

and epileptic clinical observation where neurogenesis is increased in the SGZ by epileptiform activity, neuroregeneration may be maladaptive. Using two common models of status elepticus (SE) in rodents: pilocarpine-induced and kainic acid (KA)-induced seizures, it was shown that prolonged convulsions affect not only proliferation but also the migratory behaviour of late Type III $DCX^+$ NPCs. This leads to abnormal integration of newborn dentate granule cells into the CA fields of the hippocampus (Jessberger et al., 2005; Parent et al., 2006). Further studies into the features of these cells have indicated an ability to migrate and aberrantly integrate as far as the hilar/CA3 border and yet retain granule cell intrinsic properties (Scharfman et al., 2000). These new neurons are also more hyperexcitable than normal pyramidal cells since they receive less inhibitory input (Dashtipour et al., 2001). This atypical neuronal circuitry may explain the cognitive defects seen following seizures and the susceptibility of patients to recurrent seizures (Jessberger et al., 2007b; Jessberger et al., 2007a). Thus, although the mechanism for neuronal replacement exists in the mammalian brain, we do not yet understand enough about NPC biology to enhance their specification to functional neurons following injury or disease.

### 1.5 Application of stem cell strategies in experimental models of human disease

Thus far, the predominant source of NPCs for therapeutic use has been embryonic involving transplantation strategies. These strategies have shown promise in experimental paradigms of PD and HD. Using an animal model of PD, it was shown that dopamine neurons obtained from embryonic stem cells could be successfully transplanted into the midbrain of rodents and generate new cells with electrophysiological and behavioral similarities to endogenous neurons (Kim et al., 2002). Likewise, survival and

maintenance of synaptic contacts with endogenous neurons was shown when gamma-aminobutyric acid (GABA)-ergic neurons from an immortalized striatal NSC line were transplanted into the adult brain of a HD model (Bosch et al., 2004). Transplantation through grafting of glial-restricted progenitor cells from the spinal cord of embryonic day 13.5 (E13.5) rodents and neural stem cells from the caudal neural tube of E9 rodents has shown promise to stimulate endogenous neurogenesis in the aging rodent brain (Hattiangady et al., 2007) suggesting that, given the correct cues, existing NPCs can generate new neurons *in situ.*

Cell replacement strategies have shown some promise in experimental models of stroke. In addition to the classical neurodegenerative disorders (AD, PD, HD), stroke is the third leading cause of death in North America and one of the primary causes of long-term disability and cognitive failure (Canada, 2006). Sixty percent of stroke victims require either acute or chronic care (Canada, 2006). Survivors of stroke are fraught with lifelong, devastating neurological deficits with risk doubling every 10 years after the age of 55 (Canada, 2006). Most strokes are "ischemic" meaning that there is a constriction of the cerebral vasculature or clot impeding the blood supply to the brain. Current drug treatment is only beneficial to a handful of stroke victims because it must be administered within three hours of the onset of ischemia (Donnan et al., 2008). It is aimed at restoring blood supply via "clot busters," (i.e., thrombolytics such as tissue plasminogen activator (tPA)), however, there are no effective strategies that promote neuroprotection or neuroregeneration following stroke. Experimental cell replacement strategies have been used with success in mice following hypoxia-ischemia (HI) insult with NPCs showing regenerative capability through reconstitution of lost tissue, establishment of neuronal

connections, and a decrease in secondary events such as inflammation (Park et al., 2002). These cells were obtained from the external germinal layer of neonatal mouse cerebellum and immortalized into a multipotent C17.2 NSC line (Snyder et al., 1995). Also, recent studies in which rodents were subjected to transient focal ischemia by MCAO have highlighted the potential of NPC therapy in improving morbidity following stroke. A reduction of infarct volume coincided with an increase in endogenous NPC proliferation in the SVZ and striatum following intranasal administration of FGF-2, a neurotrophic factor, in rats three hours following ischemia and daily for three days thereafter (Ma et al., 2008). Similarly, regeneration of functional hippocampal CA1 pyramidal neurons by endogenous progenitors was detected 28 days following transient ischemia of the forebrain of rats (Nakatomi et al., 2002). Here, growth factors EGF and FGF-2, given via osmotic mini-pumps infusing into the lateral ventricle, increased neuroregenerative capacity but had no neuroprotective effects on damaged neurons. Most recently, Daadi et al. (2008) have elegantly described the isolation and maintenance of NSCs from human embryonic stem cells (hESC). These cells were transplanted into mice one week following MCAO. The majority of grafted cells migrated to the stroke-damaged tissue and were able to improve sensorimotor function (Daadi et al., 2008).

While these studies are promising, a growing body of literature, primarily relying upon experimental models of epileptic seizure (reviewed above), also provides evidence for detrimental effects associated with stimulating adult neurogenesis that may exacerbate pre-existing neurological conditions. This disconnect between experimental models highlight a lack of knowledge in NPC biology that has hindered the development of an efficient cell replacement strategy. We are still in the early days of neuroregenerative

medicine and its promise has yet to be evaluated extensively. Indeed, very little is known about the behavior of NPCs in injured tissue, which raises safety concerns regarding potential side effects including tumor development following transplantation (Miller, 2006). As well, characterization of endogenous adult NPCs would allow for their mobilization following injury or disease thereby eliminating the rejection potential of exogenous cell replacement strategies.

### 1.6 The role of the microenvironment in regulating NPC fate: Connexin-mediated communication

Along with the changes in gene expression that must occur concomitantly with commitment to a given cell lineage, the microenvironment is key to directing progenitor cell proliferation, migration and specification. Indeed, it is believed that defining membrane-bound or diffusible factors that affect the neurogenic process will be instrumental in clarifying many of the questions surrounding NPC potential and behaviour. One such study performed by Song and colleagues in 2002, demonstrated the importance of both paracrine factors and juxtacrine cell-cell interactions, suggesting active rather than supportive roles for astrocytes in the commitment of neuronal progeny (Song et al., 2002). One robust form of cell-cell communication is connexin-mediated communication and represents the focus of this thesis.

Connexins are the structural units of transmembrane channels that directly link cytoplasm of adjacent cells allowing for bidirectional passage of small molecules $\leq$1 kDa (Fig 1.4, reviewed in (Connors and Long, 2004)). Oligomers of six connexin proteins form a connexon or hemichannel. When present in non-junctional membranes, these connexons allow for the passage of small molecules between cells and the extracellular

**Figure 1.4 Structural organization of gap junctions.**

Gap junctions are created by the docking of two connexons (hemichannels) at adjacent plasma membranes. There are three known types of gap junctions depending on their connexin subunit composition: (1) homotypic; composed of 12 identical connexins, (2) heterotypic; composed of two homotypic connexons made up of different connexins, and (3) heteromeric; composed when a heterotypic connexon docks with any other partner. Each hemichannel is composed of six connexin subunit proteins. Each subunit consists of four transmembrane proteins with two extracellular loops (E1, E2) and three intracellular components; a cytoplasmic loop, amino and carboxy termini[1].

[1] Figures from this chapter are included in a manuscript in preparation that will be submitted to the journal Stem Cell Reviews: C.D. Sorbara, V. Peshdary, and S.A.L. Bennett (2008) Connexin-mediated control of neurogenesis and gliogenesis. This figure was kindly provided by my co-author V. Peshdary.

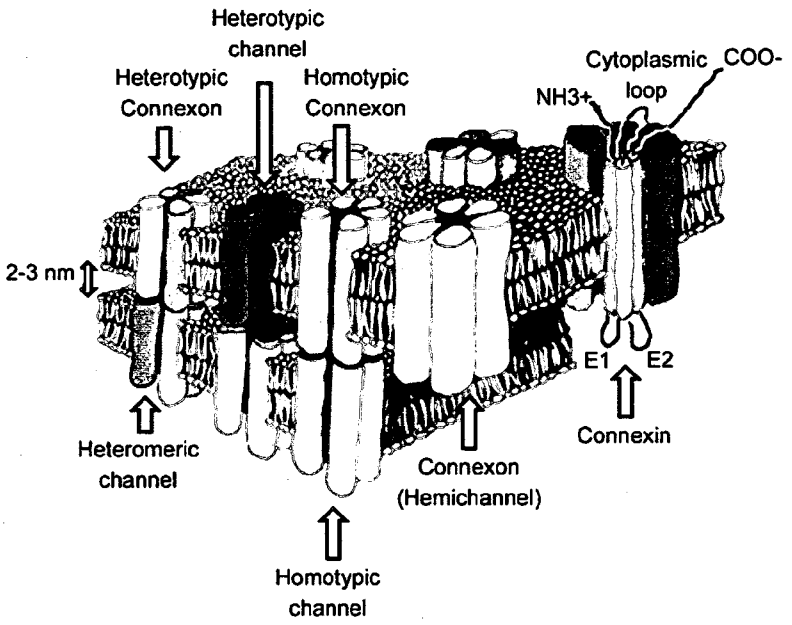
Heterotypic
channel
Heterotypic
Connexon
Homotypic
Connexon
Cytoplasmic
loop
COO-
NH3+
2-3 nm
E1
E2
Connexin
Heteromeric
channel
Connexon
(Hemichannel)
Homotypic
channel

**Figure 1.4**

space (reviewed in (Connors and Long, 2004)). When two connexons expressed by adjacent cells along junctional membranes dock, they form a functional intercellular channel that allows for the passive diffusion of second messengers, ions, and metabolites up to 1000 daltons in size. Clusters of these channels form the morphologically defined gap junction. To date, 20 connexin isoforms have been identified in the mouse genome and 21 in the human genome (Sohl and Willecke, 2003). They are denoted by the abbreviation 'Cx' followed by their molecular weight in kilodaltons. The tertiary structure of connexins includes four transmembrane domains, two extracellular loops and two intracellular regions: an amino and carboxy terminal. The least homologous of these regions is the carboxy terminal, thus, C-terminal variations in sequence and length result in the many connexin subtypes (reviewed in (Wei et al., 2004)). Based on this variety of structural building blocks, the docking of two hemichannels can be either homotypic or heterotypic, meaning that its isoform composition may be comprised of identical or different connexin subunits, respectively (Sohl et al., 2005). As well, these channels can be dynamically regulated through post-translational modification, specifically phosphorylation, assembly and disassembly (Lampe and Lau, 2000, 2004).

Given the number of connexin isoforms, it is not surprising that nearly every tissue or organ expresses at least one type of connexin, and in most cases, more than one connexin family member is present. The presence of multiple connexin family members in a single tissue allows for functional compensation in cases of genetic deletion or mutation, which can lead to serious dysfunctions and even death but also regulates the specificity of molecular passage with channel permeability defined by their connexins constituents (reviewed in (Rozental et al., 2000a; Rozental et al., 2000b)). Examples of

connexin-related abnormalities are seen developmentally where Cx26, Cx43, and Cx45 deletions are embryonic lethal due to transplacental defects (Gabriel et al., 1998), cardiac malformation (Reaume et al., 1995) and vascular defects (Kruger et al., 2000), respectively. The exact role of connexins in CNS development will be discussed in more detail below.

In the adult CNS, connexins are expressed in most neural tissue including the hypothalamus, cortex, striatum, cerebellum, retina and hippocampus and in all cell types: neurons, astrocytes, oligodendrocytes and microglia (reviewed in (Rouach et al., 2002)). Using freeze-fracture replica immunogold labeling and measuring electrical coupling between cultured cells, cellular syncytiums in the CNS have been detected through gap junction communication. To this end, gap junctions were identified between astrocytes/astrocytes, astrocytes/oligodendrocytes and microglia/neurons (Rash et al., 2001; Dobrenis et al., 2005). As well, different classes of intercellular channels exist between glial cells depending on the connexin isoform composition, furthering the complexity of this network (Altevogt and Paul, 2004; Theis et al., 2005). Although chemical transmission is often the more publicized form of communication in the CNS, electrical transmission and metabolic coupling also represent distinct and equally important modes of cell-cell communication.

Connexins are necessary for normal myelination in the CNS and expression of multiple connexins, namely Cx29, Cx32 and Cx47, is a general property of myelinating glial cells. Mutations in Cx32 lead to X-linked Charcot-Marie-Tooth disease, a demyelinating peripheral neuropathy (Scherer et al., 1998; Menichella et al., 2003). Interestingly, CNS myelination deficits are only observed following deletion of two of

the three "oligodendrocytic connexins." In the absence of Cx32, expression of Cx29 was unaltered (Altevogt et al., 2002) and the distribution of both connexins are non-overlapping with Cx29 found in inner membrane of large myelin sheaths and Cx32 found in the outer membrane (Kleopa et al., 2004). Meanwhile, Cx47 is expressed in the majority of oligodendrocytes and often colocalizes with Cx32 in oligodendrocyte somata. When both Cx32 and Cx47 are deleted in rodents, they die five to six weeks postnatally as a result of suffering severe CNS demyelination leading to tremors and seizures (Menichella et al., 2003).

With respect to neurons, Cx36 null mutants, wherein an exclusively neuronal connexin was deleted, exhibit impairment in learning and memory processes believed to be due to the loss of synchronization in gamma oscillations although a role for Cx36 in neurogenesis has yet to be assessed (Frisch et al., 2005). Little is known about the role of this connexin over the course of neurogenesis beyond evidence that Cx36 is dynamically regulated during corticogenesis (reviewed below). In this thesis, I will investigate whether Cx36 plays a role in determining post-natal NPC fate in uninjured hippocampus and following seizure-induced loss of CA3 pyramidal neurons in injured hippocampus or, alternatively whether Cx36 function is restricted to mature neuronal subtypes and impacts upon their survival following injury.

### 1.7 Connexin-mediated control of NPC fate

The role of gap junctions in mammalian neocortical development is well accepted. In order for NPC differentiation and cortical migration to occur, NPCs and their immature neuronal progeny must be able to respond to factors within their environment that elicit regionalization to enable the different cortical layers to specialize.

Connexin-mediated communication provides continuity between developing cortical "units" by allowing the passage of signals and electrical activity prior to the formation of synaptic connections (Peinado, 2001). As well, NPC migration is, in part, signalled by connexon docking, surprisingly based not on their channel-forming properties, but upon the adhesive nature of gap junctions elaborated by migrating NPC progeny and their radial glial guides. This adhesion may, in turn, contribute to intracellular signal transduction through protein-protein interaction with the C-terminal tails of the connexins composing each connexon and cytoskeletal proteins (Elias et al., 2007).

The dynamic spatial and temporal pattern of connexin expression during mouse neocortical development, between E11 and E17, has been elucidated providing further evidence of connexin-mediated control of migration, proliferation, and regional specialization. Cell clustering within the ventricular zone at this time was found to occur amongst radial glia and neural precursor cells that expressed Cx26 or Cx43 (Bittman et al., 1997; Bittman and LoTurco, 1999). Also found was a lack of coupling between Cx26 and Cx43 connexons, suggesting compartmentalization of groups of cells depending on connexon expression within different phases of the cell cycle. Finally, pharmacological blocking of these channels results in a halt of cell cycle progression (Bittman and LoTurco, 1999). More recent studies evaluating connexin expression at E14, 16 and 18 during mouse neocortical development have demonstrated that Cx26, Cx36, Cx37, Cx43 and Cx45 are expressed embryonically, whereas Cx30 and Cx32 are absent (Cina et al., 2007). Expression of Cx26 and Cx37 was uniform throughout all cortical layers while Cx36 and Cx43 were preferentially seen in the cortical plate and ventricular zone. Temporally, Cx45 expression was the highest at E18 (Cina et al., 2007). This dynamic

expression patterns is likely indicative of connexin-specific function that will require further elucidation.

Certainly, gap junctional communication figures prominently in the control of embryonic NPC fate. This function was determined using the aforementioned neurosphere assay combined with dye coupling experiments using the membrane impermeable dye, Lucifer Yellow. Here, junctional coupling was prominent between NSCs within the spherical aggregates cultured from the mouse embryonic SVZ cells. Upon differentiation and migration, only astrocytes were coupled, and blockage of coupling by addition of 18-ß-glycyrrhetinic acid decreased viability of NSCs (Duval et al., 2002). Other studies have also found that gap junctions, specifically Cx43, are expressed at high levels in NSCs in the developing rodent brain (Bittman and LoTurco, 1999) and their expression along with other connexins decreases when the cells become committed to a given lineage (Rozental et al., 1998; Boucher and Bennett, 2003). Consequently, Cx43 was required to maintain NSCs in a proliferative, self -renewing state, and when inhibited, cells either die or differentiate (Cheng et al., 2004).

Our laboratory has used the neurosphere model to determine the repertoire of connexins expressed by hippocampal-derived postnatal NPCs at the mRNA and protein levels (Imbeault et al., 2008). My contribution to this study is presented in Chapter 2. A gamut of connexins was detected with expression dependent upon culture conditions again underlying the interdependent nature of the microenvironment control of NPC fate and capacity to communicate (Imbeault et al., 2008). In particular, Cx26, Cx29, Cx30, Cx37, Cx40, Cx43, Cx45 and Cx46 mRNA and protein were detected in neurospheres. Cx29 was co-localized with protein markers for glial progenitors, while the remaining

connexins were co-localized with the neural progenitor marker nestin. The expression of connexins was altered when neurospheres were induced to differentiate on an extracellular matrix, suggesting an underlying mechanism by which connexin expression may be dynamically regulated as NPCs migrate through different cerebral compartments. Key to this thesis, we found that Cx36 mRNA but not protein was expressed when NPCs were expanded in suspension as neurospheres with protein expression induced when cultures were exposed to laminin apparently expressed by immature $TuJ1^+$ neurons (Imbeault et al., 2008). This observation provided the initial rationale for pursuing the role of Cx36 in terminal commitment of neurons during post-natal neurogenesis in uninjured and injured tissue.

One means of elucidating connexin-specific roles in hippocampal neurogenesis is through the use of "gene targeting" technology. With availability of null-mutant mouse models for various connexin family members, it is becoming increasingly evident that gap junctions also play a role in adult neurogenesis and gliogenesis *in vivo*. One such study demonstrated that Cx26 was localized to the adult rat subpial and subependymal layers, an area with high regenerative capacity. Here, Cx26 was directly associated with astrocytes expressing FGF-2, a potent regulator of neural cell proliferation and differentiation, suggesting a relationship between the expression of gap junctions and the specification of NPC fate (Mercier and Hatton, 2001). Spatial-temporal expression of Cx45 suggests that, while it may play a global role early in brain development, restricted expression to the thalamus, the CA3 region of the hippocampus, and the cerebellum in the adult suggest a role in neuronal function and possibly neurogenic response to CA3 injury (Maxeiner et al., 2003). Our laboratory has shown that Cx32 is expressed not only

by oligodendrocytes but also in subsets of early oliogodendrocyte progenitors restricted to the hippocampus (Melanson-Drapeau et al., 2003). Deletion of Cx32 caused an increase in the proliferation capacity of the oligodendrocyte progenitor pool in the immediate vicinity of the SGZ and subsequent malfunction in the ability of these cells to terminally differentiate (Melanson-Drapeau et al., 2003). Unpublished data from our laboratory has also implicated Cx32 in the control of neuronal replacement following excitotoxic injury. Loss of Cx32 enabled endogenous oligodendrocyte progenitor cells to effectively replace lost hippocampal pyramidal neurons, resulting in restoration of behavioural indices of learning and memory (Melanson-Drapeau et al., 2008). Together, transgenic mouse studies and *in vitro* neurosphere assays from our laboratory as well as support from previous work has laid the framework for a model of neurogenesis and gliogenesis that now includes connexins as markers for many NPCs and differentiated cell types (Fig 1.5).

Another influential partner regulating NPC fate are astroglia. Astrocytes are believed to regulate NPC maintenance and proliferation (Doetsch et al., 1999; Lim and Alvarez-Buylla, 1999), neuronal specification (Song et al., 2002; Kornyei et al., 2007), neuronal maturation (Christopherson et al., 2005), and synaptic integration (Hama et al., 2004; Ullian et al., 2004; Christopherson et al., 2005). Astrocytes are not, for the most part, electrically excitable, and ATP-mediated calcium signaling is the prominent intercellular signaling pathway. Astrocytes release ATP through connexin (and possibly pannexin) hemichannels (Cotrina et al., 1998) and connexins play a major role in mediating adhesive contacts between astrocytes as well as between neurons and the endothelial lining of the vasculature (Elias et al., 2007). Recently, direct evidence of the

**Figure 1.5 Model of connexin expression during neurogenesis and gliogenesis.**

Connexins are widely distributed throughout NPCs during neurogenesis and gliogenesis as depicted in this model. Cx32 is found in oligodendrocyte progenitor cells (left panel) and also appear in mature oligodendrocytes along with Cx29 and Cx47. Cx36 and Cx45 are both expressed in mature neurons (middle panel) while Cx26, Cx30 and Cx43 are found in astrocytes (right panel). Type I (putative NSCs) also express the three astrocytic connexins. (see text for specific references).

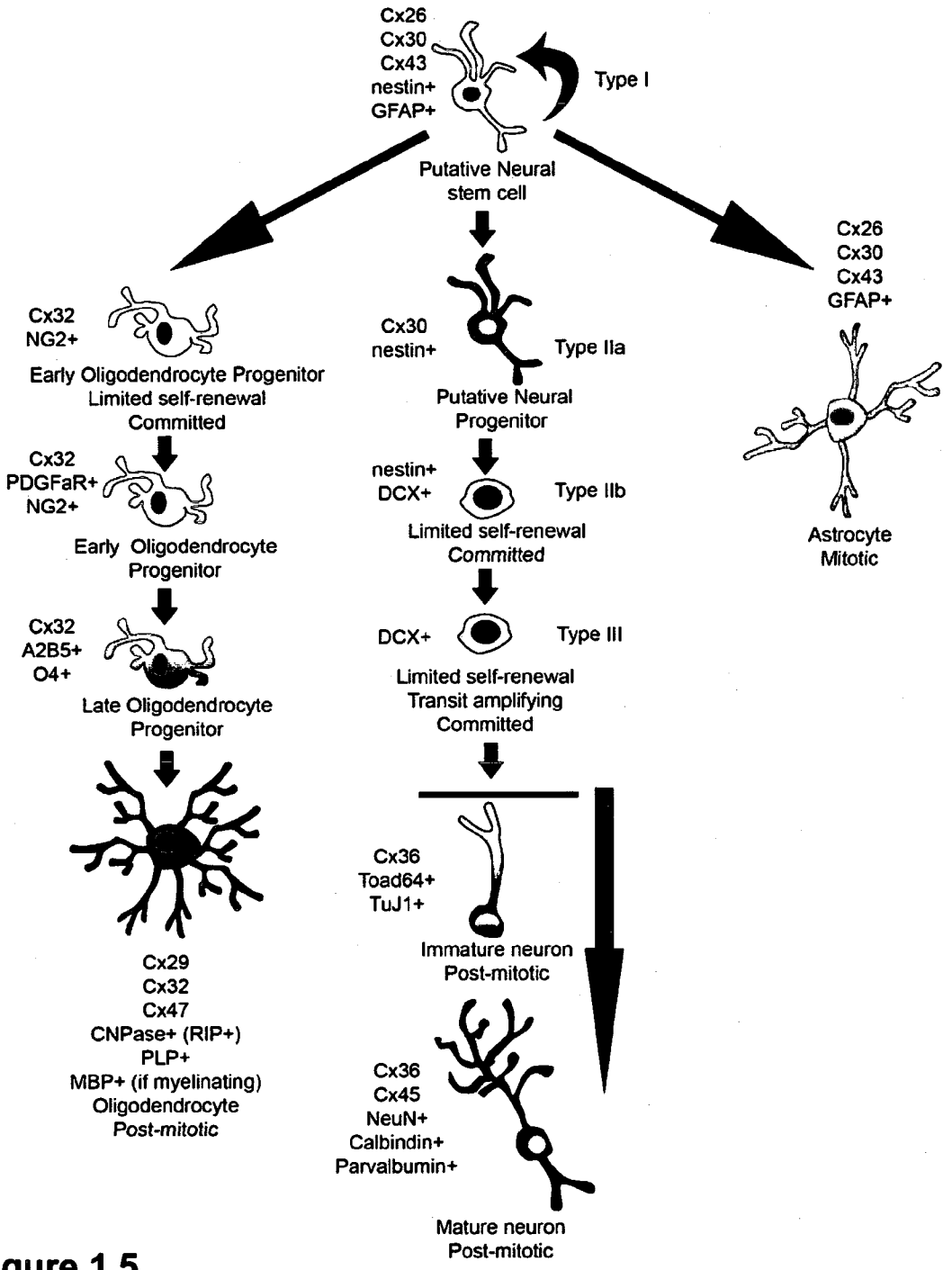
Cx26
Cx30
Cx43
nestin+
GFAP+
Type I
Putative Neural
stem cell
Cx32
NG2+
Early Oligodendrocyte Progenitor
Limited self-renewal
Committed
Cx32
PDGFaR+
NG2+
Early Oligodendrocyte
Progenitor
Cx32
A2B5+
O4+
Late Oligodendrocyte
Progenitor
Cx29
Cx32
Cx47
CNPase+ (RIP+)
PLP+
MBP+ (if myelinating)
Oligodendrocyte
Post-mitotic
Cx30
nestin+
Type IIa
Putative Neural
Progenitor
nestin+
DCX+
Type IIb
Limited self-renewal
Committed
DCX+
Type III
Limited self-renewal
Transit amplifying
Committed
Cx36
Toad64+
TuJ1+
Immature neuron
Post-mitotic
Cx36
Cx45
NeuN+
Calbindin+
Parvalbumin+
Mature neuron
Post-mitotic
Cx26
Cx30
Cx43
GFAP+
Astrocyte
Mitotic

**Figure 1.5**

ability of Cx43 hemichannels to permeate ATP was demonstrated (Kang et al., 2008). In this study, single-channel recordings of Cx43-expressing C6 glioma cells along with luminescence imaging of ATP showed that the opening of Cx43 hemichannels was linked to the release of ATP, and that this efflux transmitted current through the hemichannel. Similar properties, although infrequent, were seen from astrocytes derived from hippocampal slices of P8-P12 rats (Kang et al., 2008). Thus, it is hypothesized that hemichannel formation by astrocytes can alter the neurogenic niche through release of ATP with potential effects on neurogenesis.

Cx43-expressing astrocytes also exert neuroprotective properties following cerebral insult. Neuroprotection is believed to occur via metabolic coupling with neurons allowing for passage of glucose (Tsacopoulos and Magistretti, 1996) and the secretion of neuroactive and neurotrophic substances (Mattson et al., 1997). Consequently following MCAO in a rodent, the loss of Cx43 either globally or astrocyte-specific caused increased apoptosis in lesions, and an increase in infarct volume and size (Nakase et al., 2003a; Nakase et al., 2003b). Recently, it was found that Cx43 might also decrease neuronal injury through a gap-junction independent function. More specifically, this is believed to be through protein-protein interactions with the Cx43 C-terminus. Using a mouse model in which Cx43 proteins lacked the C-terminus (Maass et al., 2004), an increase in cerebral damage was seen following MCAO injury (Bechberger, 2007). Furthermore, primary astrocyte cultures isolated from these mice showed decreases in calcium wave propagation, combined with a reduction in dye coupling (Bechberger, 2007).

In addition to their roles in gap junction intercellular communication (GJIC), a recent study from Spray and Iacobas has introduced the concept of connexin-dependent

transcellular networks that control the transcriptome. In this way, cells that are coupled together respond in a synchronized fashion through changes in gene expression (Iacobas et al., 2007). Single genes that induce or repress neurogenesis, for example, can be altered through connexin-mediated coupling in glial or neuronal synctia, which would allow for coordinated spatial-temporal manipulation of NPC fate.

### 1.8 Pannexins: The new connexins

Finally, it must be acknowledged that connexins are not the only protein subunits capable of modulating intercellular communication. An emerging player in the field of juxtacrine communication are pannexins although this "new" family of connexin-like proteins has yet to be investigated in the context of neurogenesis. As such, we expanded our investigation of Cx36 to include early characterization of pannexins in postnatal NPCs in this thesis. Gap junctions are considered to be a feature of all multicellular organisms with innexins being the invertabrate ortholog of chordate-specific connexins and homologous to pannexins, found throughout most metazoan species (Panchin et al., 2000; Baranova et al., 2004). As such, pannexins are structurally similar to connexins (athough divergent in sequence), possessing four predicted transmembrane regions, two extracellular loops and intracellular N- and C-termini. There are three isoforms: pannexin 1,2, and 3 (Panx1/Panx2/Panx3). Only Panx1 and Panx2 are expressed in the CNS with Panx2 distribution exclusively restricted to CNS tissue. Panx1 and 2 expressions overlap with that of subsets of connexins in specific brain regions with a Panx1 exhibiting an ability to function similar to connexins, including gap junction and hemichannel formation (reviewed in (Litvin et al., 2006; Shestopalov and Panchin, 2008)). Interestingly, there is no evidence that Panx2 forms functional hemichannels.

The expression of Panx2 mRNA and protein changes following ischemic insult in the adult rat: Panx2 decreases in neurons and can be detected *de novo* in astroglia (Zappala et al., 2007). Expression and potential function in NPCs has yet to be assessed.

### 1.9 Rationale, Hypothesis and Objectives

The partnership between connexins and neurogenesis is significant although not fully understood. Based on evidence described previously with respect to Cx36 spatio-temporal and cell type expression, I initially hypothesized that Cx36-mediated signalling, whether through GJIC or the other forms of gap junction protein-mediated communication reviewed above, is involved in the regulation of NPC number and fate in the postnatal mammalian brain. Given that Panx2 shares similar expression patterns to Cx36 in mature neurons and represents a new "neuronal" gap junction-like protein with unknown function, I also hypothesized that Panx2 may also direct NPC fate to a neuronal lineage. To test these hypotheses, I employed a loss of function approach, using Cx36 null-mice to determine whether the loss of Cx36 expression by terminally differentiated neurons and/or by NPC populations altered neurogenesis *in vitro* and *in vivo*. Since there are no null mice available for Panx2, I investigated its expression in NPC subsets derived from the postnatal hippocampus. In this thesis, I describe data demonstrating that Cx36 likely accelerates NPC commitment to a neuronal lineage, and in doing so, may play a role in regulating the number of neuronal progeny generated over the course of neurogenesis and/or their survival following excitotoxic injury. This conclusion is based on the results of four specific aims:

(1) Establish when Cx36 is first expressed over the course of neurogenesis *in vitro*

(2) Assess the impact of Cx36 null-mutation on the intrinsic capacity of NPCs to respond to neurogenic stimuli *in vitro*

(3) Determine whether Cx36 alters the ability of hippocampal progenitors to proliferate and differentiate following excitotoxic injury or impacts upon the survival of terminally differentiated neurons

(4) Determine whether postntatal NPCs express Panx2 over the course of neurogenesis *in vitro*

Together, these studies represent a key step towards understanding the mechanisms involved in neuroregeneration and, more specifically, the impact of factors such as connexins in regulating NPC fate necessary to the development of new possible therapeutic targets for debilitating neurodegenerative diseases.

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# Chapter 2: Connexin 36 mRNA but not protein is expressed in neural stem cells in the postnatal mouse hippocampus[2]

## 2.1 Objectives of this study

The objectives of this chapter were twofold: 1) to determine whether Cx36 mRNA and protein are expressed in NPCs and to identify the NPC lineage, and 2) to establish whether this expression is under control of extracellular matrix components (ECM).

## 2.2 Statement of author contributions

Data in this chapter formed part of a paper submitted and, as of December 2008, conditionally accepted to BMC Neuroscience[2]. Only the studies relevant to this thesis performed by CDS are presented here with the following exceptions. LGG performed the reverse transcriptase-polymerase chain reaction (RT-PCR) analysis presented in Figure 2.1b, the Western analysis presented in 2.1e, and immunocytochemistry in 2.1f. The lineage data in Figure 2.2 represents pooled experiments processed and quantified by CDS, SI, and RD. This collaboration represents established protocol in the Bennett laboratory. All immunofluorescence counts are performed by at least two independent investigators one of whom is blind as to the identity of the sample being quantified. Counts are then averaged to yield one unbiased measurement per sample. RD is an honours student in the Bennett laboratory under the mentorship of CDS who performed the "double-blind" counts. Cx36 null-mutant mice were provided by DLP.

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[2] These data form part of a paper in press: S. Imbeault, L.G. Gauvin, H.D. Toeg, A. Pettit, C.D. Sorbara, L. Mighad, R. DesRoches, A. Menzies, K. Nishii, D.L. Paul, A. Simon and S.A.L. Bennett (2009) Gap junction expression and function in postnatal hippocampal neural progenitor cells is controlled by the extracellular matrix. *BMC Neuroscience*

## 2.3 Introduction

Cx36 mRNA and protein expression has previously been detected in the developing mouse cortex between E14 and E16 (Cina et al., 2007) and postnatally in mature neurons of the hippocampus and other brain regions (Belluardo et al., 2000; Condorelli et al., 2000). Its expression is developmentally regulated and correlates with the appearance of neuronal gap junction coupling (Sohl et al., 1998; Arumugam et al., 2005). These studies, however, did not investigate the expression of Cx36 in NPCs and, subsequently, its possible role in neurogenesis. The neurosphere assay is an ideal system to examine the intrinsic expression of Cx36 in free-floating conditions as well as its regulation by the microenvironment, specifically engagement with extracellular matrix components.

## 2.2 Experimental Procedure

*Mice*

$Cx36^{-/-}$ mice were kindly provided by Dr. David Paul (Harvard University) and were compared to congenic N4 wild-type (WT) littermates. Mice were kept on a 12 hr light-dark cycle and allowed food and water *ad libitum*. 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.

*Neurosphere cultures*

Cultures were prepared from P0-P3 mice pups euthanized by lethal injection with sodium pentobarbital. The brains were removed and placed in cold artificial cerebral

spinal fluid (aCSF; 26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM D-glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, pH 7.3). They were then sliced coronally (500 μm) using a Leica VT1000S vibratome and slices within the hippocampus between bregma -1.60 mm and -2.10 mm were collected. The slices were then moved to a Leica MZ6 dissecting microscope to dissect out the hippocampus and remove surrounding connective tissue. The pooled tissue was minced with a scalpel and placed in 2.5 ml of dissociation media (26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2.2H_2O$, 3.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM D-glucose, 1% penicillin/streptomycin, 0.1 % neural protease, 0.01 % papain, and 0.01% DNase I). The tissue was incubated with rotation for 1 h at 37 °C. Sterile 10 mM PBS was added and cells were centrifuged and resuspended in 5 ml of sterile PBS, dissociated by trituration, centrifuged a second time and resuspended in 3 ml of Maintenance Media consisting of Dulbecco's modified Eagle medium (DMEM/F12) with B27 supplement (100 U/ml), penicillin/streptomycin (100 U/ml) and L-glutamine (2 mM). Cultures were plated at a density of $2.5x10^5$ cells/ml in 6 cm Ultra low attachment polysterene dishes (Fisher). Growth factors, basic fibroblast growth factor (FGF-2; 20 ng/ml, Invitrogen) and epidermal growth factor (EGF; 10 ng/ml, Invitrogen) were supplemented every other day beginning on day 2 *in vitro* and cells were grown for a total fourteen days *in vitro* (DIV). Neurospheres used for immunocytochemistry were fixed with 3.7% formaldehyde for 20 min, and cryoprotected in 15% sucrose solution (15% sucrose in 10 mM PBS and 0.01% sodium azide) for at least 24 hr at 4 °C. Neurospheres were then flash-frozen with $CO_2$ gas and sections (10 μm thickness) were prepared using a cryostat (Leica Microsystems Inc.,

Richmond Hill, ON). For RNA and protein isolation using Trizol or RIPA buffer, the manufacturer's protocol was followed for suspension cultures.

*RT-PCR*

The length of the PCR products, nucleotide sequences of the primers and PCR conditions were as follows:

Cx36: 533 bp; forward 5'-AGCGGAGGGAGCAAACGAGAAG-3' and reverse 5' – CTGCCGAAATTGGGAACACTGAC-3'; one cycle 94 °C 5 min/35 cycles: 94 °C 25 s; 59 °C 50 s, 72 °C 1 min 45 s/one cycle: 72 °C 7 min.

LacZ: 179 bp; forward 5'-ACTATCCCGACCGCCTTACT-3' and reverse 5'-TAGCGGCTGATGTTGAACTG-3'; one cycle: 95 °C 15 min/34 cycles: 95 °C 30s; 65 °C 90 s; 72 °C 2 min/one cycle: 72 °C 15 min.

PLAP: 187 bp; forward 5'-GGTGAACCGCAACTGGTACT-3' and reverse 5'-TAGCGGCTGATGTTGAACTG-3'; one cycle: 95 °C 15 min/34 cycles: 95 °C 30 s; 65 °C 90 s; 72 °C 2 min/one cycle: 72 °C 15 min.

GAPDH: 292 bp; forward 5'-TGGTGCTGAGTATGTCGTGGAGT-3' and reverse 5'-AGTCTTCTGAGTGGCAGTGATGG-3'; one cycle 94 °C 5 min/35 cycles: 94 °C 25 s; 59 °C 50 s, 72 °C 1 min 45 s/one cycle: 72 °C 7 min.

The products were visualized on a 1.5% agarose gel stained with ethidium bromide.

*Western analysis*

At DIV 14 neurospheres were washed in 10 mM PBS, 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). If Western analysis was performed on cells adhered to laminin, RIPA buffer was applied directed to the plates. Cells were collected with the aid of a cell scraper (Fisher Scientific, Nepean, ON, Canada). Lysed cells were incubated on ice for 30 min and centrifuged at 13 400 x g for a further 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 15% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Fisher Scientific). Membranes were blocked at room temperature (RT) for 1 hr in blocking buffer (1% casein in PBS). Primary and secondary antibodies are listed in Table 2.1. Immunoreactivity was visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology Inc., Rockford, IL, USA).

**Table 2.1 List of Primary and Secondary Antibodies used for Immunocytochemistry**

| Antibody | Lineage Detected | Species | Source | Dilution |
|---|---|---|---|---|
| β-galactosidase | | Rabbit | Chemicon | 1:750 |
| Cx36 | | Rabbit | Zymed | 1:100 |
| DCX (doublecortin) | Type IIb/Type III neuronal progenitors | Guinea Pig | Chemicon | 1:400 |
| GFAP (glial fibrillary acidic protein) | Astrocytes/Type I neural stem cells | Rat | Zymed | 1:10 |
| | | Rabbit | Sigma | 1:50 |
| Nestin | Type I, Type IIa/b neural progenitors | Mouse | Chemicon | 1:25 |
| NG2 chondroitin sulfate proteoglycan | Glial and early oligodentrocyte progenitors | Rabbit | Chemicon | 1:100 |
| Parvalbumin | Interneurons | Mouse | Sigma | 1:1000 |
| PDGFαF (platelet-derived growth factor receptor alpha) | Late oligodendrocyte progenitors | Rat | BD Pharm | 1:333 |
| RIP | Mature oligodendrocytes | Mouse | Chemicon | 1:1000 |
| TuJ1 (class III β-tubulin) | Immature neurons | Mouse | RDI | 1:125 |
| Guinea Pig IgG | Cyanine-3 (Cy3)-conjugated | | Jackson | 1:400 |
| Mouse IgG | Cy3-conjugated | | Jackson | 1:400 |
| | Fluroscein isothiocyanate (FITC)-conjugated | | Jackson | 1:50 |
| Rabbit IgG | Cy3-conjugated | | Jackson | 1:300 |
| | FITC-conjugated | | Jackson | 1:50 |
| Rat IgG | Cy3-conjugated | | Jackson | 1:300 |
| | FITC-conjugated | | Jackson | 1:200 |

*Laminin Plating of Neurospheres*

On DIV 8, neurospheres were plated onto laminin-coated glass coverslips in 10 cm tissue culture dishes (Corning, NY, USA. Cells were grown in Maintenance Media supplemented with 20 ng/ml EGF and 10 ng/ml FGF-2 every other day for 6 days and on DIV 14 cells were fixed with 4% paraformaldehyde for 10 min, coverslips were removed and immunocytochemistry was performed.

*Immunocytochemistry*

Antibodies are listed in Table 2.1 and were diluted in Antibody buffer (3% bovine serum albumin, 0.3% Triton X-100 in 10 mM PBS). Hoechst 33258 was used as a nuclear counterstain at 1 μg/ml for neurospheres. Primary antibodies were incubated at 4° overnight. The following day, two 15 minute washes in 10 mM PBS were performed followed by a 1 hr incubation with secondary antibodies, an identical wash treatment, and coverslipping in an "antifade" mounting media (0.1% P-phenylenediamine, 90% glycerol in 10 mM PBS).

In order to analyze differentiated neurospheres, a core area was defined as the area in which one could not distinguish and count single cells while viewing under phase contrast microscopy. Five fields were then defined 15 μm from the core and analyzed for colocalization of Hoescht 33528 nuclear stain with lineage markers. This process was repeated for at least five neurospheres per condition and averaged. All counts were performed by a minimum of two and a maximum of three independent investigators blind as to the identity of the conditions.

## 2.3 Results

### 2.3.1 Cx36 mRNA is expressed by proliferating NPCs but protein is detected only when cells adhere to laminin and adopt a neuronal phenotype.

Cx36 expression was investigated using hippocampal-derived postnatal NPCs cultured from neonates P0-P3 (Fig 2.1A). Cx36 mRNA but not protein was detected in neurosphere cultures grown for 14 DIV in suspension. To assess mRNA expression, RT-PCR was performed on WT mice for Cx36 and on $Cx36^{-/-}$ mice for reporter genes. These reporter genes, placental alkaline phosphatase (PLAP) and LacZ, replace the Cx36 coding sequence in the null mouse model and allowed for characterization of Cx36 mRNA expression via histochemical detection when this model was first created (Deans et al., 2002). Here, it allowed for further verification of mRNA expression in our neurosphere cultures and indeed positive bands were seen for both markers in the $Cx36^{-/-}$ neurospheres and a positive band for Cx36 itself was seen in the WT neurospheres (Fig 2.1B,C).

The next technique that was employed to investigate Cx36 expression was immunocytochemistry. Once again, we took advantage of both mouse models. Immunostaining for Cx36 detected no positive Cx36 cells in both the WT and the null mouse (Fig 2.1D). The LacZ gene codes for the enzyme β-galactosidase and can be detected by immunocytochemistry. Despite widespread β-galactosidase expression in the $Cx36^{-/-}$ neurosphere, this is only an indication of mRNA expression as different translation factors may be at play for its protein expression compared to endogenous Cx36 (Fig 2.1D). Finally, Western blot analysis failed to detect Cx36 protein despite mRNA expression in wildtype (WT) neurospheres (Fig 2.1E). WT mouse whole brain

**Figure 2.1 Cx36 mRNA and protein expression by postnatal hippocampus-derived NPCs.**

(A) Schematic of methodology. NPCs derived from the hippocampus of P0-P3 pups were cultured as neurospheres and analyzed on DIV 12 for RT-PCR, and western blots and on DIV 14 for immunocytochemistry. (B), (C) RT-PCR of random-primed RNA extracted from 100-150 pooled neurospheres, adult WT and $Cx36^{-/-}$ brains. In (B) Cx36 transcript is seen in adult brain and neurosphere cultures ($RT^+$). Transcript is absent in $Cx36^{-/-}$ adult brain ($RT^+$). In (C) PLAP and LacZ transcript was evident in neurosphere cultures ($RT^+$). Both of these genes replaced the Cx36 coding sequence in the null-mutant mouse model. Controls for genomic contamination and artifactual priming included reactions processed in the absence of RT (RT-) or in the absence of template (NT). The same random-primed templates were amplified for GAPDH to confirm DNA integrity (lower panels). (D) Immunostaining for β-galactosidase is positive throughout serial neurosphere sections derived from $Cx36^{-/-}$ animals. Conversely, Cx36 immunostaining is negative in serial neurosphere sections from WT animals. Inset is an antibody control demonstrating that WT sections are negative for the β-galactosidase marker. (E) Cx36 protein was not detected in neurosphere suspension cultures but was detected following engagement with laminin. Cx36 protein is also seen in the adult WT brain (positive controls) but not $Cx36^{-/-}$ brain (negative control). Membranes were stripped and re-probed for actin protein as a loading control (lower panel). (F) Immunostaining shows Cx36 positive staining (right, arrow) when cells were plated on laminin and induced to adopt an immature neuronal phenotype. Phase contrast picture on left. Scale bar, 50 μm.

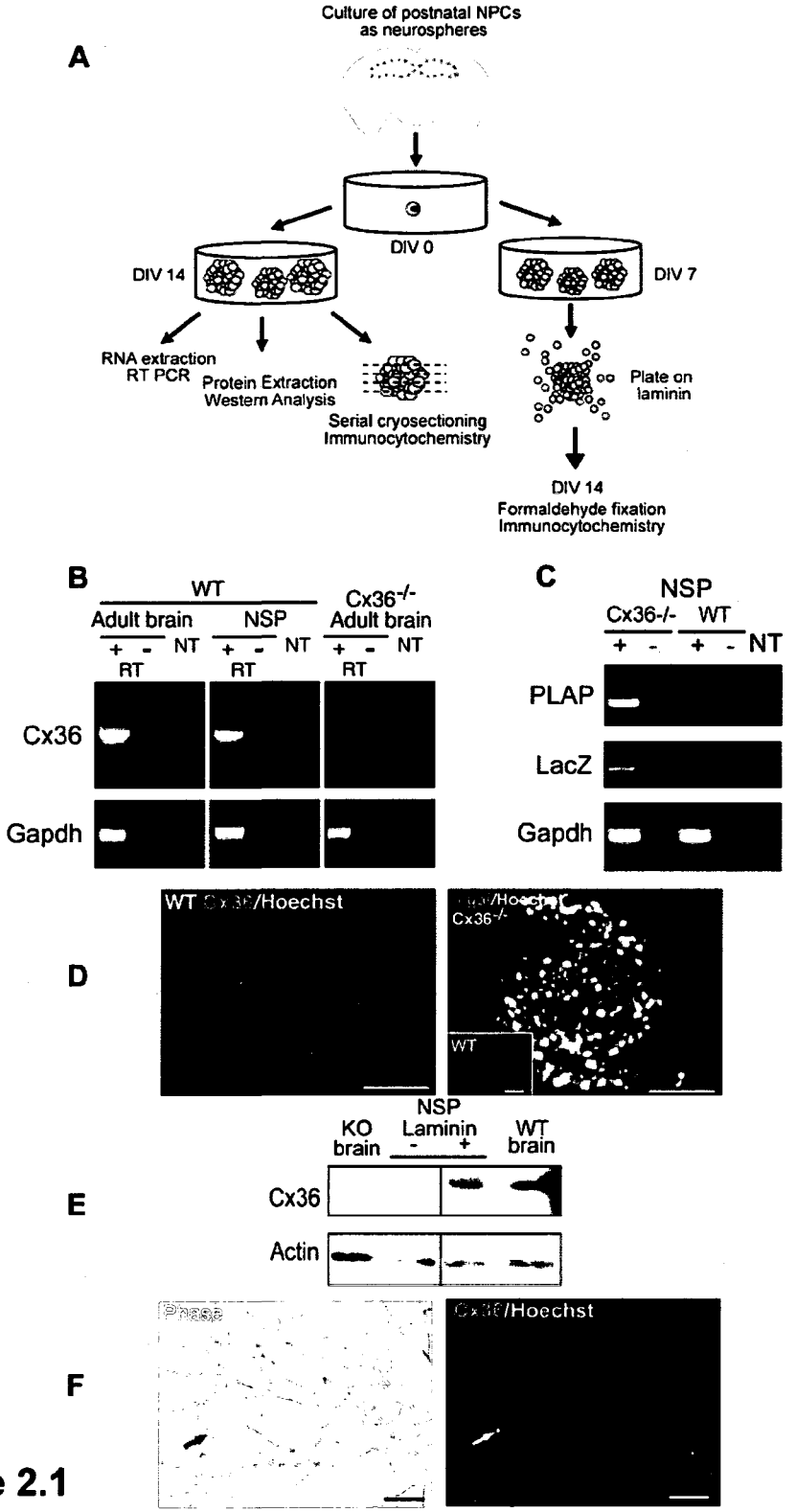
A
Culture of postnatal NPCs
as neurospheres
DIV 0
DIV 14
DIV 7
RNA extraction
RT PCR
Protein Extraction
Western Analysis
Serial cryosectioning
Immunocytochemistry
Plate on
laminin
DIV 14
Formaldehyde fixation
Immunocytochemistry
B
WT
Adult brain
NSP
Cx36-/-
Adult brain
+ - NT + - NT + - NT
RT RT RT
Cx36
Gapdh
C
NSP
Cx36-/- WT
+ - + - NT
PLAP
LacZ
Gapdh
D
WT Cx36/Hoechst
Cx36-/-
WT
E
NSP
KO brain
Laminin - +
WT brain
Cx36
Actin
F
Phase
Cx36/Hoechst

**Figure 2.1**

was used as a positive control. Meanwhile, when cells were plated on laminin and adopted a post-mitotic neuronal phenotype, a Cx36 positive band was seen by Western analysis and confirmed by Cx36 immunostaining of these cells (Fig 2.1E,F).

### 2.3.2 NPCs exposed to laminin retain a multipotential Type Ia and IIa phenotype compared to cultures proliferating in the absence of laminin

We found that NPC culture composition changes in the presence or absence of exogenous laminin, an ECM component. Antigenic lineage analysis was performed by immunofluorescence on neurospheres that were grown in suspension for 14 DIV and compared to neurospheres grown in suspension for 7 DIV followed by expansion in direct contact with laminin for an additional 7 DIV. Although neurospheres are derived from a single NPC, they spontaneously adopt different NPC lineages during proliferation and within their specialized environmental niche in a progressive manner similar to what is seen postnatally during neurogenesis and gliogenesis (Campos et al., 2004; Kempermann et al., 2004). As delineated in figure 2.2A, this lineage begins with a Type I glial fibrillary acidic protein ($GFAP^+$)/$nestin^+$ NPC and through asymmetric divisions can adopt a neuronal or glial fate eventually losing its multi-potentiality and ability for self-renewal. Upon contact with laminin, the multipotential $nestin^+$, Type IIa phenotype is better retained compared to cultures that remain in suspension (Fig 2.2B). The number of cells expressing only nestin expand with ECM contact from an average of 5.73% ± 1.26 to 17.3% ± 2. This increase is apparently at the expense of the more committed Type IIb and Type III cells, which are decreased upon ECM engagement (1.9% ± 0.8 versus 8.2± 1.8 for Type IIb cells and 0.7% ± 0.5 versus 8.3 ± 2.2 for Type III cells).

**Figure 2.2 Exposure of neurosphere cultures to laminin increases population of multipotential neural cells.**

(A) Schematic showing hippocampal NPCs lineage over the course of specification to neurons or glia. Antigenic lineage markers used in this study to identify cell populations are indicated. (B) Culture onto a laminin matrix increases the number of Type IIa cells and $NG2^{+}$ glial progenitors that are retained compared to neurospheres grown in suspension cultures concomitant to a decrease in the number of committed neuronal progenitor cells and neuroblasts. Data represent the mean of 25 counted fields (10 neurospheres, 5 representative fields per neurosphere) ± standard error of the mean (SEM). * $p<0.05$, $df = 48$, Student's *t*-test ** $p<0.01$, $df = 48$, Student's *t*-test

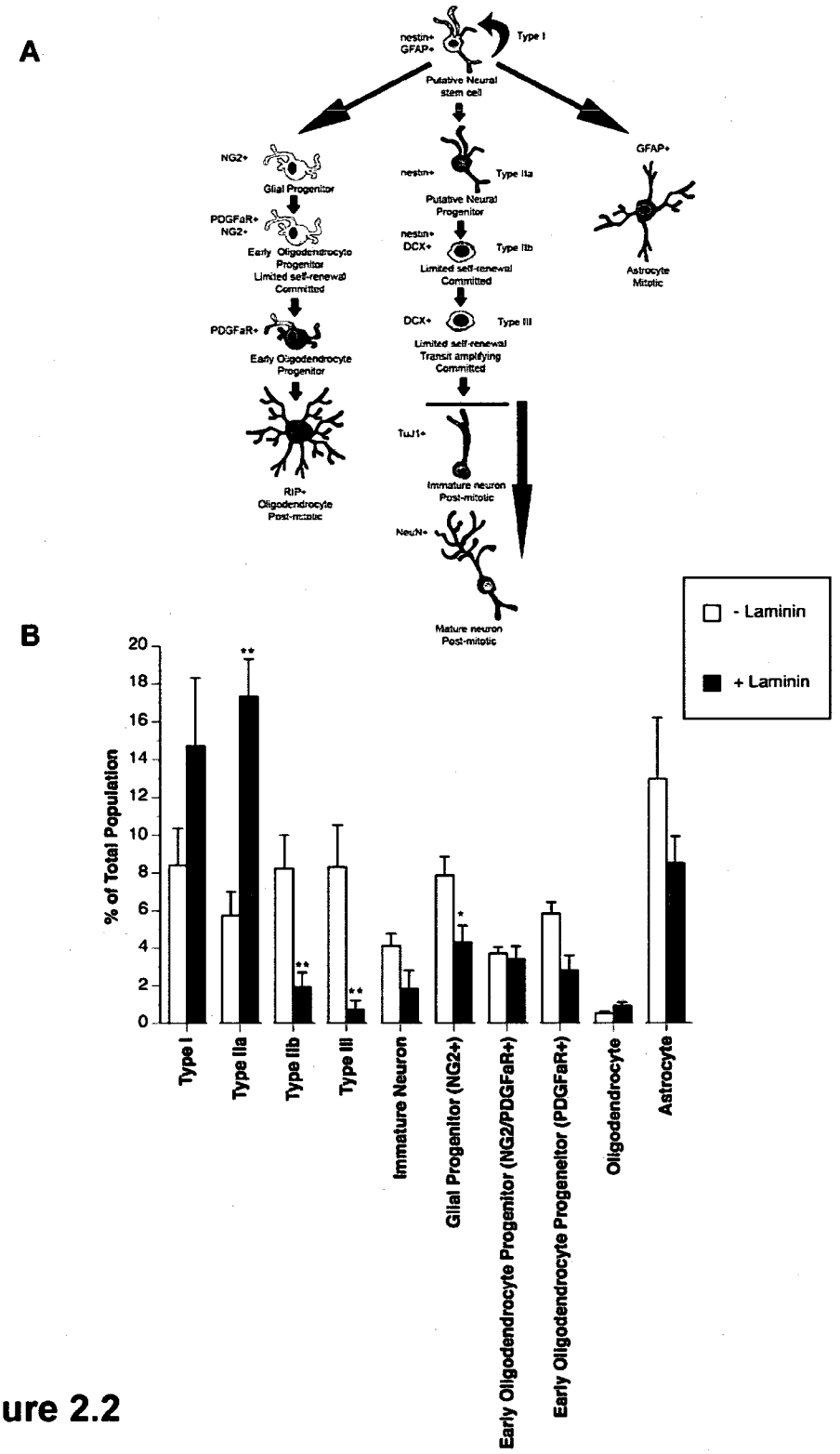
A
nestin+
GFAP+
Type I
Putative Neural
stem cell
NG2+
Glial Progenitor
PDGFaR+
NG2+
Early Oligodendrocyte
Progenitor
Limited self-renewal
Committed
PDGFaR+
Early Oligodendrocyte
Progenitor
RIP+
Oligodendrocyte
Post-mitotic
nestin+
Type IIa
Putative Neural
Progenitor
nestin+
DCX+
Type IIb
Limited self-renewal
Committed
DCX+
Type III
Limited self-renewal
Transit amplifying
Committed
TuJ1+
Immature neuron
Post-mitotic
NeuN+
Mature neuron
Post-mitotic
GFAP+
Astrocyte
Mitotic
B
- Laminin
+ Laminin
% of Total Population
0
2
4
6
8
10
12
14
16
18
20
**
**
**
*
Type I
Type IIa
Type IIb
Type III
Immature Neuron
Glial Progenitor (NG2+)
Early Oligodendrocyte Progenitor (NG2/PDGFaR+)
Early Oligodendrocyte Progenitor (PDGFaR+)
Oligodendrocyte
Astrocyte

**Figure 2.2**

Type IIb cells express both nestin and DCX while Type III cells express solely DCX. Type IIb and Type III cells are both committed toward a neuronal lineage with Type III cells defined as being migratory neuroblasts, the final stage before exiting the cell cycle and becoming immature neurons.

Together, these data suggest that laminin engagement promotes retention of a more unspecialized phenotype whereas cultures grown in suspension in the absence of laminin specify more readily to immature neurons. Further, we found that Cx36 mRNA is expressed by NPCS but that protein expression is first detected following exposure to laminin.

## 2.4 Discussion

A recent publication from our laboratory has shown for the first time that postnatal hippocampal derived NPCs express a gamut of connexins and that this expression is directly influenced by exposure to an ECM, laminin (Imbeault et al., 2008). Critical to this thesis, Cx36 mRNA only is expressed in NPCs grown in suspension for 14 DIV while *de novo* protein expression is elicited upon laminin engagement. Much work still needs to be done to decipher how ECM interactions generate inductive signals that regulate protein expression. One explanation is a direct effect of laminin/integrin signalling 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 (Lampe et al., 1998; Guo et al., 2001; Isakson et al., 2006). This study, however, is the first to demonstrate that the expression of Cx36 protein is ECM-regulated.

These findings are important in light of previous reports that two crests of Cx36 mRNA and protein expression are found in the developing murine brain: one

embryonically during the period of greatest neurogenesis in the neocortex and one postnatally, when mature neurons become coupled (Sohl et al., 1998; Gulisano et al., 2000; Prime et al., 2000; Cina et al., 2007). This *bimodal* expression may suggest that Cx36 protein expression is important during milestones of neuronal development. Following this argument, it is suggested that Cx36 mRNA expression is up-regulated upon differentiation of mouse embryonic hippocampal progenitor cells (MK31) *in vitro* and hippocampal neurons isolated from E18 mice exhibit functional coupling mediated by Cx36 (Rozental et al., 2000). Taken together, these data instigated the key experimental questions formative to my thesis: (1) does developmental loss of Cx36 alter the identity of NPCs retained in the postnatal hippocampus and (2) what role does protein expression play in hippocampal NPC proliferation, specification and differentiation.

These data also highlighted a potential disconnect between our hypothesis and Cx36 mRNA/protein phenotype that guided subsequent experiments. We found that Cx36 protein was detected in cells with an immature neuronal phenotype following laminin engagement and thus we predicted that this expression would coincide with an increase in committed NPCs to a neuronal lineage. However, upon antigenic lineage assessment of plated neurospheres, a retention of a multipotential Type IIa nestin$^{+}$ phenotype and a reduction in terminal neuronal differentiation was observed that will be further characterized in Chapters 3 and 4.

These results also suggested that a integrin signalling is important for upregulation of Cx36 protein expression, independently of a direct connexin effect on NPC fate. Indeed the pattern and expression of ECM components regulate the expansion, differentiation and migration of embryonic-derived stem cells (Leone et al., 2005;

Flanagan et al., 2006; Lathia et al., 2007). There have been many previous studies that have attempted to underline the importance of laminin as an integral ECM component for NPC proliferation and commitment, albeit with varying results depending on where the cells were derived, human versus murine as well as media components. Using hESCs, it has been shown that laminin or laminin-Matrigel had a host of effects on the culture system including regulating the expansion of NPCs and promoting differentiation to neurons and astrocytes (Flanagan et al., 2006; Ma et al., 2008). Integrin-ECM signalling, in cooperation with growth factor signalling, was also shown to play an essential role in promoting proliferation and progenitor cell survival in hESC-derived neurospheres while, debatably, maintaining the undifferentiated, nestin$^{+}$ neural stem cell populations (Leone et al., 2005; Hall et al., 2008). Here, it is clear the expression of Cx36 is under the influence of laminin, although the exact mechanism is still not understood. Together, these initial studies were pivotal in composing the rationale for studying Cx36 during postnatal hippocampal neurogenesis.

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# Chapter 3: Cx36 accelerates NPC specification over the course of neurogenesis[3]

## 3.1 Objective of this Study

In order to determine the role of Cx36 in postnatal hippocampal neurogenesis, I performed loss of function studies examining the effect of constitutive Cx36 null-mutation on proliferation and commitment of NPCs cultured *in vitro*. This chapter is presented in the form of a paper in preparation.

## 3.2 Statement of authors' contributions

CDS and SALB conceived and designed the experiments. CDS also performed all the experimental work. CDS and RD performed all of quantitative analyses with RD under the mentorship of CDS. CDS wrote the manuscript. DLP provided the null-mutant mice and assisted/hosted CDS in the preparation of Cx36 lentivirus to perform gain of function experiments that will be used to complete this paper in future experiments.

## 3.3 Introduction

Mobilizing or introducing populations of NPCs now known to exist in the adult mammalian brain, in injured tissue represents a novel, potentially transformative, approach to brain repair. Validation, however, will require a comprehensive understanding of how NPCs are directed to adopt different CNS lineages. In adult tissue,

[3] In preparation: Sorbara CD, DesRoches R, Paul DL, Bennett SAL (2008). **Connexin36 is a novel regulator of post-natal neurogenesis**

NPC specification to a specific neural lineage involves several cell intermediates, each exhibiting increasingly restricted lineage commitment and variable ability to self-replicate (Doetsch et al., 1999; Kempermann et al., 2004). The SGZ of the hippocampus is one of two well-established neurogenic niches in the adult mammalian brain. Cells born here migrate to the granule layer of the dentate gyrus where they become mature granule neurons and send axonal projections to the CA3 region (Markakis and Gage, 1999; Kempermann et al., 2003; Kempermann et al., 2004).

There are many factors that are involved in specification to a given cell lineage. Of them, the microenvironment plays an important role in directing progenitor cell proliferation, migration, and specification. In addition to paracrine factors, cell-cell interaction between progenitor cells and adjacent terminally differentiated cells has been shown to influence progenitor cell fate (Song et al., 2002). One potential signaling mechanism underlying this control is connexin-mediated communication. Oligomerization of six connexin proteins form the structural units of hemichannels or connexons in non-junctional membranes. When two connexons from apposing cells dock they form gap junction channels, providing a conduit between cells for the passive diffusion of molecules less than 1 kilodalton in size. Connexins can also elicit intracellular signaling through protein-protein interaction with their C-terminal tail (reviewed in (Connors and Long, 2004; Sohl et al., 2005)). Previous studies have provided evidence of connexin-mediated control of NPC fate. This includes changes in connexin expression and localization during development and postnatally (Maxeiner et al., 2003; Cina et al., 2007; Imbeault et al., 2008), changes in the properties of gap junction following neuronal differentiation (Rozental et al., 1998), as well as changes in

progenitor pool populations and their ability to differentiate following connexin deletion (Melanson-Drapeau et al., 2003).

Cx36, one of 21 connexin family members, is expressed by terminally differentiated neurons. Expression is a necessary element for synchronous, electrotonic coupling between neurons in many regions of the brain including the hippocampus (Deans et al., 2001; Hormuzdi et al., 2001). We have previously found that Cx36 mRNA is expressed by NPCs isolated from the hippocampi of postnatal day 0-3 (P0-3) mice and cultured as neurospheres yet protein remains undetectable until these cells are exposed to a laminin matrix and commit to a neuronal lineage (Imbeault et al., 2008)[4]. This suggests a role for Cx36 expression in the specification of NPCs. Here, we describe a series of loss of function studies designed to test this hypothesis.

### 3.4 Experimental Procedure

*Mice*

$Cx36^{-/-}$ mice were kindly provided by Dr. David Paul (Harvard University) and were compared to congenic N4 wild-type (WT) littermates. Mice were kept on a 12 hr light-dark cycle and allowed food and water *ad libitum*. 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.

*Neurosphere cultures*

[4] These data are presented in Chapter 2 of this thesis

Cultures were prepared from P0-P3 mice pups euthanized by lethal injection with sodium pentobarbital. The brains were removed and placed in cold artificial cerebral spinal fluid (aCSF; 26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM D-glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, pH 7.3). They were then sliced coronally (500 μm) using a Leica VT1000S vibratome and slices within the hippocampus between bregma -1.60 mm and -2.10 mm were collected. The slices were then moved to a Leica MZ6 dissecting microscope to dissect out the hippocampus and remove surrounding connective tissue. The pooled tissue was then minced with a scalpel and placed in 2.5 ml of dissociation media (26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2.2H_2O$, 3.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM D-glucose, 1 % penicillin/streptomycin, 0.1 % neural protease, 0.01 % papain, and 0.01 % DNase I). The tissue was then incubated with rotation for 1 hr at 37 °C. Sterile 10 mM PBS was then added and cells were centrifuged and resuspended in 5 ml of sterile PBS, dissociated by trituration, centrifuged a second time and resuspended in 3 ml of Maintenance Media consisting of Dulbecco's modified Eagle medium (DMEM/F12) with B27 supplement (100 U/ml), penicillin/streptomycin (100 U/ml) and L-glutamine (2 mM). Cultures were plated at a density of $2.5x10^5$ cells/ml in 6 cm Ultra low attachment polysterene dishes (Fisher). Growth factors, basic fibroblast growth factor (FGF-2; 20 ng/ml, Invitrogen) and epidermal growth factor (EGF; 10 ng/ml, Invitrogen) were given on day 2, 4, and 6 *in vitro* and cells were grown for eight days *in vitro* (DIV). On DIV 7, neurospheres were pulsed with bromodeoxyuridine (BrdU) at a final concentration of 20 μg/ml 24 hr prior to plating to label proliferating cells.

On DIV 8, neurospheres were plated onto laminin-coated glass coverslips in 10 cm tissue culture dishes (Corning, NY, USA) following two washes with 10 mM PBS to remove access BrdU. Cells were grown in three types of media:

1. Spontaneous: Maintenance Media without growth factor supplements
2. Mitogenic: Maintenance Media supplemented with 20 ng/ml EGF and 10 ng/ml FGF-2 every other day
3. Neurogenic: High glucose DMEM/F12 (DMEM/F12, 9.3 g/L D-glucose, 2 mM Glutamax), 6 mM Glutamax, 40 μg/ml Insulin, 40 μg/ml Apo-transferrin, 1x ITS-G, 1x MEM vitamin solution, 100 U/ml B27 supplement, 1 % penicillin/streptomycin

Neurospheres were cultured 6 days and every other day half of the media was removed and replenished with fresh media. On DIV 14 cells were fixed with 4 % paraformaldehyde for 10 min, coverslips were removed and immunocytochemistry was performed.

Alternatively, some cultures were grown in suspension for DIV 8 and were pulsed with the aforementioned amount of BrdU for 24 hr. Following this, they were washed to remove access, unincorporated BrdU, fixed with 10 % formaldehyde for 10 min and cryoprotected in 20 % sucrose solution in 10 mM PBS containing 0.001 % sodium azide for 48 hr. Cells were then frozen in sucrose blocks and sections of 10 μm thickness were prepared using a cryostat (Leica Microsystems Inc., Richmond Hill, ON) for subsequent immunocytochemistry.

*Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL)*

Apoptotic cells were detected by TdT-mediated dUTP nick end labeling. Fixed neurospheres were permeabilized by a 5 min incubation in 0.1 % Triton-X/0.1 % sodium citrate on ice. Sections were washed for 2 min in 10 mM PBS and incubated in a humidity chamber for 1 hr at 37 °C with FITC-labeled dUTP in TdT buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) and TdT according to the manufacturer's protocol (Roche). $TUNEL^+$ cells in neurospheres were calculated as a percentage of the total number of cells positive for Hoecsht staining and averaged over ten neurospheres. All counts were carried out by two independent investigators with at least one investigator blind as to the identity of the cell genotype. Counts represent the average of both investigators per field.

*In vitro immunocytochemistry*

Antibodies are listed in Table 3.1 and were diluted in Antibody buffer (3 % bovine serum albumin, 0.3 % Triton X-100 in 10 mM PBS). Hoechst 33258 was used as a nuclear counterstain at 1 μg/ml for differentiated neurospheres. Primary antibodies were incubated at 4 °C overnight. The following day, two 15 minute washes in 10 mM PBS were performed followed by an 1 hr incubation with secondary antibodies, an identical wash treatment and coverslipping in an "antifade" mounting media (0.1% P-phenylenediamine, 90 % glycerol in 10 mM PBS). For immunocytochemistry involving BrdU, neurospheres were first incubated in 2 N HCl at 37 °C for 1 hr followed by two 10 minute neutralization washes in 0.1 M borate buffer, pH 8.5 and two 5 minute washes in 10 mM PBS prior to primary antibody incubation.

In order to analyze differentiated neurospheres, a core area was defined as the area in which one could not distinguish and count single cells while viewing under phase contrast microscopy. Five fields were then defined 15 μm from the core and analyzed for colocalization of Hoescht 33528 nuclear stain with lineage markers. This process was repeated for at least five neurospheres per condition and averaged. Fold change in % BrdU was calculated as the % BrdU at DIV 14 (6 DIV after plating on laminin) divided by the average % BrdU at DIV 8 (24 h after plating on laminin) for each genotype. All counts were performed by a minimum of two and a maximum of three independent investigators blind as to the identity of the conditions as described above.

**Table 3.1 List of Primary and Secondary Antibodies used for *in vitro* Immunocytochemistry**

| Antibody | Lineage Detected | Species | Source | Dilution |
|---|---|---|---|---|
| BrdU (bromodeoxyuridine) | Cell Proliferation | Mouse | Roche | 6 μg/ml |
| DCX (doublecortin) | Type IIb/Type III neuronal progenitors | Guinea Pig | Chemicon | 1:400 |
| GFAP (glial fibrillary acidic protein) | Astrocytes/Type I neural stem cells | Rat | Zymed | 1:10 |
| | | Rabbit | Sigma | 1:50 |
| Nestin | Type I, Type IIa/b neural progenitors | Mouse | Chemicon | 1:25 |
| NeuN | Mature and newly born neurons | Mouse | Chemicon | 1:50 |
| NG2 chondroitin sulfate proteoglycan | Glial and early oligodentrocyte progenitors | Rabbit | Chemicon | 1:100 |
| PDGFαF (platelet-derived growth factor receptor alpha) | Late oligodendrocyte progenitors | Rat | BD Pharm | 1:333 |
| RIP | Mature oligodendrocytes | Mouse | Chemicon | 1:1000 |
| TuJ1 (class III β-tubulin) | Immature neurons | Mouse | RDI | 1:125 |
| Guinea Pig IgG | Cy3-conjugated | | Jackson | 1:400 |
| Mouse IgG | Cy3-conjugated | | Jackson | 1:400 |
| | FITC-conjugated | | Jackson | 1:50 |
| Rabbit IgG | Cy3-conjugated | | Jackson | 1:300 |
| | FITC-conjugated | | Jackson | 1:50 |
| Rat IgG | Cy3-conjugated | | Jackson | 1:300 |
| | FITC-conjugated | | Jackson | 1:200 |

## 3.5 Results

### 3.5.1 Neurogenic culture conditions promote differentiation of wild-type nestin$^+$ NPCs to Type III DCX$^+$ neuroblasts and TuJ1$^+$ immature neurons

In order to determine the role of Cx36 protein expression on NPC fate, postnatal hippocampal neurosphere cultures were isolated from P0-P3 WT and Cx36 $^{-/-}$ mice. We first validated our model of *in vitro* neuronal specification under culture conditions that support both Cx36 mRNA and protein expression. Postnatal NPCs, expanded in suspension as neurospheres for 8 DIV, were plated on laminin and cultured for a further 6 DIV (a) in the presence of mitogens to promote proliferation and the maintenance of a NPC phenotype, (b) in the absence of mitogens to promote spontaneous differentiation, and (c) in defined neurogenic media to promote neuronal specification. The identity of cells in each culture was assessed by immunocytochemistry using lineage-specific markers (Fig 3.1A). Spontaneous differentiating conditions was associated with a reduction in nestin$^+$ NPCs and NG2$^+$ OPCs likely as a result of death elicited by growth factor withdrawal further evidenced by the significant increase in the number of TUNEL$^+$ cells (Fig 3.1 B, Inset). Neurogenic conditions promoted the differentiation of nestin$^+$ Type I and/or IIa NPCs to DCX$^+$ Type III neuroblasts and immature neurons (Fig 3.1B). Overall, the population of DCX$^+$ neuroblasts and TuJ1$^+$ immature neurons was 28.5% ± 3.4, a significant increase from 2.5% ±1.1 in mitogenic conditions and 2.3% ± 0.3 under spontaneous conditions.

**Figure 3.1 Multi-potential post-natal hippocampal NPCs can be induced to differentiate to neurons, astrocytes, and oligodendrocytes *in vitro*.**

(A) Schematic of lineage markers used to identify glial and neuronal progenitor cells and their progression towards terminal commitment to neurons, astrocytes and oligodendrocytes, respectively. (B) Culture onto a laminin matrix with media containing neurogenic paracrine factors (insulin, transferrin, selenium, MEM vitamin solution, increased glucose concentration) increases the number of nestin$^+$ NPCs that commit to a neuronal lineage compared to cells plated on laminin with mitogenic or spontaneous media. Removal of growth factors initially proposed to elicit spontaneous differentiation resulted in a significant reduction in nestin$^+$ NPCs and NG2$^+$ OPCs likely as a result of death elicited by growth factor withdrawal evidenced by the significant increase in the number of TUNEL$^+$ cells (inset). Data represent the mean of 25 counted fields (10 neurospheres, 5 representative fields per neurosphere) ± SEM. ** $p<0.005$, df=74, One-Way ANOVA followed by Tukey test

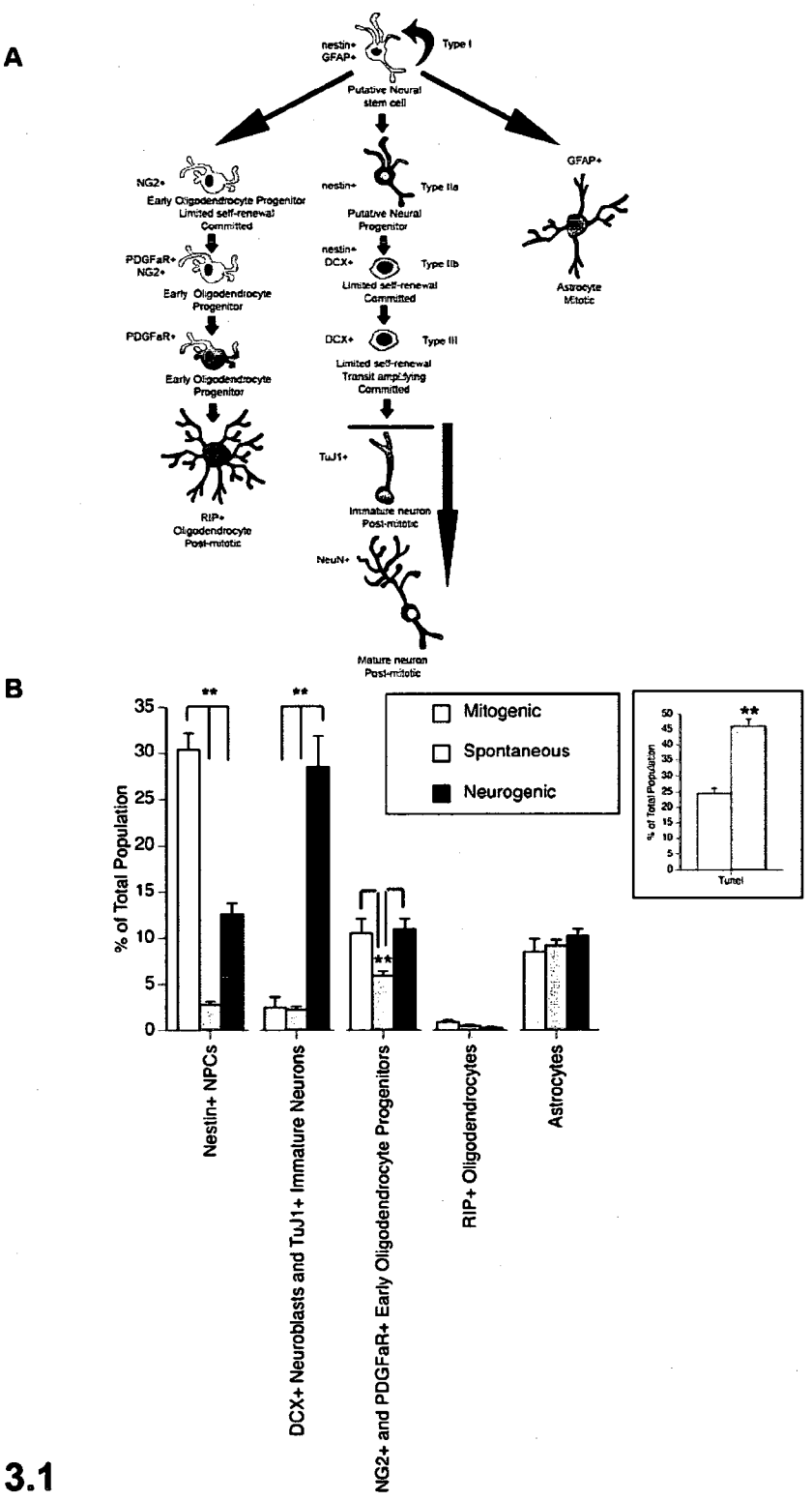

A
nestin+
GFAP+
Type I
Putative Neural stem cell
NG2+
Early Oligodendrocyte Progenitor
Limited self-renewal
Committed
PDGFaR+
NG2+
Early Oligodendrocyte Progenitor
PDGFaR+
Early Oligodendrocyte Progenitor
RIP+
Oligodendrocyte
Post-mitotic
nestin+
Type IIa
Putative Neural Progenitor
nestin+
DCX+
Type IIb
Limited self-renewal
Committed
DCX+
Type III
Limited self-renewal
Transit amplifying
Committed
TuJ1+
Immature neuron
Post-mitotic
NeuN+
Mature neuron
Post-mitotic
GFAP+
Astrocyte
Mitotic
B
Mitogenic
Spontaneous
Neurogenic
% of Total Population
Nestin+ NPCs
DCX+ Neuroblasts and TuJ1+ Immature Neurons
NG2+ and PDGFaR+ Early Oligodendrocyte Progenitors
RIP+ Oligodendrocytes
Astrocytes
Tunel

**Figure 3.1**

### 3.5.2 Loss of Cx36 enhances NPC differentiation/commitment to oligodendrocytes and/or neurons under mitogenic and spontaneous differentiation conditions *in vitro*

There was a significant increase in the commitment of NPCs to an oligodendrocyte lineage concomitant with a decrease in the number of nestin$^{+}$ NPCs and nestin-/GFAP$^{+}$ astrocytes in cultures devoid of Cx36 when grown under conditions designed to maintain NPCs in an undifferentiated and proliferative state (Fig 3.2A). In media devoid of mitogens, Cx36$^{-/-}$ cultures spontaneously differentiated to both oligodendrocytes and neurons at a greater frequency than WT cultures (Fig 3.2B). As well, there was an increase in the number of astrocytes in Cx36$^{-/-}$ cultures under spontaneous differentiation conditions. These results suggested that Cx36 itself may be involved in regulating NPC specification or the number of immature neurons and oligodendrocytes under appropriate microenvironment conditions.

### 3.5.3 Cx36 expression modulates NPC response to neurogenic stimuli *in vitro*

We next sought to determine whether loss of Cx36 alters NPC response in conditions promoting neurogenesis. Once again using our neurosphere model, cells from both WT and Cx36$^{-/-}$ cultures were plated in neurogenic media. Antigenic lineage analysis was performed after 8 days of expansion as neurospheres and a subsequent 6 DIV of culture on laminin-coated coverslips in the presence of neurogenic factors. Under these conditions, a significantly higher percentage of neuroblasts and TuJ1$^{+}$ immature neurons were evident in null-mutant compared to WT cultures (Fig 3.3A,B). This increase was accompanied a decrease in the number of proliferating nestin$^{+}$ NPCs suggesting enhanced specification and/or survival of committed neuronal progeny. There

**Figure 3.2 Oligodendrogenesis and neurogenesis is enhanced in $Cx36^{-/-}$ postnatal hippocampal neurospheres plated on laminin in mitogenic and spontaneous media**

Culture composition of $Cx36^{-/-}$ and WT postnatal hippocampal neurospheres at 14 DIV following expansion in suspension for 8 DIV and plating onto laminin for 6 DIV. In (A) cell identity was assessed following culture in mitogen-containing media. In (B) cell identity was assessed following withdrawal of growth factors thus supporting spontaneous differentiation. A significant increase in neurogenesis and gliogenesis was detected under these conditions in the absence of Cx36. Data represent the mean of 25 counted fields (10 neurospheres, 5 representative fields per neurosphere) ± standard error of the mean (SEM). ** $p<0.005$, * $p<0.01$, $df = 48$, Student's *t*-test

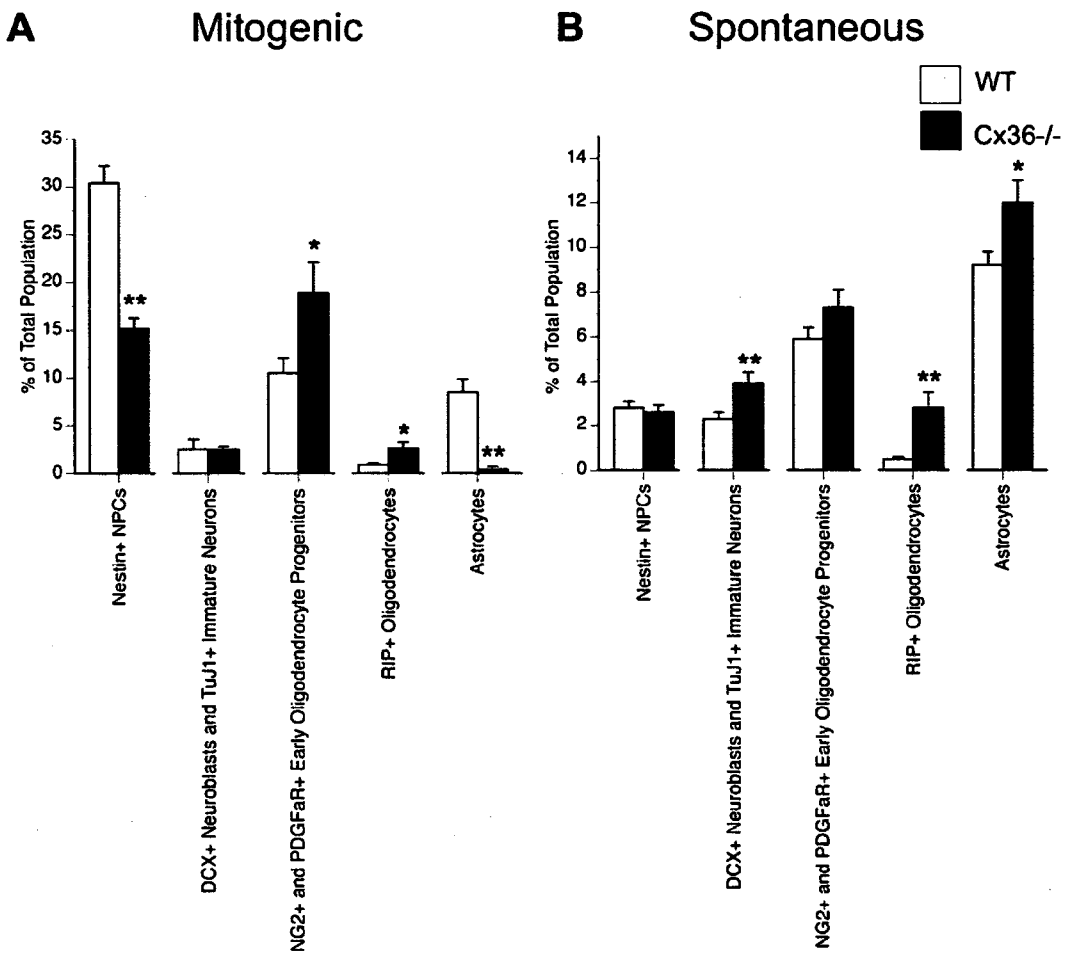

A
Mitogenic
B
Spontaneous
WT
Cx36-/-
% of Total Population
35
30
25
20
15
10
5
0
14
12
10
8
6
4
2
0
**
*
Nestin+ NPCs
DCX+ Neuroblasts and TuJ1+ Immature Neurons
NG2+ and PDGFaR+ Early Oligodendrocyte Progenitors
RIP+ Oligodendrocytes
Astrocytes

**Figure 3.2**

**Figure 3.3 Postnatal hippocampal Cx36$^{-/-}$ neurospheres differentiate to immature neurons at a higher frequency than WT mice in the presence of neurogenic stimuli**

(A) Culture composition of Cx36$^{-/-}$ and WT postnatal hippocampal neurospheres at 14 DIV following plating on laminin at 8 DIV and replacement of maintenance media with neurogenic media. Data represent the mean of 25 counted fields (10 neurospheres, 5 representative fields per neurosphere) ± SEM. (B) Representative confocal images of laminin adhered neurospheres from WT (left) and Cx36$^{-/-}$ (right) postnatal hippocampi grown in neuronal driving media. Immature neurons expressing TuJ1 are shown. Scale bars, 20 μm. ** $p<0.005$, df = 48, Student's *t*-test

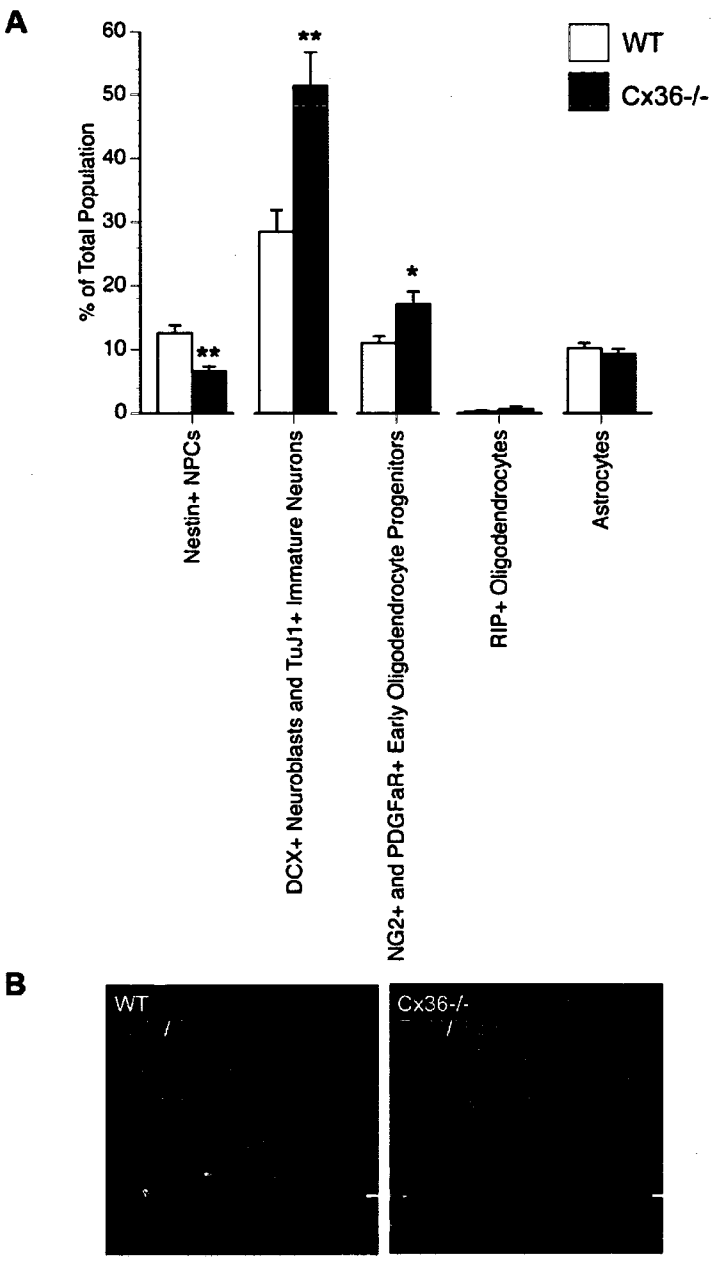

A
60
50
40
30
20
10
0
% of Total Population
**
**
*
WT
Cx36-/-
Nestin+ NPCs
DCX+ Neuroblasts and TuJ1+ Immature Neurons
NG2+ and PDGFaR+ Early Oligodendrocyte Progenitors
RIP+ Oligodendrocytes
Astrocytes
B
WT
Cx36-/-

**Figure 3.3**

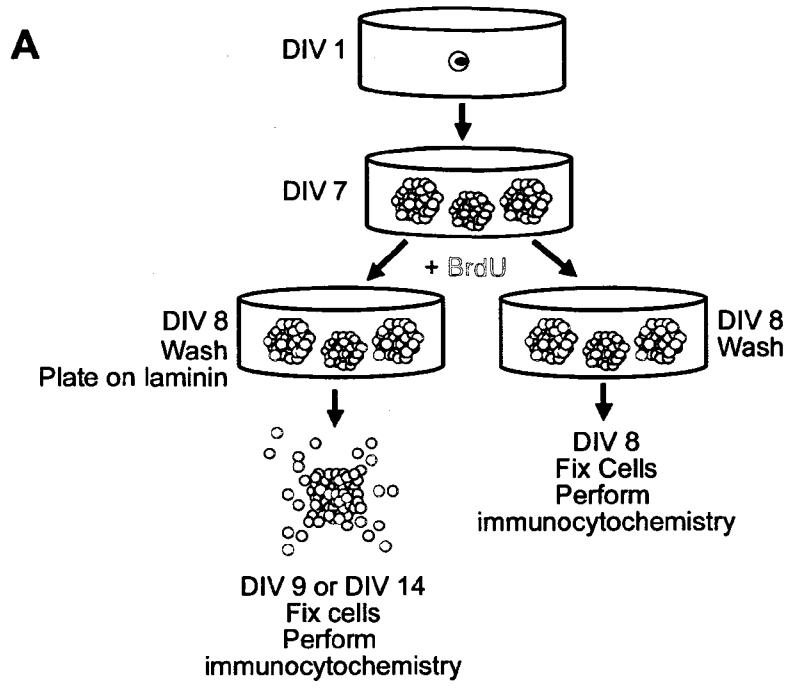
was also an appreciable increase in the number of oligodendrocyte progenitors in $Cx36^{-/-}$ cultures compared to controls (Fig 3.3A).

In order to determine the cause of this increase, we focused on characterizing NPC fate in neurogenic media, wherein the greatest increase in terminal differentiation was observed between genotypes. First, we evaluated cell proliferation in neurospheres grown as suspension. Here, Cx36 mRNA but not protein is expressed in WT cultures. As expected, no difference in BrdU labelling was detected between $Cx36^{+/+}$ and $Cx36^{-/-}$ cultures when cells were pulsed with BrdU and assessed 24 hr later (Fig 3.4). BrdU is an analog of thymidine that becomes incorporated into the DNA of cells during S phase of the cell cycle and can be detected immunochemically. The label will dissipate from the cells after approximately three cell cycles (reviewed in (Taupin, 2007)). Thus, BrdU provides information about the relative number of proliferating cells in relation to the number of times the cells divide in the presence of BrdU as well as the survival of these cells that have taken up the label.

Having determined that BrdU labels the same number of proliferating cells WT and $Cx36^{-/-}$ cultures grown in suspension, we next assessed the survival of these BrdU-labelled cells following laminin engagement shown to induce Cx36 protein expression in WT cultures (Fig 2.1). Neurospheres were pulsed for 24 hr on DIV 7 while grown in suspension. Media was removed and cultures were then plated on laminin in BrdU-free media for an additional 1 DIV or 6 DIV (Fig 3.5A). We found that more BrdU-labelled cells survived plating in null-mutant compared to WT cultures. Although we cannot rule out the possibility that this increase was due to the proliferation of labelled NPCs in $Cx36^{-/-}$ but not WT cultures, the near 4-fold increase in $BrdU^{+}$ cell number over a 24 hr

**Figure 3.4 Cx36 null mutation does not alter NPC proliferation**

(A) Neurosphere cultures were pulsed for 24 hours with 20 μg/ml of BrdU at DIV 7. On DIV 8, cultures were washed with PBS and either plated onto laminin in neurogenic media to track the fate of cells labelled in suspension (see Fig 3.5) or immediately fixed and collected for immunocytochemistry. (B) No difference in BrdU incorporation was detected between genotypes in suspension cultures expressing Cx36 mRNA but not protein (see Fig 2.1). Data shown represent the mean of 10 neurospheres/genotype ± SEM


A
DIV 1
DIV 7
+ BrdU
DIV 8
Wash
Plate on laminin
DIV 8
Wash
DIV 8
Fix Cells
Perform
immunocytochemistry
DIV 9 or DIV 14
Fix cells
Perform
immunocytochemistry

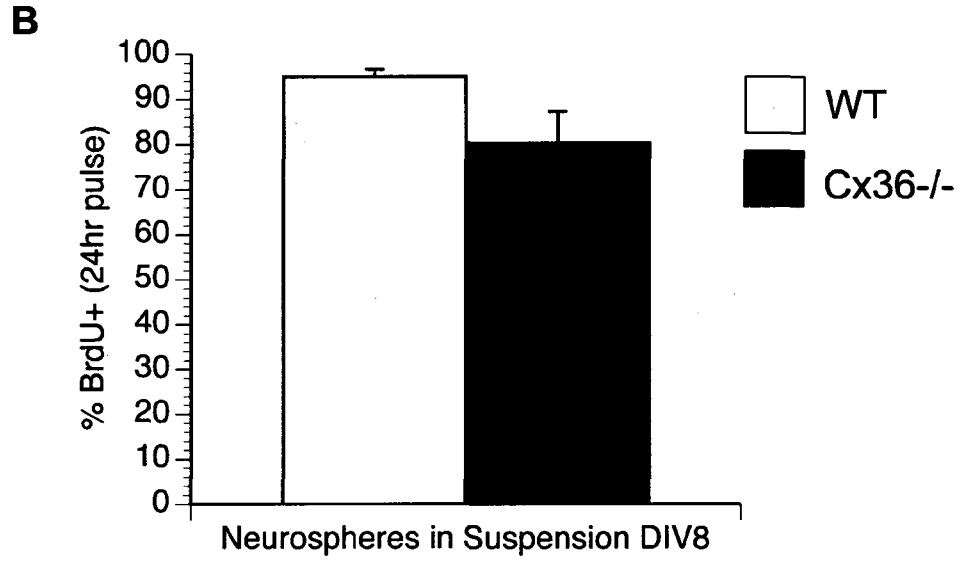

B
% BrdU+ (24hr pulse)
100
90
80
70
60
50
40
30
20
10
0
Neurospheres in Suspension DIV8
WT
Cx36-/-

**Figure 3.4**

**Figure 3.5 Cx36$^{-/-}$ neurospheres exhibit altered proliferation and survival profiles upon plating onto laminin under neurogenic conditions**

Induction of Cx36 protein by laminin engagement promotes NPC survival and/or proliferation followed by specification. We tracked the fate of cells labelled with BrdU in suspension. (A) There was a significant increase in the number of BrdU-labelled cells following plating onto laminin in neurogenic media at 1 DIV and 6 DIV. Note that this increase could be due either to (i) enhanced survival of labelled cells or (ii) enhanced proliferation as BrdU-labelled progeny arising from division of pre-labelled cells can be detected for up to 3 divisions/generations. (B) By standardizing the number of cells retaining BrdU label after DIV 6, it became evident that the same number of BrdU-labelled cells survive in Cx36$^{-/-}$ where WT cultures continued to proliferate for at least 3 division (C) The relative amount of cells labelled by TUNEL was also unchanged in Cx36 $^{-/-}$ cultures. Together, these data that loss of Cx36 may enhance cell survival upon plating on laminin (A) and accelerate neuronal specification (B) without significantly impacting upon cell death (C). Data shown represent the mean of 10 neurospheres/genotype ± SEM. * $p<0.01$, df = 48, Student's *t*-test

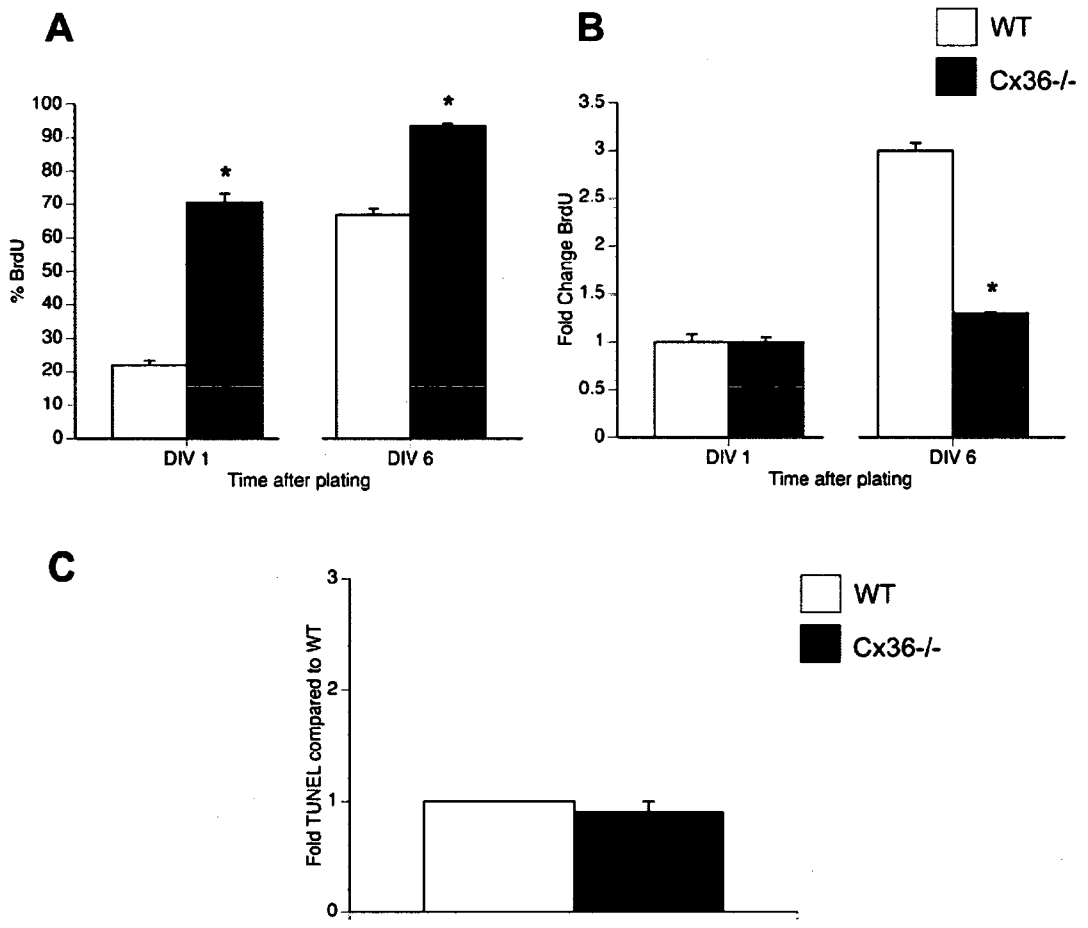

A
B
WT
Cx36-/-
100
90
80
70
60
50
40
30
20
10
0
% BrdU
*
*
DIV 1
DIV 6
Time after plating
3.5
3
2.5
2
1.5
1
0.5
0
Fold Change BrdU
*
DIV 1
DIV 6
Time after plating
C
WT
Cx36-/-
3
2
1
0
Fold TUNEL compared to WT

**Figure 3.5**

period makes this unlikely as it would represent approximately 2 doublings or an unlikely 12 cell cycles. Thus, the simplest explanation is that more BrdU-labelled cells survived plating in the absence of Cx36. This conclusion is strengthened by evidence that, where WT cultures were able to sustain limited division, as indicated by the 3-fold increase in the number of BrdU-labelled cells present over the subsequent 6 days in culture (a 1.5 doubling rate with the expected ~72 hr cell cycle), null-mutant cultures exhibited only a 1.3 fold increase in BrdU-labelled cells. Together, these data suggest that loss of Cx36 promotes more rapid terminal specification in the absence of continued proliferation (Fig 3.5B).

Alternatively, it is also possible that the rate of neuronal death was reduced in Cx36 null-mutant neurons compared to WT accounting for enhanced cell survival upon plating (Fig 3.5A) and lack of significant cell expansion (Fig 3.5B). To assess this, apoptotic death was quantified by TUNEL labelling. The number of cells undergoing apoptosis was not significantly different in the $Cx36^{-/-}$ cultures relative to WT control (Fig 3.5C). These results show that Cx36 expression modulates NPC response to neurogenic stimuli by promoting NPC commitment upon exposure to laminin, likely by inhibiting cell proliferation, without affecting cell death following initial plating.

### 3.6 Discussion

We first wanted to assess the intrinsic potential of Cx36 to restrict or enhance neurogenesis *in vitro* using neurosphere cultures of primary hippocampal NPCs isolated from WT and $Cx36^{-/-}$ neonates. Here, clusters of NPCs are grown in suspension in the presence of mitogens for 8 DIV. Following this expansion, they are transferred to plates that are coated with an extracellular matrix protein, laminin, to induce differentiation.

Previous results from our laboratory have shown that it is here, upon monolayer culture, that Cx36 protein expression is first initiated (Imbeault et al., 2008)[5].

Here, we have shown that Cx36 intrinsically promotes neurogenesis and oligodendrogenesis as its deletion had a significant effect on the number of cells that spontaneously adopted a $TuJ1^+$ neuronal phenotype or $RIP^+$ oligodendrocyte both upon differentiation following mitogen removal and when cells were exposed to strong extrinsic neurogenic stimuli. This suggests that attenuation of Cx36 could induce more efficient differentiation of NPCs toward a neuronal lineage within a permissive microenvironment. Recently, primary astrocytes isolated from neurogenic regions in the adult rodent brain were shown to modulate stem cell differentiation through expression of niche-specific factors while primary astrocytes from non-neurogenic regions had no affect (Lim and Alvarez-Buylla, 1999; Song et al., 2002; Nakayama et al., 2003; Barkho et al., 2006). Similarly, the effect of Cx36 expression on neuronal number is evident when environmental cues are favourable for differentiation.

The impact of Cx36 null-mutation on oligodendrogenesis is more complex. Loss of Cx36 also resulted in an increase in early OPCs following neurogenic induction or terminally differentiated oligodendrocytes under spontaneous differentiation conditions mediated by growth factor withdrawal. One explanation is that the neurons may in fact instruct neighbouring NPCs towards an oligodendrocyte lineage. Previously, it had been shown that neuron-enriched cultures had no effect on the promoting neurogenesis in co-culture with adult neural stem cells but did significantly promote differentiation towards oligodendrocytes (Song et al., 2002). It has also been shown that during corticogenesis,

[5] See chapter 2.

neuron and glia production proceed in an ordered manner with neurogenesis preceding oligodendrogenesis based upon extracellular cues and intrinsic property changes (Qian et al., 2000; Morrow et al., 2001). An enhanced population of neurons may therefore be involved in programming the successive differentiation of glia. This evidence suggests that the impact of Cx36 on oligodendrogenesis may be indirect, possibly due to the more rapid development of neurons and their subsequent impact of residual NPCs. It is interesting that, under mitogenic conditions, astrogenesis was reduced in null-mutant cultures whereas following growth factor removal astrogenesis was reciprocally enhanced. It is also tempting to speculate that exclusion of Cx36 protein expression may alter this inherent schedule whereby neurogenesis precedes gliogenesis so in the absence of significant paracrine stimuli, gliogenesis occurs at a higher rate in null mutant cultures compared to WT.

Thus, these dual effects may be due to loss of compartmentalization between developing neurons and NPCs within our null-mutant cultures. Compartmentalization has been shown to be important during neocortical development to assist in differentiation of subsets of cells depending on connexin coupling and expression (Bittman et al., 1997; Bittman and LoTurco, 1999). Indeed, spatiotemporal expression of Cx45 has been suggested to play a role in neuronal function (Maxeiner et al., 2003) and loss of Cx32 expression, an oligodendrocyte-specific connexin, decreases the ability of progenitor cells to terminally differentiate (Melanson-Drapeau et al., 2003). Recent microarray studies have also suggested that connexins control the expression of genes independent of the gap junction function creating networks of transcriptomes unique to

CNS cell types and perhaps serving to induce specification of progenitor cells (Iacobas et al., 2007).

We cannot rule out that the impact of Cx36 null-mutation on NPC specification is the result of compensation by functionally similar connexins. It should also be mentioned that recently discovered pannexin 1 and pannexin 2 (Panx2) proteins, structurally similar to connexins but whose function and potential gap junction properties have yet to be fully elucidated, as well as neuronal Cx45 could potentially compensate for the developmental loss of Cx36 due to the overlap in their expression in the postnatal rodent brain (Maxeiner et al., 2003; Baranova et al., 2004; Vogt et al., 2005). To provide further evidence of a direct effect of Cx36 on neuronal specification, gain of function studies are necessary. As part of my M.Sc. research, a lentiviral construct was designed in Dr David Paul's laboratory. Titres have not been sufficient to complete experiments and these studies will be pursued at a later date. As well as further assessment of Panx2 localization to define possible compensatory roles will be important and has been explored in Chapter 5.

In summary, an *in vitro* neurosphere model has shown the loss of Cx36 increases the commitment of NPCs to both neuronal and oligodendrocyte phenotypes in response to differentiation stimuli and upon plating on laminin. This suggests that Cx36 may be a key regulator NPC fate and, surprisingly that expression by immature neurons perhaps serves as signal to reduce neurogenesis in adjacent NPCs, It will be essential to establish whether this control is mediated by direct cell-cell communication or through connexin-mediated hemichannel activity.

## 3.7 References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# Chapter 4: Cx36 null-mutant protects neurons from excitotoxic challenge in the adult murine hippocampus *in vivo*

## 4.1 Objective of this Study

To further elucidate the role of Cx36 in postnatal hippocampal neurogenesis, I performed loss of function studies examining the effect of constitutive Cx36 null-mutation in the uninjured and injured adult mouse *in vivo*.

## 4.2 Introduction

Previous work within this thesis has suggested a potential role for Cx36 in regulating neurogenesis following plating on laminin and exposure to neurogenic stimuli *in vitro*. The next step, therefore, was to move to an *in vivo* model system and determine whether Cx36-mediated communication regulates NPC proliferation and specification in the adult mouse brain. A loss of function approach using $Cx36^{-/-}$ mice was employed. Null-mutant mice have been shown to exhibit impairments in the synchronization of gamma oscillations in the hippocampus, important for memory inscription and, as such, have shown deficiencies in learning and memory in a stimulus complexity-dependent manner (Hormuzdi et al., 2001; Frisch et al., 2005). No study, however, has examined whether the loss of Cx36 impacts on adult neurogenesis. In addition, if Cx36 null-mutation enhances neurogenesis *in vitro* as demonstrated in Chapter 3, then targeting expression may represent a new strategy to promote neuroregeneration following injury.

To examine this, mice were treated with kainic acid to elicit epileptiform seizures and induce excitotoxic injury of hippocampal pyramidal neurons. We have previously

shown that this paradigm results in a defined loss of neurons within the CA3a/b pyramidal cell field (Cheung et al., 2005). Cell loss triggers NPC proliferation and neuroregeneration albeit with considerable controversy as to whether the functional integration of new neurons in injured tissue is adaptive or maladaptive (Dashtipour et al., 2001; Jessberger et al., 2005; Jessberger et al., 2007b; Jessberger et al., 2007a). Here, I assessed hippocampal cell loss as well as endogenous neurogenesis in congenic WT and $Cx36^{-/-}$ mice. Unexpectedly, a role for Cx36 in neuronal survival but not NPC specification was found *in vivo* in contrast to previous *in vitro* results (Chapter 3).

## 4.3 Experimental Procedure

*Mice*

$Cx36^{-/-}$ mice were kindly provided by Dr. David Paul (Harvard University) and were compared to congenic N4 wild-type (WT) littermates derived from mating of heterozygote breeding pairs[6]. Mice were kept on a 12 hr light-dark cycle and allowed food and water *ad libitum*. All experimental protocols were approved by the Animal Care Committee of the University of Ottawa according to guidelines set forth by the

[6] **Thesis advisors note**: Ms Sorbara initially performed all of the experiments described in Chapter 4 on what was assumed to be $Cx36^{-/-}$ mice that had been backbred to for 6 generations (N6) to a C57B/6 lineage. These genotypes were based on flawed reactions performed by a colleague initially responsible for this colony. Our laboratory has a built-in system wherein animals are genotyped first upon weaning and again, upon sacrifice. Ms Sorbara discovered the error upon regenotyping all of her experimental animals after sacrifice. She was forced to repeat her experiments using N4 $Cx36^{-/-}$ mice and congenic WT animals kindly provided by Dr David Paul (Harvard University). None of her initial control animals could be included in this re-analysis due to strain and congeneity issues in seizure-induced CA3 damage. As her thesis advisor, I wish to highlight Ms Sorbara's integrity in identifying this major problem and her rigour repeating all of her experiments on a new C57BL/6 background.

Canadian Council on Animal Care.

*Tissue Preparation of Adult Mice*

Animals were killed by lethal injection with sodium pentobarbital and transcardially perfused with 10 mM phosphate buffered saline (PBS; 10 mM sodium phosphate, 154 mM sodium chloride) followed by 4% PFA in 10 mM PBS. Brains were dissected out and incubated for 24 hr in the same fixative at 4 °C. After fixation, brains were cryoprotected in 10% sucrose solution in 10 mM PBS containing 0.001% sodium azide for 48 hr. Brains were then flash-frozen with $CO_2$ gas and coronal sections (10 μm thickness) were prepared using a cryostat (Leica Microsystems Inc., Richmond Hill, ON).

*Labeling of mitotically active cells in adult mice*

In uninjured adult mice, three different halogenated thymidine analogs were used to label actively dividing cells. Bromodeoxyurdine (BrdU) was prepared at a final concentration of 50 μg/g in sterile 10 mM PBS. With equimolar delivery, replacement of the bromo group for either iodo- or chloro- moieties (IdU, CldU) allows for temporal discrimination of cell proliferation through immunofluorescent detection as previously described (Vega and Peterson, 2005). This eqimolar delivery involved the preparation of an IdU solution at 23 mg/ml and a CldU solution of 17 mg/ml. The mice then received 2.5 ml/kg of each solution for a final delivery of 57.5 mg/kg IdU or 42.5 mg/kg CldU. All of the solutions were administered intraperitoneally. Animals received two daily injections, approximately 4-5 h apart, over two consecutive days and a single injection on the third day. Mice were sacrificed 6 hr, 24 hr or 28 d after the last injection, sacrificed and paraformaldehyde-fixed as described above. Cryostat-cut sections were then

incubated at 37 °C for 1 hr in 2 N hydrochloric acid followed by neutralization in 0.1 M borate buffer, pH 8.5. BrdU incorporation was observed by immunofluorescence using mouse monoclonal anti-BrdU (Roche). CldU incorporation was detected using mouse monoclonal anti-BrdU clone B44 (BD) while IdU incorporation was detected using rat monoclonal FITC-conjugated anti-BrdU (Serotec) as described above. Dividing cells were quantified by counting thymidine analog$^+$ cells of two bilateral hippocampi in two adjacent 10 μm sections (n=4 hippocampi per animal) between bregma -1.60 mm to -2.10 mm. The region of the hippocampus assessed was an area of dentate gyrus that included the subgranular zone, the polymorphnuclear layer and the most medial tip of the CA3c. The measurement feature of OpenLab v5.08 software was used to determine the total area ($mm^2$) of each region and two independent investigators performed counts. Values were averaged to produce a single value per animal and the data was expressed as the number of BrdU$^+$, IdU$^+$ or CldU$^+$ cells per 0.1 $mm^2$.

*Excitotoxic Injury*

Adult mice received intraperitoneal injections of 15 mg/kg of kainic acid (Sigma). Seizure intensity was assessed based on a five-point behavioural scale as previously described (Bennett et al., 1995). Within five minutes of reaching status epilepticus, animals received 5 mg/kg of diazepam to limit convulsions and placed in housing without light and minimal sound and kept under close observation for 6 hr until the possibility of additional seizures has ceased. Only mice that reached stage 4 were included in this study. A total of n=15 Cx36$^{-/-}$ mice and n=12 WT mice survived and reached stage 4. Mice were euthanized with sodium pentobarbital 3, 7 or 28 days following kainic acid-

induced seizure and transcardially perfused with 10 mM PBS and 4% paraformaldehyde in 10 mM PBS as described above. Apoptotic cells were detected by TUNEL essentially as described above with the following modifications. Sections were permeabilized by a 5 min incubation in 0.1% Triton-X/0.1% sodium citrate on ice and a 2 min incubation in ethanol:acetic acid (2:1) on ice. Sections were washed for 2 min in 10 mM PBS and incubated in a humidity chamber for 1 hr at 37 °C with FITC-labeled dUTP in TdT buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) and TdT according to the manufacturer's protocol (Roche). The number of $TUNEL^+$ cells was determined as described for $BrdU^+$ cells in the hippocampus. To qualitatively assess neurodegeneration following injury, coronal sections were also stained for haematoxylin & eosin (H&E).

*Immunofluorescence*

Antibodies shown in Table 4.1 were diluted in Antibody buffer (3% bovine serum albumin, 0.3% Triton X-100 in 10 mM PBS). Primary antibodies were incubated at 4° overnight. The following day, two 15 minute washes in 10 mM PBS were performed followed by an 1 hr incubation with secondary antibodies, an identical wash treatment and coverslipping in an "antifade" mounting media (0.1% P-phenylenediamine, 90% glycerol in 10 mM PBS).

**Table 4.1 List of Primary and Secondary Antibodies used for Immunofluorescence**

| Antibody | Lineage Detected | Species | Source | Dilution |
|---|---|---|---|---|
| BrdU (bromodeoxyuridine) | Cell Proliferation | Mouse | Roche | 6 μg/ml |
| | | Mouse Clone B44 (also recognizes CldU) | BD Pharm | 1:500 |
| | | Rat –FITC (also recognizes IdU) | Serotec | 1:50 |
| NeuN | Mature and newly born neurons | Mouse | Chemicon | 1:50 |
| Parvalbumin | Interneurons | Mouse | Sigma | 1:1000 |
| Mouse IgG | Cy3-conjugated | | Jackson | 1:400 |
| | FITC-conjugated | | Jackson | 1:50 |
| Rat IgG | Cy3-conjugated | | Jackson | 1:300 |
| | FITC-conjugated | | Jackson | 1:200 |

## 4.4 Results

### 4.4.1 Cell proliferation is unchanged in the Cx36$^{-/-}$ adult dentate gyrus *in vivo*

To determine whether developmental loss of Cx36 affects hippocampal neurogenesis in the adult *in vivo*, we first examined whether there was an alteration in proliferation in the adult SGZ (Fig 4.1A). Cx36$^{-/-}$ and WT animals received five intraperitoneal injections of IdU over three consecutive days. The animals were sacrificed 24 hours followed the last instalment of IdU. There was no significant change in the frequency of IdU labelled cells in the dentate gryus between genotypes (Fig 4.1B, C). As well, there was no difference in IdU detection in the CA1 pyramidal cell field, suggesting comparable bioavailability within the hippocampal formation in both groups of animals (Fig 4.1B inset). The distribution of the labelled cells was also similar, located mostly in the SGZ (Fig 4.1C) and occasionally in the polymorphnuclear zone of the hippocampus.

Although there was no increase in progenitor cell number, we next wanted to assess whether Cx36 alters the survival of these progenitor cells. In a slight modification of the previous IdU injection paradigm, animals injected with CldU were sacrificed 28 days after labelling. The use of two different halogenated analogs allowed for the assessment of both proliferation and the survival of labelled cells within the same animal since they can be detected using two non-cross reactive antibodies. The number of CldU$^{+}$ cells retained in the dentate gyrus 28 days after initial labelling was also not significantly altered between WT and Cx36$^{-/-}$ mice (Fig 4.1D). Taken together, these data indicate that the developmental deletion of Cx36 does not impact upon NPC

**Figure 4.1 Cell proliferation and cell survival in the dentate gyrus is not altered by Cx36 null-mutation *in vivo.***

(A) Coronal schematic of hippocampus indicating CA regions, the granule layer of dentate gyrus (GrDG) and the site of adult hippocampal neurogenesis, the SGZ of the DG. Adult mice were injected with IdU or CldU over three consecutive days and sacrificed 24 hours later (for those injected with IdU) to assess cell proliferation or 28 days later (for those injected with CldU) to assess cell survival. There was no significant difference in IdU-labelled cells in the DG 24 hr after the last IdU injection between genotypes. The CA1 region was assessed as a bioavailability control (inset). (C) Representative photomicrographs of WT and $Cx36^{-/-}$ DG immunolabelled for NeuN *(red)* and IdU *(green)*. Inset depicts higher magnification of IdU labelled cells. (D) Surviving cell number 28 days after labelling once again showed no difference between WT and $Cx36^{-/-}$ mice. Percentage of reduction in labelled-cell number compared with 24 hr is indicated for each genotype. Scale bars, 50 μm. Abbreviations are as defined in Fig 1.1.

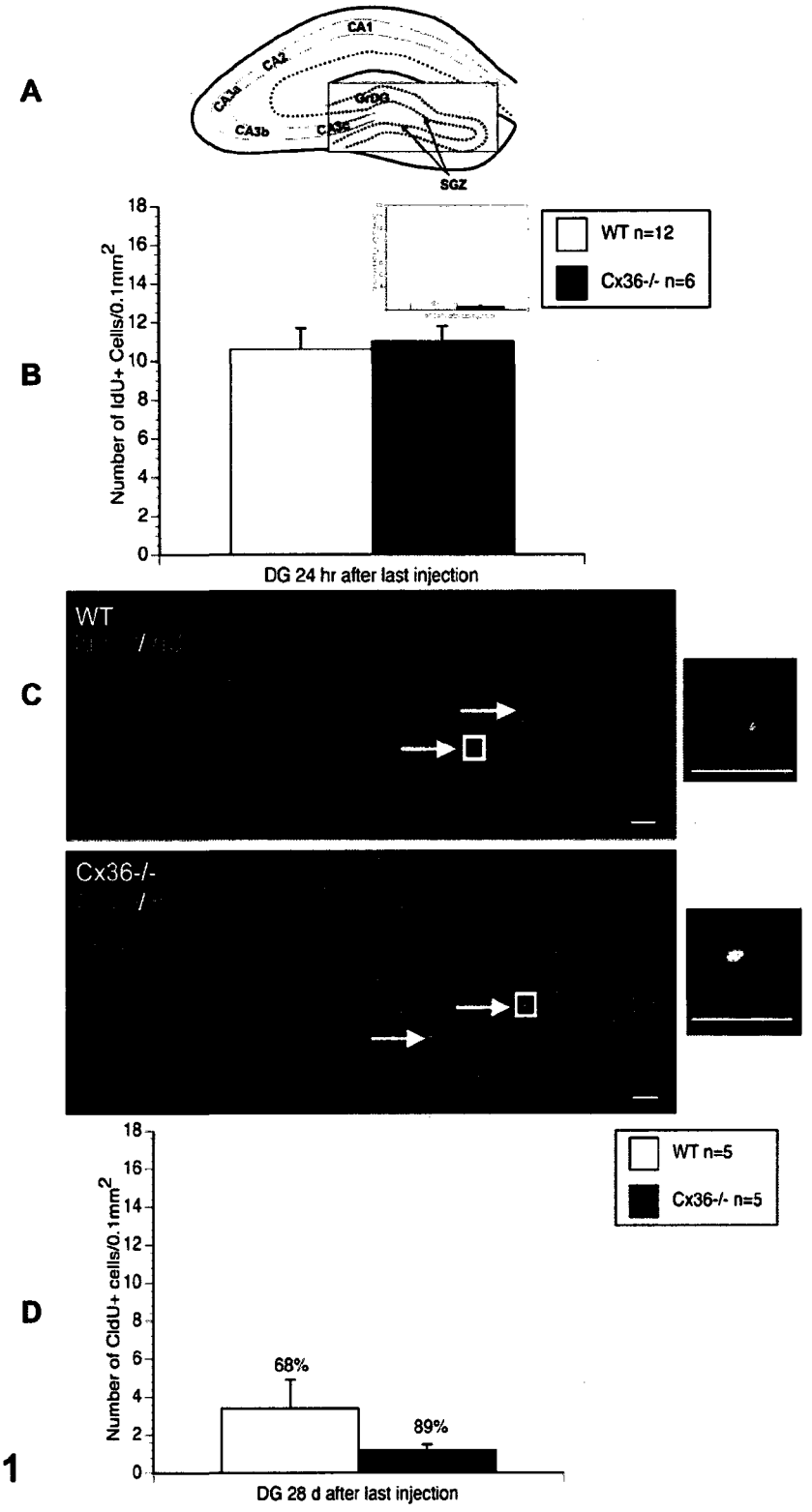
A
CA1
CA2
CA3a
CA3b
GrDG
SGZ
B
WT n=12
Cx36-/- n=6
Number of IdU+ Cells/0.1mm2
DG 24 hr after last injection
C
WT
Cx36-/-
D
WT n=5
Cx36-/- n=5
Number of CldU+ cells/0.1mm2
68%
89%
DG 28 d after last injection

**Figure 4.1**

proliferative phenotype in adult mice.

To provide a more detailed examination, we examined the number of parvalbumin$^{+}$ cells in WT and Cx36$^{-/-}$ mice. Previous analyses of Cx36 expression in the adult mouse brain had detected both mRNA and protein within cells that co-express the interneuronal marker parvalbumin in the CA region of the hippocampus (Deans et al., 2001; Wellershaus et al., 2008). As such, we sought to determine whether subsets of Cx36-expressing neurons were affected by null-mutation. No change in the number of parvalbumin$^{+}$ cells was detected in uninjured mice between genotypes (Fig 4.2).

### 4.4.2 Degeneration and regeneration in the CA3a/b pyramidal cell layer following excitotoxic injury in WT and Cx36$^{-/-}$ animals

The amount of neurogenesis occurring in a healthy, adult mouse is relatively low. We used the kainic acid-induced seizure model to enhance NPC proliferation and establish whether Cx36 impacts upon the proliferation, or specification of hippocampal NPCs following injury. This model provides the conditions to endogenously augment neurogenesis and, by doing so might perhaps reveal a phenotype for the loss of Cx36 that would otherwise not be seen under low-level neurogenesis *in vivo*. Of note, N4 WT and Cx36$^{-/-}$ littermates exhibited equivalent excitotoxin sensitivity, seizure staging and mortality rates following systemic administration of kainic acid (Table 4.2).

On initial analysis, Cx36 null mutation appeared to have no effect on the amount of hippocampal neurodegeneration observed after kainic acid administration. As expected, the number of NeuN$^{+}$ neurons was similar in the dentate gyrus at all time points

**Figure 4.2 Expression of parvalbumin$^+$ interneurons is unchanged by Cx36 null-mutation *in vivo***

Immunofluorescence for parvalbumin within the CA region of the hippocampus was performed as shown in (A) and quantified as shown in (B) to assess the number of interneurons present. No significant difference in labelled cells was found between genotypes. Inset depicts higher magnification of parvalbumin labelled cells Scale bars, 50 μm.

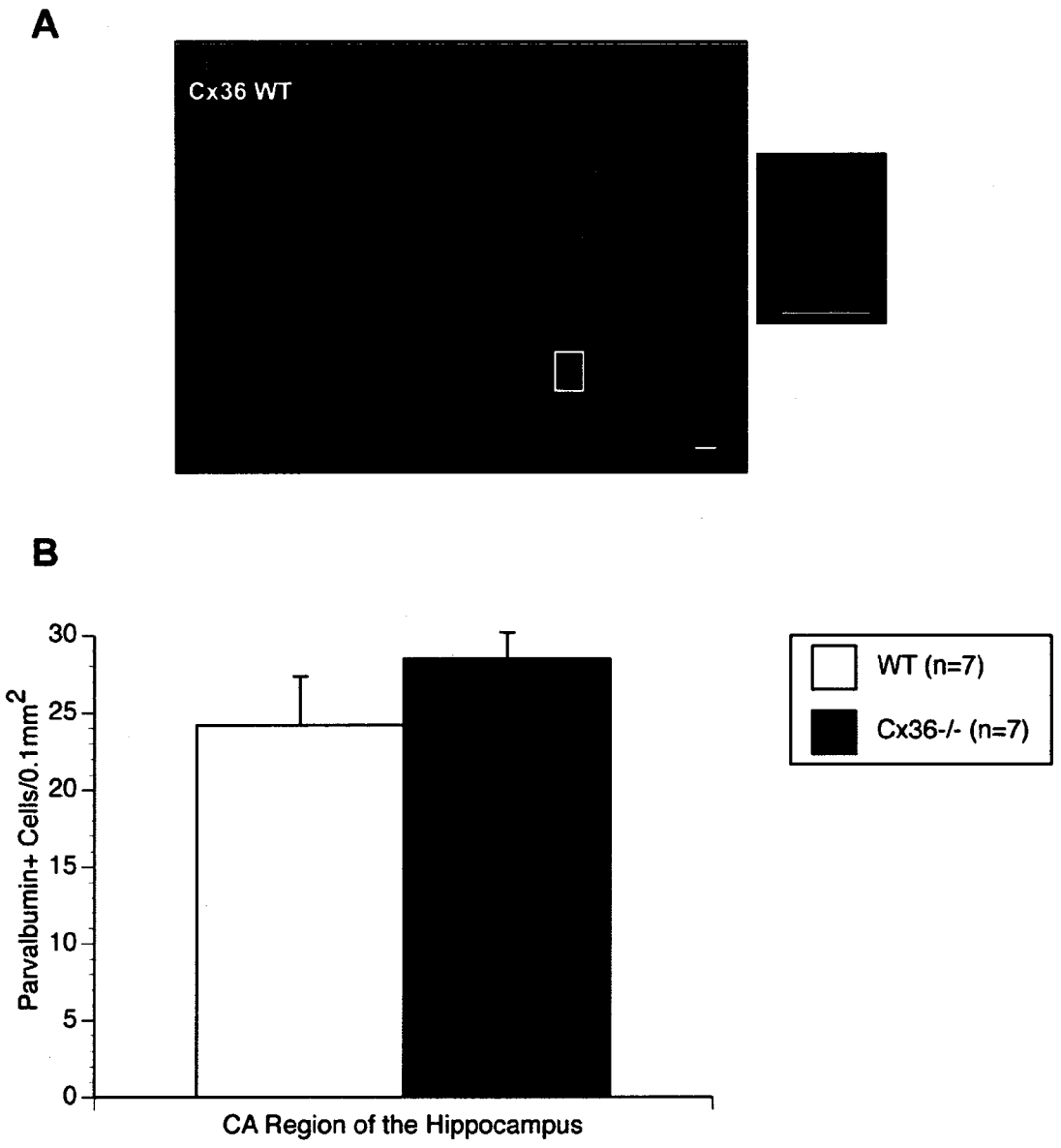
A
Cx36 WT
B
30
25
20
15
10
5
0
Parvalbumin+ Cells/0.1mm2
CA Region of the Hippocampus
WT (n=7)
Cx36-/- (n=7)

**Figure 4.2**

**Table 4.2 Sensitivity to kainic acid induced seizures is comparable between N4 WT and Cx36$^{-/-}$ mice**

| | Behavioural scale | | | | | |
|---|---|---|---|---|---|---|
| **Genotype (Dosage)** | **1** | **2** | **3** | **4** | **5 Mortality** | **Total number of mice** |
| **WT (15 mg/kg)** | 0 (0%) | 0 (0%) | 1 (8%) | 11 (84%) | 1 (8%) | 13 |
| **Cx36$^{-/-}$ (15 mg/kg)** | 0 (0%) | 0 (0%) | 0 (0%) | 15 (94%) | 1 (6%) | 16 |

Animals were injected intraperitoneally with 15 mg/kg of kainic acid and seizure intensity was staged using a five-point behavioural scale as described in Methods. Data show that this dosage triggered stage 4 seizures in the majority of mice and progressed to lethal stage 5 seizures in few subjects. Genotype had no effect on kainic acid sensitivity.

following injury and there was no significant apoptosis occurring in this region, confirming published data (Wang et al., 2005) that the GrDG is spared excitotoxic insult (Fig 4.3). Conversely, neuronal viability was significantly decreased in the CA3a/b pyramidal field 1 wk following seizure insult in both genotypes (Fig 4.4B,C). An approximate 50% decrease in the number of $NeuN^+$ cells was detected (Fig 4.4B). However, despite comparable neuronal loss 1 wk following seizure, a significant increase in the number of CA3a/b neurons was observed 4 wk following seizure in $Cx36^{-/-}$ but not $Cx36^{+/+}$ mice. In $Cx36^{-/-}$ mice, the number of $NeuN^+$ neurons was effectively restored to uninjured levels 4 wk after seizure (Fig 4.4B,C).

### 4.4.3 Lack of cell death and not enhanced regeneration underlies increased neuronal number in $Cx36^{-/-}$ after injury

The increase in $NeuN^+$ cells 4 wk following injury in the CA3 region of the $Cx36^{-/-}$ could be explained by an increase in neuronal replacement as predicted by the data presented in Chapter 3. To test this hypothesis, we evaluated the activation of NPCs in injured hippocampus at 0.5, 1 and 4 weeks following seizure. Injured mice were injected with IdU over three days and sacrificed 6 hr after their last injection (Fig 4.5A). In the dentate gyrus, the number of proliferating cells increased 0.5 wk after seizure induction, peaked 1 wk after seizure, and dropped 4 wks after kainic acid injection in WT mice. In Cx36 null-mutant mice, the number of proliferating cells increased 0.5 wk following injury, stayed constant 1 wk after seizure and decreased 4 wks after seizure induction (Fig 4.5B). Fewer cells were activated 1 wk after seizure in $Cx36^{-/-}$ mice than WT

**Figure 4.3 Kainic-acid induced seizures did not elicit damage in the dentate gyrus**

(A) $NeuN^+$ cells were quantified within the granule cell layer of the hippocampus of uninjured and injured subjects. No significant neuronal loss was seen following seizures and genotype had no effect on the quantity of $NeuN^+$ cells in this area. (B) Additionally, no significant apoptosis occurred within this region, as measured by TUNEL labelling.

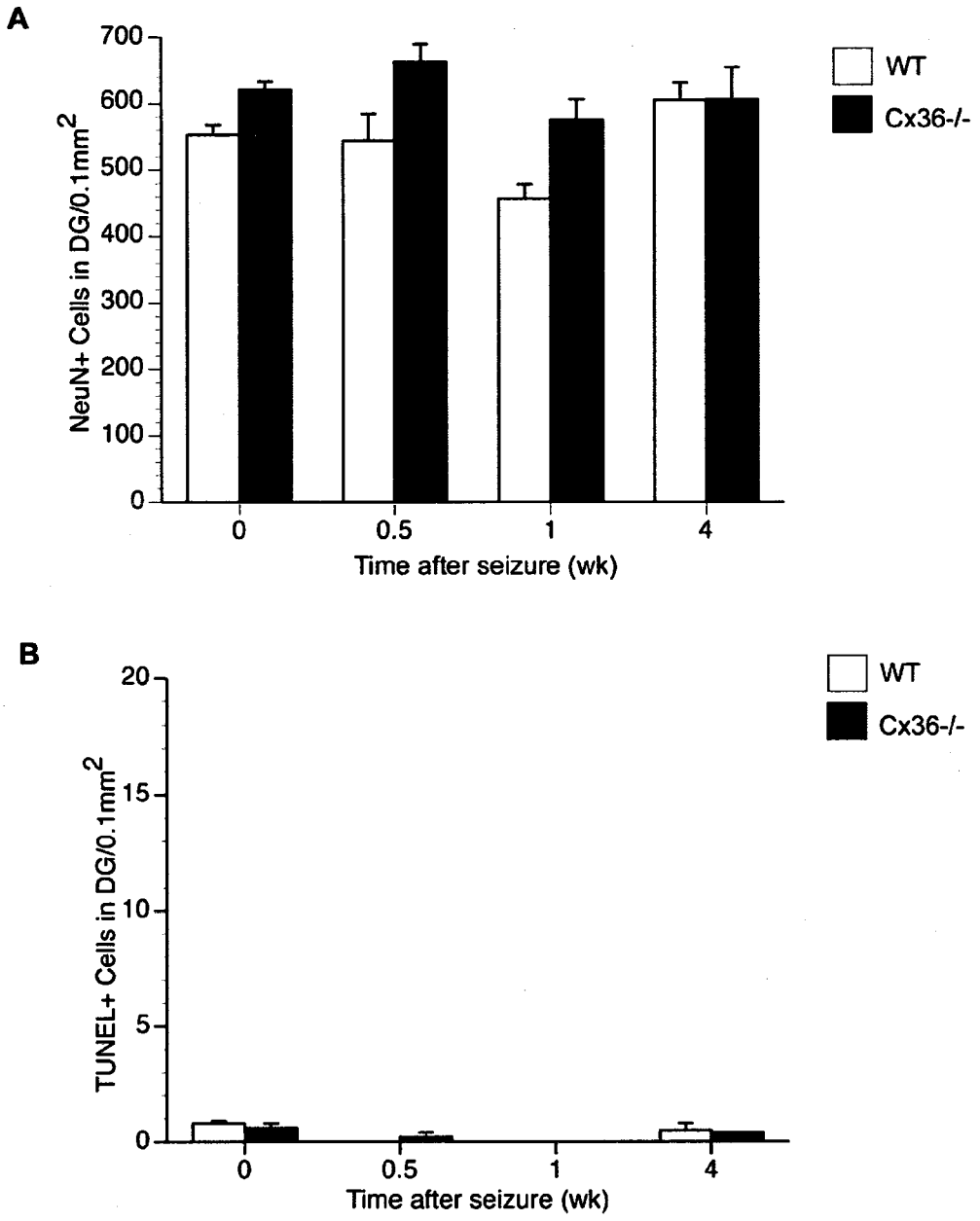
A
700
600
500
400
300
200
100
0
NeuN+ Cells in DG/0.1mm2
0
0.5
1
4
Time after seizure (wk)
WT
Cx36-/-
B
20
15
10
5
0
TUNEL+ Cells in DG/0.1mm2
0
0.5
1
4
Time after seizure (wk)
WT
Cx36-/-

**Figure 4.3**

**Figure 4.4 Degeneration and regeneration in the CA3a/b pyramidal cell layer following excitotoxic injury in WT and $Cx36^{-/-}$ animals**

(A) Coronal schematic of the hippocampus highlighting the CA3a/b region where neuronal viability following seizure induction was assessed. (B) Compromised $NeuN^{+}$ neuronal number was observed 1 wk after kainic acid administration in both genotypes. Mature neurons were restored in $Cx36^{-/-}$ but not WT mice 4 wk after seizure. (C) Representative photomicrographs of WT and $Cx36^{-/-}$ CA3a/b stained with H&E or immunolabelled for NeuN. Note the loss of NeuN immunoreactivity and apparent degenerative morphology in H&E-stained sections 0.5 and 1 wk after seizure with restoration of healthy neuronal morphology in $Cx36^{-/-}$ but not $Cx36^{+/+}$ mice 4 wks after seizure. Scale bars, 50 μm. Statistics were ANOVA followed by post-hoc Tukey test. *$p<0.05$, #$p<0.05$, **$p<0.01$. Asterisks indicate a statistically significant reduction in cell number relative to uninjured animals (0 wk).

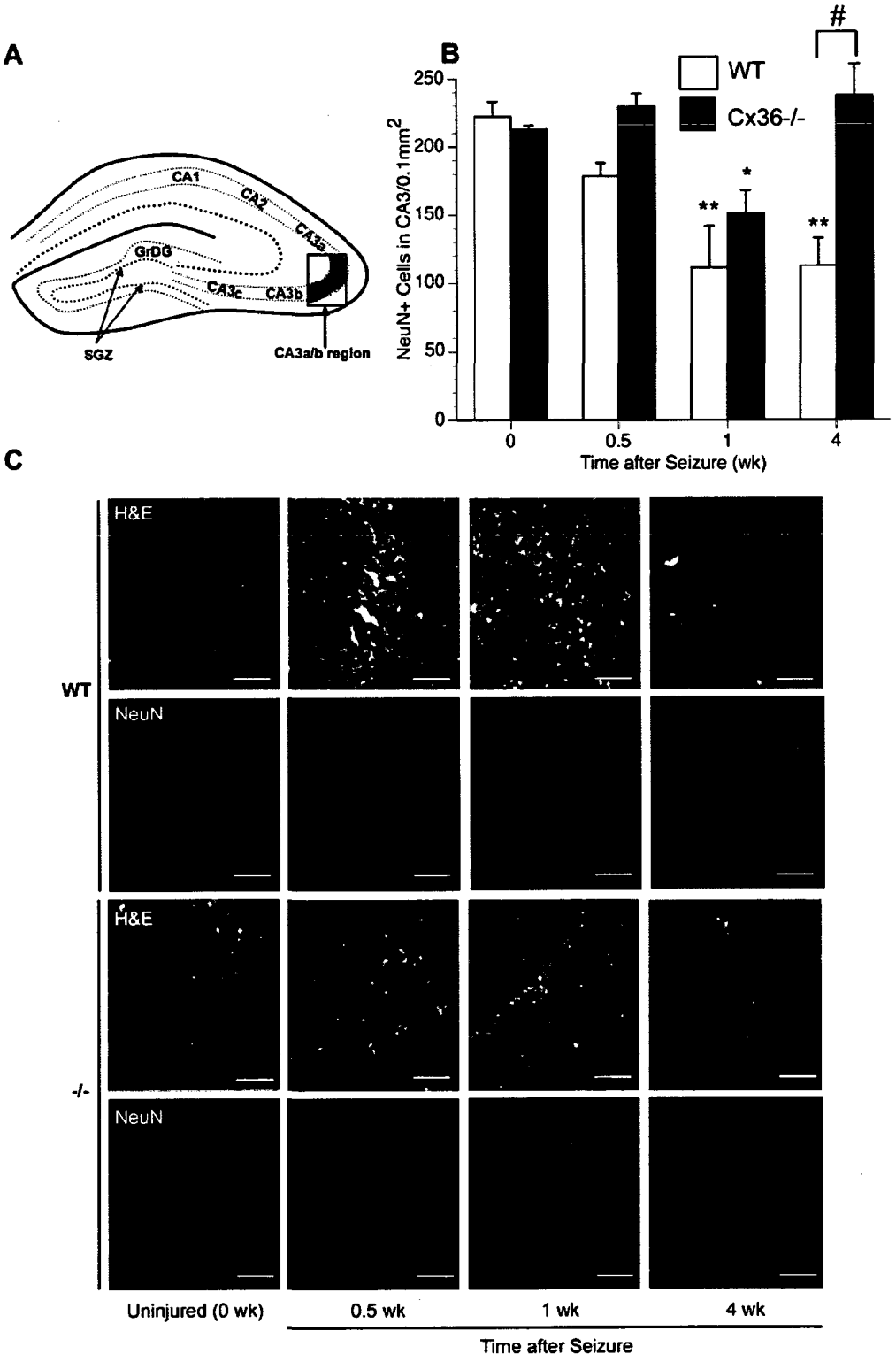
A
CA1
CA2
CA3a
GrDG
CA3c
CA3b
SGZ
CA3a/b region
B
WT
Cx36-/-
NeuN+ Cells in CA3/0.1mm2
250
200
150
100
50
0
0
0.5
1
4
Time after Seizure (wk)
#
*
**
**
C
H&E
NeuN
WT
H&E
NeuN
-/-
Uninjured (0 wk)
0.5 wk
1 wk
4 wk
Time after Seizure

**Figure 4.4**

**Figure 4.5 Loss of Cx36 does not enhance the proliferation of activated NPCs following hippocampal injury**

(A) To assess the possibility of altered neuroregeneration in the Cx36 null-mutant mouse, proliferating cells activated in the DG following injury were quantified. Mice were injected with IdU at 0 (uninjured), 0.5, 1 or 4 wks after kainic acid administration. Mice were sacrificed 6 hr after the last IdU injection. (B) Both genotypes show increased proliferating cells 0.5 wk following injury following a decrease at 4 wks following seizure induction but there is no increase in $IdU^+$ cells in $Cx36^{-/-}$ cohort compared to WT.

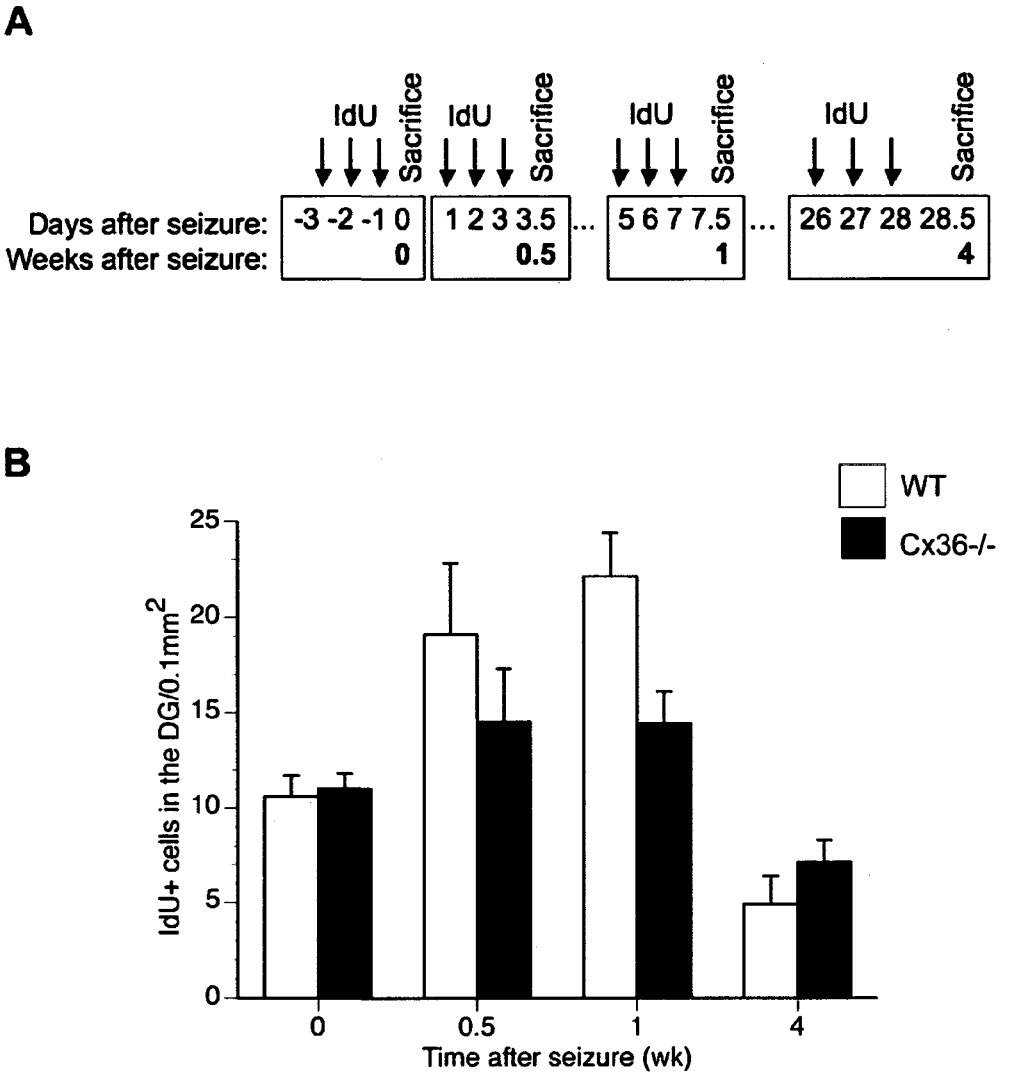
A
IdU
Sacrifice
IdU
Sacrifice
IdU
Sacrifice
IdU
Sacrifice
Days after seizure:
Weeks after seizure:
-3 -2 -1 0
0
1 2 3 3.5
0.5
5 6 7 7.5
1
26 27 28 28.5
4
B
WT
Cx36-/-
IdU+ cells in the DG/0.1mm2
25
20
15
10
5
0
0
0.5
1
4
Time after seizure (wk)

**Figure 4.5**

indicating that Cx36 deletion does not increase the proliferation of NPC activated in the SGZ following hippocampal injury.

In the absence of evidence for enhanced neurogenesis, alternatively it may be that the loss of Cx36 enhanced neuronal survival. It may be that pyramidal neurons in null-mutant mice are metabolically compromised and lose expression of NeuN following excitotoxicity but do not terminally degenerate and therefore are capable of subsequent recovery. To assess this, we once again looked at apoptosis by performing TUNEL analysis. Here, we observed that only WT animals showed significant TUNEL labelling in the CA3 region, maximal at 1 wk following injury, while $Cx36^{-/-}$ mice exhibited no DNA fragmentation indicative of apoptotic cell death (Fig 4.6A,B).

To further establish that neuronal recovery was the result of enhanced survival of mature neurons and not enhanced neurogenesis in the absence of Cx36, mice received 5 injections of BrdU over first three days following kainic acid injection and were sacrificed 6 hr or 28 d after their last injection (Fig 4.7A). The total number of $BrdU^{+}$ cells 6 hr after the last injection in both the dentate gyrus and the CA3 was not significantly different between cohorts (Fig 4.7B). Similarly, the percent of newly born BrdU-labelled cells present in the CA3 region 28 d following injury was unchanged confirming that the lack of cell death is the underlining reason for increased neuronal number in the $Cx36^{-/-}$ mouse following injury and that null mutation does not significantly impact upon the regenerative capacity of endogenous NPCs in the hippocampus (Fig 4.7C).

**Figure 4.6 Lack of cell death underlies increased neuronal number in Cx36$^{-/-}$ after injury**

Terminal degeneration was assessed TUNEL, which is indicative of DNA damage and apoptosis in the CA3a/b region. Only WT animals exhibited TUNEL$^{+}$ staining following insult as shown in representative photomicrographs in (A) and quantified in (B). Scale bars, 50 μm. Statistics were ANOVA followed by post-hoc Tukey test. *$p<0.05$, #$p<0.05$. Asterisks indicate a statistically significant increase in TUNEL$^{+}$ cells relative to uninjured animals (0 wk). # indicate a statistically significant difference between genotypes.

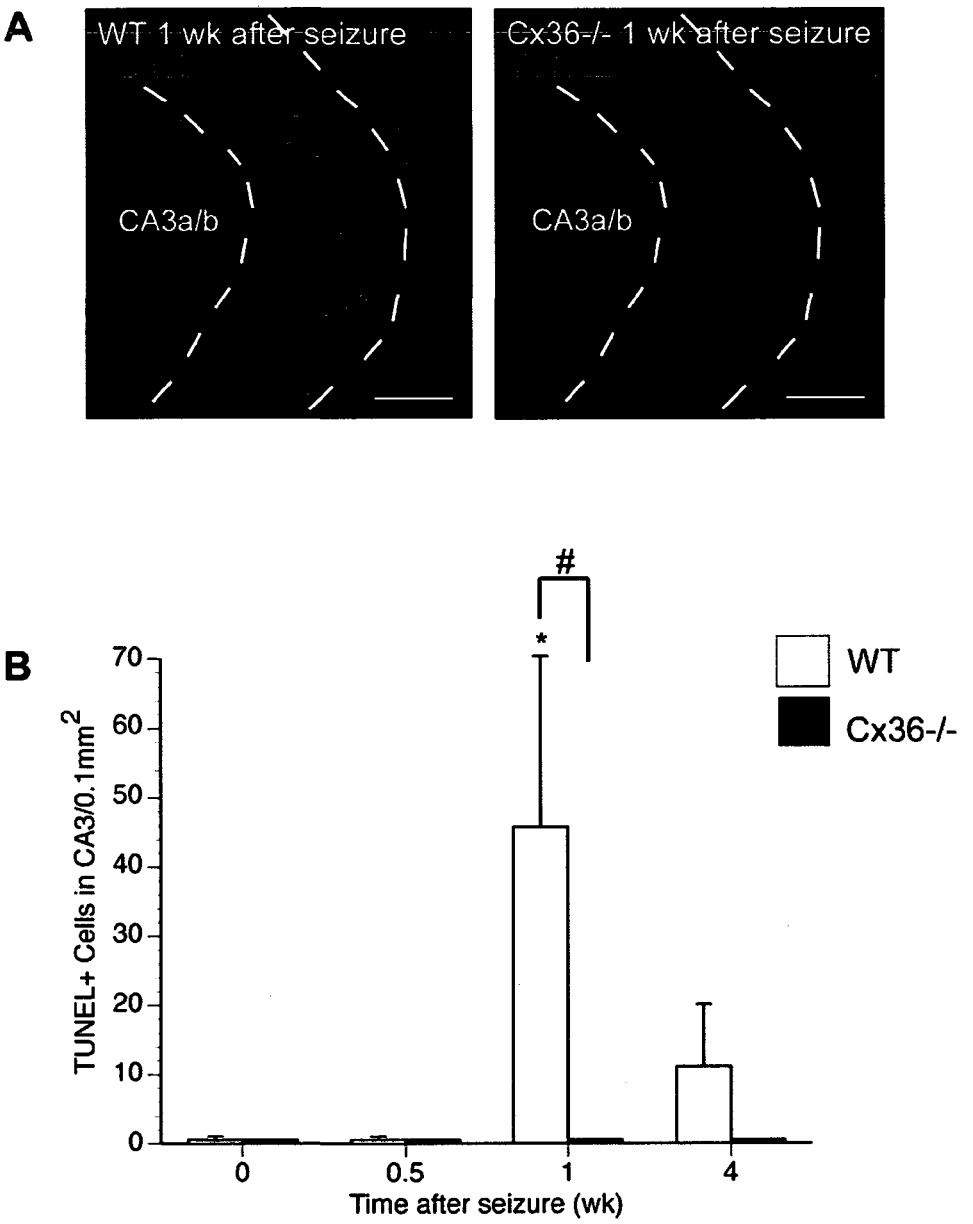
A
WT 1 wk after seizure
CA3a/b
Cx36-/- 1 wk after seizure
CA3a/b
B
#
*
WT
Cx36-/-
TUNEL+ Cells in CA3/0.1mm2
70
60
50
40
30
20
10
0
0
0.5
1
4
Time after seizure (wk)

**Figure 4.6**

**Figure 4.7 Cell birthdating and cell survival further indicate no difference in neuroregeneration between Cx36 null-mutant and WT mice**

(A) To further establish whether the increase in neuronal number in the injured CA3 in $Cx36^{-/-}$ mice was due to neuroregeneration or neuronal recovery, cell birthdating analysis coupled with cell survival assessment was performed. Animals were injected with BrdU 0.5 wk after seizure and then sacrificed 6 hr or 28 d after the last injection. The number of $BrdU^{+}$ cells surviving in the CA3a/b was quantified 28 d after labelling. Data were expressed as a percentage of the total number of labelled cells in the dentate gyrus and the CA3 pyramidal cell fields 6 hr after injection. (B) Total number of $BrdU^{+}$ cells 0.5 wk following injury was similar in both groups of animals (see also Fig. 4.3). (C) As well, cell survival of newly born neurons in the CA3a/b was not significantly different between genotypes 28 d after the last BrdU injection.

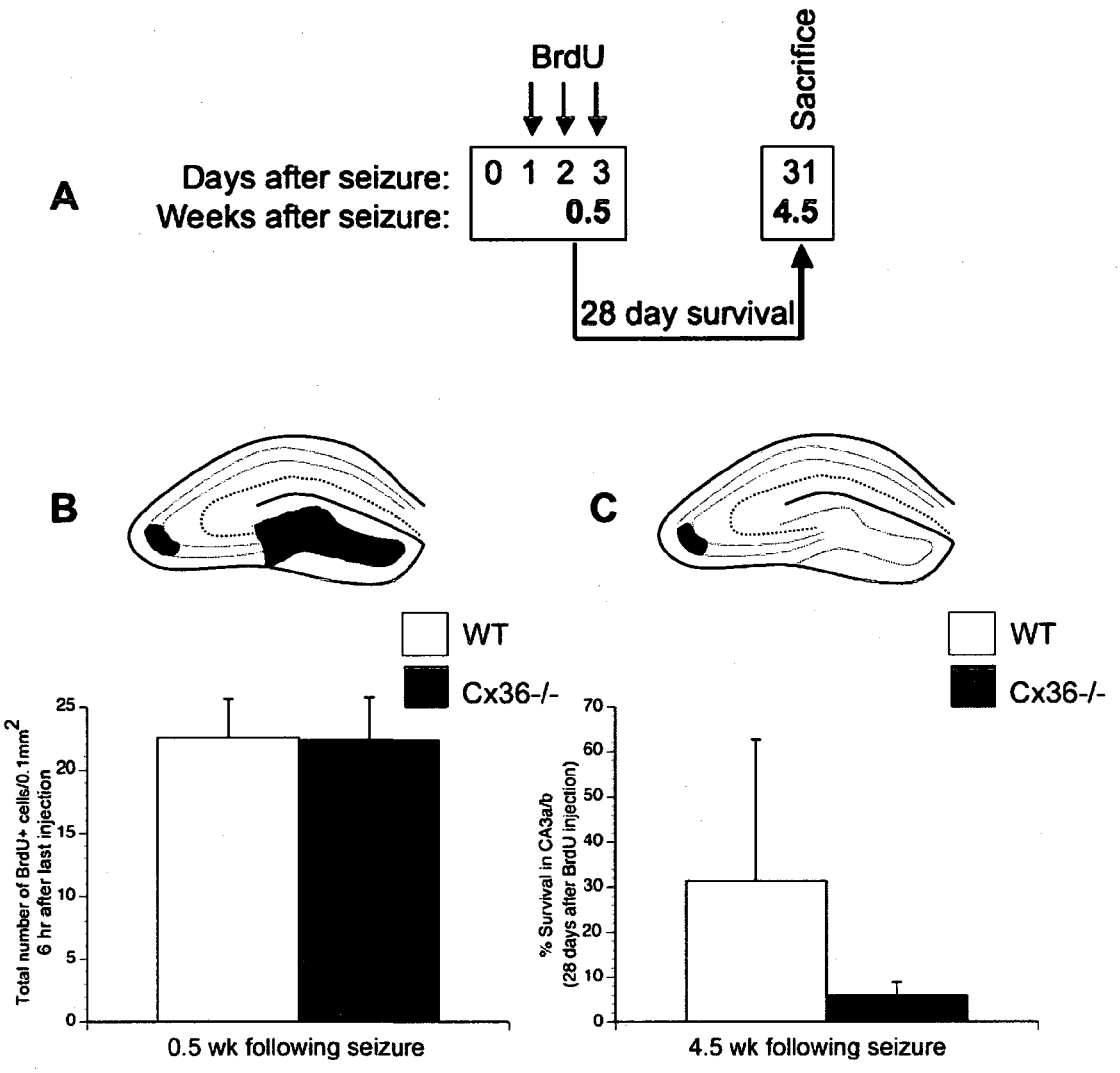
A
BrdU
Sacrifice
Days after seizure:
Weeks after seizure:
0 1 2 3
0.5
31
4.5
28 day survival
B
C
WT
Cx36-/-
Total number of BrdU+ cells/0.1mm2
6 hr after last injection
0.5 wk following seizure
% Survival in CA3a/b
(28 days after BrdU injection)
4.5 wk following seizure

**Figure 4.7**

## 4.5 Discussion

In the uninjured adult mouse, Cx36 was unable to intrinsically promote neurogenesis as neither NPC proliferation nor the survival of terminally differentiated progeny was enhanced in the dentate gyrus, the location of hippocampal neurogenesis, or the CA3a/B, the site of neuronal loss, between WT and $Cx36^{-/-}$ genotypes. This is intriguing given that *in vitro*, the loss of Cx36 increased the number of neuronal progeny following spontaneous and neurogenic-induced differentiation of hippocampal neurosphere cultures (Chapter 3). At this point, we hypothesized that perhaps the microenvironment in the uninjured animal did not provide a sufficient stimulus to detect the functional impact of loss of Cx36 function on hippocampal neurogenesis.

To provide this permissive environment, we moved to an experimental mouse model of hippocampal injury. Here, we could establish whether Cx36 expression by terminally differentiated neurons and/or by immature neuronal cell population regulates NPC proliferation or cell replacement following excitotoxic brain injury using a loss of function approach. Kainic acid injections were employed to induce epileptic seizures and subsequent neuronal damage. Kainic acid is an agonist for ionotropic glutamate receptors and the neuronal loss that occurs subsequent to administration is correlative to the expression of these receptors (reviewed in (Wang et al., 2005)). Thus, neuronal damage occurs within the CA3 subfield of the hippocampus followed by an initiation of progenitor cell proliferation within the SGZ, migration to the injured CA3 regions, and terminal differentiation (Dong et al., 2003). It is therefore an ideal *in vivo* model of neuroregeneration following neuronal damage.

Initial observations showing an increase in $NeuN^+$ neurons 4 wks following seizure induction in the Cx36 null-mutant mouse led to the hypothesis that the loss of Cx36 increases the regenerative capacity of the hippocampus following injury. This assumption was based on *in vitro* data indicating that the loss of Cx36 promoted the specification of laminin-engaged NPCs to neurons (Chapter 3). This, however, was proven incorrect when the proliferation of activated NPCs in the DG was assessed and Cx36 was shown to have no impact on activation of progenitor populations or the survival of their neuronal progeny. Surprisingly, the loss of Cx36 was found to be neuroprotective following injury. Cx36 null-mutant CA3a/b neurons were found to be resistant to excitotoxic injury exhibiting transient evidence of neurodegeneration but a lack of terminal injury thereby enabling subsequent recovery. As such, we concluded that Cx36 expression by neurons during excitotoxic challenge plays a role in mediating cell death. A potential explanation for this could arise from a recent study showing that microglia, myeloid-derived immune surveillance cells, and neurons couple through Cx36 gap junctions in hippocampal cultures *in vitro* (Dobrenis et al., 2005). Microglia can release neurotoxic factors, thereby spreading injury through the bystander effect whereby toxic metabolites are passed through Cx36-mediated gap junctions from microglia to neurons (Alvarez-Maubecin et al., 2000; Aloisi, 2001; Orellana et al., 2009). The loss of this coupling could conceivably protect the neurons from this secondary injury. As well, the spread of calcium waves through coupled cells could depolarize neurons/microglia promoting the release of more glutamate as has been shown in coupled glia (Hoffmann et al., 2003). Future co-culture experiments of neurons and microglia will help to fully elucidate this mechanism. Another explanation may lie in Cx36-mediated hemichannel

activity. Kainic acid induces glutathione efflux leading to neurotoxicity in hippocampal slices (Stridh et al., 2008). The excess glutamate as a result of excitotoxicity prevents the reuptake of cysteine, a building block of glutathione leading to oxidative stress and apoptosis. Glutathione can be released from connexin-mediated hemichannels while pharmacological inhibitors of channel function can attenuate neuronal damage mediated by glutamate *in vitro* (Wallin et al., 1999; Takeuchi et al., 2006). Cell-specific acute, rather than constitutive chronic knock down of Cx36, using shRNA prior to microglia activation in this model could be used to distinguish between these potential mechanisms. Finally, as mentioned in the previous chapter, potential compensation by Panx1 and Panx2 must also be taken into account when attempting to explain these results given their closely related cellular distribution and function in hippocampus with initial observations provided in the next chapter.

In summary, these data show that although the ability of progenitor cells to respond to extrinsic cues are affected by intrinsic determinants such as Cx36 expression *in vitro*, this regulation is not predominant *in vivo*. Rather, I show that Cx36 deletion is sufficient to protect neuronal cells from excitotoxic cell death *in vivo* suggesting a hitherto unexplored means of protecting neuron in the adult hippocampus following injury.

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# Chapter 5: Pannexin 2 is expressed by neural stem cells in the postnatal hippocampus[7]

## 5.1 Objective of this study

In order to determine whether Panx2 is also implicated in the control of NPC fate as other connexin gap junction proteins, I first sought to properly define its localization and expression in the postnatal hippocampus. This chapter is presented in the form of a paper submitted and currently under revision for resubmission in response to reviews.

## 5.2 Statement of author contributions

LAS was responsible for the conceptual design of this styd. CDS, LAS and SALB conceived and designed the experiments. CDS and LAS equally contributed to the experimental work involving antigenic analysis. LAS wrote the manuscript with CDS. LAS performed the RT-PCR and western blot in Figure 5.1 and made the supplemental movies not included in this thesis.

## 5.3 Introduction

Gap junction proteins synchronize neural stem and progenitor cells (NPC) activity, regulate metabolic coupling between NPCs and terminally differentiated "instructive cells," control the propagation of signaling waves over significant distances through the opening of hemichannels in non-junctional membranes, and initiate channel-autonomous

[7] A version of this chapter has been submitted in the Journal of Neuroscience: Swayne L, Sorbara CD, Bennett SAL (2008) Pannexin 2 is expressed by neural stem cells in the postnatal hippocampus. J Neurosci. In preparation for resubmission in response to reviews.

signaling directing NPC migration (Elias and Kriegstein, 2008). These diverse effects have been attributed to the structural subunits of gap junction channels, a family of 21 connexin proteins (Willecke et al., 2002). Recently, a second family of mammalian gap junction-like proteins, pannexins, has been identified with unknown function in the developing CNS.

Pannexins are the mammalian homologs of innexins (Panchin et al., 2000b; Baranova et al., 2004). Innexins were initially identified as functional analogs of connexin proteins in invertebrates (Phelan and Starich, 2001). There are three gap junction-like pannexin proteins. Panx1 is expressed in multiple tissues. Panx2 is restricted to the central nervous system (CNS). Panx3 is primarily found in skin and cartilage (Panchin et al., 2000b; Baranova et al., 2004). Heterologous expression studies indicate that Panx1, like connexin proteins, can form functional hemichannels in non-junctional membranes as well as gap junction intercellular channels (Bruzzone et al., 2005; Vanden Abeele et al., 2006; Huang et al., 2007). *Ex vivo* studies suggest that Panx1 hemichannel activity predominates in CNS (Thompson et al., 2006). Intriguingly, Panx1 may also form "leak channels" in the endoplasmic reticulum suggesting a physiological role for intracellular pannexin pores (Vanden Abeele et al., 2006).

The function of CNS-specific Panx2 is unknown. There is no evidence that Panx2 forms functional hemi- or intercellular channels at the plasma membrane (Bruzzone et al., 2003; Bruzzone et al., 2005). Panx2 can, however, physically interact with Panx1 and reduce hemichannel current amplitude in oocytes thereby regulating Panx1 channel activity (Bruzzone et al., 2005). In the developing CNS, Panx1 and 2 are reciprocally expressed. Panx1 mRNA levels are highest in embryonic tissue and drop in postnatal

brain, whereas Panx2 expression is first detected in neonatal tissue and rises over the course of postnatal CNS development (Vogt et al., 2005). One study suggests that Panx1 expression in neural crest-like cells is down-regulated when cells adopt a Schwann cell-like glial lineage (Ray et al., 2005); however, to our knowledge, no study has investigated whether pannexins participate in neural stem and progenitor cell signalling in the CNS.

Granule cell neurogenesis in the dentate gyrus of the hippocampal formation is maximal during the first postnatal week corresponding with the onset of Panx2 expression (Vogt et al., 2005; Rahimi and Claiborne, 2007). Here, we asked whether Panx2 plays a role in regulating neonatal hippocampal neurogenesis. We show that Panx2 is restricted to specific NPC populations during early hippocampal development, forms unique intracellular structures with rod-like morphology, and is not detected when multipotential NPCs commit to a neuronal lineage. These data provide the first evidence that Panx2 may regulate hippocampal NPC fate in neonatal tissue, contrasting with previous evidence that Panx2 is restricted to terminally differentiated neurons and glia in adult brain.

## 5.4 Experimental Procedure

*Neurosphere cultures*

C57BL/6 mouse pups aged postnatal day three (P3) were killed by lethal injection with Euthansol (Schering-Plough Canada Inc., Pointe-Claire, QC, Canada). NPCs were cultured as described in (Bennett, 2003) with some modifications. Briefly, brains were removed, immersed in ice cold artificial cerebrospinal fluid (aCSF; 26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM D-glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, pH 7.3), and serially sectioned on a Leica (Wetzlar,

Germany) VT1000S vibratome in 500 μM sections. Sections corresponding to bregma -1.60 to -2.10 were collected in aCSF and hippocampi were isolated under a Leica MZ6 dissecting microscope. Hippocampi were minced and incubated in dissociation buffer consisting of 26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$, 3.2 mM $MgCl_2$, 10 mM D-glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, pH 7.3 supplemented with 1 mg/mL neural protease, 0.1 mg/mL papain, and 0.1 mg/mL DNAse I for one hour, rotating at 37°C. Cells were washed and resuspended in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 2.7 mM KCl, 4.3 mM NaCl, pH 7.5) for trituration until the cells were completely dissociated. Single cell suspensions were cultured in medium consisting of Dulbecco's modified eagle medium/ F12 (DMEM/F12) supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 2% B27 supplement, 20 ng/mL epidermal growth factor (EGF) and 10 ng/mL basic fibroblast growth factor (FGF-2) at 37°C in 95% $O_2$/5% $CO_2$. Cultures were supplemented with EGF and FGF-2 every other day for twelve days *in vitro* (DIV). 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. All procedures were carried out in agreement with the guidelines of the Canadian Council for Animal Care and as approved by the University of Ottawa Animal Care Committee.

*RT-PCR*

Total RNA was isolated using Trizol reagent (Invitrogen). Total RNA was treated with DNase I (Promega, Madison, WI, USA). First-strand synthesis was performed using random hexamer primers (Promega) and Superscript II RT (BD

Biosciences, San Jose, CA, USA). The PCR reaction contained: 1X PCR buffer, 0.8 mM dNTPs, 1X Advantage 2 Polymerase (Clontech, Cambridge, ON, Canada) in a final volume of 25 µl. All reactions were carried out using the following cycling parameters: 94°C for 5 min, 35 cycles of 94°C for 30 sec, 57°C for 50 sec, and 72°C for 2 min, and a final step at 72°C for 7 min in a Whatman Biometra T-Gradient thermocycler (Montreal-Biotech Inc., Kirkland, QC, Canada). Primers were: 5'-GAGAAAAAGCATACCCGCCAC-3', 5'-GGTGAGCAGACATGGAATGA-3' defining a 269 bp PANX2 amplicon and 5'-TGGTGCTGAGTATGTCGTGGAGT-3', 5'-AGTCTTCTGAGTGGCAGTGATGG-3' defining a 292 bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplicon.

*Western Blotting*

Cortex, hippocampus, and liver were isolated from 3 month old C57BL/6 mice anaesthetized with Euthansol and euthanized by decapitation. Neurosphere cultures, cortex, hippocampus, and liver samples were incubated in RIPA buffer (10 mM PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 30 µl/ml aprotinin, 10 mM sodium orthovanadate, 100 µl/ml phenylmethylsulfonyl fluoride) for 30 minutes to solubilize proteins and centrifuged for 20 min at 12,000 rpm to remove cellular debris. Tissue was homogenized in RIPA buffer prior to incubation using a Tissue Tearor (Biospec Products Inc., Bartlesville, OK, USA). Protein lysates (30 µg) were separated by SDS-PAGE under reducing conditions and gels were transferred and transferred to polyvinylidene difluoride membranes (Fisher, Ottawa, Ontario). Membranes were blocked at room temperature for 1 h with 1% casein in PBS. Membranes were incubated overnight at 4°C

with primary antibody in 1% casein in 10 mM PBS supplemented with 0.1% Tween-20. Primary antibodies included anti-pannexin 2 (rabbit polyclonal diluted 0.6 μg/mL, Aviva Systems Biology, San Diego, California), anti-pannexin 2 (rabbit polyclonal 0.2 μg/ml, kindly provided by Dr Dale Laird, University of Western Ontario), and anti-Gapdh (mouse monoclonal diluted 1:2000, Imgenex, San Diego, California). Horseradish-peroxidase-conjugated secondary antibodies (rabbit and mouse IgGs) were purchased from Jackson Immunoresearch Laboratories (West Grove, Pennsylvania) and diluted 1:2000.

*Immunostaining and confocal microscopy*

Neurosphere cultures were fixed in 3.7% formaldehyde for 20 min at room temperature, washed in PBS, cryoprotected in 20% sucrose in 10 mM PBS, flash-frozen, and serially sectioned (10 μm thickness) using a cryostat (Leica CM1900). Immunocytochemistry was carried out as previously described (Melanson-Drapeau et al., 2003b). Antibodies were diluted in Ab buffer (10 mM PBS supplemented with 0.3% Triton-X-100 and 3% bovine serum albumin). Primary antibodies were: anti-pannexin 2 (rabbit polyclonal diluted 4 μg/ml, Aviva Systems Biology), anti-glial fibrillary acidic protein (GFAP; rat monoclonal diluted 1:20, Zymed, San Francisco, California), anti-nestin (mouse monoclonal diluted 1:50, Chemicon, Temecula, California), anti-doublecortin (DCX; guinea pig polyclonal diluted 1:800, Chemicon), anti-Tuj1 class III β-tubulin (mouse monoclonal diluted 1:250, RDI), anti-NG2 (rabbit polyclonal diluted 1:200, Chemicon), anti-platelet derived growth factor a receptor (PDGFαR; rat monoclonal diluted 1:300, BD Pharm), and anti-rest in peace (RIP, mouse monoclonal

diluted in 1:1000, Chemicon). Secondary antibodies were: Cy3-conjugated rabbit IgG (diluted 1:600), fluorescein isothyanate (FITC)-conjugated rabbit IgG (diluted 1:100), Cy3-conjugated guinea-pig IgG (diluted 1:800), FITC-conjugated mouse IgG (diluted 1:100), and Cy5-conjugated mouse IgG (diluted 1:400). All secondary antibodies were from Jackson ImmunoResearch. Hoechst 33258 (1 μg/mL) was used as a nuclear counterstain. Confocal imaging was performed on a Zeiss 510 META laser scanning confocal microscope captured with Zeiss LSM 5 software v4.0 SP2 (Zeiss Canada, Toronto) using an EC Plan-Neofluar 40x/1.30 Oil DIC M27 or Plan-Apochromat 63x/1.4 Oil DIC M27 objectives with a pinhole size of 1 Airy unit (or equal optical slices for multiple fluorophores) through Z-stacks in 0.5 μM optical sections. Further magnification was achieved with a 2X or 3X optical zoom. Three-dimensional digital reconstruction and AVI movies were rendered using Zeiss LSM 5 software v4.0 SP2 and Axiovision software v.4.7

### 5.5 Results

Panx2 expression in NPCs was investigated using hippocampus-derived neurosphere cultures from P3 neonates (Fig. 5.1A). Panx2 mRNA (Fig. 5.1B) and protein (Fig. 5.1C) were detected in neurosphere extracts by RT-PCR and Western blot analysis after 12 DIV. Adult cortex and hippocampus lysates served as positive controls in protein analyses. Mouse liver lysate served as a negative control for CNS-specific Panx2 expression. Panx2 protein migrated at the expected molecular weight of ~70 kDa in neonatal neurospheres, adult cortex, and adult hippocampus (Fig. 5.1B). An additional band, with an approximate mobility of 50 kDa, was observed in neurosphere samples (Fig. 5.1B, asterisks). This band was also detected using a non-commercial Panx2

**Figure 5.1 Panx2 mRNA and protein is expressed by postnatal hippocampus-derived NPCs.**

(A) Schematic of methodology. NPCs derived from the hippocampus of P3 pups were cultured as neurospheres and analyzed on DIV 12. (B) RT-PCR of random-primed RNA extracted from 100-150 pooled neurosphere cultures. Panx2 transcript was evident in neurosphere cultures (RT+, upper panel). Controls for genomic contamination and artifactual priming included reactions processed in the absence of RT (RT-) or in the absence of template (NT). The same random-primed templates were amplified for GAPDH to confirm DNA integrity (lower panel). (C) Panx2 protein (~70 kDa) was detected in neurosphere cultures and adult murine cortex and hippocampus (positive controls) but not liver (negative control). Three non-specific bands were observed in liver and neurosphere lysates (arrowheads). Two additional bands (50 and 15 kDa, asterisks) were observed in neurosphere lysates but not in the positive or negative controls. Membranes were stripped and re-probed for Gapdh protein as a loading control (lower panel).

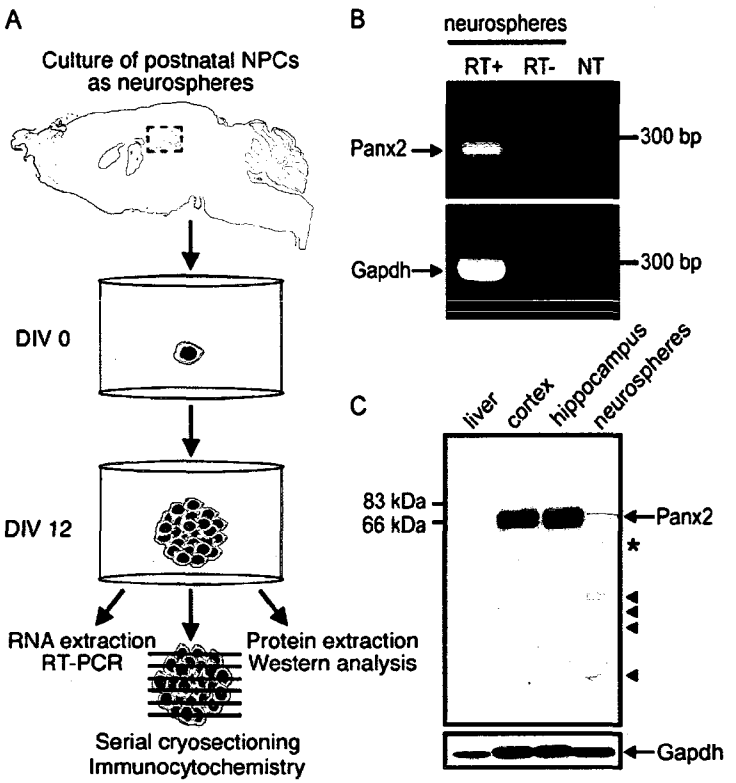

A
Culture of postnatal NPCs
as neurospheres
DIV 0
DIV 12
RNA extraction
RT-PCR
Protein extraction
Western analysis
Serial cryosectioning
Immunocytochemistry
B
neurospheres
RT+ RT- NT
Panx2
300 bp
Gapdh
300 bp
C
liver
cortex
hippocampus
neurospheres
83 kDa
66 kDa
Panx2
*
Gapdh

**Figure 5.1**

antibody provided by Dr. Dale Laird (data not shown) in both neurosphere and positive control lysates and may reflect the presence of splice variants and/or variations in post-translational modification and degradation as previously reported for Panx1 and Panx3 (Penuela et al., 2007). Four non-specific bands were also detected in the negative control and neurosphere lysates using the commercial Panx2 antibody not present in adult brain samples (Fig. 5.1B, arrowheads).

Confocal microcsopy localized Panx2 to a small subset of cells present in neurospheres after 12 DIV. Panx2 immunolocalization invariably appeared as prominent perinuclear punctate aggregates (Fig. 5.2A). Upon examination at higher magnification and with three-dimensional reconstruction, these Panx2 puncta were found to form oblong structures of approximately 1.5 μm in length with a flattened rod-like morphology (Fig. 5.2B, Supplemental Movies M1 and M2). The frequency of cells expressing Panx2 varied somewhat between cultures (Fig. 5.2C), with an average of 8 ± 2% of cells positive for Panx2 per serial neurosphere section (mean ± standard error of the mean (SEM). Interestingly, only one Panx2-positive structure was observed per cell (Supplemental Movies M5.1 and M5.2, not included in thesis).

The lineage of Panx2-positive cells was analyzed using a panel of antibodies detecting NPCs, oligodendrocyte progenitor populations (OPCs), and mature neurons, oligodendrocyes, and astrocytes (Fig. 5.3A). Although each neurosphere is derived from a single NPC, progeny spontaneously adopt distinct developmental lineages as cultures expand (Fig. 5.4A). This progression recapitulates many of the developmental stages observed *in vivo* over the course of early postnatal neurogenesis and gliogenesis (Campos, 2004; Kempermann et al., 2004). We found that Panx2 was restricted to

**Figure 5.2 Panx2 forms oblong structures in the cytosol of a small subset of NPCs in neurosphere cultures.**

(A) Panx2 protein localizes as oblong-shaped aggregates to discrete NPCs. The area outlined by the box in A is depicted in (B) as an orthogonal slice view (3X optical zoom) of serial confocal Z-stacks extracted at the levels indicated by the white lines in x-z and y-z planes. $Panx2^{+}$ oblong puncta were seen to form perinuclear rod-like structures (see also Supplemental Movies M1 and M2). Scale bars, 10 μm. (C) Quantitative analysis of the percentage of $Panx2^{+}$ cells per section. Black boxes depict Panx2-postive cells expressed as a percentage of the total number of cells per neurosphere section (n=12 cultures). Panx2 is expressed in 8.2 ± 2.1% (SEM) of $Hoechst^{+}$ cells (horizontal line).

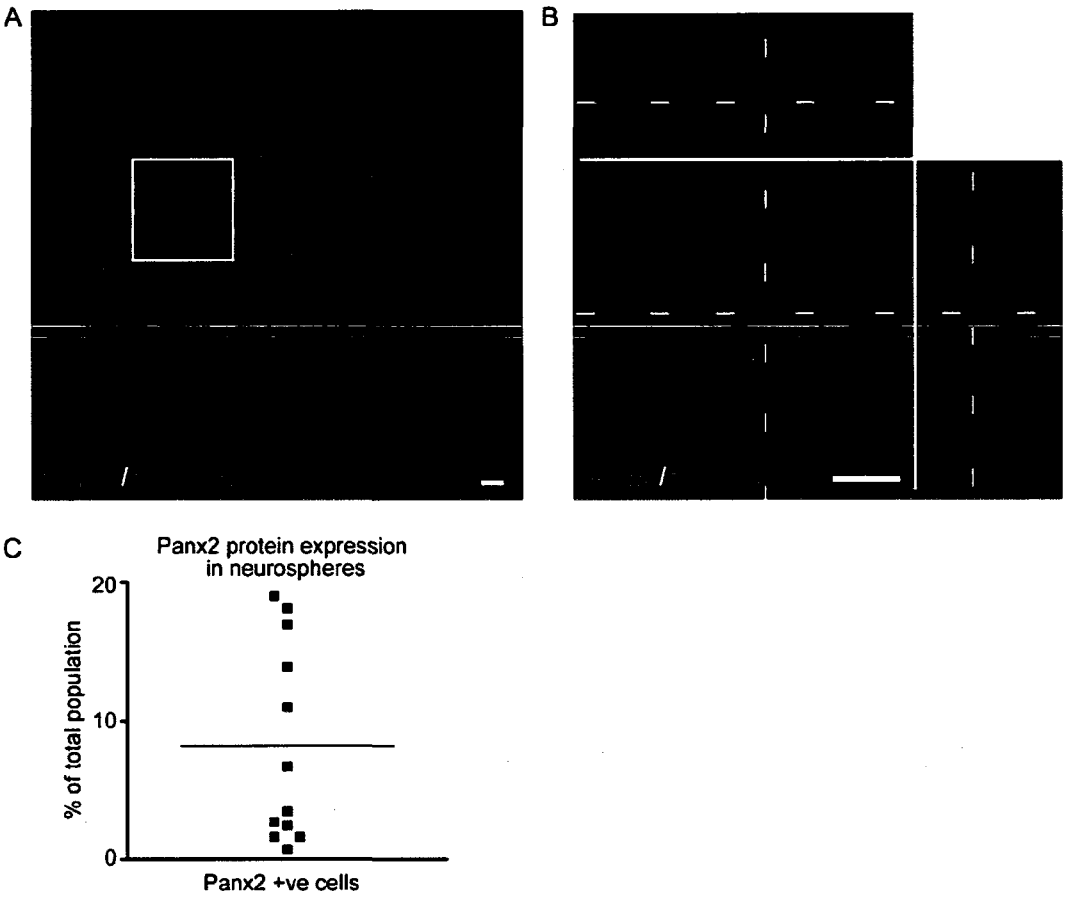
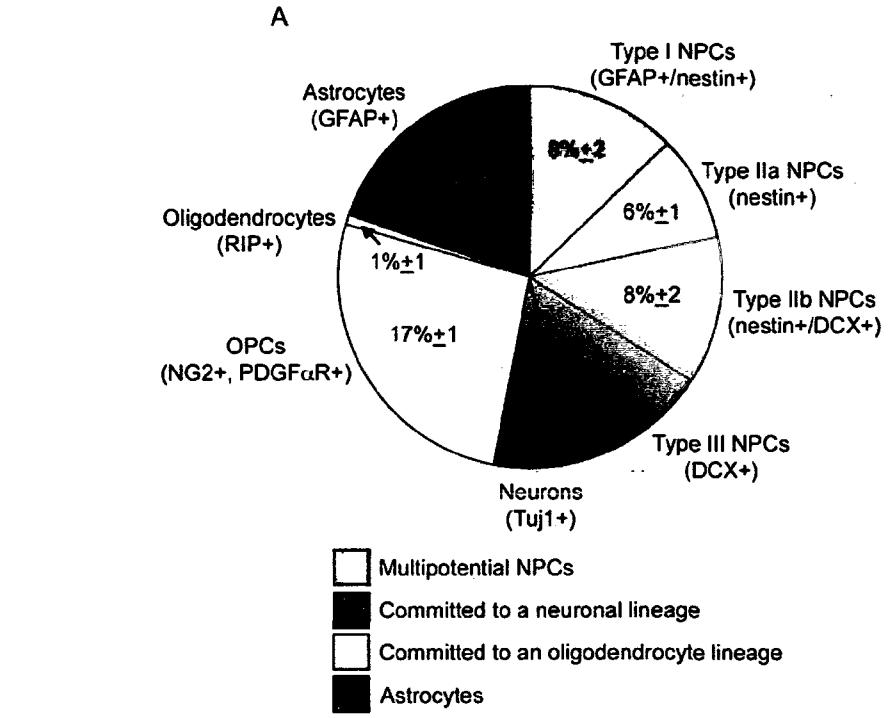

A
B
C
Panx2 protein expression
in neurospheres
20
10
0
% of total population
Panx2 +ve cells

**Figure 5.2**

**Figure 5.3 Panx2 was not detected in committed neuroblasts or post-mitotic immature neurons in hippocampal-derived neurospheres.**

(A) Culture composition of hippocampal-derived neurospheres at DIV 12. We identified 65% of the cells in culture using the antigenic markers indicated. Data represent mean of 5-15 sections counted over n=5-10 neurosphere cultures/condition ± SEM (SEM). (B) Panx2 was not detected in DCX-positive Type IIb or Type III NPCs. Arrow points to a Panx2-postive structure in a DCX-negative cell. (B) Panx2 structures were not detected in TuJ1-positive neurons. Arrow points to a Panx2-postive structure in a TuJ1-negative cell. Scale bars, 10 μm.


A
Type I NPCs
(GFAP+/nestin+)
Astrocytes
(GFAP+)
8%±2
Type IIa NPCs
(nestin+)
6%±1
Oligodendrocytes
(RIP+)
1%±1
8%±2
Type IIb NPCs
(nestin+/DCX+)
17%±1
OPCs
(NG2+, PDGFαR+)
Type III NPCs
(DCX+)
Neurons
(Tuj1+)
Multipotential NPCs
Committed to a neuronal lineage
Committed to an oligodendrocyte lineage
Astrocytes

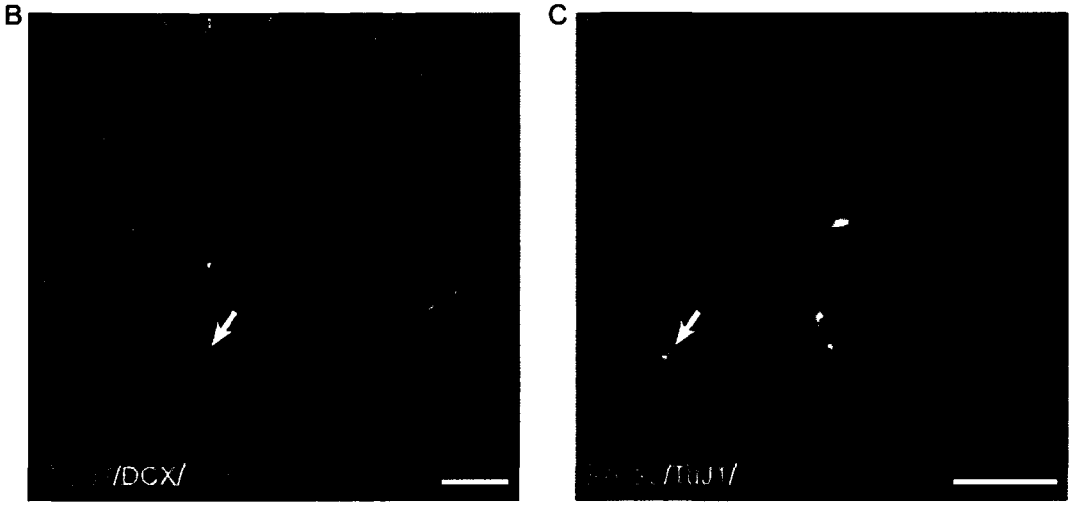

B
/DCX/
C
/TuJ1/

**Figure 5.3**

**Figure 5.4 Panx2 is expressed by Type I and IIa neural progenitor cells.**

(A) Schematic depicting the lineage progression of hippocampal NPCs over the course of specification to a neuronal or glial lineage. Antigenic lineage markers used in this study to identify cell populations are indicated. (B) Confocal imaging of Type I and IIa NPCs. Panx2 is detected in both Type I NPCs (GFAP/nestin-positive, white arrows) and Type IIa NPCs (GFAP-negative/nestin-positive, arrowheads). (C) Panx2 localizes to Type I NPCs. Orthogonal slice view (box) of serial confocal Z-stacks extracted at the levels indicated by the white lines in x-z and y-z planes demonstrates Panx2 structures in a GFAP (green) and nestin (pink) positive cell. (D) Less frequently Panx2 oblong structures were found in Type IIa NPCs (box). A Panx2-positive Type I cell can be seen in the same field (arrow). Panx2 was not detected in DCX-positive Type IIb or Type III NPCs (Fig 5.3B,C). Scale bars, 10 μm.

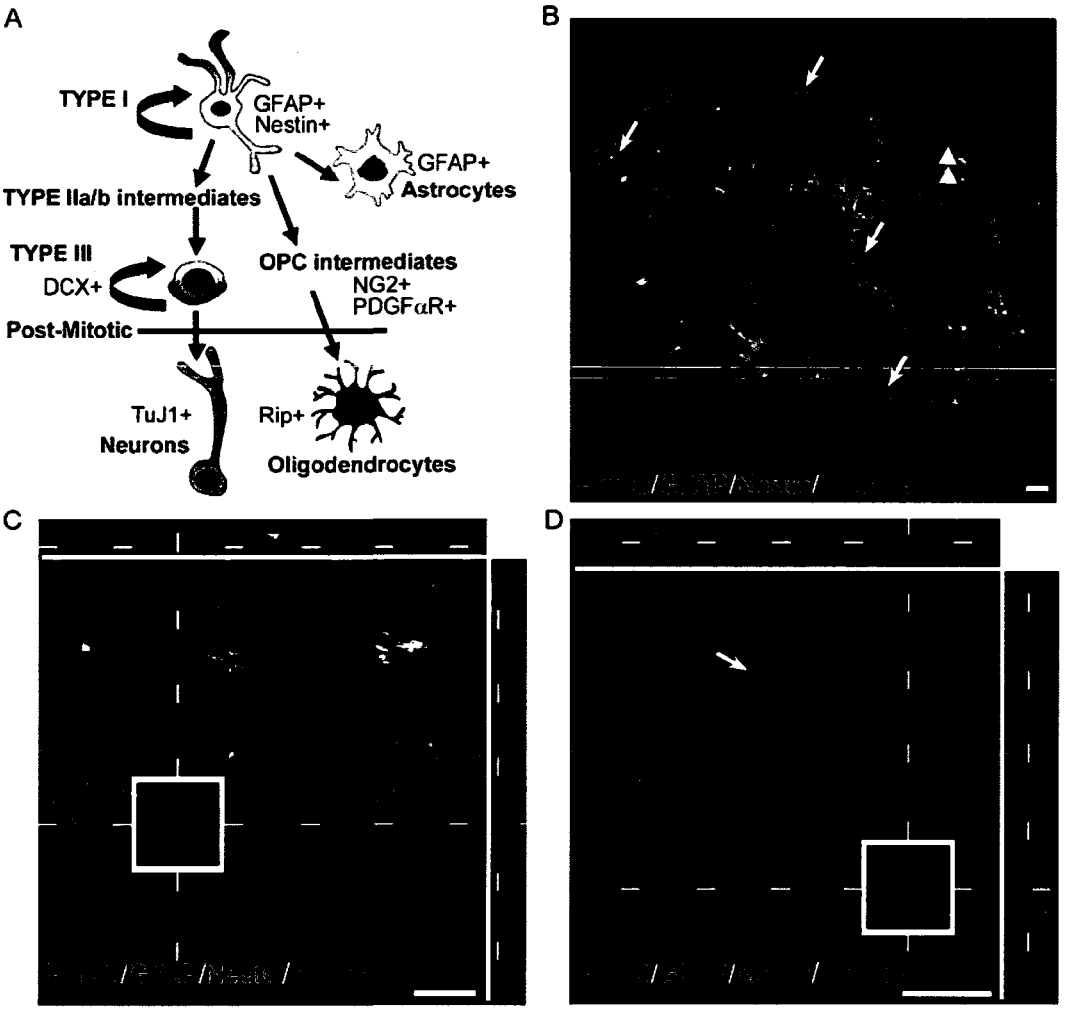

A
TYPE I
GFAP+
Nestin+
GFAP+
Astrocytes
TYPE IIa/b intermediates
TYPE III
DCX+
OPC intermediates
NG2+
PDGFαR+
Post-Mitotic
TuJ1+
Neurons
Rip+
Oligodendrocytes
B
C
D

**Figure 5.4**

subsets of nestin-positive cells (Fig. 5.4B, arrows). Rod-like structures in close proximity to nuclei were detected in both Type I NPCs expressing GFAP and nestin (Fig. 5.4C) and Type IIa cells expressing nestin but not GFAP (Fig. 5.4D). Panx2 immunostaining was not observed in DCX-positive cells or post-mitotic neurons, indicating that Panx2 is restricted to Type I and Type IIa NPCs and is not expressed in Type IIb or Type III neuronal progenitor cells (Fig. 5.3, refer to Fig. 5.4A). Panx2 protein was not expressed in mature GFAP-positive/nestin-negative astrocytes (data not shown).

## 5.6 Discussion

Our data demonstrate that Panx2 forms perinuclear rod-like structures in Type I and IIa NPCs of neonatal hippocampus. One Panx2 structure per NPC is observed. These structures are unique for a gap junction-like transmembrane protein. Further, we show that Panx2 is not detected once multipotential NPCs commit to a neuronal or glial lineage *in vitro*. Together, these data provide the first evidence implicating CNS-specific Panx2 in the specification neonatal hippocampal NPCs.

The finding that Panx2 is not detected in neurons or astrocytes *in vitro* was surprising and contrasts with previous studies indicating that Panx2 localizes to terminally differentiated neurons in the adult CNS with transient expression detected in activated astrocytes following injury (Vogt et al., 2005; Dvoriantchikova et al., 2006; Ray et al., 2006; Zappala et al., 2007; Tang et al., 2008). This disconnect is likely indicative of a shift in expression over the course of early postnatal hippocampal development. Unpublished data from our laboratory indicates that Panx2 expression shifts to mature

neurons in adult tissue with rare NPCs exhibiting Panx2 rod-like structures. This shift may indicate a terminal symmetric commitment of some Type I and II progenitor cell populations as has been suggested for connexin36. Connexin36 mRNA is initially expressed by neural stem cells but not their neuronal progeny during early corticogenesis yet transcript becomes restricted to neurons as these cells symmetrically adopt a neuronal lineage over the course of late embryonic neurogenesis (Gulisano et al., 2000b).

Our work also suggests that the dominant Panx2 isoform may change over the course of postnatal hippocampal development. Two Panx2 isoforms (70 and 50 kDa) are present with equal intensity in NPCs cultured from neonatal mice while the 70 kDa protein likely predominates in adult brain tissue. The identity of the smaller Panx2 isoform is unclear, but likely reflects either alternative splicing, as has been reported for the human Panx2 (Baranova et al., 2004) or post-translational modification. Both Panx1 and Panx3 are phosphorylated and undergo N-linked glycosylation (Penuela et al., 2007). Panx2 may undergo similar processing although the mature protein has a predicted molecular weight of 74 kDa suggesting the small fragment is more likely a splice variant or degradative product.

Finally, the perinuclear rod-like structure formed by Panx2 protein is unique among gap junction proteins. This expression profile supports electrophysiological data that Panx2 does not form functional hemichannels or gap junctions (Bruzzone et al., 2005) and suggests that the potential regulatory effects of Panx2 on Panx1 channels may not involve direct changes in channel properties at the plasma membrane as has been reported for Cx30 and Cx26 (Manthey et al., 2001). For instance, Panx2 may regulate Panx1 by sequestering Panx1 in intracellular pores. Panx1 hemichannels, unlike

connexons, are open at depolarized membrane potentials (Bruzzone et al., 2005; Locovei et al., 2006) associated with NPC activation (Walker et al., 2008). Thus, putative aggregation of Panx1 by Panx2 in rod-like structures could promote the symmetric specification of activated NPCs by preventing the formation of Panx1 hemichannels at the plasma membrane, or by establishing functional pore-like structures in intracellular membranes as has been reported in Panx1 overexpression studies (Vanden Abeele et al., 2006). Alternatively, the rod-like morphology of Panx2 aggregates may exert their own unique function. Clearly, it will be essential to determine how the dynamic expression and localization of Panx2 protein over the course of Type I and IIa NPC specification regulates pannexin channel function and granule cell neurogenesis to impact upon postnatal hippocampal development.

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# Chapter 6: General Discussion

## 6.1 Cell-cell communication and neurogenesis

The mere existence of adult neurogenesis is now undisputed but the reasons for the selectivity in which neurogenic niches reside and the molecular processes behind NPC commitment remain to be teased out. Adult neurogenesis is an individualized process where one can find cells in different stages of commitment and these cells have been shown to respond to environmental and genetic stimuli. How do these cells communicate with one another in response to extrinsic cues for regeneration and can these signals be harnessed therapeutically in non-permissive niches to replace dead or dying neurons? Membrane-bound and diffusible factors are known to regulate the behaviour of NPCs (Song et al., 2002; Barkho et al., 2006). Paracrine factors and juxtracrine cell-cell interactions play an essential role in this process. One such form of cell-cell communication is mediated by connexin or pannexin forming gap junctions. Embryonically, connexin-mediated gap junctions have been shown to couple NPCs, connexin-mediated hemichannels mediate the spread of calcium waves across progenitor cell populations, and opposing connexons act as mutual adhesive molecules aiding in neuronal migration (Bittman et al., 1997; Bittman and LoTurco, 1999; Weissman et al., 2004; Elias et al., 2007). The role of connexins in adult neurogenesis, though, is less well understood although multiple family members are expressed in all mature CNS subtypes through adulthood with expression restricted to various precursor cell populations as shown in Chapter 2 (Imbeault et al., 2008).

This goal of this thesis was to understand the function and expression of "neuronal" gap junction proteins in the context of postnatal hippocampal neurogenesis.

Specifically, I chose to study the prototypical neuronal connexin, Cx36, as well as a new, uncharacterized CNS-specific pannexin, Panx2.

### 6.2 The role of Cx36 in post-natal hippocampal neurogenesis

Cx36 expression has been well defined in mature neurons of the CNS (Condorelli et al., 2000; Deans et al., 2002) but has yet to investigated in adult NPC populations despite dynamic expression over the course of development (Gulisano et al., 2000; Cina et al., 2007). Therefore, I was driven by the hypothesis that Cx36 would (a) be expressed over the course of adult neurogenesis, and (b) promote NPC specification to a neuronal lineage. My results were both surprising and intriguing. Using the neurosphere assay as an *in vitro* model of NPCs, I was able to characterize the mRNA and protein expression of Cx36 during postnatal hippocampal neurogenesis (Chapter 2). Cx36 mRNA expression only was found in NPCs maintained as free-floating neurospheres with protein was first expressed upon engagement to a laminin matrix. Given that the expression of Cx36 was dependent upon ECM interactions, I next examined the effect of the loss of Cx36 function under various media conditions designed to ensure Cx36 protein expression while directing NPCs towards different lineages (Chapter 3). Three microenvironments were examined: 1) Mitogenic media containing growth factors and supporting NPC proliferation, 2) Spontaneous differentiation media devoid of growth factors and 3) Neurogenic media with specific factors that stimulate neuronal differentiation. Interestingly, Cx36 null mutation enhanced neurogenesis upon growth factor withdrawal. This was also seen to an even greater extent in neurogenic media and occurred concomitant with a decrease in nestin$^{+}$ NPCs. All three media conditions also showed an increase in oligodendrogenesis in the null-mutant cultures. Together, these

data supported a role for Cx36 in regulating NPC fate but surprisingly these loss of function studied suggested that Cx36 protein expression was sufficient to inhibit terminal commitment to a neuronal lineage in contrast to my original functional hypothesis.

To provide further insight, I assessed NPC proliferation and survival under neurogenic conditions using BrdU birth-dating and fate-mapping of labelled progeny. Proliferation of cells was unchanged in neurospheres derived from either genotype maintained in suspension wherein Cx36 mRNA but not protein was expressed. Laminin (and thus Cx36 protein expression) induced a dramatic difference in NPC phenotype. Survival of BrdU-labelled cells and their progeny was enhanced by loss of Cx36 and differentiation to a neuronal phenotype apparently accelerated. Of note, there was no change in cell death between genotypes during this period. Taken together, these data suggested that Cx36 regulates neurogenesis, surprisingly, by inhibiting neuronal commitment and maintaining NPC proliferation in a microenvironment-specific manner. Arguably, this function could serve to promote neuronal specialization whereby only subsets of Cx36-expressing neurons are allowed to develop into mature neurons. Thus, Cx36 may be involved in denoting cells as neurons thereby instructing adjacent residual cells to restrict the production of more neurons. Certainly, the expression of Cx36 protein in the adult brain is restricted to specific subtypes. Using animal models in which Cx36 was either conditionally or constitutively knocked-out, reporter expression was found to co-localize with cell markers of interneurons (Deans et al., 2002; Wellershaus et al., 2008). Cx45, another neuron-specific connexin, is also known to specify to certain neuronal subtypes in the adult brain and it would be interesting to see what effects its deletion would have on neuronal commitment in neurospheres (Maxeiner et al., 2003).

The finding presented here could reveal an important, novel function for connexins and the specification of mature cell subtypes in the adult mammalian brain.

One could speculate many possible mechanisms for these results. Diffusable factor released through Cx36-mediated hemichannels may induce progenitor cells to maintain pluripotency at the expense of neuronal fate commitment thus providing a competitive advantage to immature $Cx36^{+}$ neurons that inhibits the specialization of their neighbours. The loss of Cx36 causes a subsequent loss of extrinsic cues for the proliferation of adjacent cells leading to an increase in differentiation and commitment to neurons in the presence of a permissive microenvironment. In support of this proposed mechanism, aberrant intercellular communication has been shown previously to be detrimental to maintaining proliferation of ES cells leading to the upregulation of differentiation markers and marked morphological changes (Todorova et al., 2008).

Surprisingly, the increase in neurogenesis detected in Cx36 null-mutant cultures was accompanied by an increase in oligodendrogenesis. Likely, this effect is indirect perhaps with the increased number of immature neurons providing a conditioned environment for oligodendrocyte differentiation as seen previously in co-cultures of primary neurons and adult neural stem cells (Song et al., 2002). Changes in astrogenesis following deletion of Cx36 were even more surprising. Under mitogenic condition there was a significant decrease in astrocytes yet under spontaneous conditions, the exact opposite was observed. Astrocytes are also known as regulators of neurogeneisis, instructing stem cells to adopt a neuronal cell fate (Song et al., 2002). Again, one could speculate that loss of paracrine signalling between Cx36 expressing cells and astrocytes may lead to aberrant gliogenesis here under differentiation conditions. Deciphering

whether Cx36 intercellular channel or hemichannel activity represents the crucial next step in mechanistic evaluation of connexin-mediated communication in NPCs.

With this phenotype in hand, I next sought to investigate the impact of loss of Cx36 function on adult neurogenesis *in vivo* (Chapter 4). In the uninjured adult mouse, there was no difference in NPC proliferation or survival in Cx36 null-mutant mice compared to congenic WT adults. As of yet, we cannot rule out functional changes in the connectivity of immature neurons generated continually in the SGZ over the course of mammalian life span. As a result of hippocampal neurogenesis, newborn granule neurons migrate to the granule cell layer of the dentate gyrus and have arborizations and electrophysiology similar to mature neurons within two months of birth (Zhao et al., 2006). Future experiments have been planned to see if, following the loss of Cx36, these newborn neurons no longer make correct connections with CA3 pyramidal cells or whether their migration through the granule cell layer is compromised in otherwise healthy animals.

To evaluate the null-mutant phenotype following injury, I then moved to a kainic-acid induced excitotoxic injury model reasoning that if the loss of Cx36 enhances neurogenesis *in vitro*, this may prove valuable for neuroregeneration following hippocampal injury. Unexpectedly, loss of Cx36 was found to promote survival of mature neurons without significant impact upon the neuroregenerative capacity of adult NPCS. Following seizures, neurogenesis increases within the hippocampus but has been shown to occur with significant abnormalities, including dispersion of the DGC layer, ectopic localization of NPCs and erroneous mossy fiber connections with the CA3 (Jessberger et al., 2005; Parent et al., 2006). This abnormal circuitry development has

therefore been suggested to contribute to the evolution of initial seizure-induced hippocampal injury into chronic epilepsy and cognitive impairment (Jung et al., 2004; Jessberger et al., 2007). Suppression of abnormal neurogenesis after seizures has been considered to reduce epileptogenesis and prevent subsequent cognitive impairment. These studies used different means to ablate neurogenesis, such as using mitotic inhibitors, low dose gamma radiation and enzymatic depolysialyation of neural cell adhesion molecule (NCAM) potentially leading to unknown side effects due to their non-selectivity and, as such, have shown contrasting results (Jung et al., 2004; Pekcec et al., 2007; Wyckhuys et al., 2007). Cx36 represents a potentially selective therapeutic target to promote neuronal survival. By doing this, it will reduce aberrant neurogenesis and perhaps subsequent recurrent seizures and cognitive abnormalities.

The disconnect between the *in vitro* and *in vivo* data could also prove beneficial in aiding the use of NPCs for potential therapeutic targets in cell replacement strategies. Transiently knocking down Cx36 *in vitro*, for example, could help stimulate neuronal differentiation to produce sufficient quantities of neurons for transplantation. Recent strategies for neurodegenerative cell therapy have suggested that transplantations involving more committed NPCs and/or post-mitotic neuronal cells are better to decrease the incidence tumour formation, thereby allowing this commitment to occur *in vitro* (Sanchez-Pernaute et al., 2008; Wernig et al., 2008).

Summarizing the results, loss of Cx36 increases NPC commitment to neurons and oligodendrocytes *in vitro* while providing neuroprotection to mature neurons following hippocampal injury *in vivo* (Fig 6.1). Cx36-mediated communication may restrict the capacity of NPCs to commit to a neuronal lineage within permissive microenvironments.

**Figure 6.1 Model of NPC commitment following loss of Cx36**

Depiction of adult hippocampal neurogenesis and gliogenesis summarizing the effect of Cx36 null mutation on NPC fate *in vitro* and following hippocampal injury *in vivo*. Under specific microenvironment conditions, laminin engaged neurospheres at 14 DIV show an increase (red square) or decrease (blue square) in the total population of specific progenitors and post-mitotic cells. Mitogenic, spontaneous and neurogenic conditions all show an increase in oligodendrocyte progenitors following constitutive deletion of Cx36. Spontaneous and neurogenic conditions show an increase in $DCX^+$ neuroblasts and $TuJ1^+$ immature neurons. Mitogenic and neurogenic conditions also show a decrease in $nestin^+$ NPCs. Finally, mitogenic conditions produce an increase in astrocytes while spontaneous conditions produce a decrease in this cell type. Following hippocampal injury, the loss of Cx36 protects neurons from apoptotic cell death without showing a change in neuroregenerative capacity (green circle).

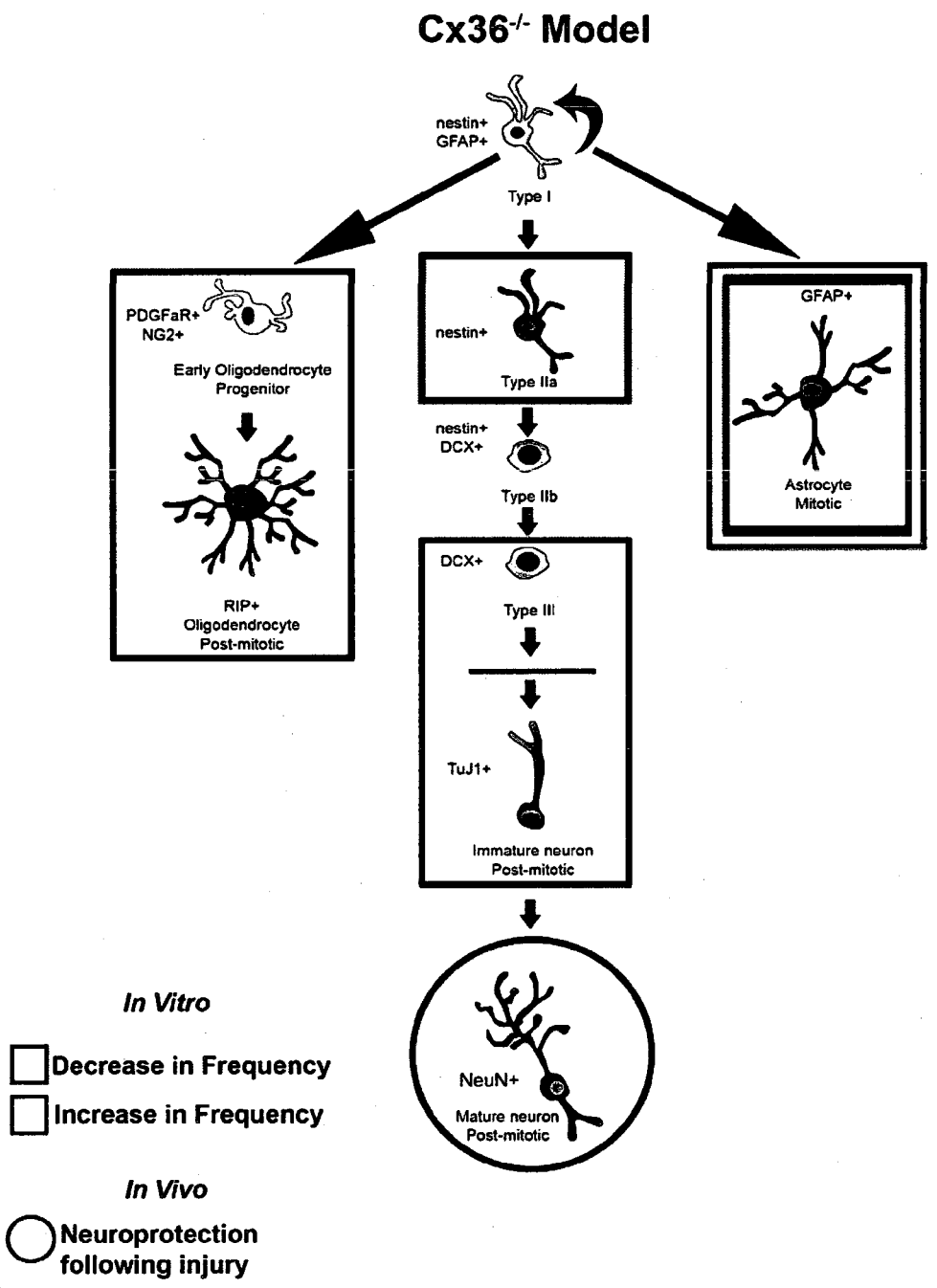
Cx36-/- Model
nestin+
GFAP+
Type I
PDGFaR+
NG2+
Early Oligodendrocyte
Progenitor
RIP+
Oligodendrocyte
Post-mitotic
nestin+
Type IIa
nestin+
DCX+
Type IIb
DCX+
Type III
TuJ1+
Immature neuron
Post-mitotic
GFAP+
Astrocyte
Mitotic
NeuN+
Mature neuron
Post-mitotic
In Vitro
Decrease in Frequency
Increase in Frequency
In Vivo
Neuroprotection
following injury

**Figure 6.1**

Furthermore, I speculate that Cx36 may propagate the spread of neurotoxic factors following excitotoxic injury by creating connections with adjacent immune responsive cells. Together, these results implicate the role of Cx36 in directing NPC fate *in vitro* and regulating neuronal survival *in vivo.*

### 6.3 Characterizing Panx2 expression in NPCs

The second aspect of my thesis, looked defining the expression of Panx2 during postnatal hippocampal neurogenesis as a basis for future functional studies. Panx2 is CNS specific but has yet unknown function in that evidence of functional hemi- or intercellular homomeric channel activity at the plasma membrane remains elusive (Bruzzone et al., 2005; Shestopalov and Panchin, 2008). Previous literature reported that Panx2 mRNA is found in neonatal tissue and rises over the course of development (Vogt et al., 2005). Furthermore, protein expression is found in most mature neurons of the CNS (Vogt et al., 2005; Zappala et al., 2007). Therefore I hypothesized a similar protein expression profile in neurospheres as Cx36, that is, exclusively in immature, committed neuronal cells. Again unexpectedly, I was able to find Panx2 protein expression in DIV 14 neurospheres in suspension and moreover, it was co-localized to Type IIa/b NPCs, that is nestin$^+$/GFAP$^+$ cells or nestin$^+$/GFAP- cells (Chapter 5). Quantitative analysis by flow cytometry is currently underway to validate the immunocytochemistry. Preliminary analysis shows neurospheres expressing Panx2 at a frequency of approximately 4%. In addition, Panx2 formed cytoplasmic rod-like structures in neurospheres morphologically distinct from other gap junction proteins as shown by confocal microscopy. These structures co-localize to markers of the endoplasmic reticulum (ER) and not to plasma membrane or nuclear membrane markers. Current work in our laboratory by colleague

Dr Leigh Anne Swayne has corroborated these findings, showing Panx2 colocalizing with ER and golgi marker by protein fractionation of neurospheres followed by Western blotting. These data is in accordance with the aforementioned studies that were unable to observe functional gap junction channel properties of Panx2 *in vitro* and suggests a potential regulatory mechanism for sequestering of proteins such as Panx1. New data for our laboratory not included in this thesis also shows that Panx2 expression shift to mature neurons in the adult while maintaining their rod-like structure. This may indicate a terminal asymmetric division of Type I and IIa NPCs as has once been hypothesized as the reason for the diverse embryonic and post natal expression of Cx36 (Gulisano et al., 2000). Thus, this study establishes an interesting basis for functional analysis of the role of Panx2 in postnatal hippocampal neurogenesis that justifies future mechanistic evaluation.

### 6.4 Significance of Research: Implications for neurodegenerative disorders and neural replacement strategies

With the incidence of neurodegenerative disease increasing with age, this confluence of demographics and disease susceptibility represents a challenge to both the medical community and Western society at large (Elbaz and Moisan, 2008; Salloway, 2008). Despite this, the current spectrum of treatment options for neurodegenerative diseases remains inadequate. However, in a possible boon to research, recent progress resulted in the discovery of a renewable source of neurons in the adult mammalian brain, an environment once believed to be incapable of generating new neurons. This untapped source of neural precursor cells provides a new resource to replace dead or damaged neurons and raises the possibility of a new hope for cell-replacement strategies as a

treatment option for neurodegenerative disease (Emsley et al., 2005; Colucci-D'Amato and di Porzio, 2008). By understanding the basic biological processes of neuro-cellular activation, migration, survival, and specification, it may become possible to isolate and perpetuate progenitor cell populations in sufficient quantities to either generate "type-specific" neurons for transplantation or stimulate endogenous populations by use of proliferation and differentiation-promoting factors. As discussed above, this thesis has implicated, for the first time, a Cx36 and Panx2 proteins in the regulation of NPC architecture (i.e., lineage commitment) and function. Future investigation into the factors and signaling pathways affected by their expression will need to be elucidated and studied for the ultimate goal of being useful in therapeutic strategies discussed above.

## 6.5 References


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# Appendix 1: Cirriculum vitae

## ACADEMIC BACKGROUND

| | | |
|---|---|---|
| Dec 2007 | **HARVARD UNIVERSITY**<br>• Invited to study and collaborate with Dr. David Paul in the Department of Neurobiology, Harvard Medical School<br>• Developed a lentivirus expressing connexin36 | Boston, MA |
| 2006-2009 | **UNIVERSITY OF OTTAWA**<br>• Masters of Science, Biochemistry - Completion March 2009<br>• Courses Completed: NSC 5102, Cellular an Molecular Neuroscience and BCH 8105, Advanced Topics in the Molecular Biology of Human Disease<br>• Member of the Neural Regeneration Laboratory under the supervision of Dr. Steffany Bennett | Ottawa, ON |
| 2002-2006 | **UNIVERSITY OF WESTERN ONTARIO**<br>• Obtained undergraduate degree in the Bachelor of Medical Science Honours Biochemistry Program with distinction<br>• Dean's Honour Roll, averaging above 90% in all years attended | London, ON |
| 1997-2002 | **CENTENNIAL SECONDARY SCHOOL**<br>• Graduated with Honours and received Ontario Secondary School Diploma<br>• Ontario Scholar | Welland, ON |

## WORK EXPERIENCE

| | | |
|---|---|---|
| Jan-Jan 2009 | **TEACHERS' SCIENCE AND TECHNOLOGY OUTREACH PROGRAM**<br>• Created lesson plans, laboratory experiments and taught one-hour lectures for grade seven students at Henry Munro Public School<br>• Topics included, "Energy and Control: Heat" and "Material and Mixtures: Structural Strength and Stability" | Ottawa, ON |
| Jan-May 2008 | **DR. ILONA SKERJANC, UNIVERSITY OF OTTAWA**<br>• Teaching assistant for biochemistry undergraduate course entitled, "Cell Regulation and Control" (BCH 4125)<br>• Marked assignments and exams, proctored exams, tutored students | Ottawa, ON |
| Summer 2006 | **DR. STEFFANY BENNETT RESEARCH LAB, UNIVERSITY OF OTTAWA**<br>• Investigated the role of connexin protein in regenerating damaged brain tissue using mouse models<br>• Recipient of NRL-Bennett Summer Research Award | Ottawa, ON |

Summer 2005-May 2006 **DR. MEGAN DAVEY RESEARCH LAB, UNIVERSITY OF WESTERN ONTARIO** London, ON

- Recipient of NSERC Undergraduate Student Research Award
- Investigated the role of Mcm 3 and Mcm 5 in the initiation of DNA replication
- Performed Bradford, helicase, and ATPase assays, Western Blots and various protein purification protocols
- Also completed Honour's level thesis project at the same facility

Summer 2003 – 2006 **CONFERENCE SERVICES, UNIVERSITY OF WESTERN ONTARIO** London, ON

- Conference Assistant and promoted to Conference Secretary during second year of employment
- Reservations Clerk at Essex Hall Bed and Breakfast during third year of employment
- Facilitated the co-ordination of conferences on campus using campus facilities
- Involved in integration of services of various campus departments

## HIGHLIGHTS OF VOLUNTEER EXPERIENCE

2008 **CANADA-WIDE SCIENCE FAIR, YOUTH SCIENCE FOUNDATION CANADA** Ottawa, ON

- Served on Judging Panel in the Life Sciences Junior Division May 12-15

2007 **THE CANADIAN - IRONMAN DISTANCE DUATHLON, TRIATHLON** Ottawa, ON

- Helped distribute race-chips, supervise water stations, distribute medals to finishers and look after athletes post-race

2005 **SCIENCE PEER MENTOR, LEADERSHIP AND MENTORSHIP PROGRAM (LAMP)** London, ON

- Provide first-year students with a strong sense of both academic and community support through guidance and mentorship
- Organize events and volunteer opportunities for students to help enhance academic success and aid in transition to university life
-

2004 **FRONTIER COLLEGE: "STUDENTS FOR LITERACY"** London, ON

- Helped organize and run homework programs, as well as bake, art and fitness activities for public school children

2003 **ST. JOSEPH'S HEALTH CARE UNIT, MOUNT HOPE CENTER FOR LONG TERM CARE** London, ON

- Recreation Event Coordinator – Organized local field trips, leisure games and visitations to improve the patients' quality of stay

## AWARDS

- Natural Sciences and Engineering Research Council of Canada (NSERC) Post Graduate Studies - *Canadian Graduate Scholarship Masters – 2006, 2007*
- Neural Regeneration Laboratory Scholarship – 2006, 2007
- Strategic Areas of Development Admission Scholarship – 2006
- University of Ottawa Excellence Scholarship – 2006, 2007
- University of Western Ontario Biochemistry 420b Excellence Award - 2006
- University of Western Ontario Biochemistry In-Course Scholarship Year IV - 2006
- NSERC Undergraduate Student Research Award – 2005, 2006 (not held)
- Biochemistry Summer Research Studentship Award – 2005

## PUBLICATIONS

- **C.D. Sorbara**, L. Melanson-Drapeau, V. Peshdary and S.A.L. Bennett (2008) Connexin-mediated control of neurogenesis and gliogenesis. *Curr Stem Cell Res Ther.* Invited Submission. Under editorial review.
- L. Swayne, **C.D. Sorbara**, and S.A.L. Bennett (2008) Pannexin 2 is expressed by neural progenitor cells in the postnatal hippocampus. *J Neurosci.* In preparation for resubmission in response to reviews.
- S. Imbeault, L.G. Gauvin, H.D. Toeg, A. Pettit, **C.D. Sorbara**, L. Mighad, A. Menzies, K. Nishii, D.L. Paul, A. Simon and S.A.L. Bennett (2009) Gap junction protein expression and function in postnatal hippocampal neural progenitor cells is controlled by the extracellular matrix. *BMC Neuroscience.* In press.

## ORAL COMMUNICATIONS

- C.D. Sorbara, R. DesRoches, D.L Paul, and S.A.L. Bennett (2009) Connexin 36 null mutant regulates NPC specification over the course of post-natal hippocampal neurogenesis *in vitro* and protect neurons from excitotoxic challenge in the adult murine hippocampus *in vivo*. *Poster presentation to be given at the International Gap Junction Conference, Arizona (July 2009)*
- C.D. Sorbara, D.L. Paul, and S.A.L. Bennett (2008) Connexin 36 is implication in control of post-natal murine neural progenitor cell survival and specification. Neuroscience 2008, Poster presentation given at the Society for Neuroscience Annual Meeting, Washington, USA (November 2008)
- Adult Neural Stem Cells and Mental Illness. Lecture prepared and given for BCH 4009 Undergraduate Course at the University of Carleton, Ottawa, ON (November 2007)
- C.D Sorbara, S.N. Whitehead, D.L Paul, and S.A.L. Bennett (2007) Connexin 36 is implicated in neural progenitor cell proliferation and specification in the adult mouse brain. Oral presentation given at the International Gap Junction Conference, Copenhagen, Denmark (August 2007)

## LABORATORY TECHNIQUES

- Mammalian tissue culture and primary culture, in situ hybridization, extraction and isolation of DNA, RNA and protein from rodent tissue, PCR analysis, cloning, lentiviral construction, DNA electrophoresis, flow cytometry of neurospheres using a Beckman Coulter FC500 Flow Cytometer, immunohistochemistry, TUNEL/Fluorojade B assays, epifluorescent and confocal microscopy using a Zeiss 510 META laser SCM
- Mouse handling: maintenance, cytological smear (estrous cycle), genotyping, intraperitoneal injections, kainic acid seizure induction, transcardial perfusion and decapitation, tissue cutting (cryostat, vibratome), neurosphere assays, tissue isolation, blood pressure readings
- Computer skills: Microsoft Office, Openlab, Clone Manager, DNAStar, Adobe Photoshop, DeltaGraph, EndNote

## INTERESTS

- Boston Marathon Qualifier, May 2008
- Completed 2007 Niagara Falls International Marathon and 2007, 2008 ING National Capital Marathon as a member of "Team Parkinson's"
- Completed 2008, 2007 HBC Run for Canada, and 2007 Mitsubishi Ottawa City Chase
- Completed Tier1 of the UWO Leadership Education Program 2006
- Enrolled in UWO Mini Medical School 2005, 2004, 2003
- Member of Kensal Park Women's Soccer League, 2005