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**Enhancement of neural cell regeneration and survival by altering  
gap junctional communication following brain injury**

**Mario Y. Morin**

Thesis submitted to the  
Faculty of Graduate and Postdoctoral Studies  
In partial fulfillment of the requirements  
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## Thesis Abstract

The adult brain is particularly susceptible to injury given that it lacks significant regenerative capacity. The presence of rare neural stem and progenitor cells - cells capable of self-renewal and specification to a neuronal or a glial lineage - in specific areas of the adult central nervous system (CNS) offers new hope for therapeutic cell replacement. Our laboratory has previously implicated connexin32 (Cx32) in participating in neural progenitor cell fate determination to an oligodendrocyte lineage (Melanson-Drapeau et al., 2003; Hebb et al., submitted). Based on these previous findings, I hypothesized that neuronal regeneration might be increased in Cx32 knockout (KO) mice following hippocampal injury. Kainic acid (KA)-induced epileptic seizure was used to elicit loss of hippocampal neurons and study subsequent progenitor cell proliferation and specification to neurons and oligodendrocytes in the hippocampus of adult wild type (WT) and Cx32KO mice. The kinetics of hippocampal cellular degeneration and regeneration were established following seizure by qualitative and quantitative histological analyses. I found that the viability of the granule cell population in the dentate gyrus (DG) was compromised following excitotoxic challenge but that damaged cells recovered four weeks post-injury without undergoing significant cell death. Substantial neuronal loss with both apoptotic and non-apoptotic characteristics was detected in the CA3 field of the hippocampus 0.5 weeks post-injury in both genotypes. Significant progenitor cell amplification (proliferation) was observed in the CA3 field at the same time period. Progenitor cells in Cx32KO mice preferentially committed to a neuronal

lineage, increasing neuroregeneration 4.5 weeks following injury. The Morris water maze, a hippocampal dependent spatial memory task, was used to test the functionality of these new neurons four weeks following KA-induced injury. I show that uninjured WT and Cx32KO mice exhibit comparable indices of spatial learning and memory. Following KA-induced seizure, WT mice are behaviourally impaired while Cx32KO mice are not, providing strong evidence of enhanced functional neuroregeneration in the absence of Cx32. Finally, to provide a tool to track the fate of neural progenitors destined to express Cx32 over time following injury, a new transgenic marker mouse, that expresses the enhanced green fluorescent protein (EGFP) in place of Cx32, was engineered for use in future studies.

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## List of Abbreviations

AD	Alzheimer's disease
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AOI	area of interest
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
bp	base pair
BrdU	5-bromo-2-deoxyuridine
cAMP	adenosine 3', 5'-cyclic monophosphate
CCAC	Canadian Council on Animal Care
cGMP	guanosine 3', 5'-cyclic monophosphate
CL	cytoplasmic loop
CMTX	X-linked Charcot-Marie Tooth disease
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
DG	dentate gyrus
DIG	digoxigenin
Cx	connexin
dNTP	deoxynucleotide triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
DEPC	diethylpyrocarbonate
DTA	diphtheria toxin A
E	extracellular loop
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ERK	extracellular signal-regulated kinase
ES cell	embryonic stem cell
FJB	Fluoro-Jade B
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GJIC	gap junctional intercellular communication
H&E	Hematoxylin and Eosin
HRP	horseradish peroxidase
KA	kainic acid
kDa	kilo Dalton
KO	knockout (null mutant)
lacZ	beta galactosidase
LRD	lysinated rhodamine dextran
MANOVA	multiple analysis of variance
MAPK	mitogen-activated protein kinase
MF	mossy fibre
MGPY	Methyl green and Pyronin-Y
MRRC	Mental Retardation Research Center
NaCl	sodium chloride

NF	nuclease free
NMDA	N-methyl-D-aspartate
NPC	neural progenitor cell
NSC	neural stem cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF $\alpha$ R	platelet-derived growth factor alpha-receptor
PGK	phosphoglycerate kinase
PMSF	fluorosulfonylmethylbenzene
PNS	peripheral nervous system
PP	perforant pathway
PVDF	polyvinylidene difluoride
RNase	ribonuclease
SC	Schaffer's collateral
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of measurement
SGZ	subgranular zone
SVZ	subventricular zone
Taq	<i>thermus aquaticus</i>
TdT	terminal deoxynucleotidyl transferase
TE	tris EDTA
TOAD-64	Turned on after division (64 kDa)
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol
TUJ1	$\beta$ -tubulin Type III
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling
WT	wild type

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# Chapter 1: Thesis Introduction

## 1.1 Past and present of neurodegenerative diseases

Nearly a century ago, Alois Alzheimer reported the presence of senile plaques and neurofibrillary tangles in the brain of a middle-aged patient suffering from memory deficits and cognitive dysfunctions (Alzheimer et al., 1995). Shortly after, in 1912, Friedrich Lewy reported the presence of small aggregates in the brain that are now referred to as Lewy bodies, the neuropathological characteristic of Parkinson's disease, in a patient suffering from motor function impairment (Forman et al., 2004). These two publications were among the first systematic assessments of neurodegenerative disease.

Neurodegeneration is characterized by a progressive loss of neuronal cells in specific regions of the brain (Limke and Rao, 2003). A wide variety of neurodegenerative conditions such as Alzheimer's disease (AD) or epilepsy often result in severe loss of neurons and functionality in the hippocampus, a region critical for the encoding and retrieval of memory (Nakagawa et al., 1999). In AD, progressive synaptic failure followed by neuronal loss in hippocampus and other related memory regions are purported to underlie the loss of memory (Mikkonen et al., 2001). In epilepsy, strong depolarization of excitatory synapses in the hippocampus and other foci can trigger generalized seizure activity in the organism (Scharfman, 2000). Because of its rich content of glutaminergic neurons, the hippocampus not only plays a major role in the aetiology of epileptic seizure, but is also profoundly damaged by sustained seizure activity (Dalby and

Mody, 2001). Many have postulated that glutaminergic excitotoxicity contributes to neuronal loss in multiple neurodegenerative conditions such as ischemia (Won et al., 2002), epilepsy (Schwarcz et al., 1984; Kelsey et al., 2000; Campbell and Hablitz, 2005), AD (Mattson, 1997; Tannenberg et al., 2004) and Parkinson's disease (Olney et al., 1990; Przedborski, 2005).

Considering the currently aging population and the increase in life expectancy, neurodegenerative disorders are an escalating concern for the medical field. The majority of treatments aim at reducing the rate of neuronal injury and/or boosting function of residual cells. The recent discovery of neural stem (NSCs) and progenitor cells (NPCs) in the adult mammalian CNS brings new hope for therapeutic cell replacement given their potential to restore damaged brain tissue. Mobilizing endogenous NSCs and NPCs or transplanting endogenous populations represents a new therapeutic target to help individuals suffering from neurodegenerative conditions (Gage et al., 1998; Prochiantz, 2000). Validation of this promise, however, will require a thorough understanding of the mechanisms underlying NSC commitment, specification, and integration in injured and uninjured brain.

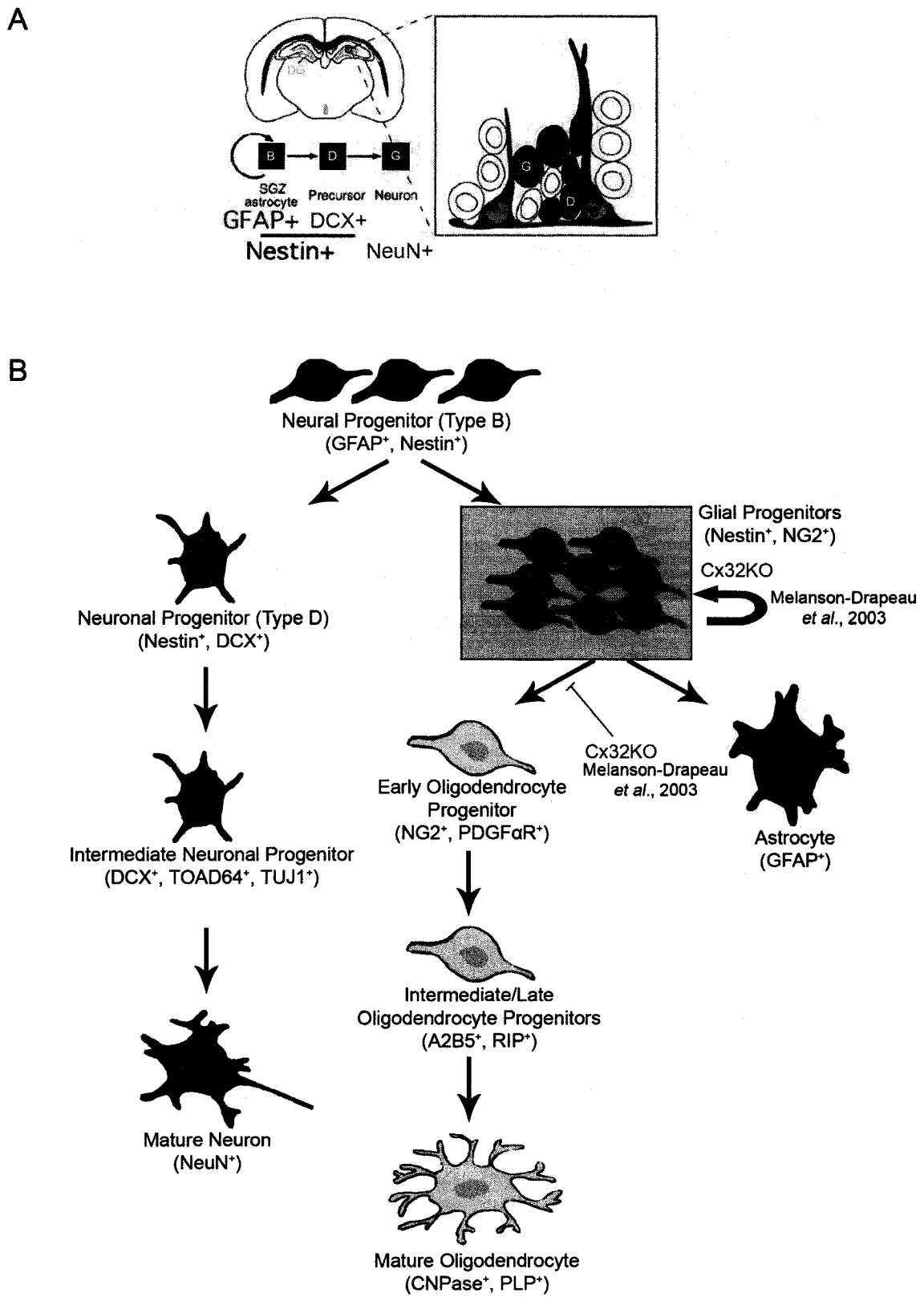
## **1.2 Stem and progenitor cell based therapy for neuronal impairment**

NSCs and NPCs are, by definition, precursor cells capable of self-renewal and of generating neurons or glia (Reynolds and Weiss, 1992). NSCs have the capacity for unlimited proliferation and are likely only present in the adult forebrain (Morshead and van der Kooy, 1992; Morshead et al., 1994; van der

Kooy and Weiss, 2000; Morshead and van der Kooy, 2004). NPCs have a more restricted proliferative potential and represent the primary multipotential cell type in the hippocampus (Kempermann et al., 2004). As illustrated in Figure 1.1, progenitor cells found in the adult hippocampus progress through a well-defined step-wise lineage commitment in which type B progenitor cells (glial fibrillary acidic protein positive (GFAP<sup>+</sup>) and Nestin<sup>+</sup>) divide asymmetrically. Their progeny specifies either to Type D (DCX<sup>+</sup>) neuronal progenitors and then to neurons or to NG2<sup>+</sup> progenitors and then to oligodendrocytes (Doetsch, 2003). Intermediate NG2<sup>+</sup>/nestin<sup>+</sup> “glial” progenitor cells may also be multipotential and able to generate a series of intermediate progeny to oligodendrocytes, possibly astrocytes, or more rarely, commit to a neuronal lineage (Belachew et al., 2003).

Recent studies indicate that it may be possible to increase the rate of endogenous regeneration of the brain by transplanting exogenous NPCs and NSCs, delivering growth factors that promote NPC and NSC expansion, or mobilizing endogenous NPC and NSC populations to partially restore damaged neuronal circuitry (Magavi et al., 2000; Nakatomi et al., 2002; Arlotta et al., 2003; Chiba et al., 2003; Limke and Rao, 2003; McBride et al., 2004). All of these approaches possess numerous advantages, such as the potential to expand the cells prior to transplantation and the ability to manipulate differentiation, but careful attention to their disadvantages is critical. For example, following transplantation, immune rejection of the transplanted cells, failure to thrive, or alternatively, uncontrolled cell proliferation leading to tumor formation could very well negate the promise of therapeutic neuroregeneration.

**Figure 1.1: Progenitor cell specification in the SGZ of the adult hippocampus.** Section (A) was modified from Doetsch *et al.*, 2003. (A) Coronal view of the hippocampus, identifying cell types and neuronal differentiation in the SGZ. Nestin<sup>+</sup>/GFAP<sup>+</sup> progenitor cells give rise to intermediate neuronal precursors (Nestin<sup>+</sup>/DCX<sup>+</sup>) which in turn generate new neurons (NeuN<sup>+</sup>). (B) Specification of multipotential progenitors in the SGZ. Type B progenitors cells (GFAP<sup>+</sup> and Nestin<sup>+</sup>) divide asymmetrically to produce type D (DCX<sup>+</sup>) neuronal progenitors and then neurons, or NG2<sup>+</sup> glial progenitors and then through a series of intermediate progeny to oligodendrocytes or astrocytes.



**Figure 1.1**

Early clinical trials indicate that precursor cell transplantation has the potential to improve neurological function in diseased brain (Kondziolka et al., 2000; Meltzer et al., 2001; Nelson et al., 2002). However, our knowledge about how NSC and NPC fate are regulated in the adult brain following injury is still very limited. As indicated above, exploring the promise of effective cell replacement therapy will require a comprehensive understanding of basic NSC and NPC biology (Lindvall et al., 2004).

The largest populations of putative multipotential NSCs and NPCs in adults – cells capable of specification to either neurons or glia – are found in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the DG (Gage, 2000; Jin et al., 2001; Johansson, 2003). In the SVZ, these populations of “unspecialized cells” represent only 0.2-0.4% of the cells found in the adult forebrain subependymal (Morshead et al., 2002). In other brain regions, proliferating cells primarily give rise to glia (Horner et al., 2000; Kornack and Rakic, 2001; Rakic, 2002), or arguably, they retain their multipotentiality but remain quiescent during adulthood in uninjured tissue (Limke and Rao, 2003). A growing body of information, although controversial (Seaberg and van der Kooy, 2003; Morshead and van der Kooy, 2004), suggest that the potential to generate new neurons may not be an intrinsic property of NSCs and NPCs residing in the SGZ and SVZ, but rather is the result of environmental control exerted by surrounding cells on neural precursor cells present within these neurogenic regions (Kondo and Raff, 2000a; Magavi et al., 2000; Temple, 2001; Lie et al., 2002; Song, 2002).

### **1.3 Connexin-mediated control of stem and progenitor cells**

Both paracrine and juxtacrine signalling mechanisms have been implicated in controlling the fate of NPCs. However, the signalling mechanisms essential for this control have only begun to be elucidated (Drago et al., 1991; Ghosh and Greenberg, 1995; LoTurco et al., 1995; Magavi et al., 2000; Temple, 2001; Lie et al., 2002; Song, 2002). Gap junctional intercellular communication (GJIC) is implicated in various cellular activities, including the regulation of NSC and NPC activation in the developing nervous system (Belliveau and Naus, 1994; Rozental et al., 1995; Rozental et al., 1998; Bani-Yaghoub et al., 2000; Cheng et al., 2004).

The role of gap junctions was first illustrated by the finding of electrical coupling between excitable cells in the crayfish giant motor axon (Furshpan and Potter, 1959). Since then, gap junctions have been implicated in a multitude of processes such as rhythmic contractions of the heart (Severs et al., 2004), synchrony of neuronal populations in the CNS (Long et al., 2002), neuroprotectants (Perez Velazquez et al., 2003), and directing NPC fate towards different CNS lineages (Trosko et al., 2000; Melanson-Drapeau et al., 2003). Given this multiplicity of activities, mechanistic and regulatory mechanisms underlying gap junctional control of cellular regulation is receiving increasing attention (LoTurco and Kriegstein, 1991; Fulton, 1995; Trosko et al., 2000; Melanson-Drapeau et al., 2003; Cheng et al., 2004)

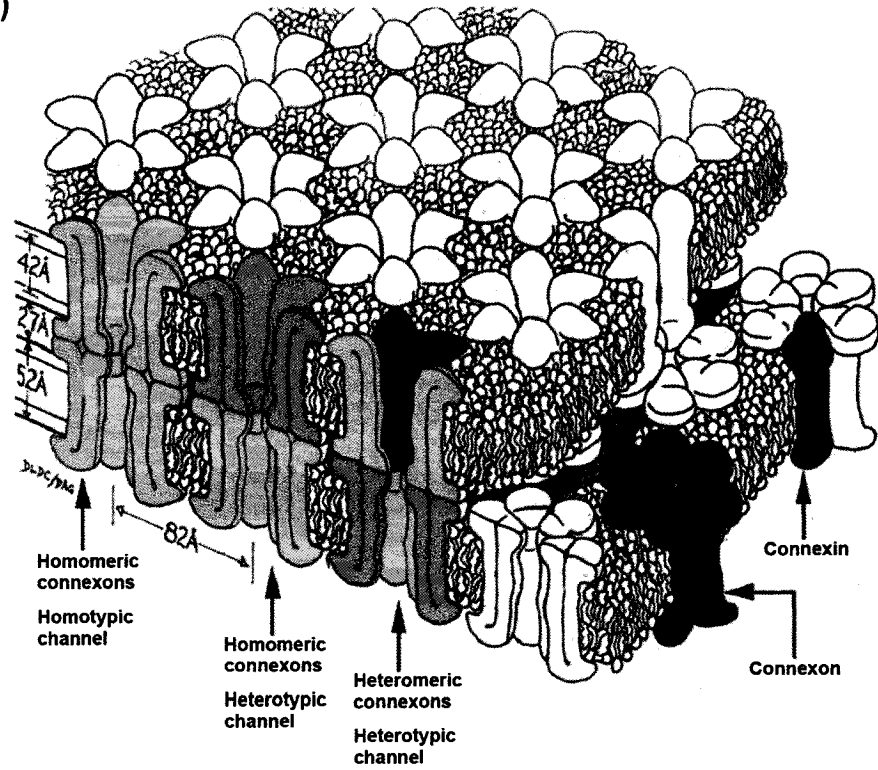
Gap junctions can either connect two adjacent cells via electrical coupling allowing the passage of ions such as  $\text{Ca}^{2+}$  and  $\text{Na}^+$  through the axial pore, or via

biochemical coupling allowing the passage of second messengers such as adenosine 3', 5'-cyclic monophosphate (cAMP) and guanosine 3', 5'-cyclic monophosphate (cGMP) between cells. A gap junction is defined by a cluster of intercellular channels, located at the plasma membranes of two adjacent cells (Figure 1.2 – A). These channels provide an axial hydrated pore smaller than 1 kilo Dalton (kDa) between two cells (Goodenough et al., 1996). Each channel is composed of two connexons – a hexamer of Cxs – elaborated at the membrane of adjacent cells. Different structural arrangements – defined by the identity of the Cx subunits – of the connexons hemichannels are possible. As illustrated in Figure 1.2 – A, a connexon that is composed of one type of Cx characterized as homomeric, while a composition of more than one Cx subunit characterizes it as heteromeric (Evans and Martin, 2002). A channel that is formed by the alignment of two connexons that have the same Cx composition is homotypic, while one that is composed of two connexons with differing Cx composition is heterotypic (Evans and Martin, 2002). The Cx subunits of these channels determine the specificity of molecular passages between cells, and thus regulate signalling events through gap junctions.

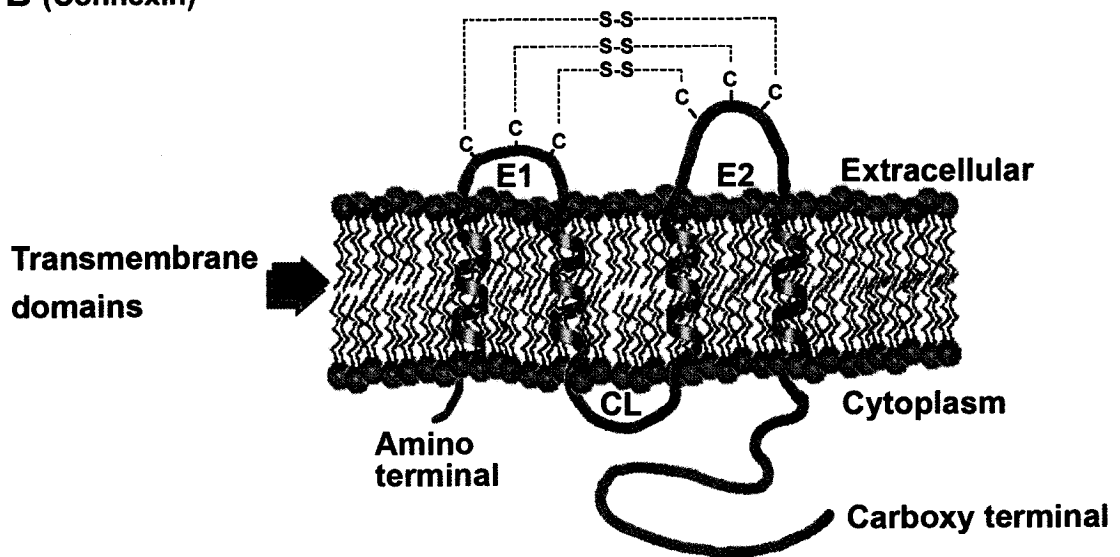
Proteins from the Cx superfamily are characterized by three cytoplasmic domains (N-terminal, cytoplasmic loop (CL) and C-terminal), two extracellular loops (E1 and E2), and four transmembrane domains (Figure 1.2 – B). While the greatest sequence homology resides in E1 and E2, each containing three conserved cysteine residues involved in the formation of disulfide bonds between E1 and E2, the CLs, and the carboxy-terminal regions possess the greatest

**Figure 1.2: Schematic representation of a gap junction and their connexin subunits.** The schematics of a gap junction (A) were modified from (Goodenough et al., 1996). (A) The connexon (dark green) is composed of six connexins (blue). Structural arrangements of the connexons subunits or the axial channels are represented. A connexon composed of one type of connexin forms a homomeric connexon. A connexon composed of more than one type of connexin constitutes a heteromeric connexon. A channel that is formed by the alignment of two connexons with the same connexin composition produces a homotypic channel. A channel that is formed by the alignment of two connexons with differing Cx identities between connexons forms a heterotypic channel. (B) Connexins are characterized by three cytoplasmic domains (N-terminal, cytoplasmic loop (CL) and C-terminal), two extracellular loops (E1 and E2) each containing three cysteine residues involved in disulfide bond formation, and four transmembrane domains.

**A (Gap junction)**



**B (Connexin)**



**Figure 1.2**

sequence variability (Goodenough et al., 1996). The members of the Cx family are highly homologous within vertebrates, and most lack introns in their coding sequences, with the exception of Cx36, Cx39, and Cx57 (Sohl and Willecke, 2003). Expression is highly cell- and tissue-specific (Bruzzone et al., 1996).

Typically, one connexon docks with another connexon elaborated by an adjacent cell. However, gated connexons in non-junctional membranes have also been reported. Open Cx32, Cx26, Cx43, and Cx45-containing hemichannels in non-junctional membranes facilitate passage of metabolites, biologically active lipids, and second messengers to and from extracellular space (Castro et al., 1999; Kamermans et al., 2001; Stout et al., 2002; Valiunas, 2002; Cherian et al., 2003). The opened or closed state of the connexon is regulated by a variety of cellular processes, such as pH,  $\text{Ca}^{2+}$ , and phosphorylation (De Pina-Benabou et al., 2001). These regulatory mechanisms, in turn, lead to changes in the conformational arrangements of Cxs that form the connexon, and result in altered signalling (Bruzzone et al., 1996). Previous studies have suggested that the opening and closing of hemichannels in non-differentiated cells in neurogenic regions of the brain regulate their proliferative state. Calcium waves mediated by hemichannels, extracellular ATP, the metabotropic ATP receptor  $\text{P2Y}_1$ , and intracellular  $\text{Ca}^{2+}$  release from  $\text{IP}_3$ -sensitive intracellular storages have been identified as important players in radial glia cell maintenance in the cell cycle by progressing into S phase rather than enter  $\text{G}_0$  and differentiate into postmitotic cells (Weissman et al., 2004). Other studies have suggested that the open states of hemichannels can contribute to cell survival via

the activation of extracellular signal-regulated kinases and mitogen-activated protein kinases (ERKs and MAPKs) and Src (Goodenough and Paul, 2003).

Exploration of hemichannel mediated communication and its implications remains in its early days.

The current nomenclature for Cxs refers to their respective molecular weight (kDa). However, because of complexities caused by the increasing number of discovered Cxs with similar molecular weight, the nomenclature is presently under revision (International Gap Junction Conference 2005, Whistler, BC, Canada). To date, 21 human genes and 20 mouse genes have been identified in the Cx family (Willecke et al., 2002; Sohl and Willecke, 2003, 2004). The members of this family are highly cell-type and tissue specific (Saez et al., 2003). At least twelve Cxs (Cx26, Cx29, Cx30, Cx31.9, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx47, Cx57) are expressed in the mammalian CNS and peripheral nervous system (PNS) (Rouach et al., 2002). Five of these Cxs (Cx30, Cx37, Cx43, Cx45, and Cx26) have been identified in embryonic, teratocarcinoma-derived, or immortalized progenitor cells (Bani-Yaghoub et al., 1997; Bittman and LoTurco, 1999; Rozental et al., 2001; Duval et al., 2002; Boucher and Bennett, 2003; Cheng et al., 2004; Weissman et al., 2004).

#### **1.4 Cx32 – Structure, function, and its role in NPC fate**

One of the very first pathological conditions to be associated with Cx dysfunction in the CNS and PNS is X-linked Charcot-Marie-Tooth disease (CMTX), a peripheral neuropathy caused by a series of mutations in the Cx32

gene (Bergoffen et al., 1993; Neuberg and Suter, 1999; Seeman et al., 2001). The mutated protein alters signalling among Schwann cells in the PNS. This change contributes to the aetiology of CMTX, characterized by demyelination of peripheral neuronal axons (White and Paul, 1999). The absence (or change) of coupling between the different layers of the myelin sheath in the PNS results in progressive loss of myelin – clinically characterized by motor speed reduction, and eventually axonal death – leading to the loss of conductivity of the affected motor units. In the CNS, however, pathology is minimal and is restricted to a mild dysmyelination (Bergoffen et al., 1993; Taylor et al., 2003). A Cx32 null mutant mouse was generated in Dr. Klaus Willecke's laboratory in 1996 (Nelles et al., 1996). Yet, these KO animals do not develop CMTX-like symptoms beyond mild reduction in peripheral nerve velocity conductance or any obvious behavioural abnormality (Nelles et al., 1996), suggesting that pathology does not result for loss of Cx32 function but more precisely to altered signalling function of mutant protein (Menichella et al., 2003).

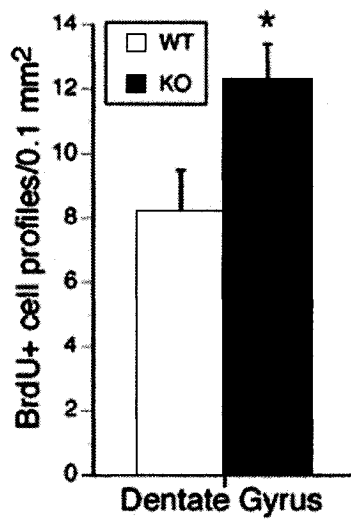
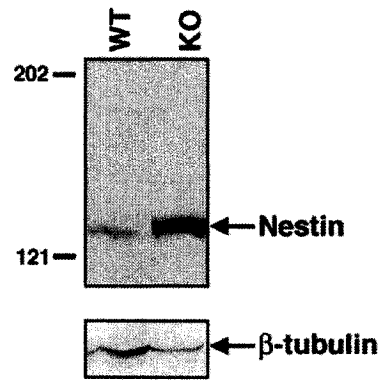
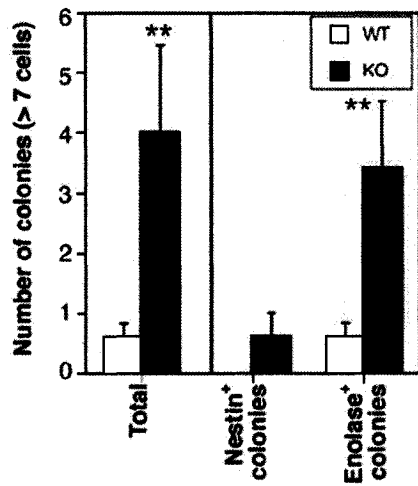
The Cx32 protein is expressed in hepatocytes, secretory acinar cells, Schwann cell in the PNS, oligodendrocytes in the CNS, and NG2<sup>+</sup> progenitor cells in the SGZ and polymorphonuclear layer of the hippocampus (Melanson-Drapeau et al., 2003; Oyamada et al., 2005). The Cx32 gene is located on the X chromosome, and its genetic structure in mice primarily consists of four exons (1, 1A, 1B, and 2), where the transcription/translation of exon 2 generates the functional Cx32 protein (Oyamada et al., 2005). Three main transcripts, resulting from alternative splicing, have been identified for the mouse Cx32 locus: E1/E2,

E1A/E2, and E1B/E2 (Sohl et al., 2001). Interestingly, the E1A/E2 transcript has only been identified in embryonic stem (ES) cells (Sohl et al., 2001). The transcriptional activity of Cx32 is dependent upon promoter interaction with transcription factors that can be cell-type independent such as SP1 (Bai et al., 1993; Piechocki et al., 2000; Field et al., 2003), or cell-type dependent such as HNF-1 (Koffler et al., 2002), Mist1 (Rukstalis et al., 2003), Sox10 (Bondurand et al., 2001; Houlden et al., 2004), and estrogen (Hebb *et al.*, submitted). The identity of the Cx32 promoters remains elusive, but the emergence of new tools, has and will continue to contribute to our understanding of Cx regulation.

As illustrated in Figure 1.3, previous studies in our laboratory have shown the presence of an enriched pool of proliferating NG2<sup>+</sup> progenitors in the SGZ of Cx32KO mice (Melanson-Drapeau et al., 2003). The rapid turnover of these progenitor cells in the SGZ is most likely due to a failure to differentiate into mature oligodendrocytes, indicating that Cx32 is likely required for specification of at least a subset of NG2<sup>+</sup> progenitor cells (Melanson-Drapeau et al., 2003). Relevant to this thesis, when progenitor cells from Cx32KO mice were cultured *in vitro*, they demonstrated an enhanced capacity to proliferate when treated with basic fibroblast growth factor (bFGF) and were capable of differentiation into mature neurons when treated with brain-derived neurotrophic factor (BDNF) (Melanson-Drapeau et al., 2003). However, in the uninjured brain, this subset of progenitor cells fails to differentiate, and their increased rate of proliferation is balanced by an increased rate of apoptosis (Melanson-Drapeau et al., 2003).

**Figure 1.3: Cx32KO mice have an enriched pool of progenitors that exhibits increased neurogenic potential *in vitro* residing in the SGZ.**

These figures were obtained from previously published work from our laboratory (Melanson-Drapeau et al., 2003). (A) Immunofluorescence detection BrdU from WT and Cx32KO mice that were injected with BrdU (labels proliferating cells) is shown, indicating greater number of proliferating progenitor cells in the DG of the Cx32KO hippocampus. (B) BrdU+ cells were quantified in the DG of WT and Cx32KO mice. This correlated previous observations, indicating more proliferating progenitor cells in the DG in absence of Cx32. (C) Cellular identity of these cells expressed in greater numbers was confirmed as precursor cells by immunoblotting for Nestin. (D) Dentate gyrus progenitor cells from WT and Cx32KO mice were cultured *in vitro*; Cx32KO SGZ progenitors demonstrate an enhanced capacity to proliferate when treated with bFGF and were capable of differentiation into mature neurons when treated with BDNF.

**A****B****C****D****Figure 1.3**

## 1.5 Thesis objectives

Based on these findings, I hypothesized that reducing Cx32 expression following injury might facilitate neuronal specification of NG2<sup>+</sup> progenitor cells and promote neuronal cell replacement in injured brain given the loss of neurons and requirement for cell replacement not present in uninjured tissue. To test this hypothesis, I subjected adult Cx32KO mice and their congenic WT controls to excitotoxic injury and assessed hippocampal injury at the molecular level and functional regeneration in a behavioural paradigm of learning and memory. Over the course of this work, a number of problems were identified in the detection of Cx32 protein using existing antibodies. To address these issues, I created a Cx32 null-mutant “marker mouse” in which the Cx32 coding region was replaced with the EGFP coding region.

These studies were made possible without developmental confounds of Cx null-mutation on embryonic survival given the profile of Cx expression over the course of CNS development. The repertoire of Cxs expressed by NSCs and NPCs during mammalian development changes in parallel with major events in neural development, such as specification to glial or neuronal lineages (Guthrie and Gilula, 1989; Fulton, 1995; Bittman and LoTurco, 1999). Altering Cx expression has shown to modify proliferative and/or specification cellular responses in embryonic NPCs (Rozental et al., 1998; Rozental et al., 2000; Cheng et al., 2004). As such, caution must be applied when assessing adult neurogenesis in an animal that has developed devoid of an embryonically relevant Cx. However, Cx32 is the only Cx in the CNS in which protein is

expressed exclusively postnatally (Dermietzel et al., 1989; Belliveau et al., 1991). Thus, I chose first to evaluate the impact of Cx32 null-mutation on adult neurogenesis following brain injury in lieu of other Cx KO animals available in our laboratory.

## **Chapter 2: Kinetics of neurodegeneration and regeneration in the adult Cx32 null-mutant mouse hippocampus following excitotoxic injury**

### **Abstract**

This chapter focuses on elucidating the kinetics of neurodegeneration and regeneration following KA-induced excitotoxic injury in the adult male mouse hippocampus. Following kainate administration, WT and Cx32KO mice undergo comparable neuronal death in the CA3a/b pyramidal cell field, but not in the DG region of the hippocampus. Data in this chapter also show that NPCs activated 0.5 week after injury in the hippocampus specify preferentially to neurons in the CA3a/b region resulting in an increased number of mature neurons 4.5 weeks following injury in absence of Cx32. The level of Cx32 in WT animals increased transiently following seizure induction, correlating with progenitor specification to NG2<sup>+</sup> progenitors or mature oligodendrocytes. These data implicate Cx32 as a switch regulator inhibiting neurogenesis in NPCs residing in the SGZ.

### **Publications resulting from studies described in this chapter and chapter 3**

Melanson-Drapeau L\*, Morin M.Y.\*, Haykal S., Boucher S, Whitehead S, Pelletier L., Paul D.L., Bennett S.A.L. Connexin-mediated control of neurogenesis following excitotoxic injury, in preparation \* both authors will have contributed equally

### **Statement of author contributions**

Lysanne Melanson-Drapeau, a PhD student in our laboratory processed the majority of the brain tissue used for histological analysis. MGPY staining of brain sections was performed by Louise Pelletier, a research technician in the Pathology department of the University of Ottawa. Siba Haykal, a summer student in our laboratory, performed the Fluoro-Jade B staining and analysis, and contributed to the quantitation of MGPY-stained brain sections. Shawn Whitehead, a post-doctoral fellow in our laboratory, contributed to the lineage analysis presented in Figure 2.4. All other data were generated over the course of this thesis.

## 2.1 Introduction

The adult human brain is now known to support neuronal and glial stem and progenitor cells - cells with varying capacities for self-renewal and specification to neurons, oligodendrocytes, and astrocytes. As discussed in Chapter 1, the hippocampus is one of two regions in the uninjured adult brain capable of significant neurogenesis. Neuronal replacement, albeit limited, is induced following extensive injury to the hippocampus (Nakagawa et al., 2000; Dash et al., 2001; Chirumamilla et al., 2002; Dong et al., 2003; Picard-Riera et al., 2004; Jessberger et al., 2005). If we are to expand this regenerative capacity, a thorough understanding of how activated progenitor cells are directed to a neuronal lineage in adult injured brain is essential.

Excitotoxicity, in part, underlies cell death in various neurodegenerative conditions including ischemia (Won et al., 2002), epilepsy (Schwarcz et al., 1984; Kelsey et al., 2000; Campbell and Hablitz, 2005), AD (Mattson, 1997; Tannenberg et al., 2004) and Parkinson's disease (Olney et al., 1990; Przedborski, 2005). This particular type of cellular injury is initiated by over-activation of N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors in response to excess glutamate. In animal models of disease, the extent of excitotoxic cell death has been shown to be highly influenced by many variables such as animal strain (Schauwecker, 2003), cell type (Malva et al., 1998), and age (Kesslak et al., 1995; Sutherland et al., 1996). For example, pyramidal cells in the CA3 region of

the hippocampus are rich in glutamate receptors of kainate type and, as such, are particularly susceptible to injury (Malva et al., 1998).

Here, I used the KA model of epileptic seizure to induce neuronal loss in the hippocampus of WT and Cx32KO mice and to investigate the regulatory role of Cx-mediated control on activation of progenitor cell population following injury. As reviewed in Chapter 1, colleagues in the Bennett laboratory have previously shown that the expression of Cx32 in the uninjured hippocampus is initiated when a subset of NG2<sup>+</sup> progenitors commit to the oligodendrocyte lineage. Expression is retained throughout specification to an oligodendrocyte lineage, generating a subpopulation of Cx32-expressing oligodendrocytes (Melanson-Drapeau et al., 2003). The same study demonstrated that NPCs extracted from the SGZ of Cx32KO mice exhibit an enhanced capacity for *in vitro* neurogenesis (Melanson-Drapeau et al., 2003). Based on these data and supporting literature, I hypothesized that, in the absence of Cx32, NG2<sup>+</sup> progenitor cells may mobilize more effectively to regenerate pyramidal neurons in the hippocampus following excitotoxic injury in adult male mice.

## **2.2 Materials and Methods**

### **2.2.1 Generation of Cx32KO mice**

Breeding pairs of Cx32KO mice in a mixed genetic background of 129SVJ1 and C57Bl/6 (Charles River Laboratories, Wilmington, DE) were obtained from Dr. Klaus Willecke at the Universitat Bonn, in Germany (Nelles et al., 1996). Genetic background homogeneity between WT and Cx32KO mice was insured by backcrossing the Cx32KO transgenic mouse line in pure C57Bl/6 mice for 13 generations (Melanson-Drapeau et al., 2003).

### **2.2.2 Animal treatments**

Adult C57Bl/6 WT and Cx32KO male mice ranging from 2-5 months of age were used in this study. The mice were individually housed at least 2 weeks prior to the experiments, maintained on a 12/12 light/dark cycle, and provided with food and water *ad libitum*. All procedures were in agreement with the standards instated by the Canadian Council on Animal Care (CCAC) and the policy on animals used in research and teaching at the University of Ottawa (University Policy 31).

### **2.2.3 Kainate-induced excitotoxic injury**

Adult male mice were initially administered KA intraperitoneally (25 mg/kg, OPIKA-1; Ocean Produce Int., Shelburne, Nova Scotia, Canada) and monitored for behavioural indices of seizure activity. Prior to the injection, the KA was reconstituted in 10 mM phosphate buffered saline (PBS, 10 mM sodium

phosphate and 154 mM sodium chloride (NaCl)) to a final concentration of 5 mg/ml. Following the injection, the subjects were monitored and scored for seizure activity according to a previously published five-point behavioral scale (McIntyre et al., 1982; Bennett et al., 1995): 0, no response; 1, increased frequency of "freezing" and "wet-dog shakes"; 2, weak clonic convulsions with rearing; 3 single severe convulsion with loss of postural control; 4, frequent limbic convulsions; and 5, severe limbic seizures with respiratory difficulty. Animals that reached stage 4 seizure activity were injected with diazepam (5 mg/kg) to limit seizure duration and prevent physical trauma. Animals who did not reach stage 4 were not included in the study because of insufficient and/or inconsistent hippocampal damage among the experimental subjects. The mice were continuously monitored 4 hours following KA-induced seizure, and vital signs were monitored twice per day for a period 5 days post-injury.

#### **2.2.4 Histology and immunofluorescence**

Control animals and animals sacrificed 0.5, 1, 2, and 4.5 weeks after KA-induced seizure were injected intraperitoneally with a lethal dose of sodium pentobarbital. Animals were perfused with a transcardial injection of 10mM PBS followed by 3.7% paraformaldehyde. Brains were removed, post-fixed for 24 h, and transferred to a 20% sucrose solution in 10 mM PBS containing 0.001% sodium azide. Coronal brain sections (10  $\mu$ m) were cut in series with a cryostat (Leica Microsystems Inc., Richmond Hill, Ontario, Canada) and processed for molecular analysis of brain sections.

Brain sections were histologically stained with methyl green pyronin Y (MGPY) as described previously (Moffitt, 1994), hematoxylin and eosin (H&E) or cresyl violet as described previously (Bennett et al., 1995). In MGPY-stained sections, the DNA is stained blue and mostly restricted to the nucleus, while the RNA is stained pink and generally localized to the cytoplasm. Cells that were defined as viable exhibited a light blue nucleus, confirming nuclear integrity, and a light pink cytoplasm, indicative of RNA transcription. Cells with an hyperchromatic nucleus and/or lacking cytoplasmic (RNA) staining were identified as degenerating cells (Al-Hazzaa and Bowen, 1998).

The DG and CA3a/b regions of the hippocampus from stained brain sections were photomicrographed with a Leica DMXRA2 microscope equipped with a digital video camera (Leica Microsystems Inc.), and analyzed with the computer software Openlab 3.1.7 (Improvision, Lexington, MA). The layer thickness, the total cell number, the number of degenerating cells, and the number of viable cells were assessed in the superior limb of the granule cell layer of the DG, and the CA3a/b regions of the MGPY-stained hippocampal brain sections. Cell counts were performed within a predetermined area of interest (AOI) by two independent investigators, blinded to animal group identity, using the Advanced Measurement Module of Openlab 3.1.7, and the data were expressed as cell number/0.1 mm<sup>2</sup>. Data from both investigators was averaged for each animal to generate a single parameter per animal, and the genotype averages were calculated for each of the time points mentioned above.

Quantitative observations were confirmed by qualitative assessment of

H&E- and cresyl violet-stained adjacent sections. In H&E and cresyl violet stained sections, pyknotic, eosinophilic or hyperchromatic cells were defined as degenerating cells. Cells with oval nuclei, prominent nucleoli lacking eosinophilic cytoplasm and homogeneously stained by hematoxylin or cresyl violet were defined as healthy cells (Bennett et al., 1995; Bennett et al., 1998). Edematous tissue was identified as areas exhibiting macroscopic swelling and vacuolization in H&E and cresyl violet stained sections (Bennett et al., 1995).

Terminally differentiated neurons, immature oligodendrocytes, and mature oligodendrocytes in the hippocampal formation were identified and quantitated in a double blind fashion, to eliminate observer bias, by immunofluorescence detection using monoclonal mouse anti-NeuN (Chemicon, Temecula, CA, USA; 1:100), polyclonal anti-NG2 (Chemicon; 1:200), and monoclonal mouse anti-2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; Sigma, St. Louis, MO, USA; 1:100) antibodies respectively. The expression of Cx32 was identified and quantitated as the percent immunofluorescent area using a monoclonal anti-Cx32 antibody (Zymed, San Francisco, CA, USA; 1:250). Secondary antibody was a Cy3-conjugated monoclonal anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA; 1:800). Quantitative analyses were performed with the Advanced Measurement Module of Openlab 3.1.7 as described above, and expressed as cells per 0.1mm<sup>2</sup> for NeuN and percent fluorescent area for Cx32.

### **2.2.5 BrdU labelling and lineage analysis**

Mice were intraperitoneally injected with 5-bromo-2-deoxyuridine (BrdU; Roche Applied Science, Indianapolis, IN, USA; 50 µg/g in sterile 10 mM PBS, pH 7.0). Animals received two daily injections (4-5 h apart) over two consecutive days and a single injection on the third day. The first BrdU injection was performed at the time of KA injection, and the last was performed 2 days after. Animals were sacrificed 24 hours to determine the number of surviving BrdU<sup>+</sup> cells or 4 weeks after the injections to determine the identity of the specifying progenitor cells. BrdU incorporation was detected by immunofluorescence using mouse anti-BrdU (Roche Applied Science; 6 µg/ml). Cell identify was established by analysis of sections double-labelled for BrdU and GFAP (Sigma; 1:800), CNPase (Sigma; 1:100), NeuN (Chemicon; 1:100), or NG2 (Chemicon; 1:200) 4 weeks after the last BrdU injection. The labelled cells were quantitated in a double blind fashion using the Advanced Measurement Module of OpenLab 3.17 software as described in (Melanson-Drapeau et al., 2003). The total area (mm<sup>2</sup>) of each region was determined using OpenLab 3.17 Measurement Module. Data were expressed as the number of BrdU<sup>+</sup> cell profiles per 0.1 mm<sup>2</sup>.

### **2.2.6 *In Situ* Hybridization to PDGFαR transcript**

The number of oligodendrocytes expressing platelet-derived growth factor alpha-receptor (PDGFαR) mRNA was assessed by *in situ* hybridization in uninjured control animals and injured animals 4.5 weeks after KA administration in both genotype (n=5 / time point / genotype; N=20). Fresh brain sections were

equilibrated to room temperature 30 minutes before the hybridization. The digoxigenin (DIG)-labelled PDGF $\alpha$ R probe (graciously provided by Dr. Valerie Wallace at the Ottawa Health Research Institute; 1:2000) was thawed quickly and diluted into hybridization solution (200 mM NaCl, 9 mM 2-amino-2-(hydroxymethyl) propane-1,3-diol-hydrochloric acid (Tris-HCl) pH 7.5, 1 mM Tris Base, 5 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 5 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM ethylenediaminetetraacetic acid (EDTA), 50% deionized formamide, 10% dextran sulfate, 1 mg/ml yeast rRNA, 0.02% BSA, 0.02% Ficoll<sup>™</sup>, and 0.02% polyvinyl pyrrolidone in diethylpyrocarbonate (DEPC)-treated H<sub>2</sub>O). The probe was denatured at 70°C for 10 minutes. The sections were covered with the hybridization solution (100  $\mu$ l / section), coverslipped, and incubated overnight in a sealed chamber containing 50% formamide/1X salt at 65°C. The sections were washed once for 15 minutes and twice for 30 minutes with mild shaking at 65°C in washing buffer (1x SSC, 50% formamide, and 0.1% Tween-20 in dH<sub>2</sub>O). The sections were equilibrated twice for 30 minutes in 1X MABT (0.1 M maleic acid, 0.8 M NaOH, 0.25 M NaCl, 0.5% Tween-20) at room temperature. The slides were dried around the sections, and incubated in blocking solution (20% heat-inactivated sheep serum, 2% blocking reagent (Roche Applied Science), and 1X MABT in dH<sub>2</sub>O) in a humid chamber containing PBS for 1 hour at room temperature. The blocking solution was removed and replaced by anti-DIG antibody (Roche Applied Science; 1:1500) in blocking solution. The sections were incubated overnight at 4°C in a humid chamber containing PBS. The sections were washed 5 times for 20 minutes in 1X MABT at room temperature with mild shaking. The sections

were equilibrated two times for 10 minutes in staining buffer (100 mM NaCl, 50 mM MgCl<sub>2</sub>, 100 mM Tris pH 9, 0.1% Tween-20) with shaking at room temperature. The sections were incubated overnight in the dark at room temperature in staining buffer containing nitro-blue tetrazolium chloride (NBT; 4.5 µl/ml) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt in 67% DMSO (BCIP; 3.5 µl/ml) for colour reaction. The reaction was stopped with several washes in PBS, and the sections were mounted and coverslipped with glycerol/PBS 1:1, and sealed. The sections were processed as described above for photomicrographs and single cell quantitation.

### **2.2.7 Assessment of neuronal degeneration**

Neuronal death occurring in the hippocampus following KA-induced excitotoxicity in mice was assessed using terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) nick end labelling (TUNEL) for apoptotic-like cell death assessment and Fluoro-Jade B (FJB) for neuronal degeneration assessment.

For TUNEL labelling, the sections were first permeabilized by a 5-minute incubation in a 0.1% sodium citrate and 0.1% Triton X-100 solution on ice, and a 2 min incubation in an ethanol:acetic acid solution (2:1) on ice. The sections were rinsed twice for 2 minutes in 10 mM PBS, and incubated for 1 hr at 37°C with FITC-conjugated dUTP in TdT buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride) and TdT (1:10) as suggested by the manufacturer (Roche Applied Science). Sections were washed three times

for 5 minutes in 10 mM PBS, coverslipped with Vectashield (Vector Laboratories Inc., Burlingame, CA), sealed, and visualized on a Leica DMRXA2 epifluorescent microscope. The cells were quantitated as TUNEL-positive cells per 0.1mm<sup>2</sup> in the CA3a/b pyramidal cell field and the DG granule cell layer of the hippocampus as described above.

For FJB staining, the sections were permeabilized in a 1% NaOH and 80% ethanol (EtOH) solution for 5 minutes, a 70% EtOH solution for 2 minutes, and distilled water for 2 minutes. The sections were then stained with a 0.06% (w/v) potassium permanganate solution for 10 minutes, rinsed in distilled water for 2 minutes, and incubated in the FJB staining solution (0.001% (w/v) FJB in 0.1% acetic acid) for 30 minutes at room temperature. The sections were washed three times for 1 minute in distilled water, one time for 5 minutes in 70% EtOH, one time for 5 minutes in 95% EtOH, one time for 5 minutes in 100% EtOH, dried, mounted in Permount (Fisher Scientific Ltd., Nepean, Ontario, Canada), and visualized on a Leica DMRXA2 epifluorescent microscope (Leica Microsystems Inc.).

### **2.2.8 Western blot analyses**

Uninjured control mice and mice sacrificed 0.5, 1, 2, and 4 weeks following KA-induced excitotoxicity were euthanized with a lethal dose of sodium pentobarbital. Three mouse hippocampi were pooled to each time point (N=15 mice/genotype). Hippocampi were dissected in chilled PBS under a Leica dissection microscope. The hippocampal tissue was homogenized in 3 ml of

RIPA buffer (10 mM PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 30 µl/ml aprotinin, 10 mM sodium orthovanadate, 100 µl/ml phenylmethylsulfonyl fluoride 0.1% sodium dodecyl sulphate (SDS)) per gram of tissue with a Tissue Tearor (Fisher Scientific Ltd.) homogenizer. For each millilitre of RIPA used, the following protease inhibitors were added prior to homogenization: 1 µl of 1M sodium monofluoride, 10 µl of 10 mg/ml fluorosulfonylmethylbenzene (PMSF), 10 µl of 100mM Na orthovanadate and 30 µl of stock aprotinin. Samples were incubated for 30 minutes on ice, and transferred to 1.5ml microtubes for a 30-minute centrifugation (16 000 rpm) at 4°C. Protein concentration in the supernatant containing the total cell lysate was established using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The protein samples were then stored at -80°C until electrophoresis.

Aliquots containing 30 µg of hippocampal proteins, from each time point and both genotype mentioned were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on 12.5% (w/v) polyacrylamide gels containing 0.1% SDS and electroblotted to a polyvinylidene difluoride (PVDF) Immobilon membranes (Amersham-Pharmacia Biotech, Baie d'Urfé, Quebec, Canada) for Western Blot analysis. The PVDF membranes were blocked in 1% heat-denatured casein in 10 mM PBS for 1 hour. The membranes were then incubated overnight at 4°C in primary with primary antibodies, monoclonal mouse anti-Cx32 (Zymed; 1:250), monoclonal mouse anti-CNPase (Sigma; 1:1000), or monoclonal mouse anti-actin (Sigma; 1:1000), diluted in the blocking solution. The membranes were then incubated with a horseradish

peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch; 1:2000). Immunoreactive bands were identified using the SuperSignal West Pico Chemiluminescence substrate (Pierce, Rockford, IL, USA) and X-ray film imaging.

### **2.2.9 Statistical analyses**

The data presented in this chapter are shown as the mean and the standard error of measurement (SEM). The experimental groups were analyzed with a student t-test, a one way analysis of variance (ANOVA) followed by a multiple comparisons versus control group (uninjured) with a Bonferroni t test or Tukey's *post hoc* test, or with a multivariate analysis of variance (MANOVA) followed with a Tukey's *post hoc* test as appropriate. The comparisons were performed with  $\alpha$  values set at 0.05. Statistical analyses were either executed using the computer software Systat 5.2.1 or SigmaStat (Systat Software Inc., California, USA).

## **2.3 Results**

### **2.3.1 Comparable sensitivity and seizure activity following KA injection**

The Cx32KO mutant mice were obtained in a mixed genetic background of C57BL/6 and 129/SVJ1 (Nelles et al., 1996). As reported previously, C57BL/6 mice exhibit a relatively high resistance to excitotoxic injury, while 129/SVJ1 mice are more sensitive to this type of injury (Schauwecker, 2003). In addition, it has been reported that mice from the 129/SVJ1 strain exhibit a reduced potential for NPC activation in comparison to other strains. These strain-specific variations are critical in reason of potential masking of the effects of altering Cx status on neuroregeneration following hippocampal injury (Kempermann et al., 1997).

To address this issue, null-mutant mice were backbred for 13 generations into C57BL/6 WT mice and congenic Cx32KO and WT colonies were derived from mating of N13 mice heterozygote for the Cx32 gene. In Table 2.1, we show that these N13 C57BL/6 Cx32KO and WT mice exhibit comparable seizure susceptibility, seizure staging, and mortality.

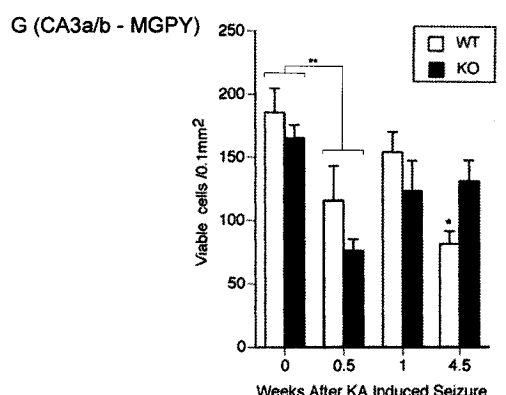
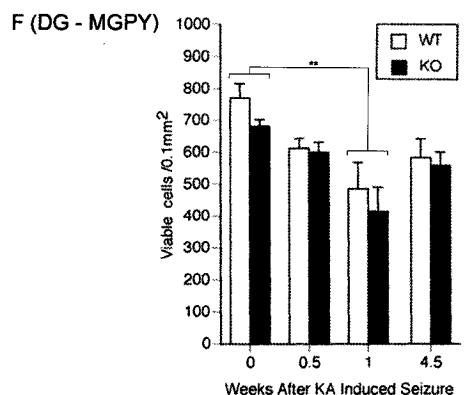
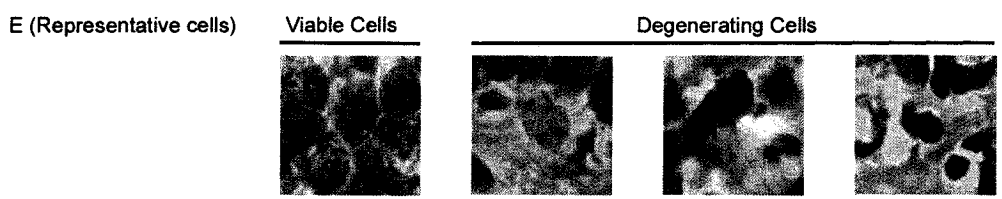
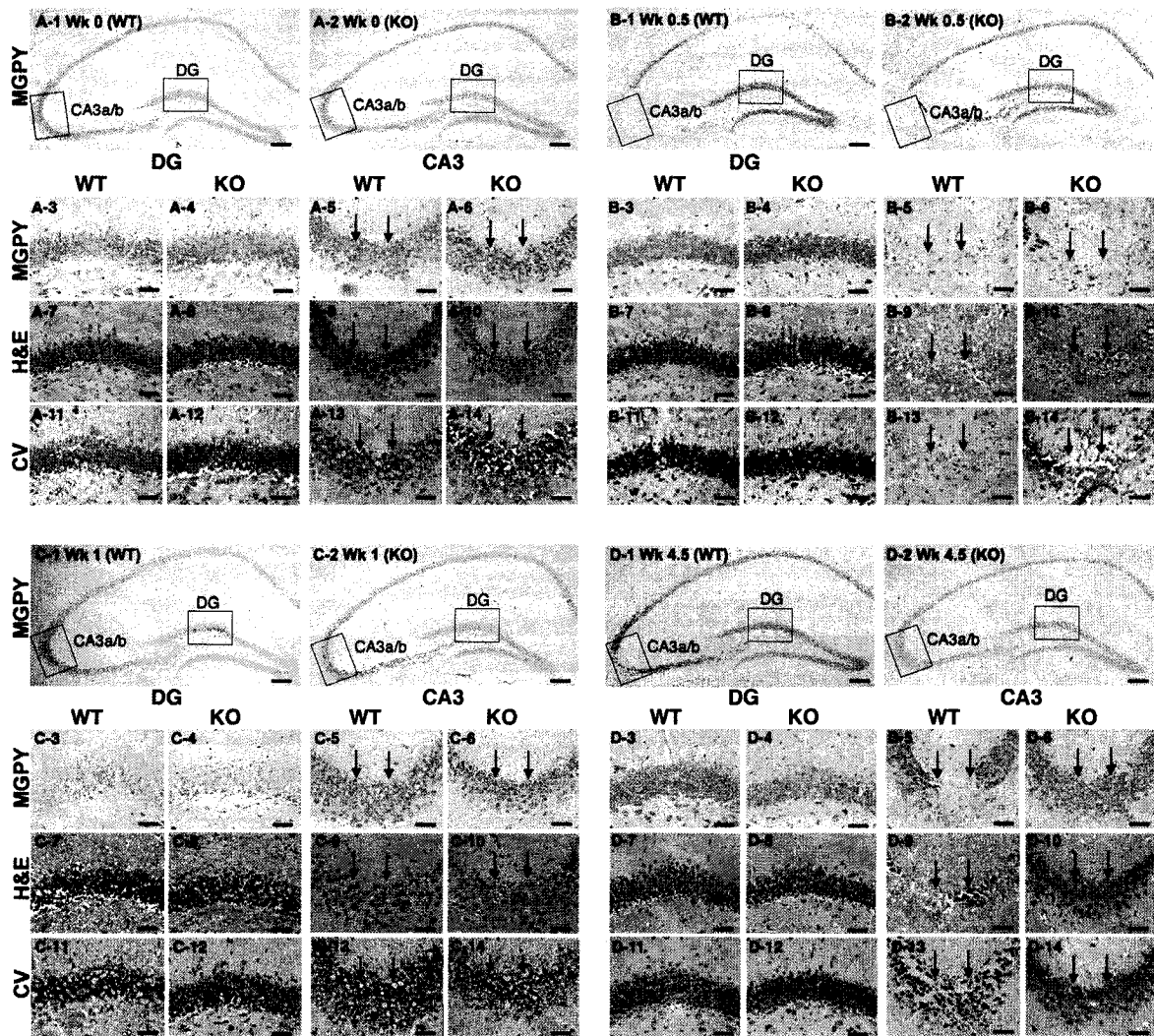
### **2.3.2 Kainate-induced neuronal loss in the CA3a/b region**

Both Cx32KO and WT mice exhibited a significant loss of cell viability in the DG and the CA3a/b regions of the hippocampal formation as assessed by MGPY, H&E, and Cresyl violet staining. Viable cell number was significantly reduced in the DG 1 week-, and in the CA3a/b region 0.5 week following KA administration (Figure 2.1 – F, G; \*  $p < 0.01$ ).

**Table 2.1: Sensitivity to kainic acid and seizure activity in WT and Cx32KO adult male mice.** Data represent the percentage of mice that arrested at each of the 5 behavioural stages described in Materials and Methods. Only animals that reached Stage 4 were analyzed in this study.

<b>Genotype</b>	<b>Stage (%)</b>				<b>Mortality (Stage 5)</b>	<b>Total # of mice</b>
	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>		
<b>WT</b>	3	4	2	72	19	72
<b>Cx32KO</b>	2	1	7	73	17	84

**Figure 2.1: Histological assessment of neurodegeneration and neuroregeneration in WT and Cx32KO hippocampus following excitotoxic injury.** Photomicrographs of 5x hippocampus stained with MGPY (WT: A1, B1, C1, D1; KO: A2, B2, C2, D2) were taken from mice sacrificed at 0, 0.5, 1 and 4.5 weeks following injury. Note that the 0 time period refers to uninjured control animals. Photomicrographs of 40x DG stained with MGPY (WT: A3, B3, C3, D3; KO: A4, B4, C4, D4), H&E (WT: A7, B7, C7, D7; KO: A8, B8, C8, D8), and CV (WT: A11, B11, C11, D11; KO: A12, B12, C12, D12), and of the CA3a/b region stained with MGPY (WT: A5, B5, C5, D5; KO: A6, B6, C6, D6), H&E (WT: A9, B9, C9, D9; KO: A10, B10, C10, D10), and CV (WT: A13, B13, C13, D13; KO: A14, B14, C14, D14) taken from mice sacrificed at 0.5, 1 and 4.5 weeks following injury are also shown. Representative cells indicating viable or degenerating cells are shown (E). The number of viable cells was assessed from MGPY stained sections in the DG (F) and CA3a/b (G) regions of the hippocampus. Arrows point to the CA3a/b regions exhibiting significant cell injury following kainate administration. Data were analyzed by MANOVA and *post hoc* Tukey tests. Statistically significant degeneration is indicated by \* (\* p<0.05, \*\* p<0.01). Scale bars: 5x, 150  $\mu$ m; 40x, 50  $\mu$ m. Abbreviations: WT, wild type; KO, Cx32KO; MGPY, methyl green pyronin Y; H&E, hematoxylin and eosin; CV, cresyl violet; DG, dentate gyrus; CA3a/b, pyramidal layer.



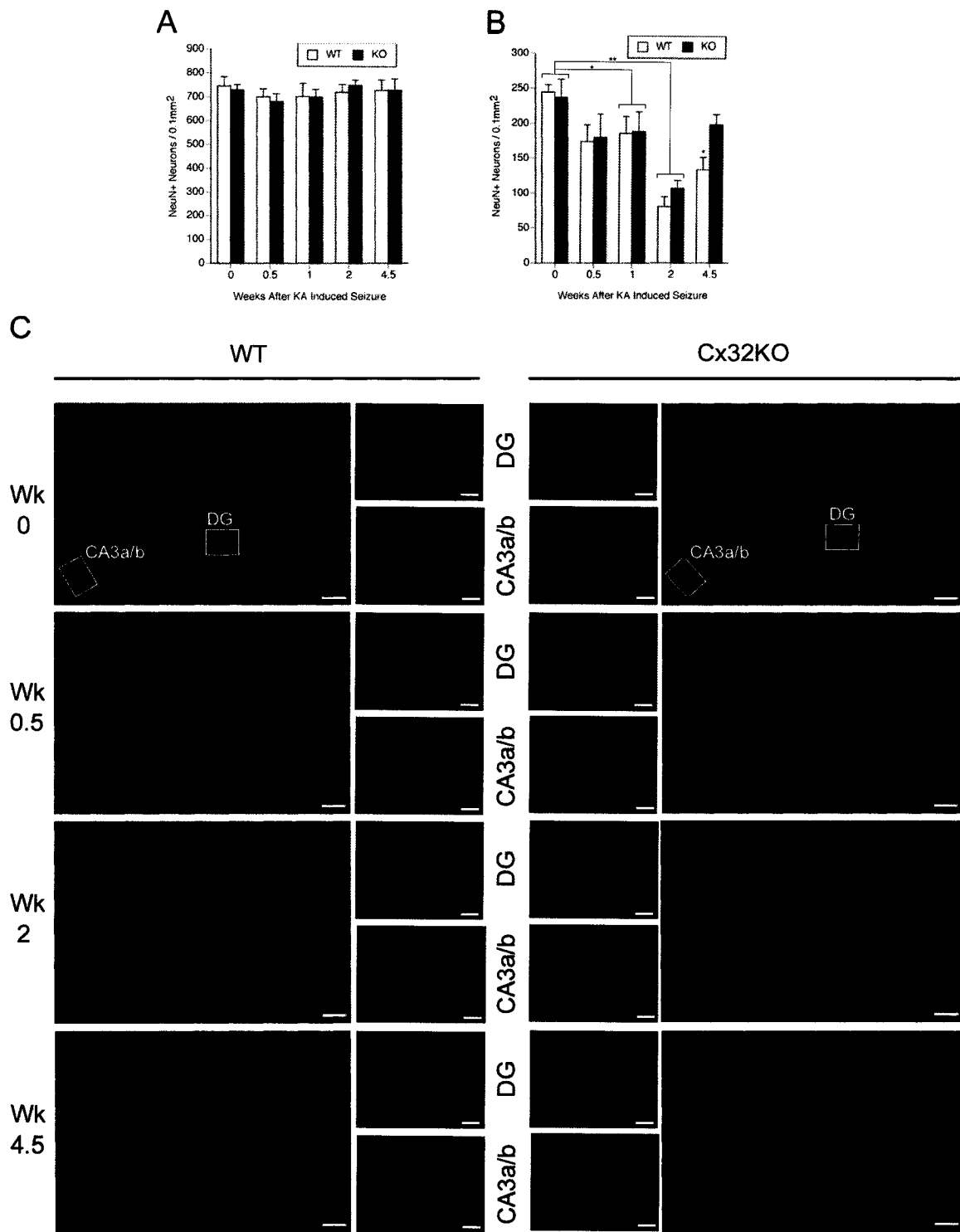
**Figure 2.1**

To establish whether compromised cell viability translated into significant neuronal loss, neuronal number was established by quantifying NeuN<sup>+</sup> neuronal nuclear profiles (Figure 2.2). No change in mature neuronal number was detected in the DG following kainate seizure. However, a 30% decrease in neuronal number was detected 1 and 2 weeks following injury (Figure 2.2 – B, \*  $p < 0.05$ ), and a > 50% decrease in neuronal number compared to uninjured controls 2 weeks post-injury (Figure 2.2 – B, \*\*  $p < 0.01$ ).

Neuronal death was confirmed using TUNEL to identify cells undergoing apoptotic-like DNA fragmentation and FJB to identify degenerating neurons. Consistent with NeuN immunolabelling, significant apoptotic-like death was not observed in the DG of the hippocampus (Figure 2.3 – A, B) with the exception of a small increase in TUNEL labelling that approached statistical significance. We have previously shown that this increase apoptotic-like death localizes to hyperproliferating NG2<sup>+</sup> progenitors in uninjured Cx32KO mice (Melanson-Drapeau et al., 2003).

A significant increase in the numbers of TUNEL<sup>+</sup> cells (Figure 2.3 – C, \*  $p < 0.05$ ) and FJB<sup>+</sup> neurons (Figure 2.3 – D, \*  $p < 0.05$ ) was observed 0.5 weeks following injury in the CA3a/b region of the hippocampus in both WT and Cx32KO mice. Interestingly, the number of cells undergoing apoptotic-like cell death and neuronal degeneration 4.5 weeks after kainate injection remained higher in WT as compared to Cx32KO mice although this latter difference approached but did not reach statistical significance ( $\sim p = 0.08$ ).

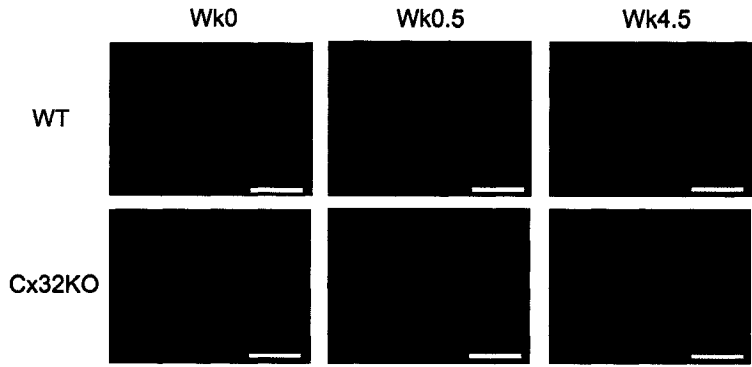
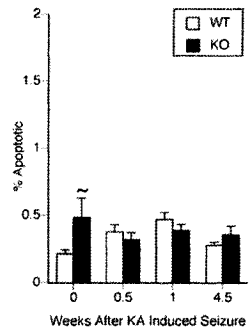
**Figure 2.2: Neuronal degeneration and regeneration in the CA3a/b pyramidal cell field of the Cx32KO and WT hippocampus following excitotoxic injury.** Mature neurons were identified qualitatively and quantitatively by NeuN immunofluorescence. Neurons were quantified from 40x photomicrographs of the dentate gyrus (A) and CA3a/b pyramidal cell field (B) in both WT and Cx32KO mice sacrificed at various time points following injury. Note that the 0 time period refers to uninjured control animals. Representative photomicrographs are shown for both the DG and CA3a/b pyramidal layer are shown (C). Data were analyzed by MANOVA and *post hoc* Tukey tests. Significant neuronal loss was observed 1 (\* $p < 0.05$ ) and 2 (\*\* $p < 0.01$ ) weeks after seizure in both genotype. Neurons in the CA3a/b region of the WT hippocampus fail to fully regenerate (\* $p < 0.05$ ) while neurons in Cx32KO mice regenerate to a level comparable to their uninjured controls. Scale bars: 5x, 200  $\mu\text{m}$ ; 40x 50  $\mu\text{m}$ .



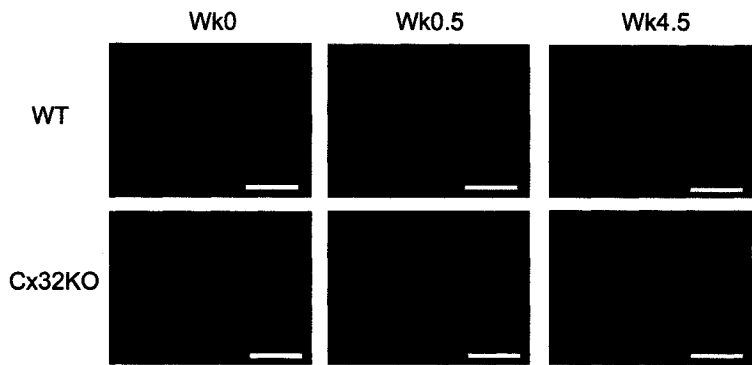
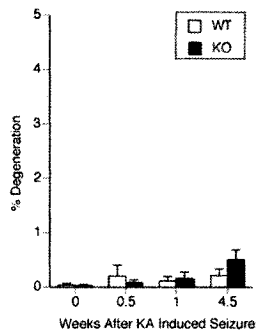
**Figure 2.2**

**Figure 2.3: Neuronal death in the mouse hippocampus following excitotoxic injury.** Apoptotic-like cell death was assessed by TUNEL in the DG (A) and CA3a/b (C) regions of the hippocampus of WT and Cx32KO mice following excitotoxic injury. The number of degenerating neuronal cells was also assessed by Fluoro-Jade B (FJB) staining in the DG (B) and CA3a/b (D) regions of the hippocampus of WT and Cx32KO mice following excitotoxic injury. Data are expressed as the number of TUNEL<sup>+</sup> or FJB<sup>+</sup> cells per field relative to the number of NeuN<sup>+</sup> cells counted in immediately adjacent sections. Data were analyzed by MANOVA and *post hoc* Tukey test. Significant neuronal death was observed 0.5 week following injury in the CA3a/b pyramidal cell field (\**p*<0.05). Scale bars: 50  $\mu$ m.

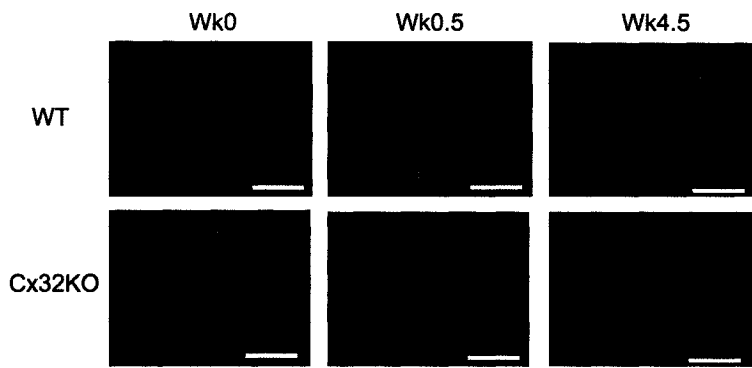
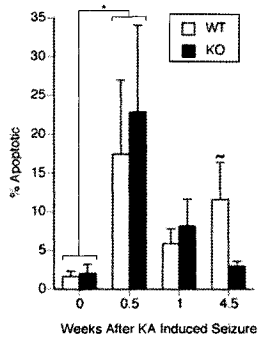
### A (DG - TUNEL)



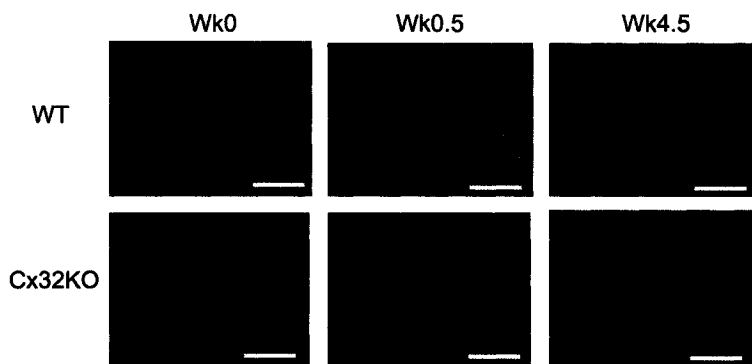
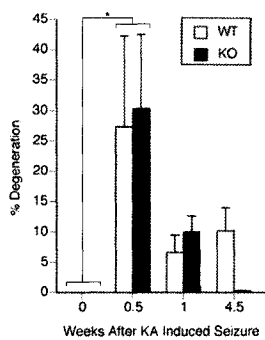
### B (DG - FJ)



### C (CA3a/b - TUNEL)



### D (CA3a/b - FJ)



**Figure 2.3**

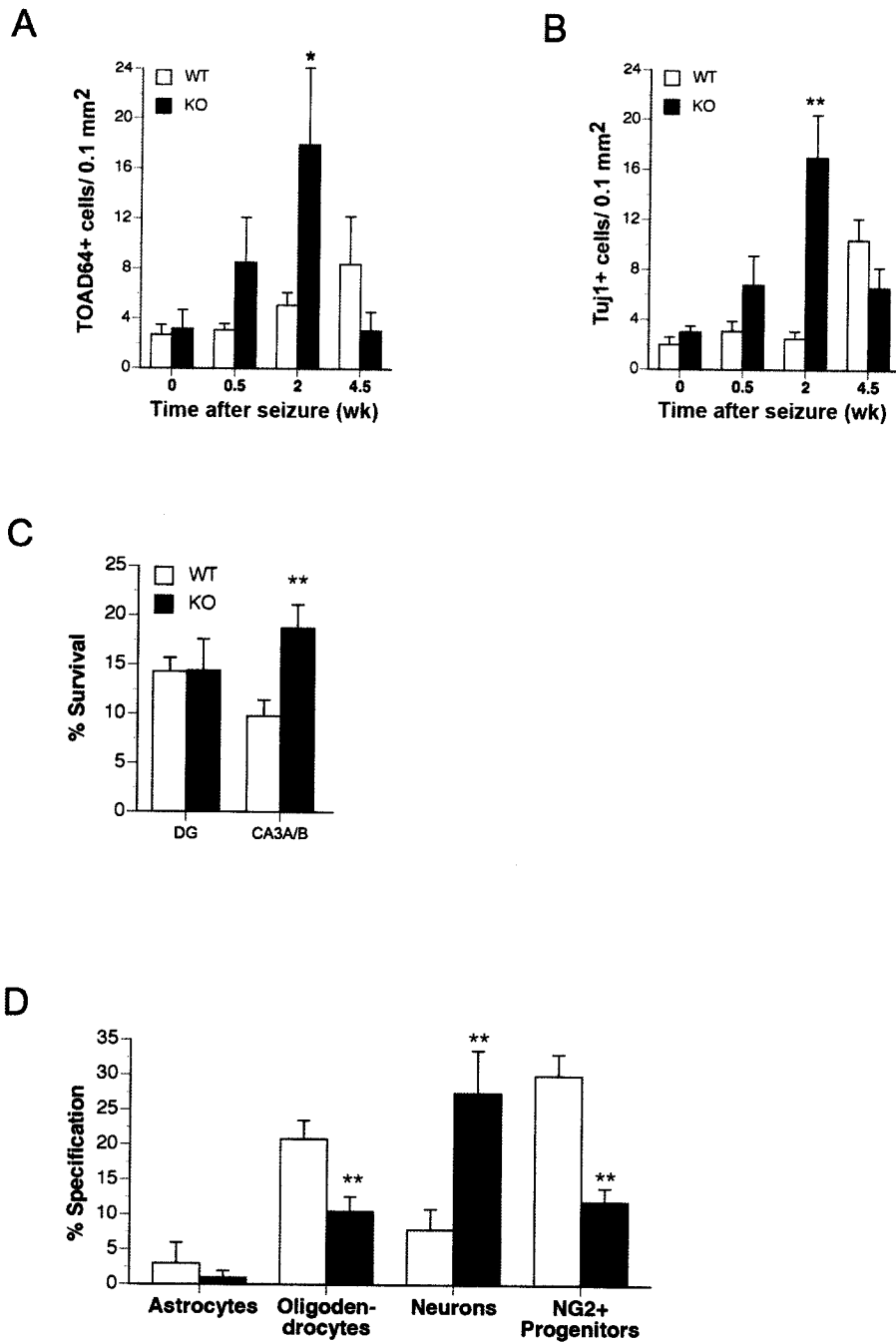
Together, these data demonstrate that, while cell viability is compromised in the granule cell layer of the DG, NeuN<sup>+</sup> neurons survive kainate challenge and do not undergo terminal degeneration as assessed by TUNEL and FJB staining. Conversely, significant neuronal death is detected in the CA3a/b cell field as indicated by a loss in cell viability and significant neuronal apoptosis and degeneration 0.5 week after seizure with dying neurons cleared from injured tissue within two weeks of kainate administration.

### **2.3.3 Neuronal replacement following excitotoxic injury is enhanced in absence of Cx32**

Despite extensive neuronal death in the CA3a/b pyramidal layer of both genotypes, viable MGPY<sup>+</sup> and NeuN<sup>+</sup> neuronal number were effectively restored in Cx32KO mice 4.5 wk after kainate seizure (Figure 2.1 and 2.2). A transient restoration in cell viability was detected in WT mice (Figure 2.1) but MGPY<sup>+</sup> and NeuN<sup>+</sup> cell number remained compromised 4.5 wk after seizure (Figure 2.1 and 2.2). A significant increase in the 64 kDa Turned on after division protein (TOAD-64)<sup>+</sup> and  $\beta$ -tubulin Type III (TuJ1<sup>+</sup>) cells, two markers of immature neurons rarely observed in uninjured adult brain (< 3 cells/0.1 mm<sup>2</sup>), two weeks after seizure (Figure 2.4 – A, B) preceded the appearance of new NeuN<sup>+</sup> neurons observed 4.5 weeks after KA administration in Cx32KO mice (Figure 2.2).

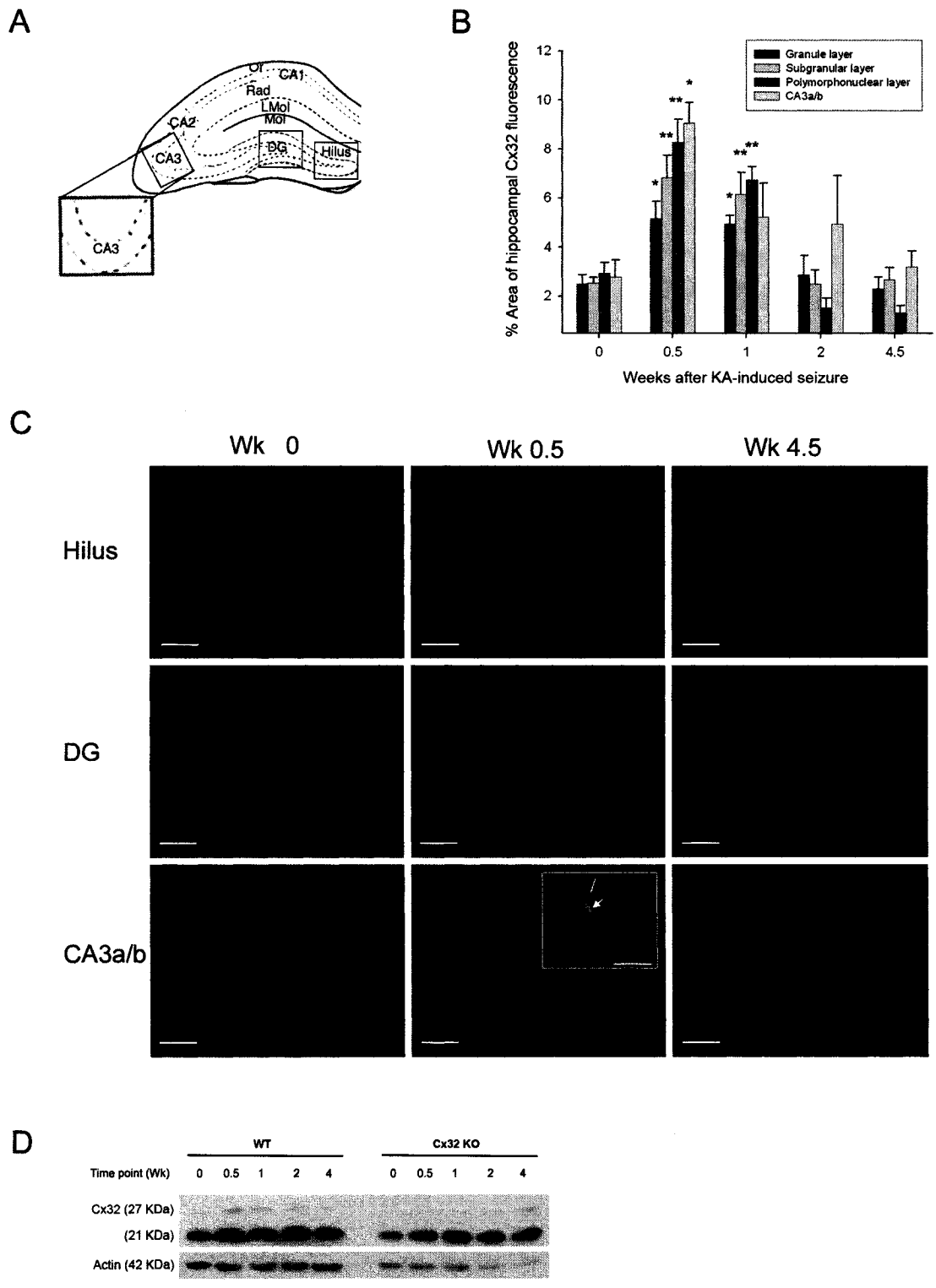
To evaluate the source of these newly formed neurons, mice were injected with BrdU, a thymidine analogue that incorporates into dividing progenitor cells, 5 times over 3 days at 0.5 week after kainate administration.

**Figure 2.4: Neuronal specification of CA3a/b NG2<sup>+</sup> progenitor cells following excitotoxic injury.** Immature neurons were identified quantitatively in the CA3a/b region of the hippocampus by (A) TOAD64 and (B) Tuj1 immunofluorescence in both WT and Cx32KO mice sacrificed at various time points following injury. Note that the 0 time period refers to uninjured control animals. Significant increase in the number of newly born neurons is observed 2 weeks following seizure. The source of newly formed neurons was assessed by BrdU labelling of progenitor cells activated 0.5 weeks following seizure. Mice were injected with BrdU 5 times over 3 days at 0.5 week after kainate administration. Mice were sacrificed 24 hours or 4 weeks after the last BrdU injection. The survival of BrdU<sup>+</sup> cells is represented as the percentage of BrdU<sup>+</sup> cells 4 weeks after injection relative to the number of BrdU<sup>+</sup> cells 24 hours after injection. No difference was observed in survival between genotype in the DG, but a significant increase in the survival of BrdU<sup>+</sup> cells was observed in the CA3a/b pyramidal cell field of Cx32KO mice (C). Lineage analysis was performed by double labelling using BrdU and GFAP (astrocytes), CNPase (oligodendrocytes), NeuN (neurons), or NG2 (progenitor cells) 4 weeks after the last BrdU injection (D). Neuronal specification was significantly increased in the Cx32KO CA3a/b as compared to their WT controls (\*\* p<0.01), while WT mice, the majority of cells proliferating 0.5 week after seizure either remained NG2<sup>+</sup> progenitors or specified to oligodendrocytes (\*\* p<0.01). Data were analyzed by a Student t-test or a MANOVA and post hoc Tukey test (\*p<0.05, \*\*p<0.01).



**Figure 2.4**

**Figure 2.5: Hippocampal expression of Cx32 in uninjured and injured mice.** Schematic of the adult mouse hippocampus are represented, identifying the regions of interest (A). Quantitative immunofluorescence data are presented for the hilus, the dentate gyrus, and the CA3a/b regions of the hippocampus (B) at various time points following KA-induced seizure, and qualitative photomicrographs are presented for each of these regions. Data were analyzed with a one-way ANOVA and a multiple comparisons versus control group (uninjured animals) using a Tukey *post hoc* test with  $\alpha$  values set at 0.05 (\* $p < 0.05$ ; \*\* $p < 0.01$ ). (C). Cx32 was mainly localized to NG2<sup>+</sup> cells 0.5 week following KA administration (C - inset). Western blot analysis of hippocampal Cx32 levels at various time points following injury. Controls include analysis of Cx32 null-mutant lysates. Each time point contains proteins extracted from hippocampi pooled from three animals. Actin was used as a loading control. Note the appearance of a 27 kDa artifactual band following KA treatment in the Cx32KO.



**Figure 2.5**

Animals were sacrificed 24 hours to determine the number of surviving BrdU<sup>+</sup> cells or 4 weeks after the injections to determine the identity of the specifying progenitor cells. No difference in percent survival of dividing cells in the DG was observed (Figure 2.4 – C). Cells actively proliferating 0.5 weeks after KA administration in the CA3a/b region of the hippocampus were retained at a significantly higher extent in Cx32KO mice (Figure 2.4 – C, \*\* p<0.01), thus suggesting that these progenitor cells might be the source of the new neurons as mentioned above. To verify this, sections were double-labelled for BrdU and GFAP (astrocytes), CNPase (oligodendrocytes), NeuN (neurons), or NG2 (progenitor cells) 4 weeks after the last BrdU injection (Figure 2.4 – D). Neuronal specification was significantly increased in the Cx32KO CA3a/b as compared to their WT controls (Figure 2.4 – D, \*\* p<0.01). On the other hand, in WT mice, the majority of cells proliferating 0.5 week after seizure either remained NG2<sup>+</sup> progenitors or specified to oligodendrocytes (Figure 2.4 – D, \*\* p<0.01).

#### **2.3.4 Hippocampal expression of Cx32 following excitotoxicity**

To provide mechanistic insight into how Cx32 regulates neurogenesis, Cx32 protein expression and localization was assessed following seizure by immunoblotting and immunofluorescence (Figure 2.5). Western blot analysis revealed low levels of Cx32 protein in uninjured animals and a transient increase in Cx32 protein expression (27 kDa band) in hippocampal lysates peaking 0.5 week following injury. Controls included immunoblots of Cx32 null-mutant hippocampal protein. It should be noted that existing Cx antibodies are

notoriously cross-reactive and our laboratory routinely confirms the specificity of all reagents with the cogent null-mutant control. We have screened eight different Cx32 antibodies (Chemicon, Zymed, 4 generated in-house) and have found that only two of these reagents (Zymed monoclonal and one in-house polyclonal reagent) do not cross-react with a 27 kDa band in Cx32 null-mutant hippocampus. However, our in-house polyclonal reagent is not an effective tool for immunofluorescence. As such, the Zymed monoclonal was used in this study. As we have previously demonstrated, no signal was detected in Cx32 null-mutant control tissue (0) at 27 kDa. However, a cross-reactive species at 27 kDa was detected at low levels in samples isolated 1 and 2 week(s) after seizure readily apparent in samples isolated 4.5 weeks after seizure in Cx32 null-mutant mice. A 21 kDa cross-reactive band was evident in all samples. In Appendix A, I describe the creation of a new mouse model to address this problem of Cx32 antibody.

Cautious interpretation suggests that Cx32 protein levels increase 0.5 week after seizure in WT hippocampus. This transient increase in Cx32 expression was confirmed with immunofluorescence labelling of brain sections. A significant increase in Cx32 immunofluorescence was observed throughout the layers of the DG and in the CA3a/b at 0.5 week after seizure. Negative controls included analysis of null-mutant sections and immunodetection with secondary antibody alone. These controls are not trivial as excitotoxicity can increase immunofluorescence background. Moreover, KA-induced seizure promotes blood-brain barrier breakdown (Bennett et al 1999). As we are using a

monoclonal antibody, infiltration of circulating murine IgGs through a compromised blood-brain barrier could lead to secondary antibody cross-reaction. As with Western analysis, some artifactual immunoreactivity was detected 4.5 weeks after seizure in Cx32KO controls (data not shown). At no point did we observed immunoreactivity with the secondary antibody alone controls in both genotypes (data not shown).

We localized Cx32 in the CA3a/b to mature CNPase<sup>+</sup> oligodendrocytes in uninjured tissue. At 0.5 week after kainate administration, Cx32 was also detected at the membrane of NG2<sup>+</sup> progenitor cells activated in the CA3a/b, stratum lucidum, and stratum radiatum (Figure 2.5 - inset). By 4.5 weeks after seizure, Cx32 was expressed primarily by CNPase<sup>+</sup> oligodendrocytes resident in the CA3a/b.

### **2.3.5 Potential role of Cx32 in modulating NPC-oligodendrocyte**

#### **intercellular communication that direct NG2<sup>+</sup> progenitor cell fate**

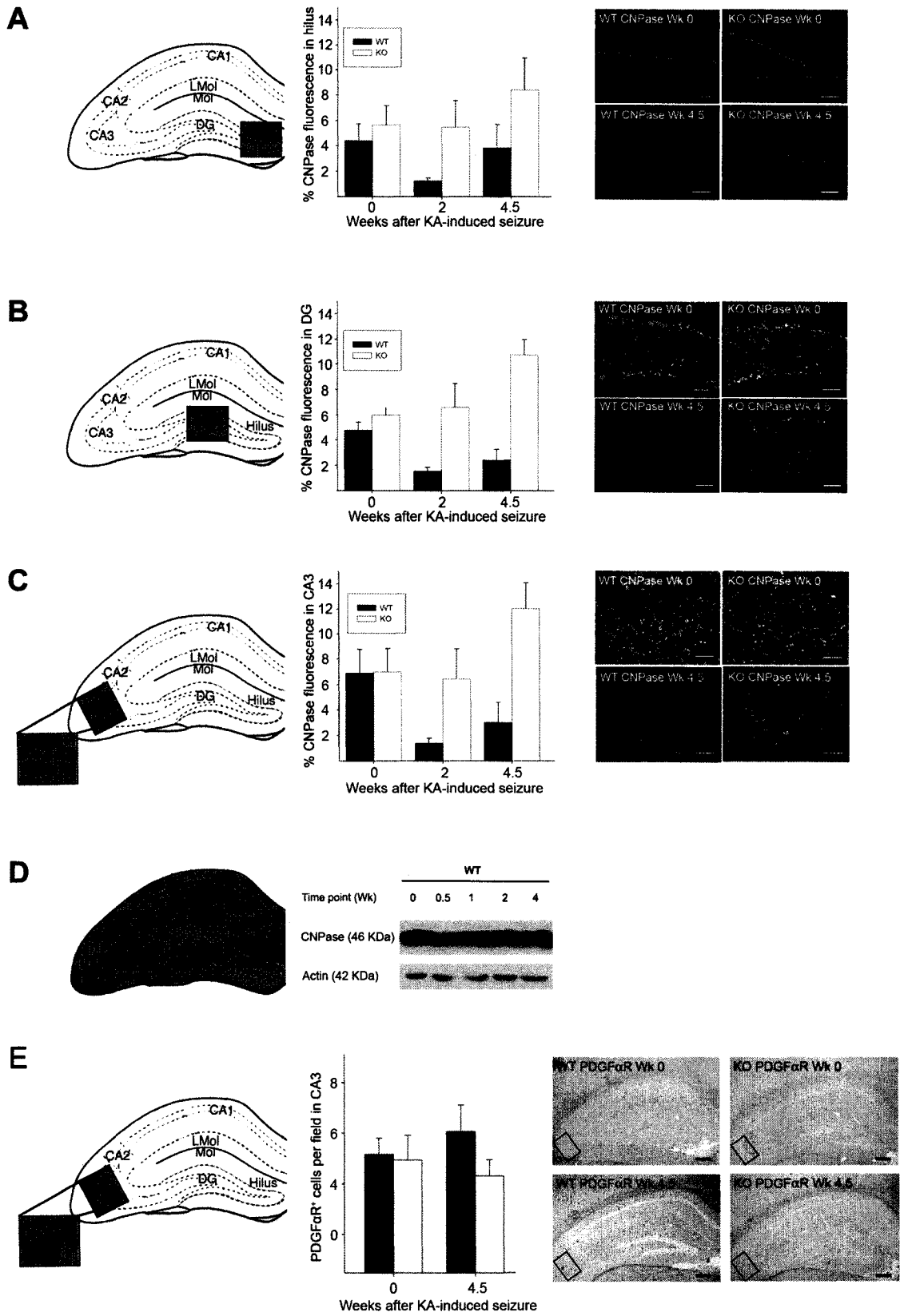
NG2<sup>+</sup> progenitor cells normally specify to oligodendrocytes in uninjured brain tissue (Nishiyama et al., 1996; Dawson et al., 2000; Nishiyama, 2001; Varlet, 2005). A subset of cells have recently been shown to be inherently multipotential (Belachew et al., 2003). Based on our *in vivo* findings, my colleagues and I hypothesized that expression of Cx32 by NG2<sup>+</sup> progenitor cells themselves and/or by neighbouring “instructive” oligodendrocytes inhibits neurogenesis and restricts NG2<sup>+</sup> specification to an oligodendrocyte lineage. In the absence of Cx32, NG2<sup>+</sup> progenitor cells are capable of neuronal

specification. To test this hypothesis, Lysanne Melanson-Drapeau, a Ph.D candidate in our laboratory, demonstrated that primary progenitor cells isolated from the hippocampus of Cx32KO mice exhibited an enhanced capacity for neurogenesis when cultured *in vitro* (Melanson-Drapeau et al., 2003). She then performed gain of function studies overexpressing Cx32 in these null-mutant progenitor cell cultures and demonstrated that ectopic expression of Cx32 inhibits neurogenesis. Shawn Whitehead, a post-doctoral fellow in the Bennett laboratory, followed the specification of progenitor cells labelled with BrdU in the CA3a/b of Cx32KO mice at 0.5 week after seizure and has shown that proliferating cells activated by seizure in CA3a/b, identified as NG2<sup>+</sup> progenitor cells, terminally specify to neurons to a greater extent in Cx32KO mice comparatively to their WT controls. These data are not included in my thesis as I was not the primary investigator involved. I followed the argument that loss of Cx32 by instructive oligodendrocytes in the CA3a/b alters the fate of activated NG2<sup>+</sup> progenitor cells.

Excitotoxic injury results in degeneration of not only pyramidal neurons but also myelinating oligodendrocytes in the major fibre tracks (Miller et al., 2005; Salter and Fern, 2005) albeit with some controversy (Wosik et al., 2004). To our knowledge, the fate of non-myelinating oligodendrocytes along the mossy fibre (MF) pathway and in the stratum lucidum, and in the CA3a/b pyramidal cell layer following KA-induced seizure has not been determined. As indicated in Figure 2.3, a second wave of cell loss, albeit at lower levels than that observed 0.5 week after seizure, is detected by TUNEL and FJB 4.5 week after seizure.

To establish whether oligodendrocytes are compromised following KA-induced seizure, CNPase immunoreactivity was assessed at 0 (uninjured controls), 2 and 4.5 week(s) after KA administration. A decrease in CNPase immunoreactivity was detected in WT but not Cx32KO mice 2 weeks following seizure in the hilus (Figure 2.6 – A), the DG (Figure 2.6 – B), and the CA3a/b (Figure 2.6 – C) regions of the hippocampus. These data suggested that oligodendrocytes were compromised in WT mice but not in Cx32KO mice. To verify this reduction in CNPase immunoreactivity in WT animals, immunoblot analysis of CNPase from WT hippocampal cell lysates was performed. The level of hippocampal CNPase expression remained unchanged from uninjured controls (Figure 2.6 – D), suggesting either a) no change in oligodendrocyte number or b) epitope masking. To reconcile this disconnect, *in situ* hybridization for PDGF $\alpha$ R mRNA was performed in uninjured controls and 4.5 weeks following KA administration on WT and Cx32KO brain sections. No difference in the total number of oligodendrocytes was found between uninjured and injured WT and Cx32KO mice (Figure 2.6 – E), suggesting that the reduction in CNPase labelling resulted from epitope masking or an overall reduction in CNPase protein expression rather than a decrease in the number of oligodendrocytes in the hippocampus following KA-induced seizure.

**Figure 2.6: Excitotoxicity does not result in hippocampal oligodendrocyte death in WT or Cx32KO mice.** Oligodendrocyte viability and number in the hippocampus were qualitatively and quantitatively evaluated at 0 (uninjured controls), 2, and 4.5 weeks following KA administration by CNPase immunofluorescence in the hilus (A), the dentate gyrus (B), and the CA3a/b (C) regions of the hippocampus. CNPase immunoreactivity decreased 2 weeks following seizure in WT, but not Cx32KO mice and remained low in the DG and CA3 (B,C). This reduction was not confirmed by Western analysis of whole hippocampal lysates (D). Data represent hippocampal lysates from n=3 animals per time point. No variation in the level of hippocampal CNPase immunoreactivity was observed (D). The total number of oligodendrocytes was assessed in the CA3 using PDGF $\alpha$ R *in situ* hybridization (E). No change in the total number of oligodendrocytes was observed following excitotoxic injury, regardless of genotype. Scale bars: 200  $\mu$ m (E); 50  $\mu$ m (A-C).



**Figure 2.6**

## 2.4 Discussion

The experiments in the present chapter implicate Cx32 as a regulatory switch directing the fate of NG2<sup>+</sup> progenitor cells activated by excitotoxic seizures towards either a neuronal or an oligodendrocyte lineage. Data presented in this thesis demonstrate that WT and Cx32KO mice exhibit comparable neuronal death in the CA3a/b pyramidal cell field following systemic injection of KA. Granule neurons in the DG were transiently compromised but survived excitotoxic insult consistent with previous literature. It should be noted that in rats, granule neurons have been shown to be terminally compromised following KA-induced seizure (Kesslak et al., 1995; Covolan et al., 2000) albeit with some controversy (Becker et al., 1999). However, this regional susceptibility in pyramidal neurodegeneration following KA-induced neurodegeneration is consistent with previous findings indicating that pyramidal cells from in the CA3 region express high levels of kainate receptors (Sperk, 1994). Furthermore, CA3 pyramidal neurons make numerous excitatory synaptic connections between cells in the same field as well as projecting to CA1, while pyramidal cells in the CA1 field or DG of the hippocampal complex are not extensively interconnected (Lee et al., 2004) resulting in a higher excitatory activation of CA3 pyramidal cells following KA administration.

Remarkably, neuronal replacement was more effective in the absence of Cx32. NeuN<sup>+</sup> cell number was restored in Cx32 null-mutant but not WT mice and was preceded by an increase in the number of immature neurons indicative of neurogenesis. Cx32 protein levels transiently increased following kainate

administration, peaking 0.5 week after seizure, although these results must be interpreted with caution given a low level of antibody cross-reactivity at 4.5 weeks after seizure in Cx32 null-mutant mice. The increase in Cx32 localized to activated NG2<sup>+</sup> progenitor cells proliferating 0.5 week after seizure activity in the CA3a/b cell field. Data not presented in this thesis performed by my colleagues in the Bennett laboratory indicate that these NG2<sup>+</sup> cells are the source of the new neurons in the Cx32KO mice.

Alternatively, the impact of genetic differences between null-mutant mice and WT controls could influence the rate of neurogenesis observed after kainate seizure. Given the strain-specific differences in the levels of excitotoxic injury discussed previously, and the mixed genetic background of our Cx32KO mice, we backbred our colony for 13 generations into C57BL/6 lineage. Furthermore, the mice were monitored daily for a period of one month following the KA administration, and no recurrent seizure activity was observed, as previously reported in rats (Sperk, 1994; Stubbley-Weatherly et al., 1996), regardless of genotype. The fact that seizure survival and intensity following administration of KA in congenic null-mutant and WT animals are comparable as is neurodegeneration, and the absence of recurrent seizure activity provide strong evidence that and the enhanced neuroregeneration cannot be attributed to strain differences between the two genotypes.

It is well documented that brain insult, notably KA-induced seizures, activates endogenous progenitor cell populations (Bengzon et al., 1997; Parent et al., 1997; Arvidsson et al., 2001; Jin et al., 2001; Parent and Lowenstein,

2002; Liu et al., 2003a; Parent, 2003). These stimulated progenitor cells in the injured brain, amplify, and migrate from the SGZ to the granule cell layer, hilus or molecular cell layer (Wang et al., 2004), where they can differentiate into granule neurons (van Praag et al., 2002; Bonde et al., 2006) or inhibitory interneurons (Liu et al., 2003b), and finally project their axons and integrate in the neuronal circuitry (Zhao et al., 2006). The functional implication of these newly formed neurons in the adult injured hippocampus, however, remains unclear (Bonde et al., 2006).

More recently, some research groups have shown that activation CA3 neuronal regeneration might be the result of progenitor cells residing in the CA3 being triggered by seizure activity (Dong et al., 2003), while others have suggested migration of progenitor cells from other regions to repair local damage (Parent et al., 2006). Here, I identified resident NG2<sup>+</sup> progenitor cells as activated in the CA3 following KA seizure and, in collaboration with my colleagues, we have determined that these cells are the main effectors of neurogenesis in the CA3 region of the hippocampus following KA-induced neuronal loss. NG2<sup>+</sup> expression is commonly associated with commitment to an oligodendrocyte lineage (Nishiyama et al., 1996; Dawson et al., 2000; Nishiyama, 2001; Varlet, 2005). A number of reports, however, indicate that these cells retain a multipotential capacity and are capable of neurogenesis (Kondo and Raff, 2000b; Belachew et al., 2003; Aguirre and Gallo, 2004; Aguirre et al., 2004; Dayer et al., 2005). The mechanisms directing NG2<sup>+</sup> cell fate to a neuronal or glial lineage have yet to be identified.

We found that activated NG2<sup>+</sup> progenitors in WT mice express Cx32. In Cx32 null-mutant mice, these cells appear capable of neurogenesis (Melanson-Drapeau et al., 2003). My colleague Shawn Whitehead has further demonstrated that progenitor cells in the CA3a/b labelled with BrdU at 0.5 week after seizure specify to neurons in Cx32 null-mutant but not WT mice, while Lysanne Melanson-Drapeau, a Ph.D candidate in the Bennett laboratory, has demonstrated that Cx32 overexpression in null-mutant progenitor cultures inhibits neurogenesis. Together, these data in context with the increase in neuronal replacement documented in this thesis implicate Cx32 expression as key event in NG2<sup>+</sup> progenitor cell commitment.

While this study does not directly address how Cx32 functions, a number of possibilities can be postulated. Earlier studies have implicated direct cell communication in stem and progenitor cell differentiation. In 2002, Song *et al*, reported that direct contact between stem cells and astrocytes instructs stem cells for neuronal commitment, and that direct contact between stem cells and neurons instructs stem cells for oligodendroglial commitment (Song et al., 2002). The interest generated by this particular study raised a number of important questions in the field. Most relevant to this study, what is the outcome direct contact between stem cells and oligodendrocytes? A Ph.D student in the Bennett Laboratory, Sophie Imbeault, is currently exploring this experimental question by investigating oligodendrocyte primary cultures obtained from various Cx-null mutants. Previous studies have indicated that connexons at the plasma membrane of oligodendrocytes, containing the Cx32 subunit, have the ability to

couple with other connexons at the plasma membrane of other oligodendrocytes or astrocytes. Connexons can be formed by various arrangements of the Cx32, Cx29 and Cx47 protein subunits in oligodendrocytes and Cx26, Cx30 and Cx43 protein subunits in astrocytes (Nagy and Rash, 2003). This heterogeneous Cx composition of oligodendrocytic and astrocytic connexons give rise to multiple possibilities of interactions between activated Cx32<sup>+</sup>/NG2<sup>+</sup> progenitors in the CA3a/b region of the hippocampus and these mature instructive cells via gap junctional communication. It has been proposed that calcium wave propagation between precursor cells stimulate cellular proliferation (Weissman et al., 2004) while others argue that an increase in the intracellular concentration of Ca<sup>2+</sup> increases neuronal differentiation through activation of the Ras/MAPK pathway (Finkbeiner and Greenberg, 1996). Calcium waves could potentially be propagated between NG2<sup>+</sup> cells and instructive cells to regulate their fate by direct passage of Ca<sup>2+</sup> through gap junctions (electrical coupling), or by the passage of second messengers (biochemical coupling), such as IP<sub>3</sub>, that could in turn increase the cytoplasmic concentration of Ca<sup>2+</sup> (Kandler and Katz, 1998). Previous unpublished studies in the Bennett laboratory indicate functional redundancy and compensatory gene expression in the adult Cx32KO mouse hippocampus. In absence of Cx32, Cx47 is upregulated. The loss of Cx32 in these cells does not necessarily implicate the loss of their gap junctional coupling, but most likely alteration of signalling properties. Thus, in absence of Cx32, calcium wave propagation between the NG2<sup>+</sup> progenitors in the CA3a/b

region of the hippocampus could be disrupted, and result in altered differentiation and/or proliferation properties.

Direct cell coupling between the progenitor cell population and instructive cells represent a strong potential regulatory mechanism in the frame of this thesis, but other mechanism could also be implicated. As reviewed in Chapter 1, Cx mediated communication can also result in exchanges between the extracellular environment and the cytoplasm of cells expressing hemichannels composed of these Cxs (Bennett et al., 2003; Goodenough and Paul, 2003). Progenitor cells form functional hemichannels in non-junctional membranes, and blocking this activity pharmacologically or by altering Cx expression promotes neuronal specification (Boucher and Bennett, 2003). Human NT2 precursor cells are capable of hemichannel activity, but this activity is lost over the course of specification (Boucher and Bennett, 2003). The opening of hemichannel in mature cells in the neurogenic niche might act as an ionic or nucleophilic sink (Kamermans et al., 2001), and prevent a cytoplasmic increase of ATP, adenosine, and  $Ca^{2+}$ , thus prevent neurogenesis and promote gliogenesis, given that these are essential to ensure neuronal specification. Hemichannel activity has also been associated as a signalling pathway leading to the release of ATP in non-excitabile cells in the CNS (Stout et al., 2002). Thus, changing the Cx composition of oligodendroglial hemichannels could alter the availability of ATP, and thus affect neurogenesis.

As reviewed above, progenitor cells are highly sensitive to their microenvironment. Spatial buffering of potassium efflux following neuronal

excitation has long been attributed strictly to astrocytes, but the mechanism by which astrocytes uptake potassium remained undetermined (Kofuji and Newman, 2004; Kamasawa et al., 2005). Oligodendrocytes have recently been identified as key players in potassium siphoning, allowing the passage of potassium from the periaxonal region to the astrocytes (Rasband, 2004), via Cx32- and Cx47-mediated gap junctional coupling of oligodendrocytes and astrocytes (Menichella et al., 2003; Kamasawa et al., 2005). Altering the Cx expression in these mature glial cells, notably Cx32 in oligodendrocytes, might result in the loss of this spatial buffering in the hippocampus (Sohl et al., 2004; Kamasawa et al., 2005; Theis et al., 2005). This could lead to sustained increased extracellular concentration of potassium. Such a rise in the extracellular potassium concentration could in turn affect the resident NG2<sup>+</sup> progenitor cells, and direct them towards a neuronal fate and increase neurogenesis in absence of Cx32.

Alternatively, the increased CA3 pyramidal cell regeneration reported in absence of Cx32 may not reflect a change in NPC specification or survival. The loss of Cx32 may rather facilitate or inhibit the survival of terminally differentiated cells, namely non-myelinating oligodendrocytes within the damaged hippocampus that may affect the toxic environment to which regenerating neurons are exposed. It is interesting that we see a second wave of apoptotic death 4.5 weeks post seizure in WT but not KO mice. A significant number of studies have implicated Cx-mediated communication apoptosis, via a spread of toxic metabolites called the “bystander effect” (Mesnil et al., 1996; Lin et al., 1998; Andrade-Rozental et al., 2000; Condorelli et al., 2003; Kalvelyte et al.,

2003; Udawatte and Ripps, 2005). At no point do we detect Cx32 in neurons in the pyramidal cell layer, thus it may be that the loss of this partner population, likely the non-myelinating oligodendrocytes in the hippocampus, impacts upon the survival of the newly born neurons.

To provide mechanistic insight into the potential role of oligodendrocytes in providing support for the newly formed neurons, the impact of excitotoxic injury on oligodendrocyte number was assessed. We found that expression of CNPase was reduced *in situ* in WT but not Cx32KO mice. These data suggested that oligodendrocytes might be compromised following seizure possibly because of oligodendrocyte-oligodendrocyte coupling or hemichannel activity. Closer examination revealed that the overall level of CNPase remained constant as assessed by Western blot analysis and the absolute number of oligodendrocytes expressing PDGF $\alpha$ R transcript did no change following seizure in either genotype. These data suggest either a decrease in protein expression or masking of the CNPase epitope *in situ* in WT but not Cx32KO mice. This in turn may indicate a change in protein-protein interaction or, at least, a reduction in metabolic activity in oligodendrocytes expressing Cx32 following injury. While we cannot conclude that loss of Cx32 compromises viability of potentially instructive oligodendrocytes, these data do raise the possibility that oligodendrocyte metabolism is altered by the presence or absence of Cx32 that could conceivably impact on the fate of neighbouring NG2<sup>+</sup> cells activated following injury.

The research presented in this chapter shows that neuronal degeneration following KA-induced excitotoxic injury occurs mainly in CA3a/b neurons of the

hippocampus 0.5 week after KA administration in WT and Cx32KO mice. Following this extensive neuronal loss, NG2<sup>+</sup> progenitor cells residing in the CA3a/b region are activated and progress to immature TOAD64<sup>+</sup> and TUJ1<sup>+</sup> neuronal progenitors, to mature NeuN<sup>+</sup> neurons at a greater extent in absence of Cx32. The level of Cx32 in WT animals increased transiently, correlating with progenitor specification to NG2<sup>+</sup> progenitors or mature oligodendrocytes. These data implicate Cx32 as a switch regulator inhibiting neurogenesis in NPCs residing in the SGZ. Some mechanistic insights were provided in this chapter, but much remains undetermined. Assessing the expression of other oligodendroglial Cxs (Cx47 and Cx29) following excitotoxic injury in the Cx32KO mouse would provide interesting information regarding functional redundancy and potentially implications of other Cxs in progenitor cell regulation. Assessing neuronal functionality at the molecular level, via MF sprouting or electrophysiology, and at the behavioural level could further substantiate this potential approach for potential stem cell therapy following neuronal loss in the adult brain.

## **Chapter 3: Behavioural assessment of functional neuronal regeneration**

### **Abstract**

Experiments in Chapter 2 have shown that neuronal regeneration in the CA3a/b region of the hippocampus occurs at a greater extent following excitotoxic injury in absence of Cx32. Whether these newly formed neurons translate into functional memory encoding, however, remained undetermined. This chapter first analyzes a variant of the Morris water maze to determine whether the task exhibits enough specificity and sensitivity to detect CA3a/b specific neuronal impairment, using the WT mice 4 weeks post-injury, given their incomplete neuronal regeneration as reported in Chapter 2. The neuronal impairment in these mice was reflected as a behavioural impairment in the Morris water maze. Motivational or motoric impairment confounds were not present during the study. Following validation of the behavioural task, Cx32KO mice were evaluated in the Morris water maze 4 weeks following injury in comparison to their uninjured controls. Most interestingly, Cx32KO mice that were injured prior to the Morris water maze performed comparably to their uninjured controls, indicating functionality of the regenerated pyramidal neurons in the CA3a/b region of the hippocampus reported in Chapter 2.

### **Publications resulting from studies described in this chapter**

Morin M.Y., Vanderleest E., Toeg H., Midzic I., Messier C., Bennett S.A.L.. Behavioural assessment of spatial memory performance as an indicator of chronic neuronal impairment in the mouse hippocampus following excitotoxic injury (In preparation for Behavioral Brain Research).

### **Statement of author contributions**

Erin Vanderleest and Hadi Toeg, two honours student in our laboratory contributed during the Morris water maze trials. Ines Midzic, a summer student in our laboratory contributed to cutting the brains following perfusions and analyzing the data. Tanya Bossy, a student in Dr. Claude Messier's laboratory, assisted in establishing the conditions and criteria appropriate to the study of C57Bl/6 mice in the Morris water maze. Jessica Lallier, a research assistant in our laboratory, assisted in the perfusion of the animals.

### **3.1 Introduction**

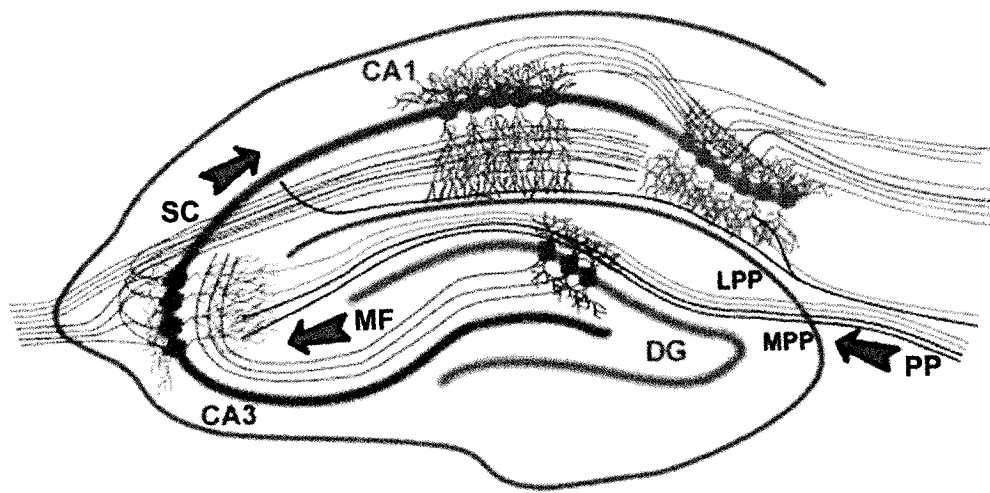
The hippocampus is a critical player in the encoding of associative long-term memory (Forwood et al., 2005; Leutgeb et al., 2005). This limbic brain region is mainly composed of three different neuronal pathways (Johnston et al., 1992): the perforant pathway (PP), the MF pathway, and the Schaffer's collateral pathway (SC; Figure 3.1). The PP is responsible for the connection between the parahippocampal cortex and the DG of the hippocampus. The MF pathway is responsible for establishing functional neuronal connections between the granule neurons of the DG and the CA3 pyramidal cells of the hippocampus. The SC pathway is responsible for neuronal connections between the pyramidal cells of the CA3 and CA1 regions of the hippocampus.

Interruption of this pathway has been closely associated with memory loss notably anterograde amnesia, first reported in humans with patient H.M. following a medial temporal lobectomy (Scoville and Milner, 1957; Corkin, 2002).

Moreover, the presence of "place cells" that are required to process spatial memory in the hippocampus have been identified (O'Keefe and Dostrovsky, 1971; Hartley et al., 2005). The presence of functional and molecular differences between the sub-regions of the hippocampus emphasizes key regional differences in both cell types and cell interactions that must be functionally recapitulated if neuroregenerative strategies are to be successful.

A number of behavioural paradigms have been used to monitor the function and integrity of hippocampus in experimental models of human disease. Of these paradigms, the Morris water maze has been frequently used in

**Figure 3.1: Schematics of the major intrahippocampal pathways involved in learning and memory.** Input from the Entorhinal Cortex forms connections with the Dentate Gyrus (DG) and CA3 pyramidal neurons via the Perforant Path (PP). Neurons in the CA3 region receive input from the DG via the mossy fibre (MF) pathway, and project to CA1 pyramidal neurons via the Schaffer Collateral (SC) pathway. Neurons in the CA1 also receive input from the Perforant Pathway, and project their axons to the Subiculum (Sb), which in turn project back to the EC, creating a loop. This figure was modified from the following source: <http://www.bris.ac.uk/Depts/Synaptic/info/pathway/hippocampal.htm>



**Figure 3.1**

behavioural neuroscience to study spatial memory and navigation associated with hippocampal activity (Morris, 1984; Gilbert et al., 2000; D'Hooge and De Deyn, 2001; Van Dam et al., 2005). Multiple studies indicate that performance in the water maze is dependent upon both endogenous variables such as sex (Berger-Sweeney et al., 1995; Bucci et al., 1995), age (Chapillon and Roulet, 1996), strain (Schimanski and Nguyen, 2004), nutrition (Bedi, 1992; Bellush et al., 1996), and stress (de Quervain et al., 1998; Holscher, 1999), and exogenous variables such as the characteristics of the apparatus, the training procedures, and the experimental paradigms (D'Hooge and De Deyn, 2001). Consequently, it is critical that experimenters pay close attention to these multiple parametric factors in both the murine model and the experimental paradigm when optimizing the behavioural task to study hippocampal function in transgenic mice with mixed backgrounds.

In the present chapter, uninjured and injured C57Bl/6 adult mice were first submitted to a variant of the Morris water maze in order to establish the sensitivity of the task for the specific neuronal deficit identified in the CA3a/b pyramidal cell field of the hippocampus 4 weeks following excitotoxic challenge. Following validation of the paradigm with WT mice, the behavioural functionality of the regenerated neuronal population in the CA3a/b pyramidal cell field of Cx32KO mice 4 weeks post-injury, as described in Chapter 2, was evaluated. Here, we show that the increased neuronal regeneration in absence of Cx32 results in functional improvement of spatial memory performance as compared to their WT controls. This provides greater evidence that the neurons regenerated

in absence of Cx32 following excitotoxic injury possess comparable functional characteristics to the uninjured resident neuronal population in the CA3a/b region of the hippocampus.

## **3.2 Material and Methods**

### **3.2.1 Animal treatments**

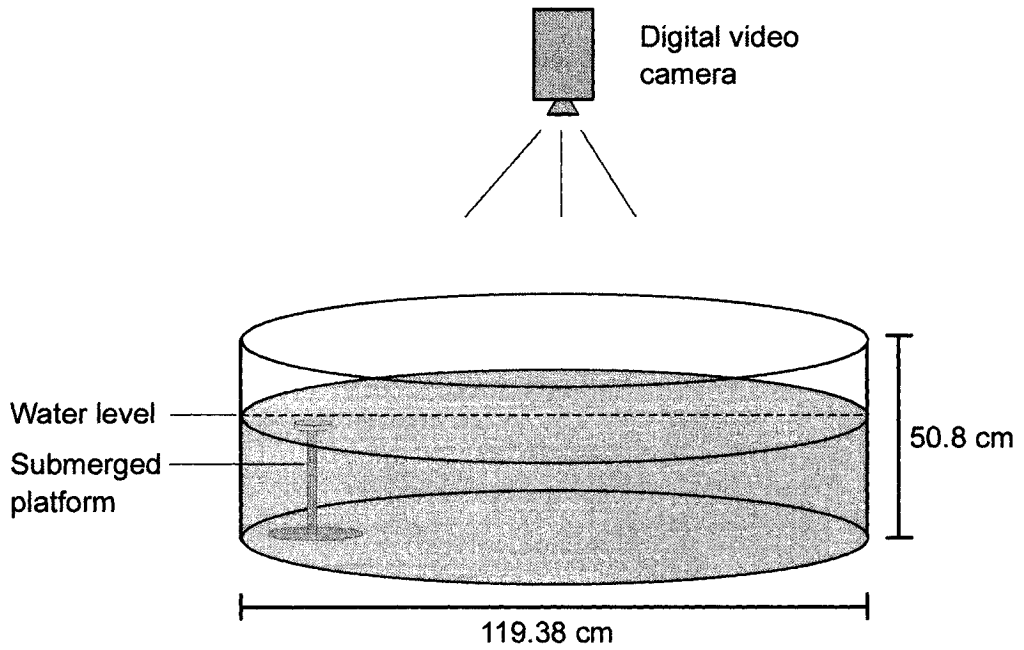
Adult congenic C57Bl/6 male mice ranging from 2-5 months of age (N=38) were used in this study, and divided into four experimental groups: WT uninjured (n=11), WT injured (n=11), Cx32KO uninjured (n=7), and Cx32KO injured (n=8). One Cx32KO mice was removed from study because of ocular infection, which could have affected navigational performance in the water maze. The mice were individually housed 2 weeks prior KA administration, maintained on a 12/12 light/dark cycle, and provided with food and water *ad libitum* throughout testing. Animals were subjected to KA-induced seizures 4 weeks prior to the Morris water maze paradigm, as described in Chapter 2. Uninjured controls included uninjured age-matched animals. All procedures were in agreement with the guidelines of the CCAC and the policy for animals use in research and teaching at the University of Ottawa (University Policy 31).

### **3.2.2 Behavioural analysis with the Morris water maze**

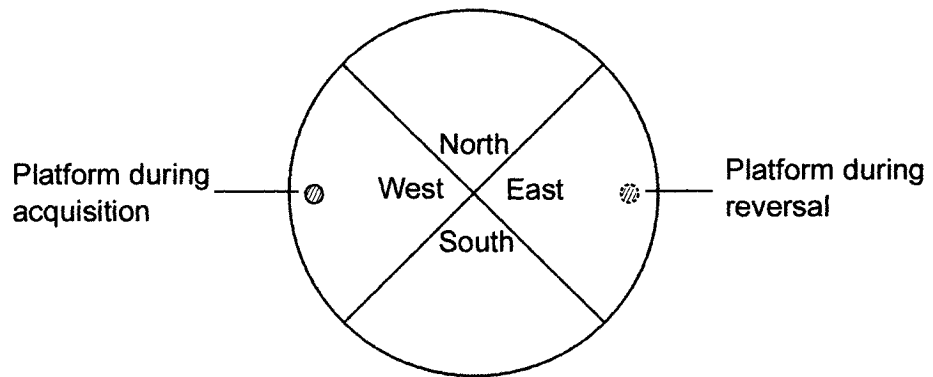
Four weeks following excitotoxic injury, the animals were submitted to 10 consecutive days of trials in the Morris water maze (Figure 3.2 – C). The water maze consisted of a circular plastic pool (119.38 cm in diameter and 50.8 cm in height) divided in 4 virtual quadrants and filled with water that was rendered opaque white with non-toxic water-soluble paint (Figure 3.2 – A). The water temperature was maintained approximately at  $22 \pm 1^{\circ}\text{C}$  to promote swimming activity in the mice.

**Figure 3.2: Schematic representation of the Morris Water Maze setup and timeline of experimental procedures.** The water maze trials were performed in Dr. Claude Messier's laboratory at the University of Ottawa. All trials were recorded with a digital camera located above the circular pool (A). The pool was surrounded by black curtains to reduce visual cues for the animals. The water maze was divided into 5 different zones (North, South, East, West, and Platform) during the trials, with the platform zone varying between the acquisition phase and the reversal phase (B). The animals were injected with KA. Four weeks after KA injection, kainate-treated or control (uninjured) animals were tested in the water maze (C).

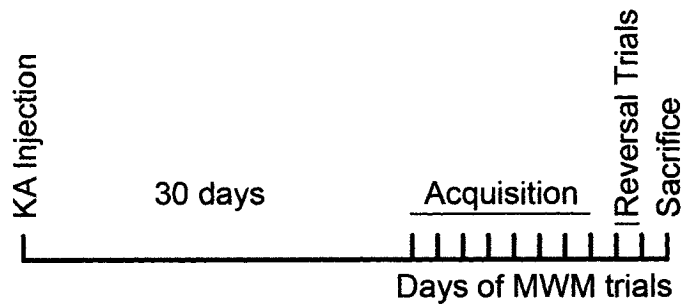
**A (Schematics of the Morris Water Maze apparatus)**



**B (Division of the Morris Water Maze Arena)**



**C (Kinetics of Experimental Paradigm)**



**Figure 3.2**

The water level was approximately 1 cm above a small circular white platform of 5 cm in diameter, which was placed in the western quadrant during the acquisition phase and the eastern quadrant for the reversal phase of the water maze paradigm. The maze was surrounded by four identical black curtains, and no additional cues were available to the mice except those provided by the curtains themselves and the slight variations in lighting. Contrary to other strains, these cues are sufficient for C57BL/6 mice to navigate in the water (Ammassari-Teule and Caprioli, 1985; Van Dam et al., 2005).

During the first 8 days of the water maze paradigm (acquisition period), the mice were inserted by the tail into the water, facing the perimeter of the pool in the western quadrant of the water maze (Figure 3.2 – B). The time to find and attain the hidden platform for at least 2 seconds (escape latency) was assessed. Each day of trial consisted of four trials of 60 seconds per day, with a 60 seconds rest period between each trial. On the 9th day of trial, the platform was removed from the pool, and the mice inserted into the water maze for a free swim period of 60 seconds. One hour following the probe trial, the platform was inserted in the eastern quadrant of the water maze, at 180 degrees from its original position, and the mice were submitted to the 4-trial per day paradigm for two consecutive days, starting on day 9 of the Morris water maze paradigm, to assess reversal learning performance.

All trials were recorded with a digital camera located above the water tank, and analyzed on a computer with Ethovision (Noldus, Leesburg, VA, USA). Once the mice found the location of the platform in the maze, they were left on it

for period of 10 seconds; if the subjects did not find the location of the platform within 60 seconds, they were guided to it, where they remained for 10 seconds. After each trial, the mice were dried, and returned to their home cage that was placed on a heating pad. All the animals were anesthetised with sodium pentobarbital 24 hours after the last day of the Morris water maze paradigm, and transcardially perfused with 10 mM PBS and 4% paraformaldehyde in 10 mM PBS.

### **3.2.3 Statistical analyses**

Data are presented as the mean  $\pm$  SEM. The WT and Cx32KO experimental groups were analyzed with either a Student t test or a two way repeated measures ANOVA as appropriate. Following detection of a significant F value (main effect); individual comparisons were made using a Tukey *post hoc* test with  $\alpha$  value set at 0.05. Statistical analyses were performed with SigmaStat (Systat Software Inc.).

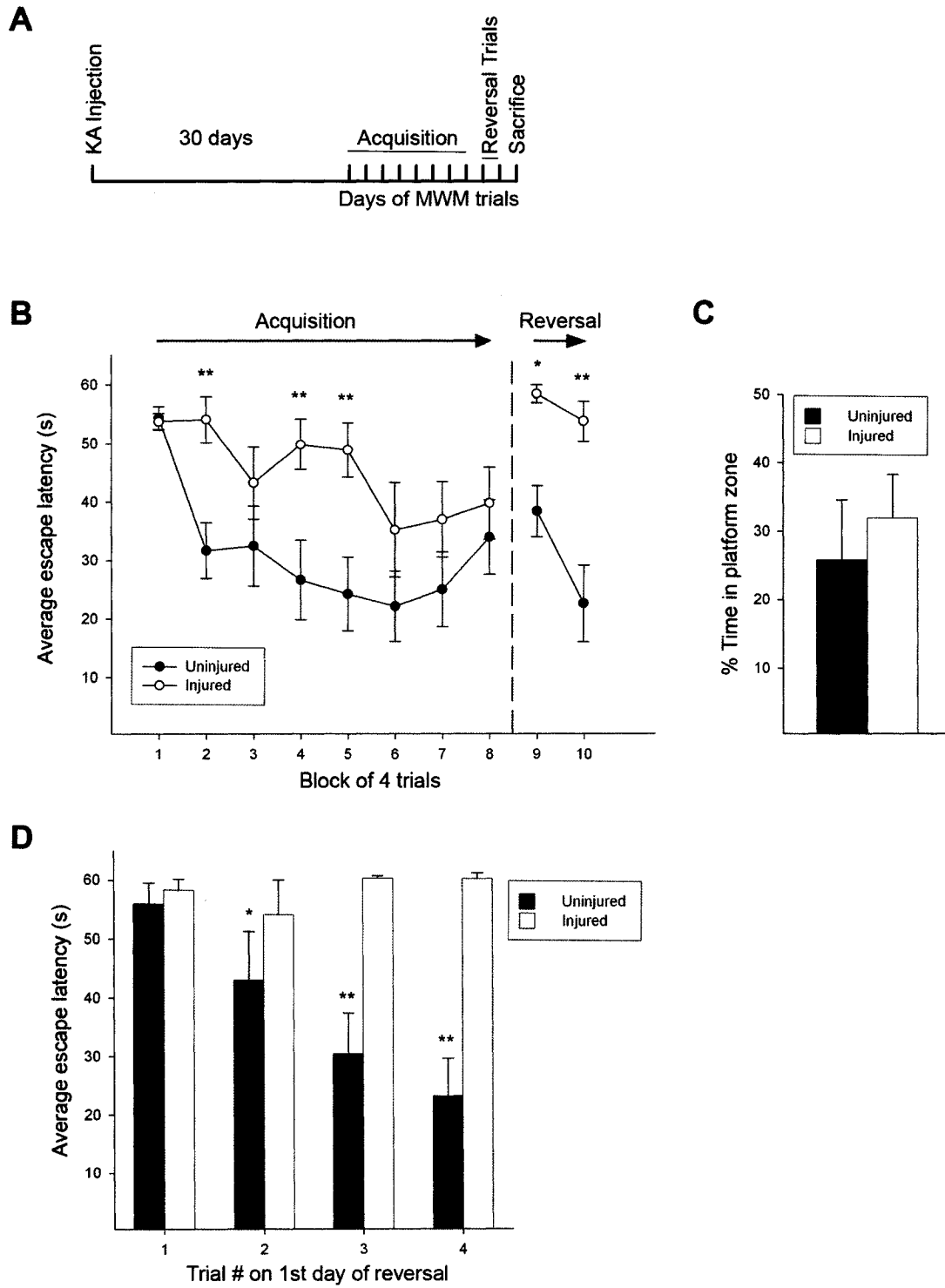
### **3.3 Results**

#### **3.3.1 WT mice are functionally impaired in the Morris water maze 5 weeks following kainate administration**

As indicated in Chapter 2, WT mice exhibit incomplete regeneration of neurons in the CA3a/b cell field 4.5 wks after KA-induced seizure (Figure 2.1; Figure 2.2). My first objective was to determine whether the Morris water maze paradigm was sensitive enough to detect this region-specific neuronal deficit in the hippocampus.

Four weeks following KA administration, WT mice were tested in the Morris water maze as illustrated in Figure 3.1 – C. A main effect was observed between the two experimental groups ( $F_{(1,14)}=8.179$ ,  $P=0.013$ ) and among the different levels of the day of trial ( $F_{(9,126)}=7.531$ ,  $P<0.001$ ). The injured animals demonstrated a significantly slower rate of learning the position of the platform during the acquisition phase when compared to their uninjured controls from the same day of trial (Figure 3.3 – B;  $p<0.01$ ). Despite the slower rate of learning the spatial location of the platform, both groups reached criteria by the 8<sup>th</sup> day of testing. Learning was validated by the comparable amount of time spent in the platform zone during the probe trial (Figure 3.3 – C). The injured animals demonstrated a particular difficulty at learning the new position of the platform as compared to their uninjured controls during the first (Figure 3.3 – B;  $p<0.05$ ) and the second (Figure 3.3 – B;  $p<0.01$ ) days of the reversal phase of the water maze paradigm.

**Figure 3.3: Impairment of spatial memory performance in the Morris Water Maze in adult C57Bl/6 mice 5 weeks after excitotoxic injury.** Adult male mice were injected with kainic acid, aged for 30 days, tested in the Morris water maze for 10 consecutive days, and sacrificed 24 hours later (A). The mean latency of 4 trials to find the platform spatial location is represented for 8 days of acquisition and 2 days of reversal (B). The mice were submitted to a probe trial on day 9, and the percentage of time spent in the original platform quadrant was assessed (C). The four trials during the first day of reversal are represented in order to illustrate a new learning curve in non-injured animals (D). The two groups were analyzed using a two way repeated measures analysis of variance (ANOVA) with one factor repetition followed with a multiple comparisons versus control group (uninjured animals) using a Tukey *post hoc* test with  $\alpha$  values set at 0.05 (\* $p < 0.05$ ; \*\* $p < 0.01$ ).

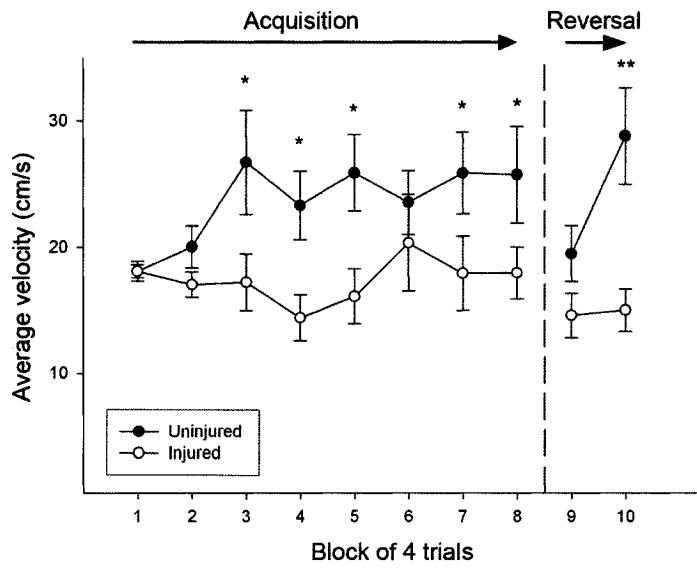
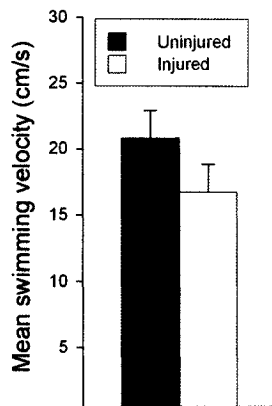
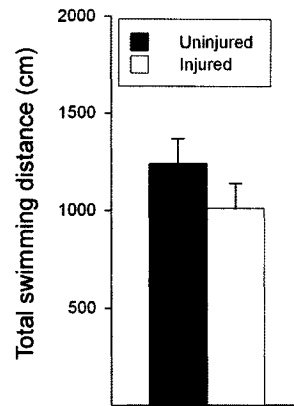


**Figure 3.3**

On the first day of reversal, a learning curve was observed in the uninjured animals over the four trials (Figure 3.3 – D;  $p < 0.05$  or  $p < 0.01$ ). However, this learning curve was not observed in the injured animals, where the escape latency remained unchanged from the first trial up to the fourth trial.

To insure that the spatial memory deficits observed in injured WT mice were not caused by motor or motivational deficits, swimming velocity and the swimming distance were assessed. For the swimming velocity during the acquisition period of the water maze, a main effect was observed between the two experimental groups ( $F_{(1,14)} = 6.072$ ,  $P = 0.027$ ) and among the different levels of the day of trial ( $F_{(9,126)} = 2.380$ ,  $P = 0.016$ ). The initial velocity of the two groups was comparable on the first day of trial (Figure 3.4 – A), and during the probe trial (Figure 3.4 – B), suggesting the absence of a motor deficit in the injured animals. The two experimental groups also showed comparable swimming distances during the probe trial (Figure 3.4 – C), correlating the absence of motor or motivational deficits. Some differences in the swimming velocity were observed between the two groups during the acquisition and reversal periods of the water maze paradigm (Figure 3.4 – A), but these variations were correlated with quicker escape latency in most cases (Figure 3.3 – B). Taken together, these data demonstrate that behavioural impairment of injured WT mice in the Morris water maze is caused by spatial memory deficits, rather than motor skill or motivational discrepancies.

**Figure 3.4: Motor activities of adult C57Bl/6 mice in the water maze 5 weeks after excitotoxic injury.** The mean swimming velocity of 4 trials to find the platform spatial location in the water maze is represented for 8 days of acquisition and 2 days of reversal learning (A). The mice were submitted to a probe trial on day 9, and the mean swimming velocity (B) and the total swimming distance (C) were assessed in absence of the platform. The two groups were analyzed using a two way repeated measures analysis of variance (ANOVA) with one factor repetition followed with a multiple comparisons versus control group (uninjured animals) using a Tukey *post hoc* test with  $\alpha$  values set at 0.05 (\* $p < 0.05$ ; \*\* $p < 0.01$ ).

**A****B****C****Figure 3.4**

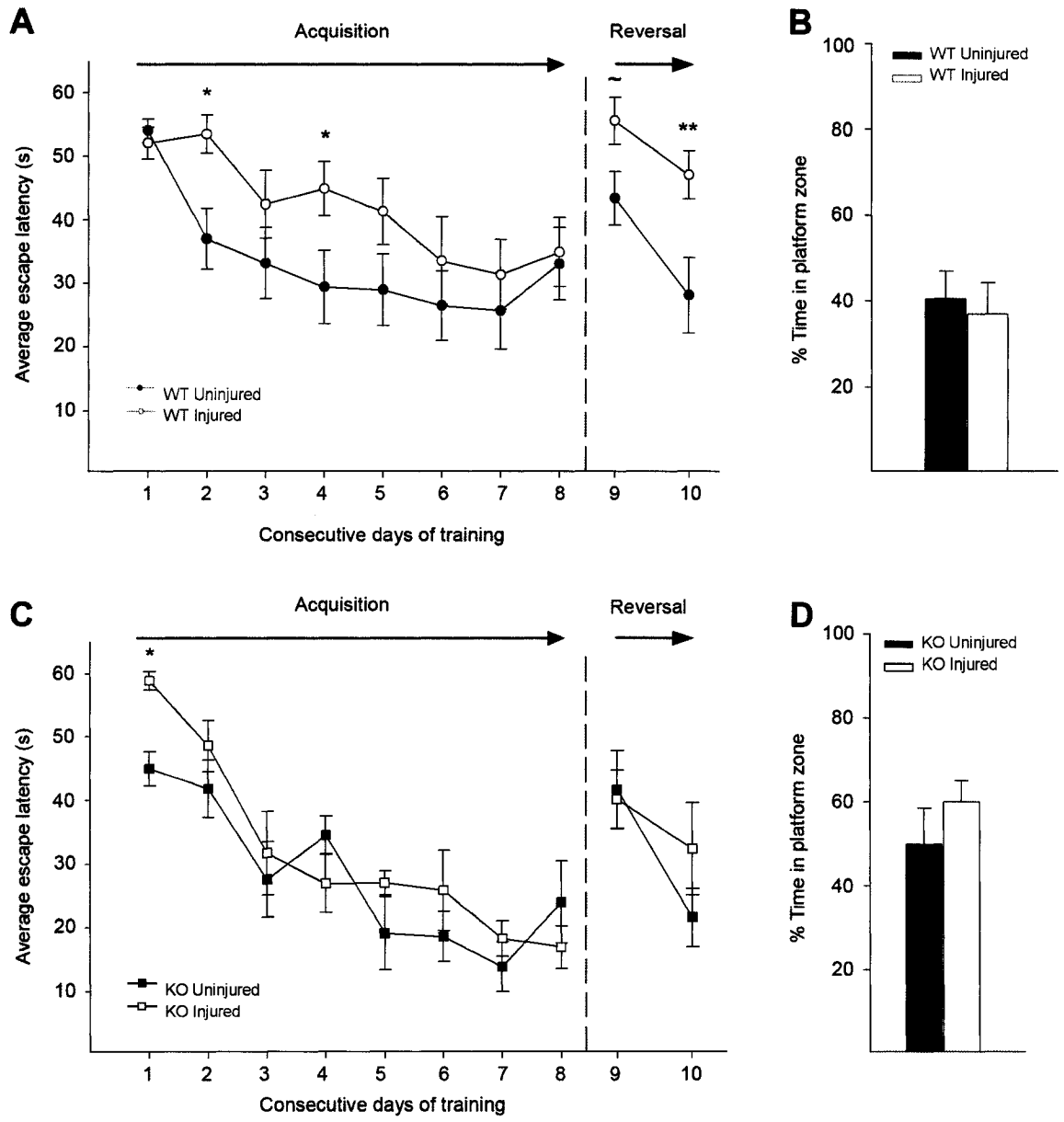
### **3.3.2 Hippocampal function is effectively restored in Cx32KO mice following seizure**

After validation of the Morris water maze paradigm to detect functional impairment of CA3a/b neurons, Cx32KO and WT mice were tested using the same behavioural paradigm to ascertain the functionality of the enhanced neuronal regeneration in the Cx32KO mice 4 weeks following excitotoxic injury, previously discussed in Chapter 2.

During the acquisition phase of the water maze, WT injured mice showed a significantly slower rate of learning comparatively to their uninjured controls, as indicated by significant “group within day” differences on the second and fourth days of the paradigm (Figure 3.5 – A; \*  $p < 0.05$ ), consistent with the data presented in Section 3.3.1. Conversely, injured Cx32KO mice were impaired on the first day of the paradigm but showed dramatic improvement with equivalent learning curves as uninjured mice (Figure 3.5 – C; \*  $p < 0.05$ ). All four groups had learned the spatial location the hidden platform within the water maze on the 8<sup>th</sup> day of trial, as indicated by comparable escape latencies on the 8<sup>th</sup> day of trial and comparable percentages of time spent in the western quadrant of the maze during the probe trial (Figure 3.5 – B, D).

During the reversal phase of the water maze, WT injured mice were significantly impaired at learning the new spatial location of the platform within the circular pool as compared to their uninjured controls (Figure 3.5 – A; ~  $p = 0.065$ ; \*\*  $P < 0.01$ ). Injured Cx32KO mice demonstrated spatial memory encoding performance comparable to their uninjured controls (Figure 3.5 – C).

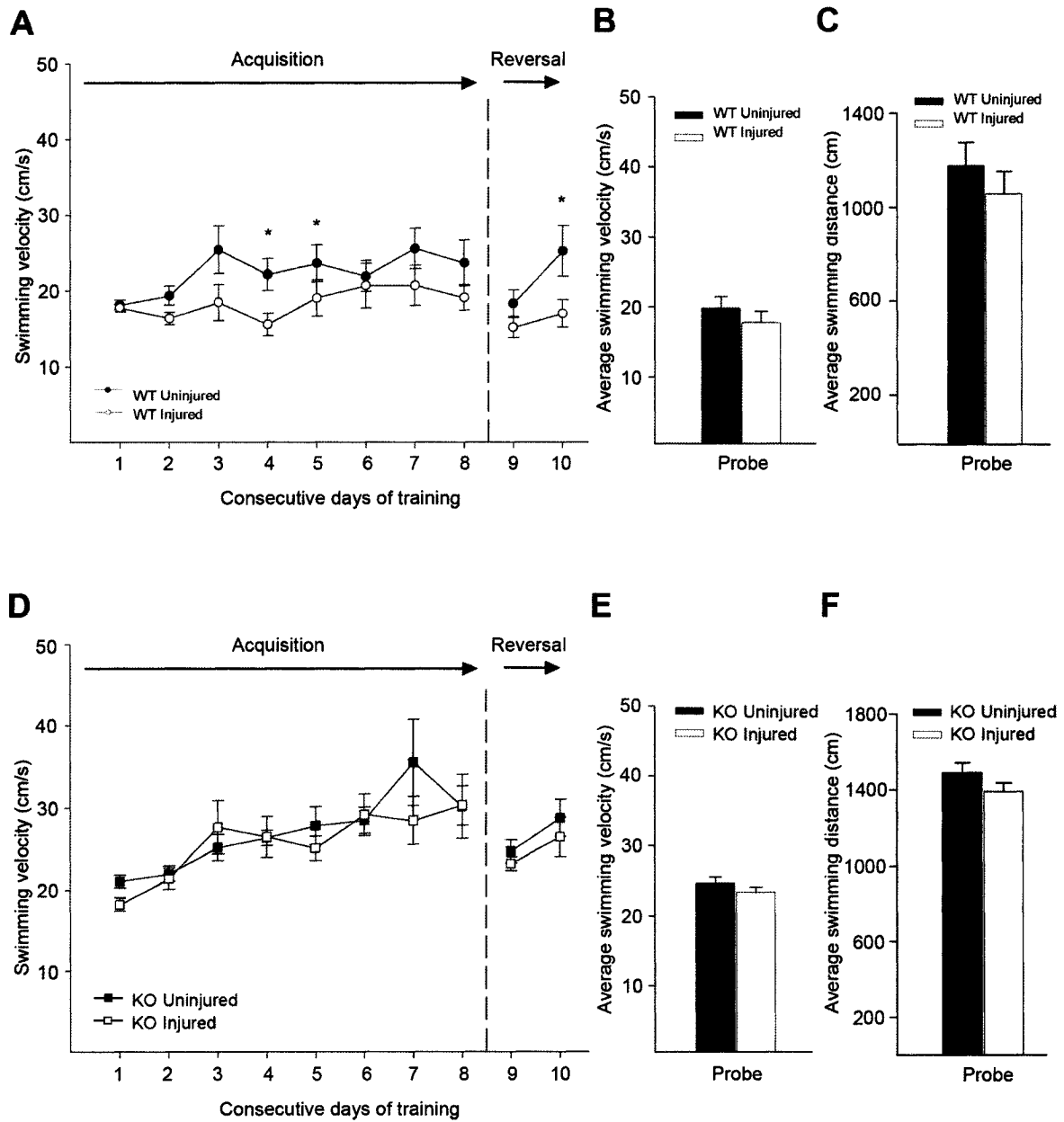
**Figure 3.5: Spatial memory performance in the Morris Water Maze following excitotoxic injury in WT and Cx32KO adult male mice.** The mean latency to find the platform spatial location of four trials per day is represented for 8 days of acquisition and 2 days of reversal for WT (A) and Cx32KO (C) mice. The mice were submitted to a probe trial on day 9, and the percentage time spent in the original platform zone was assessed for both WT (B) and Cx32KO (D) mice. The data were analyzed by two way repeated measures ANOVA with one factor repetition followed with a multiple comparisons versus control group (uninjured) using a Tukey *post hoc* test with  $\alpha$  values set at 0.05. Significant interactions of group within days are indicated (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; ~ $p = 0.065$ ).



**Figure 3.5**

As reported in section 3.3.1, motor or motivational deficits were not confounding factors during the study. The significant differences between WT injured and uninjured animals observed on the fourth and fifth day of acquisition, and on the second day of reversal (Figure 3.6 – A; \*  $p < 0.05$ ) were closely correlated with the variations in escape latencies reported above (Figure 3.5 – A). No significant differences in the swimming velocity of Cx32KO injured and uninjured mice were observed. Furthermore, the average swimming velocity (Figure 3.6; WT – B, Cx32KO – E) and the average swimming distance (Figure 3.6; WT – C, Cx32KO – F) during the probe trial were comparable within the WT and Cx32KO groups. These data suggest that neurons regenerated with greater efficiency in the CA3a/b region of the Cx32KO mouse hippocampus (Chapter 2) integrate and allow functional hippocampal encoding of learning and memory.

**Figure 3.6: Motoric performance in the Morris Water Maze following excitotoxic injury in WT and Cx32KO adult male mice.** The mean swimming velocity of four trials per day within the water maze is represented for 8 days of acquisition and 2 days of reversal for WT (A) and Cx32KO (D) mice. The mice were submitted to a probe trial on day 9, during which the average swimming velocity for the WT (B) and the Cx32KO (E) mice was assessed. The average swimming distance was also assessed for the WT (C) and the Cx32KO (F) mice. The data were analyzed by two way repeated measures ANOVA with one factor repetition followed with a multiple comparisons versus control group (uninjured) using a Tukey *post hoc* test with  $\alpha$  values set at 0.05. Significant interactions of group within days are indicated (\*  $p < 0.05$ ).



**Figure 3.6**

### 3.4 Discussion

The experiments presented in this chapter first aimed at determining whether KA-induced excitotoxic injury to the CA3a/b region of the C57Bl/6 male mouse hippocampus manifested as a behavioural impairment the Morris water maze, a spatial memory-dependent task. Given the relative resistance of adult C57Bl/6 mice to KA-induced seizure (Schauwecker, 2003), and their previously reported high performance in the Morris water maze (Ammassari-Teule and Caprioli, 1985; Van Dam et al., 2005), the difficulties associated with assessing pyramidal cell function are not trivial. The Morris water maze has previously been identified as an adequate behavioural task to identify CA3 synaptic activity (Florian and Roulet, 2004). However, these experiments consisted of CA3 stereotaxic injections of diethyldithiocarbamate, a chelator inhibiting synaptic transmission, in adult C57Bl/6 mice and behavioural assessment 1 week following surgery. In our experiments, the mice were injured with KA and tested in the water maze one month following administration of the excitotoxin; thus requiring further validation of the behavioural task. Following the validation of our Morris water maze paradigm to detect CA3a/b pyramidal cell damage, the behavioural task was used to determine whether the enhanced neuronal regeneration in the Cx32KO mouse following KA-induced seizure described in Chapter 2 translated into restoration of spatial learning and memory. Here, we show that neurons generated in absence of Cx32 are indeed functional.

Previous studies have shown that some of the intermediate neuronal progeny generated in the DG following seizure migrate from the SGZ to the

granule cell layer, hilus or molecular cell layer (Wang et al., 2004), where they can differentiate into granule neurons (van Praag et al., 2002; Bonde et al., 2006) or inhibitory interneurons (Liu et al., 2003b), and project their axons to integrate in the hippocampal neuronal circuitry (Zhao et al., 2006). While some have identified that these newly born neurons form aberrant connections within the hippocampus (Parent et al., 1997), their functionality remains unclear, (Bonde et al., 2006). Others have proposed that these newly born neurons do not normally integrate in the neuronal circuitry of the hippocampus and contribute to pathogenesis such as epilepsy (Parent and Lowenstein, 2002; Jessberger et al., 2005). Previous work indicates that the hippocampal dependent process of spatial memory encoding mainly involves the CA3 region of the hippocampus, while the retrieval process involves other intrahippocampal cells (Kunec et al., 2005). This finding provides substantial evidence indicating that hippocampal dependent spatial memory reflects CA3 functionality. Here, we report that newly formed neurons in the CA3 region of the hippocampus normally integrate in the hippocampal circuitry as demonstrated by behavioural functionality. We show that, in the absence of Cx32, behavioural performance is comparable to uninjured animals 0.5 week following seizure. These data provide strong evidence that the appropriate neuronal subtype is likely regenerated following seizure.

The improvement in spatial memory aptitude in the Morris water maze in Cx32KO mice following injury could have been the result of enhanced motoric performance by these mice. This hypothesis is counterintuitive given the role of

Cx32 mutation in CMTX, which is characterized by peripheral motor impairments. In humans, individuals affected by this hereditary peripheral neuropathy suffer from progressive demyelination, resulting in distal muscle weakness and amyotrophy, and decreased or absent tendon reflexes (Vance, 1991; Harding, 1995; Suter and Snipes, 1995; Ressot et al., 1998). Previous studies have identified motoric impairment following excitotoxic injury in rodents in the earlier stages of injury (Gerber and O'Shaughnessy, 1986; O'Shaughnessy and Gerber, 1986; Bennett et al., 1995). In the present study, the animals were aged for four weeks following the excitotoxic injury, thus allowing limited neuronal regeneration to occur as mentioned above and any excitotoxic impairment of peripheral motor performance to dissipate. The swimming velocity of the mice during the first day of the acquisition phase (Figure 3.4 – A), and during the probe trial (Figure 3.4 – B) of the water maze paradigm were comparable between the WT uninjured and WT injured experimental groups, thus suggesting no major motor deficit in the injured animals. Some differences in swimming velocities were observed between the injured and uninjured groups in both genotypes during the later days of the acquisition phase and during the reversal phase. These variations are not likely due to motor deficits, but rather to spatial memory performance, given that these discrepancies were mostly always associated with the differences in escape latencies discussed above. Intuitively, mice that have acquired the location of the platform will take less time to reach it, and thus result in a greater distance per time ratio. These data highlight the fact that Cx32 null-mutation does result in significant motoric impairment whether in uninjured or injured

tissue. Interestingly, these data provide further evidence that CMTX is not due to loss of Cx32 function but rather to altered channel function resulting from mutations, as previously proposed by previous groups (Menichella et al., 2003; Scherer et al., 2005).

This study also adds to the existing examination of behavioural impairments associated with Cx null-mutations. Previous studies have demonstrated that removal of some of these critical subunits in intercellular communication result in various behavioural alterations. For example, the Cx36 null mutant (Cx36 is expressed in neurons) exhibit memory impairment, thus implicating this particular protein in learning and memory processes (Frisch et al., 2005). Others have shown the implication of Cx43 in motor abilities, exploratory behaviour and (Frisch et al., 2003; Theis et al., 2003), and Cx30 in emotionality (Dere et al., 2003). In our study, it is worth mentioning that Cx32KO mice appear to show a slight improvement in spatial memory acquisition as compared to WT animals (Figure 3.5; comparing uninjured animals on graph A and C on the first day of acquisition). These differences are not likely to be the result of motoric performance variations, given their comparable swimming velocity on that day of acquisition (Figure 3.6). Despite this minor genotypic difference, the two uninjured groups showed comparable learning curves throughout the acquisition and reversal phase of the water maze, thus validating the task for studying spatial memory learning and retention in the study.

Taken together, the data presented in this chapter provide novel understanding of the implication of Cx32 in hippocampal-related behaviour. We have shown that long term excitotoxic injury to the CA3 pyramidal cell field of the adult C57Bl/6 mouse hippocampal complex significantly impairs spatial memory encoding, and can thus be detected by assessment of spatial memory performance with a variant of the Morris water maze paradigm. Most interestingly, the data demonstrate that reducing Cx32-mediated communication during progenitor activation following excitotoxic injury to the adult hippocampus induces CA3a/b neuronal replacement that translates into functional behavioural indices of learning and memory.

## Chapter 4: Thesis discussion and conclusions

The studies presented in this thesis intended to evaluate the *in vivo* neurogenic potential of NG2<sup>+</sup> progenitors following excitotoxic hippocampal injury in absence of Cx32, given the previously reported implication of Cx32 in NPC cell specification (Melanson-Drapeau et al., 2003). Using Cx32 null mutant and excitotoxicity mouse models, we demonstrated that hippocampal neuronal degeneration occurs mainly in CA3a/b region 0.5 week after KA administration, regardless of genotype. Following this extensive neuronal loss, we showed that NPCs are locally activated and progress to immature TOAD64<sup>+</sup> and TUJ1<sup>+</sup> neuronal progenitors 2 weeks after the injury, and finally to mature NeuN<sup>+</sup> neurons at a greater extent in absence of Cx32 4.5 weeks after the injury. My colleagues and I identified these progenitors as NG2<sup>+</sup> cells activated in the CA3a/b following injury. I demonstrated that these newly born neurons in the Cx32KO mouse exhibit behavioural functionality with the use of the Morris water maze, after confirming CA3 specificity and adequate injury sensitivity for this behavioural task one month following injury in adult C57Bl/6 male mice. Our data implicate Cx32 as a switch regulator inhibiting neurogenesis in NPCs residing in the SGZ.

As discussed in Chapter 2, the level of Cx32 protein transiently increased in response to KA. Previous work in the Bennett laboratory have shown that overexpression of Cx32 in both primary progenitor cells cultured from postnatal hippocampus and teratocarcinoma cells, inhibits neurogenesis (Boucher and

Bennett, 2003). Together, these data lead me to speculate that Cx32 expression in adult NPCs promotes oligodendrocytic specification and inhibits neurogenesis. This line of evidence provides important understanding of the role of Cxs in progenitor cell fate in adulthood, a process previously implicated during development in the embryonic brain (Rozental et al., 1998; Rozental et al., 2000; Cheng et al., 2004).

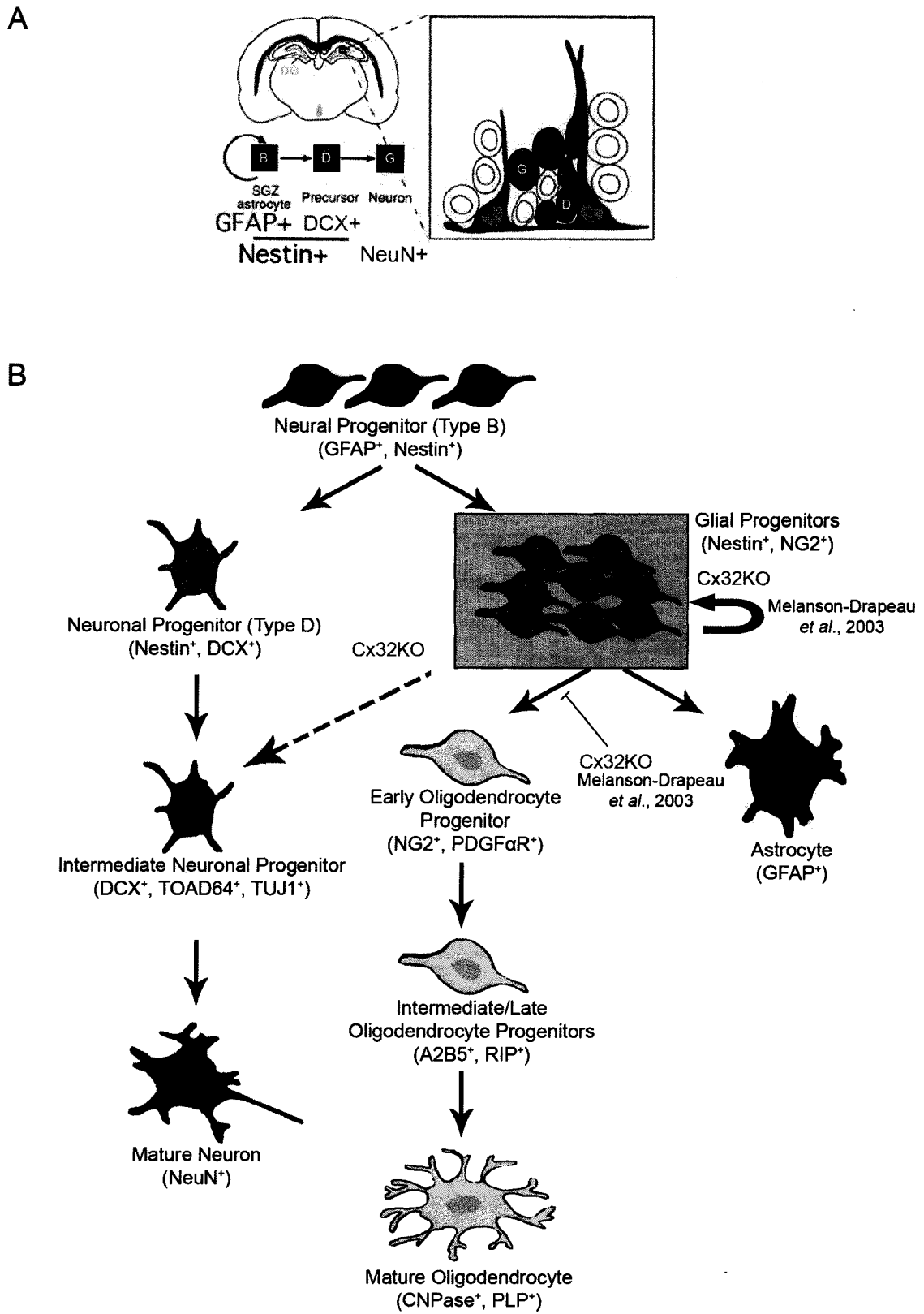
Given the critical implication of Cx32 in progenitor cell specification and the poor detection methods currently available to assess the protein levels of Cx32, a transgenic mouse model, in which the EGFP marker protein replaced the Cx32 coding region at the start codon, was engineered (Appendix A) to assess accurately the levels of Cx32 during these crucial cellular regulation events. This new marker mouse will not only allow us to accurately determine when and where Cx32 is expressed during neural precursor fate determination, but will also greatly contribute to other research in the Cx field.

In addition, we also approached the effect of excitotoxic insult on the survival of oligodendrocytes in the hippocampus. Previous studies have implicated oligodendrocytic death in neurodegenerative conditions such multiple sclerosis (Miller, 2005; Frohman et al., 2006), AD (de Leeuw et al., 2004; Miller, 2005; Sjobeck et al., 2005a, b) and epilepsy (Dong and Greenough, 2004; Meier et al., 2004). Excitotoxicity and oligodendrocyte degeneration have been implicated in the pathology of these diseases (Bu et al., 2001; Miller et al., 2005; Salter and Fern, 2005) and the role of oligodendrocytes in providing trophic support to neurons have been recently reported (Du and Dreyfus, 2002; Wilkins

et al., 2003; Williams et al., 2005). The studies presented Chapter 2 demonstrate that following KA-induced excitotoxicity, oligodendrocytes might undergo metabolic changes, but these changes do not translate into oligodendrocyte death in the adult C57Bl/6 mouse hippocampus, supporting previous studies (Wosik et al., 2004). However, in absence of Cx32 no changes in metabolic activity or death of oligodendrocytes were observed, suggesting a potential role for Cx32 mediated GJIC in the synchrony of the non-myelinating oligodendrocyte population residing in the hippocampus. Given the previous implication of oligodendrocytic death in the pathology of neurodegenerative diseases, metabolic coupling via Cx32 mediated GJIC between oligodendrocytes could potentially play a role in their degeneration and/or death.

The work described in this thesis suggests that NG2<sup>+</sup> progenitor cells residing in the SGZ of the adult hippocampus exhibit multipotential capacities *in vivo*, (i.e. the ability to specify to a neuronal lineage *in vivo*; Figure 4.1). These findings are preceded by *in vitro* studies (Belachew et al., 2003; Aguirre and Gallo, 2004; Aguirre et al., 2004; Dayer et al., 2005), and are of great importance for therapeutic cell replacement research in the adult brain. Ultimately, reducing Cx32-mediated communication during progenitor activation following neuronal injury in the adult hippocampus “activates” the multipotentiality the endogenous pool of NG2<sup>+</sup> progenitors in the hippocampus, increases the efficiency of neuronal replacement in the damaged hippocampal fields, and translates into hippocampal behavioural functionality. Our studies represent the first report suggesting that this multipotency in the resident NG2<sup>+</sup> progenitors in the adult

**Figure 4.1: Progenitor cell specification in absence of Cx32 in the SGZ of the adult hippocampus.** Section (A) was modified from Doetsch *et al.*, 2003. (A) Coronal view of the hippocampus, identifying cell types and neuronal differentiation in the SGZ. Nestin<sup>+</sup>/GFAP<sup>+</sup> progenitor cells give rise to intermediate neuronal precursors (Nestin<sup>+</sup>/DCX<sup>+</sup>) which in turn generate new neurons (NeuN<sup>+</sup>). (B) Specification of multipotential progenitors in the SGZ. Type B progenitors cells (GFAP<sup>+</sup> and Nestin<sup>+</sup>) divide asymmetrically to produce type D (DCX<sup>+</sup>) neuronal progenitors and then neurons, or NG2<sup>+</sup> glial progenitors that specify to oligodendrocytes or astrocytes in presence of Cx32 or retain their multipotentiality in absence of it.



**Figure 4.1**

hippocampus is regulated by Cx32. Most importantly, we introduce a potential avenue for further therapeutic exploration to treat hippocampal neurodegeneration seen in many neurodegenerative conditions such as epilepsy (Kelsey et al., 2000; Scharfman et al., 2000) and AD (Mikkonen et al., 2001). Therapeutic applications for this multipotency activation of NG2<sup>+</sup> hippocampal progenitors using Cx32 manipulation will require an efficient knockdown and delivery strategies. One possible method to reduce the expression of Cx32 *in vivo* in order to increase neuronal specification is using RNA silencing strategies *in vivo* (Xia et al., 2002). Ongoing research in the Bennett laboratory is investigating a potential reoviral vector for gene delivery to NPCs in the adult brain. Before extending these findings to therapeutic applications, however, a number of questions remain to be answered. A conditional null-mutant mouse for Cx32 in which the expression of Cx32 could be removed specifically in progenitor cells would provide substantial insight into the mechanism of Cx32-mediated progenitor specification, given that the protein is mainly expressed in resident oligodendrocytes, which could in turn aid in neuronal regeneration.

Finally, we validated a variant of the Morris water maze as an efficient tool to investigate the functionality of neuronal regeneration in the adult hippocampus. Previous groups had used the water maze to investigate CA3 functionality, but these studies were performed at the time of maximal injury following stereotaxic injections into the hippocampus. (Florian and Rouillet, 2004). Our studies, however, assessed functional hippocampal behaviour with the Morris water maze one month following the injury, and demonstrated that the task exhibits enough

sensitivity for this purpose and high specificity to CA3 directed hippocampal injury. These findings will provide an adequate tool to study the long term functionality of neuronal regeneration.

In conclusion, the work presented in this M.Sc. thesis contributes to our current knowledge of adult hippocampal progenitor specification and directly implicates Cx32 as a key regulatory protein inhibiting neurogenesis. As discussed in Chapter 2, the mouse model of excitotoxic injury used in this study reflects the pathology of various neurodegenerative diseases. Our demonstration of improved neuronal regeneration and functionality by altering Cx expression represents an important avenue for progenitor and stem cell therapy. Novel insight in our understanding of how Cx32-mediated communication affects neurogenesis following brain injury was put forward. *In situ* manipulation of Cx expression in the adult brain represent a potential mean of controlling progenitor fate in the adult brain and improve the efficiency and safety of clinical therapy in the future.

## REFERENCES

- Aguirre A, Gallo V (2004) Postnatal neurogenesis and gliogenesis in the olfactory bulb from NG2-expressing progenitors of the subventricular zone. *J Neurosci* 24:10530-10541.
- Aguirre AA, Chittajallu R, Belachew S, Gallo V (2004) NG2-expressing cells in the subventricular zone are type C-like cells and contribute to interneuron generation in the postnatal hippocampus. *J Cell Biol* 165:575-589.
- Al-Hazzaa AA, Bowen ID (1998) Improved cytochemical methods for demonstrating cell death using LR White as an embedding medium. *Histochem J* 30:897-902.
- Alzheimer A, Stelzmann RA, Schnitzlein HN, Murtagh FR (1995) An English translation of Alzheimer's 1907 paper, "Über eine eigenartige Erkrankung der Hirnrinde". *Clin Anat* 8:429-431.
- Ammassari-Teule M, Caprioli A (1985) Spatial learning and memory, maze running strategies and cholinergic mechanisms in two inbred strains of mice. *Behav Brain Res* 17:9-16.
- Andrade-Rozental AF, Rozental R, Hopperstad MG, Wu JK, Vrionis FD, Spray DC (2000) Gap junctions: the "kiss of death" and the "kiss of life". *Brain Res Brain Res Rev* 32:308-315.
- Arlotta P, Magavi SS, Macklis JD (2003) Induction of adult neurogenesis: molecular manipulation of neural precursors in situ. *Ann N Y Acad Sci* 991:229-236.
- Arvidsson A, Kokaia Z, Lindvall O (2001) N-methyl-D-aspartate receptor-mediated increase of neurogenesis in adult rat dentate gyrus following stroke. *Eur J Neurosci* 14:10-18.
- Bai S, Spray DC, Burk RD (1993) Identification of proximal and distal regulatory elements of the rat connexin32 gene. *Biochim Biophys Acta* 1216:197-204.
- Bani-Yaghoub M, Bechberger JF, Naus CC (1997) Reduction of connexin43 expression and dye-coupling during neuronal differentiation of human NTera2/clone D1 cells. *J Neurosci Res* 49:19-31.
- Bani-Yaghoub M, Felker JM, Sans C, Naus CC (2000) The effects of bone morphogenetic protein 2 and 4 (BMP2 and BMP4) on gap junctions during neurodevelopment. *Exp Neurol* 162:13-26.
- Becker AJ, Gillardon F, Blumcke I, Langendorfer D, Beck H, Wiestler OD (1999) Differential regulation of apoptosis-related genes in resistant and vulnerable subfields of the rat epileptic hippocampus. *Brain Res Mol Brain Res* 67:172-176.
- Bedi KS (1992) Spatial learning ability of rats undernourished during early postnatal life. *Physiol Behav* 51:1001-1007.
- Belachew S, Chittajallu R, Aguirre AA, Yuan X, Kirby M, Anderson S, Gallo V (2003) Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. *J Cell Biol* 161:169-186.

- Belliveau DJ, Naus CC (1994) Cortical type 2 astrocytes are not dye coupled nor do they express the major gap junction genes found in the central nervous system. *Glia* 12:24-34.
- Belliveau DJ, Kidder GM, Naus CC (1991) Expression of gap junction genes during postnatal neural development. *Dev Genet* 12:308-317.
- Bellush LL, Wright AM, Walker JP, Kopchick J, Colvin RA (1996) Caloric restriction and spatial learning in old mice. *Physiol Behav* 60:541-547.
- Bengzon J, Kokaia Z, Elmer E, Nanobashvili A, Kokaia M, Lindvall O (1997) Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. *Proc Natl Acad Sci U S A* 94:10432-10437.
- Bennett MV, Contreras JE, Bukauskas FF, Saez JC (2003) New roles for astrocytes: gap junction hemichannels have something to communicate. *Trends Neurosci* 26:610-617.
- Bennett SA, Stevenson B, Staines WA, Roberts DC (1995) Periodic acid-Schiff (PAS)-positive deposits in brain following kainic acid-induced seizures: relationships to fos induction, neuronal necrosis, reactive gliosis, and blood-brain barrier breakdown. *Acta Neuropathol (Berl)* 89:126-138.
- Bennett SA, Tenniswood M, Chen JH, Davidson CM, Keyes MT, Fortin T, Pappas BA (1998) Chronic cerebral hypoperfusion elicits neuronal apoptosis and behavioral impairment. *Neuroreport* 9:161-166.
- Berger-Sweeney J, Arnold A, Gabeau D, Mills J (1995) Sex differences in learning and memory in mice: effects of sequence of testing and cholinergic blockade. *Behav Neurosci* 109:859-873.
- Bergoffen J, Scherer SS, Wang S, Scott MO, Bone LJ, Paul DL, Chen K, Lensch MW, Chance PF, Fischbeck KH (1993) Connexin mutations in X-linked Charcot-Marie-Tooth disease. *Science* 262:2039-2042.
- Bittman KS, LoTurco JJ (1999) Differential regulation of connexin 26 and 43 in murine neocortical precursors. *Cereb Cortex* 9:188-195.
- Bonde S, Ekdahl CT, Lindvall O (2006) Long-term neuronal replacement in adult rat hippocampus after status epilepticus despite chronic inflammation. *Eur J Neurosci* 23:965-974.
- Bondurand N, Girard M, Pingault V, Lemort N, Dubourg O, Goossens M (2001) Human Connexin 32, a gap junction protein altered in the X-linked form of Charcot-Marie-Tooth disease, is directly regulated by the transcription factor SOX10. *Hum Mol Genet* 10:2783-2795.
- Boucher S, Bennett SA (2003) Differential connexin expression, gap junction intercellular coupling, and hemichannel formation in NT2/D1 human neural progenitors and terminally differentiated hNT neurons. *J Neurosci Res* 72:393-404.
- Bruzzone R, White TW, Paul DL (1996) Connections with connexins: the molecular basis of direct intercellular signaling. *Eur J Biochem* 238:1-27.
- Bu J, Akhtar N, Nishiyama A (2001) Transient expression of the NG2 proteoglycan by a subpopulation of activated macrophages in an excitotoxic hippocampal lesion. *Glia* 34:296-310.
- Bucci DJ, Chiba AA, Gallagher M (1995) Spatial learning in male and female Long-Evans rats. *Behav Neurosci* 109:180-183.

- Campbell S, Hablitz JJ (2005) Modification of epileptiform discharges in neocortical neurons following glutamate uptake inhibition. *Epilepsia* 46 Suppl 5:129-133.
- Castro C, Gomez-Hernandez JM, Silander K, Barrio LC (1999) Altered Formation of Hemichannels and Gap Junction Channels Caused by C-Terminal Connexin-32 Mutations. *J Neurosci* 19:3752-3760.
- Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. *Science* 263:802-805.
- Chapillon P, Roulet P (1996) Use of proximal and distal cues in place navigation by mice changes during ontogeny. *Dev Psychobiol* 29:529-545.
- Cheng A, Tang H, Cai J, Zhu M, Zhang X, Rao M, Mattson MP (2004) Gap junctional communication is required to maintain mouse cortical neural progenitor cells in a proliferative state. *Dev Biol* 272:203-216.
- Cherian PP, Cheng B, Gu S, Sprague E, Bonewald LF, Jiang JX (2003) Effects of mechanical strain on the function of Gap junctions in osteocytes are mediated through the prostaglandin EP2 receptor. *J Biol Chem* 278:43146-43156.
- Chiba S, Iwasaki Y, Sekino H, Suzuki N (2003) Transplantation of motoneuron-enriched neural cells derived from mouse embryonic stem cells improves motor function of hemiplegic mice. *Cell Transplant* 12:457-468.
- Chirumamilla S, Sun D, Bullock MR, Colello RJ (2002) Traumatic brain injury induced cell proliferation in the adult mammalian central nervous system. *J Neurotrauma* 19:693-703.
- Condorelli DF, Trovato-Salinaro A, Mudo G, Mirone MB, Belluardo N (2003) Cellular expression of connexins in the rat brain: neuronal localization, effects of kainate-induced seizures and expression in apoptotic neuronal cells. *Eur J Neurosci* 18:1807-1827.
- Corkin S (2002) What's new with the amnesic patient H.M.? *Nat Rev Neurosci* 3:153-160.
- Covolan L, Ribeiro LT, Longo BM, Mello LE (2000) Cell damage and neurogenesis in the dentate granule cell layer of adult rats after pilocarpine- or kainate-induced status epilepticus. *Hippocampus* 10:169-180.
- Dalby NO, Mody I (2001) The process of epileptogenesis: a pathophysiological approach. *Curr Opin Neurol* 14:187-192.
- Dash PK, Mach SA, Moore AN (2001) Enhanced neurogenesis in the rodent hippocampus following traumatic brain injury. *J Neurosci Res* 63:313-319.
- Dawson MR, Levine JM, Reynolds R (2000) NG2-expressing cells in the central nervous system: are they oligodendroglial progenitors? *J Neurosci Res* 61:471-479.
- Dayer AG, Cleaver KM, Abouantoun T, Cameron HA (2005) New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. *J Cell Biol* 168:415-427.
- de Leeuw FE, Barkhof F, Scheltens P (2004) White matter lesions and hippocampal atrophy in Alzheimer's disease. *Neurology* 62:310-312.

- De Pina-Benabou MH, Srinivas M, Spray DC, Scemes E (2001) Calmodulin kinase pathway mediates the K<sup>+</sup>-induced increase in Gap junctional communication between mouse spinal cord astrocytes. *J Neurosci* 21:6635-6643.
- de Quervain DJ, Roozendaal B, McGaugh JL (1998) Stress and glucocorticoids impair retrieval of long-term spatial memory. *Nature* 394:787-790.
- Deans MR, Gibson JR, Sellitto C, Connors BW, Paul DL (2001) Synchronous activity of inhibitory networks in neocortex requires electrical synapses containing connexin36. *Neuron* 31:477-485.
- Dere E, De Souza-Silva MA, Frisch C, Teubner B, Sohl G, Willecke K, Huston JP (2003) Connexin30-deficient mice show increased emotionality and decreased rearing activity in the open-field along with neurochemical changes. *Eur J Neurosci* 18:629-638.
- Dermietzel R, Farooq M, Kessler JA, Althaus H, Hertzberg EL, Spray DC (1997) Oligodendrocytes express gap junction proteins connexin32 and connexin45. *Glia* 20:101-114.
- Dermietzel R, Traub O, Hwang TK, Beyer E, Bennett MV, Spray DC, Willecke K (1989) Differential expression of three gap junction proteins in developing and mature brain tissues. *Proc Natl Acad Sci U S A* 86:10148-10152.
- D'Hooge R, De Deyn PP (2001) Applications of the Morris water maze in the study of learning and memory. *Brain Res Brain Res Rev* 36:60-90.
- Doetsch F (2003) The glial identity of neural stem cells. *Nat Neurosci* 6:1127-1134.
- Dong H, Csernansky CA, Goico B, Csernansky JG (2003) Hippocampal neurogenesis follows kainic acid-induced apoptosis in neonatal rats. *J Neurosci* 23:1742-1749.
- Dong WK, Greenough WT (2004) Plasticity of nonneuronal brain tissue: roles in developmental disorders. *Ment Retard Dev Disabil Res Rev* 10:85-90.
- Drago J, Murphy M, Carroll SM, Harvey RP, Bartlett PF (1991) Fibroblast growth factor-mediated proliferation of central nervous system precursors depends on endogenous production of insulin-like growth factor I. *Proc Natl Acad Sci U S A* 88:2199-2203.
- Du Y, Dreyfus CF (2002) Oligodendrocytes as providers of growth factors. *J Neurosci Res* 68:647-654.
- Duval N, Gomes D, Calaora V, Calabrese A, Meda P, Bruzzone R (2002) Cell coupling and Cx43 expression in embryonic mouse neural progenitor cells. *J Cell Sci* 115:3241-3251.
- Evans WH, Martin PE (2002) Gap junctions: structure and function (Review). *Mol Membr Biol* 19:121-136.
- Field JM, Tate LA, Chipman JK, Minchin SD (2003) Identification of functional regulatory regions of the connexin32 gene promoter. *Biochim Biophys Acta* 1628:22-29.
- Filippov MA, Hormuzdi SG, Fuchs EC, Monyer H (2003) A reporter allele for investigating connexin 26 gene expression in the mouse brain. *Eur J Neurosci* 18:3183-3192.

- Finkbeiner S, Greenberg ME (1996) Ca(2+)-dependent routes to Ras: mechanisms for neuronal survival, differentiation, and plasticity? *Neuron* 16:233-236.
- Florian C, Roulet P (2004) Hippocampal CA3-region is crucial for acquisition and memory consolidation in Morris water maze task in mice. *Behav Brain Res* 154:365-374.
- Forman MS, Trojanowski JQ, Lee VM (2004) Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs. *Nat Med* 10:1055-1063.
- Forwood SE, Winters BD, Bussey TJ (2005) Hippocampal lesions that abolish spatial maze performance spare object recognition memory at delays of up to 48 hours. *Hippocampus* 15:347-355.
- Frisch C, De Souza-Silva MA, Sohl G, Guldenagel M, Willecke K, Huston JP, Dere E (2005) Stimulus complexity dependent memory impairment and changes in motor performance after deletion of the neuronal gap junction protein connexin36 in mice. *Behav Brain Res* 157:177-185.
- Frisch C, Theis M, De Souza Silva MA, Dere E, Sohl G, Teubner B, Namestkova K, Willecke K, Huston JP (2003) Mice with astrocyte-directed inactivation of connexin43 exhibit increased exploratory behaviour, impaired motor capacities, and changes in brain acetylcholine levels. *Eur J Neurosci* 18:2313-2318.
- Frohman EM, Racke MK, Raine CS (2006) Multiple sclerosis--the plaque and its pathogenesis. *N Engl J Med* 354:942-955.
- Fulton BP (1995) Gap junctions in the developing nervous system. *Perspect Dev Neurobiol* 2:327-334.
- Furshpan EJ, Potter DD (1959) Transmission at the giant motor synapses of the crayfish. *J Physiol* 145:289-325.
- Gage FH (2000) Mammalian neural stem cells. *Science* 287:1433-1438.
- Gage FH, Kempermann G, Palmer TD, Peterson DA, Ray J (1998) Multipotent progenitor cells in the adult dentate gyrus. *J Neurobiol* 36:249-266.
- Gerber GJ, O'Shaughnessy D (1986) Comparison of the behavioral effects of neurotoxic and systemically toxic agents: how discriminatory are behavioral tests of neurotoxicity? *Neurobehav Toxicol Teratol* 8:703-710.
- Ghosh A, Greenberg ME (1995) Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. *Neuron* 15:89-103.
- Gilbert TH, Hannesson DK, Corcoran ME (2000) Hippocampal kindled seizures impair spatial cognition in the Morris water maze. *Epilepsy Res* 38:115-125.
- Goodenough DA, Paul DL (2003) Beyond the gap: functions of unpaired connexon channels. *Nat Rev Mol Cell Biol* 4:285-294.
- Goodenough DA, Goliger JA, Paul DL (1996) Connexins, connexons, and intercellular communication. *Annu Rev Biochem* 65:475-502.
- Guthrie SC, Gilula NB (1989) Gap junctional communication and development. *Trends Neurosci* 12:12-16.

- Hadjantonakis AK, Gertsenstein M, Ikawa M, Okabe M, Nagy A (1998) Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech Dev* 76:79-90.
- Harding AE (1995) From the syndrome of Charcot, Marie and Tooth to disorders of peripheral myelin proteins. *Brain* 118 ( Pt 3):809-818.
- Hartley M, Taylor N, Taylor J (2005) Subfield variations in hippocampal processing-components of a spatial navigation system. *Neural Netw* 18:611-619.
- Holscher C (1999) Stress impairs performance in spatial water maze learning tasks. *Behav Brain Res* 100:225-235.
- Horner PJ, Power AE, Kempermann G, Kuhn HG, Palmer TD, Winkler J, Thal LJ, Gage FH (2000) Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. *J Neurosci* 20:2218-2228.
- Houlden H, Girard M, Cockerell C, Ingram D, Wood NW, Goossens M, Walker RW, Reilly MM (2004) Connexin 32 promoter P2 mutations: a mechanism of peripheral nerve dysfunction. *Ann Neurol* 56:730-734.
- Jessberger S, Romer B, Babu H, Kempermann G (2005) Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. *Exp Neurol* 196:342-351.
- Jin K, Minami M, Lan JQ, Mao XO, Batteur S, Simon RP, Greenberg DA (2001) Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. *Proc Natl Acad Sci U S A* 98:4710-4715.
- Johansson CB (2003) Mechanism of stem cells in the central nervous system. *J Cell Physiol* 196:409-418.
- Johnston D, Williams S, Jaffe D, Gray R (1992) NMDA-receptor-independent long-term potentiation. *Annu Rev Physiol* 54:489-505.
- Kalvelyte A, Imbrasaite A, Bukauskiene A, Verselis VK, Bukauskas FF (2003) Connexins and apoptotic transformation. *Biochem Pharmacol* 66:1661-1672.
- Kamasawa N, Sik A, Morita M, Yasumura T, Davidson KG, Nagy JI, Rash JE (2005) Connexin-47 and connexin-32 in gap junctions of oligodendrocyte somata, myelin sheaths, paranodal loops and Schmidt-Lanterman incisures: implications for ionic homeostasis and potassium siphoning. *Neuroscience* 136:65-86.
- Kamermans M, Fahrenfort I, Schultz K, Janssen-Bienhold U, Sjoerdsma T, Weiler R (2001) Hemichannel-Mediated Inhibition in the Outer Retina. *Science* 292:1178-1180.
- Kandler K, Katz LC (1998) Coordination of neuronal activity in developing visual cortex by gap junction-mediated biochemical communication. *J Neurosci* 18:1419-1427.
- Kelsey JE, Sanderson KL, Frye CA (2000) Perforant path stimulation in rats produces seizures, loss of hippocampal neurons, and a deficit in spatial mapping which are reduced by prior MK-801. *Behav Brain Res* 107:59-69.

- Kempermann G, Kuhn HG, Gage FH (1997) Genetic influence on neurogenesis in the dentate gyrus of adult mice. *Proc Natl Acad Sci USA* 94:10409-10414.
- Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. *Trends Neurosci* 27:447-452.
- Kesslak JP, Yuan D, Neeper S, Cotman CW (1995) Vulnerability of the hippocampus to kainate excitotoxicity in the aged, mature and young adult rat. *Neurosci Lett* 188:117-120.
- Koffler LD, Fernstrom MJ, Akiyama TE, Gonzalez FJ, Ruch RJ (2002) Positive regulation of connexin32 transcription by hepatocyte nuclear factor-1alpha. *Arch Biochem Biophys* 407:160-167.
- Kofuji P, Newman EA (2004) Potassium buffering in the central nervous system. *Neuroscience* 129:1045-1056.
- Kondo T, Raff M (2000a) Oligodendrocyte precursor cells reprogrammed to become multipotential stem cells. *Science* 289:1754-1757.
- Kondo T, Raff M (2000b) Oligodendrocyte Precursor Cells Reprogrammed to Become Multipotential CNS Stem Cells. *Science* 289:1754-1757.
- Kondziolka D, Wechsler L, Goldstein S, Meltzer C, Thulborn KR, Gebel J, Jannetta P, DeCesare S, Elder EM, McGrogan M, Reitman MA, Bynum L (2000) Transplantation of cultured human neuronal cells for patients with stroke. *Neurology* 55:565-569.
- Kornack DR, Rakic P (2001) Cell proliferation without neurogenesis in adult primate neocortex. *Science* 294:2127-2130.
- Kruger O, Plum A, Kim JS, Winterhager E, Maxeiner S, Hallas G, Kirchhoff S, Traub O, Lamers WH, Willecke K (2000) Defective vascular development in connexin 45-deficient mice. *Development* 127:4179-4193.
- Kunec S, Hasselmo ME, Kopell N (2005) Encoding and retrieval in the CA3 region of the hippocampus: a model of theta-phase separation. *J Neurophysiol* 94:70-82.
- Kunzelmann P, Blumcke I, Traub O, Dermietzel R, Willecke K (1997) Coexpression of connexin45 and -32 in oligodendrocytes of rat brain. *J Neurocytol* 26:17-22.
- Lee I, Yoganarasimha D, Rao G, Knierim JJ (2004) Comparison of population coherence of place cells in hippocampal subfields CA1 and CA3. *Nature* 430:456-459.
- Leutgeb S, Leutgeb JK, Moser MB, Moser EI (2005) Place cells, spatial maps and the population code for memory. *Curr Opin Neurobiol*.
- Li X, Wang W, Lufkin T (1997) Dicistronic LacZ and alkaline phosphatase reporter constructs permit simultaneous histological analysis of expression from multiple transgenes. *Biotechniques* 23:874-878, 880, 882.
- Lie DC, Dziewczapolski G, Willhoite AR, Kaspar BK, Shults CW, Gage FH (2002) The adult substantia nigra contains progenitor cells with neurogenic potential. *J Neurosci* 22:6639-6649.
- Limke TL, Rao MS (2003) Neural stem cell therapy in the aging brain: pitfalls and possibilities. *J Hematother Stem Cell Res* 12:615-623.

- Lin JH, Weigel H, Cotrina ML, Liu S, Bueno E, Hansen AJ, Hansen TW, Goldman S, Nedergaard M (1998) Gap-junction-mediated propagation and amplification of cell injury. *Nat Neurosci* 1:494-500.
- Lindvall O, Kokaia Z, Martinez-Serrano A (2004) Stem cell therapy for human neurodegenerative disorders-how to make it work. *Nat Med* 10 Suppl:S42-50.
- Liu H, Kaur J, Dashtipour K, Kinyamu R, Ribak CE, Friedman LK (2003a) Suppression of hippocampal neurogenesis is associated with developmental stage, number of perinatal seizure episodes, and glucocorticosteroid level. *Exp Neurol* 184:196-213.
- Liu S, Wang J, Zhu D, Fu Y, Lukowiak K, Lu YM (2003b) Generation of functional inhibitory neurons in the adult rat hippocampus. *J Neurosci* 23:732-736.
- Long MA, Deans MR, Paul DL, Connors BW (2002) Rhythmicity without synchrony in the electrically uncoupled inferior olive. *J Neurosci* 22:10898-10905.
- LoTurco JJ, Kriegstein AR (1991) Clusters of coupled neuroblasts in embryonic neocortex. *Science* 252:563-566.
- LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR (1995) GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron* 15:1287-1298.
- Magavi SS, Leavitt BR, Macklis JD (2000) Induction of neurogenesis in the neocortex of adult mice. *Nature* 405:951-955.
- Malva JO, Carvalho AP, Carvalho CM (1998) Kainate receptors in hippocampal CA3 subregion: evidence for a role in regulating neurotransmitter release. *Neurochem Int* 32:1-6.
- Mattson MP (1997) Advances fuel Alzheimer's conundrum. *Nat Genet* 17:254-256.
- McBride JL, Behrstock SP, Chen EY, Jakel RJ, Siegel I, Svendsen CN, Kordower JH (2004) Human neural stem cell transplants improve motor function in a rat model of Huntington's disease. *J Comp Neurol* 475:211-219.
- McDevitt MA, Shivdasani RA, Fujiwara Y, Yang H, Orkin SH (1997) A "knockdown" mutation created by cis-element gene targeting reveals the dependence of erythroid cell maturation on the level of transcription factor GATA-1. *Proc Natl Acad Sci U S A* 94:6781-6785.
- McIntyre DC, Nathanson D, Edson N (1982) A new model of partial status epilepticus based on kindling. *Brain Res* 250:53-63.
- Meier S, Brauer AU, Heimrich B, Nitsch R, Savaskan NE (2004) Myelination in the hippocampus during development and following lesion. *Cell Mol Life Sci* 61:1082-1094.
- Melanson-Drapeau L, Beyko S, Dave S, Hebb AL, Franks DJ, Sellitto C, Paul DL, Bennett SA (2003) Oligodendrocyte progenitor enrichment in the connexin32 null-mutant mouse. *J Neurosci* 23:1759-1768.
- Meltzer CC, Kondziolka D, Villemagne VL, Wechsler L, Goldstein S, Thurlborn KR, Gebel J, Elder EM, DeCesare S, Jacobs S (2001) Serial [18F]

- fluorodeoxyglucose positron emission topography after human neuronal implantation for stroke. *Neurosurgery* 49:586-591.
- Menichella DM, Sellito C, Postma FR, Goodenough DA, Paul DL (2001) Normal oligodendrocyte-dependent myelination requires Cx32 and Cx46.6. International Gap Junction Conference, Honolulu.
- Menichella DM, Goodenough DA, Sirkowski E, Scherer SS, Paul DL (2003) Connexins are critical for normal myelination in the CNS. *J Neurosci* 23:5963-5973.
- Mesnil M, Piccoli C, Tiraby G, Willecke K, Yamasaki H (1996) Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. *Proc Natl Acad Sci U S A* 93:1831-1835.
- Mikkonen M, Soininen H, Alafuzof I, Miettinen R (2001) Hippocampal plasticity in Alzheimer's disease. *Rev Neurosci* 12:311-325.
- Miller BA, Sun F, Christensen RN, Ferguson AR, Bresnahan JC, Beattie MS (2005) A sublethal dose of TNF $\alpha$  potentiates kainate-induced excitotoxicity in optic nerve oligodendrocytes. *Neurochem Res* 30:867-875.
- Miller G (2005) Neuroscience. The dark side of glia. *Science* 308:778-781.
- Moffitt P (1994) A methyl green-pyronin technique for demonstrating cell death in the murine tumour S180. *Cell Biol Int* 18:677-679.
- Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods* 11:47-60.
- Morshead CM, van der Kooy D (1992) Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain. *J Neurosci* 12:249-256.
- Morshead CM, van der Kooy D (2004) Disguising adult neural stem cells. *Curr Opin Neurobiol* 14:125-131.
- Morshead CM, Benveniste P, Iscove NN, van der Kooy D (2002) Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. *Nat Med* 8:268-273.
- Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D (1994) Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron* 13:1071-1082.
- Nagy JI, Rash JE (2003) Astrocyte and oligodendrocyte connexins of the glial syncytium in relation to astrocyte anatomical domains and spatial buffering. *Cell Commun Adhes* 10:401-406.
- Nakagawa E, Aimi Y, Yasuhara O, Tooyama I, Shimada M, McGeer PL, Kimura H (2000) Enhancement of progenitor cell division in the dentate gyrus triggered by initial limbic seizures in rat models of epilepsy. *Epilepsia* 41:10-18.
- Nakagawa Y, Nakamura M, McIntosh TK, Rodriguez A, Berlin JA, Smith DH, Saatman KE, Raghupathi R, Clemens J, Saido TC, Schmidt ML, Lee VM, Trojanowski JQ (1999) Traumatic brain injury in young, amyloid-beta peptide overexpressing transgenic mice induces marked ipsilateral

- hippocampal atrophy and diminished Abeta deposition during aging. *J Comp Neurol* 411:390-398.
- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 110:429-441.
- Nelles E, Butzler C, Jung D, Temme A, Gabriel HD, Dahl U, Traub O, Stumpel F, Jungermann K, Zielasek J, Toyka KV, Dermietzel R, Willecke K (1996) Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin32-deficient mice. *Proc Natl Acad Sci U S A* 93:9565-9570.
- Nelson PT, Kondziolka D, Wechsler L, Goldstein S, Gebel J, DeCesare S, Elder EM, Zhang PJ, Jacobs A, McGrogan M, Lee VM, Trojanowski JQ (2002) Clonal human (hNT) neuron grafts for stroke therapy: neuropathology in a patient 27 months after implantation. *Am J Pathol* 160:1201-1206.
- Neuberg DH, Suter U (1999) Connexin32 in hereditary neuropathies. *Adv Exp Med Biol* 468:227-236.
- Nishiyama A (2001) NG2 cells in the brain: a novel glial cell population. *Hum Cell* 14:77-82.
- Nishiyama A, Lin XH, Giese N, Heldin CH, Stallcup WB (1996) Co-localization of NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells in the developing rat brain. *J Neurosci Res* 43:299-314.
- Odermatt B, Wellershaus K, Wallraff A, Seifert G, Degen J, Euwens C, Fuss B, Bussow H, Schilling K, Steinhauser C, Willecke K (2003) Connexin 47 (Cx47)-deficient mice with enhanced green fluorescent protein reporter gene reveal predominant oligodendrocytic expression of Cx47 and display vacuolized myelin in the CNS. *J Neurosci* 23:4549-4559.
- Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y (1997) 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 407:313-319.
- O'Keefe J, Dostrovsky J (1971) The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res* 34:171-175.
- Olney JW, Zorumski CF, Stewart GR, Price MT, Wang GJ, Labruyere J (1990) Excitotoxicity of L-dopa and 6-OH-dopa: implications for Parkinson's and Huntington's diseases. *Exp Neurol* 108:269-272.
- O'Shaughnessy D, Gerber GJ (1986) Damage induced by systemic kainic acid in rats is dependent upon seizure activity--a behavioral and morphological study. *Neurotoxicology* 7:187-202.
- Oyamada M, Oyamada Y, Takamatsu T (2005) Regulation of connexin expression. *Biochim Biophys Acta* 1719:6-23.
- Parent JM (2003) Injury-induced neurogenesis in the adult mammalian brain. *Neuroscientist* 9:261-272.
- Parent JM, Lowenstein DH (2002) Seizure-induced neurogenesis: are more new neurons good for an adult brain? *Prog Brain Res* 135:121-131.
- Parent JM, von dem Bussche N, Lowenstein DH (2006) Prolonged seizures recruit caudal subventricular zone glial progenitors into the injured hippocampus. *Hippocampus* 16:321-328.

- Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci* 17:3727-3738.
- Perez Velazquez JL, Frantseva MV, Naus CC (2003) Gap junctions and neuronal injury: protectants or executioners? *Neuroscientist* 9:5-9.
- Picard-Riera N, Nait-Oumesmar B, Baron-Van Evercooren A (2004) Endogenous adult neural stem cells: limits and potential to repair the injured central nervous system. *J Neurosci Res* 76:223-231.
- Piechocki MP, Toti RM, Fernstrom MJ, Burk RD, Ruch RJ (2000) Liver cell-specific transcriptional regulation of connexin32. *Biochim Biophys Acta* 1491:107-122.
- Prasher DC (1995) Using GFP to see the light. *Trends Genet* 11:320-323.
- Prochiantz A (2000) [Neurogenesis in the adult brain: hope for brain repair?]. *Bull Acad Natl Med* 184:1181-1189; discussion 1189-1190.
- Przedborski S (2005) Pathogenesis of nigral cell death in Parkinson's disease. *Parkinsonism Relat Disord* 11 Suppl 1:S3-7.
- Rakic P (2002) Adult neurogenesis in mammals: an identity crisis. *J Neurosci* 22:614-618.
- Rasband MN (2004) It's "juxta" potassium channel! *J Neurosci Res* 76:749-757.
- Ressot C, Gomes D, Dautigny A, Pham-Dinh D, Bruzzone R (1998) Connexin32 mutations associated with X-linked Charcot-Marie-Tooth disease show two distinct behaviors: loss of function and altered gating properties. *J Neurosci* 18:4063-4075.
- Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255:1707-1710.
- Rouach N, Avignone E, Meme W, Koulakoff A, Venance L, Blomstrand F, Giaume C (2002) Gap junctions and connexin expression in the normal and pathological central nervous system. *Biol Cell* 94:457-475.
- Rozental R, Mehler MF, Morales M, Andrade-Rozental AF, Kessler JA, Spray DC (1995) Differentiation of hippocampal progenitor cells in vitro: temporal expression of intercellular coupling and voltage- and ligand-gated responses. *Dev Biol* 167:350-362.
- Rozental R, Andrade-Rozental AF, Zheng X, Urban M, Spray DC, Chiu FC (2001) Gap junction-mediated bidirectional signaling between human fetal hippocampal neurons and astrocytes. *Dev Neurosci* 23:420-431.
- Rozental R, Morales M, Mehler MF, Urban M, Kremer M, Dermietzel R, Kessler JA, Spray DC (1998) Changes in the properties of gap junctions during neuronal differentiation of hippocampal progenitor cells. *J Neurosci* 18:1753-1762.
- Rozental R, Srinivas M, Gokhan S, Urban M, Dermietzel R, Kessler JA, Spray DC, Mehler MF (2000) Temporal expression of neuronal connexins during hippocampal ontogeny. *Brain Res Brain Res Rev* 32:57-71.

- Rukstalis JM, Kowalik A, Zhu L, Lidington D, Pin CL, Konieczny SF (2003) Exocrine specific expression of Connexin32 is dependent on the basic helix-loop-helix transcription factor Mist1. *J Cell Sci* 116:3315-3325.
- Saez JC, Berthoud VM, Branes MC, Martinez AD, Beyer EC (2003) Plasma membrane channels formed by connexins: their regulation and functions. *Physiol Rev* 83:1359-1400.
- Salter MG, Fern R (2005) NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. *Nature* 438:1167-1171.
- Scharfman HE (2000) Epileptogenesis in the parahippocampal region. Parallels with the dentate gyrus. *Ann N Y Acad Sci* 911:305-327.
- Scharfman HE, Goodman JH, Sollas AL (2000) Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. *J Neurosci* 20:6144-6158.
- Schauwecker PE (2003) Genetic basis of kainate-induced excitotoxicity in mice: phenotypic modulation of seizure-induced cell death. *Epilepsy Res* 55:201-210.
- Scherer SS, Xu YT, Messing A, Willecke K, Fischbeck KH, Jeng LJ (2005) Transgenic expression of human connexin32 in myelinating Schwann cells prevents demyelination in connexin32-null mice. *J Neurosci* 25:1550-1559.
- Schimanski LA, Nguyen PV (2004) Multidisciplinary approaches for investigating the mechanisms of hippocampus-dependent memory: a focus on inbred mouse strains. *Neurosci Biobehav Rev* 28:463-483.
- Schwarcz R, Foster AC, French ED, Whetsell WO, Jr., Kohler C (1984) Excitotoxic models for neurodegenerative disorders. *Life Sci* 35:19-32.
- Scoville WB, Milner B (1957) Loss of recent memory after bilateral hippocampal lesions. *J Neurol Neurosurg Psychiatry* 20:11-21.
- Seaberg RM, van der Kooy D (2003) Stem and progenitor cells: the premature desertion of rigorous definitions. *Trends Neurosci* 26:125-131.
- Seeman P, Mazanec R, Ctvrtckova M, Smilkova D (2001) Charcot-Marie-Tooth type X: A novel mutation in the Cx32 gene with central conduction slowing. *Int J Mol Med* 8:461-468.
- Severs NJ, Coppens SR, Dupont E, Yeh HI, Ko YS, Matsushita T (2004) Gap junction alterations in human cardiac disease. *Cardiovasc Res* 62:368-377.
- Single FN, Rozov A, Burnashev N, Zimmermann F, Hanley DF, Forrest D, Curran T, Jensen V, Hvalby O, Sprengel R, Seeburg PH (2000) Dysfunctions in mice by NMDA receptor point mutations NR1(N598Q) and NR1(N598R). *J Neurosci* 20:2558-2566.
- Sjobeck M, Haglund M, Englund E (2005a) White matter mapping in Alzheimer's disease: A neuropathological study. *Neurobiol Aging*.
- Sjobeck M, Haglund M, Englund E (2005b) Decreasing myelin density reflected increasing white matter pathology in Alzheimer's disease--a neuropathological study. *Int J Geriatr Psychiatry* 20:919-926.

- Sohl G, Willecke K (2003) An update on connexin genes and their nomenclature in mouse and man. *Cell Commun Adhes* 10:173-180.
- Sohl G, Willecke K (2004) Gap junctions and the connexin protein family. *Cardiovasc Res* 62:228-232.
- Sohl G, Odermatt B, Maxeiner S, Degen J, Willecke K (2004) New insights into the expression and function of neural connexins with transgenic mouse mutants. *Brain Res Brain Res Rev* 47:245-259.
- Sohl G, Theis M, Hallas G, Brambach S, Dahl E, Kidder G, Willecke K (2001) A new alternatively spliced transcript of the mouse connexin32 gene is expressed in embryonic stem cells, oocytes, and liver. *Exp Cell Res* 266:177-186.
- Song H, Stevens CF, Gage FH (2002) Astroglia induce neurogenesis from adult neural stem cells. *Nature* 417:39-44.
- Song H, Stevens C.F., Gage F.H. (2002) Astroglia induce neurogenesis from adult neural stem cells. *Nature* 417:39-44.
- Sperk G (1994) Kainic acid seizures in the rat. *Prog Neurobiol* 42:1-32.
- Stout CE, Costantin JL, Naus CC, Charles AC (2002) Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. *J Biol Chem* 277:10482-10488.
- Stubley-Weatherly L, Harding JW, Wright JW (1996) Effects of discrete kainic acid-induced hippocampal lesions on spatial and contextual learning and memory in rats. *Brain Res* 716:29-38.
- Suter U, Snipes GJ (1995) Biology and genetics of hereditary motor and sensory neuropathies. *Annu Rev Neurosci* 18:45-75.
- Sutherland GR, Dix GA, Auer RN (1996) Effect of age in rodent models of focal and forebrain ischemia. *Stroke* 27:1663-1667; discussion 1668.
- Tannenberg RK, Scott HL, Westphalen RI, Dodd PR (2004) The identification and characterization of excitotoxic nerve-endings in Alzheimer disease. *Curr Alzheimer Res* 1:11-25.
- Taylor RA, Simon EM, Marks HG, Scherer SS (2003) The CNS phenotype of X-linked Charcot-Marie-Tooth disease: more than a peripheral problem. *Neurology* 61:1475-1478.
- Temple S (2001) The development of neural stem cells. *Nature* 414:112-117.
- Teubner B, Odermatt B, Guldenagel M, Sohl G, Degen J, Bukauskas F, Kronengold J, Verselis VK, Jung YT, Kozak CA, Schilling K, Willecke K (2001) Functional expression of the new gap junction gene connexin47 transcribed in mouse brain and spinal cord neurons. *J Neurosci* 21:1117-1126.
- Theis M, Sohl G, Eiberger J, Willecke K (2005) Emerging complexities in identity and function of glial connexins. *Trends Neurosci* 28:188-195.
- Theis M, Jauch R, Zhuo L, Speidel D, Wallraff A, Doring B, Frisch C, Sohl G, Teubner B, Euwens C, Huston J, Steinhauser C, Messing A, Heinemann U, Willecke K (2003) Accelerated hippocampal spreading depression and enhanced locomotory activity in mice with astrocyte-directed inactivation of connexin43. *J Neurosci* 23:766-776.

- Trosko JE, Chang C-C, Wilson MR, Upham B, Hayashi T, Wade M (2000) Gap Junctions and the Regulation of Cellular Functions of Stem Cells during Development and Differentiation. *Methods* 20:245-264.
- Udawatte C, Ripps H (2005) The spread of apoptosis through gap-junctional channels in BHK cells transfected with Cx32. *Apoptosis* 10:1019-1029.
- Valiunas V (2002) Biophysical Properties of Connexin-45 Gap Junction Hemichannels Studied in Vertebrate Cells. *J Gen Physiol* 119:147-164.
- Van Dam D, Lenders G, De Deyn PP (2005) Effect of Morris water maze diameter on visual-spatial learning in different mouse strains. *Neurobiol Learn Mem.*
- van der Kooy D, Weiss S (2000) Why stem cells? *Science* 287:1439-1441.
- van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. *Nature* 415:1030-1034.
- Vance JM (1991) Hereditary motor and sensory neuropathies. *J Med Genet* 28:1-5.
- Varlet P (2005) [Histology and oligodendrogenesis of glial cells]. *Neurochirurgie* 51:229-238.
- Wang YL, Sun RP, Lei GF, Wang JW, Guo SH (2004) [Neurogenesis of dentate granule cells following kainic acid induced seizures in immature rats]. *Zhonghua Er Ke Za Zhi* 42:621-624.
- Weissman TA, Riquelme PA, Ivic L, Flint AC, Kriegstein AR (2004) Calcium Waves Propagate through Radial Glial Cells and Modulate Proliferation in the Developing Neocortex. *Neuron* 43:647-661.
- White TW, Paul DL (1999) Genetic diseases and gene knockouts reveal diverse connexin functions. *Annu Rev Physiol* 61:283-310.
- Wilkins A, Majed H, Layfield R, Compston A, Chandran S (2003) Oligodendrocytes promote neuronal survival and axonal length by distinct intracellular mechanisms: a novel role for oligodendrocyte-derived glial cell line-derived neurotrophic factor. *J Neurosci* 23:4967-4974.
- Willecke K, Eiberger J, Degen J, Eckardt D, Romualdi A, Guldenagel M, Deutsch U, Sohl G (2002) Structural and functional diversity of connexin genes in the mouse and human genome. *Biol Chem* 383:725-737.
- Williams G, Williams EJ, Maison P, Pangalos MN, Walsh FS, Doherty P (2005) Overcoming the inhibitors of myelin with a novel neurotrophin strategy. *J Biol Chem* 280:5862-5869.
- Won SJ, Kim DY, Gwag BJ (2002) Cellular and molecular pathways of ischemic neuronal death. *J Biochem Mol Biol* 35:67-86.
- Wosik K, Ruffini F, Almazan G, Olivier A, Nalbantoglu J, Antel JP (2004) Resistance of human adult oligodendrocytes to AMPA/kainate receptor-mediated glutamate injury. *Brain* 127:2636-2648.
- Xia H, Mao Q, Paulson HL, Davidson BL (2002) siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol* 20:1006-1010.
- Zernicka-Goetz M, Pines J, Ryan K, Siemering KR, Haseloff J, Evans MJ, Gurdon JB (1996) An indelible lineage marker for *Xenopus* using a mutated green fluorescent protein. *Development* 122:3719-3724.

Zernicka-Goetz M, Pines J, McLean Hunter S, Dixon JP, Siemering KR, Haseloff J, Evans MJ (1997) Following cell fate in the living mouse embryo. *Development* 124:1133-1137.

Zhao C, Teng EM, Summers RG, Jr., Ming GL, Gage FH (2006) Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. *J Neurosci* 26:3-11.

## **Appendix A: Engineering a marker mouse for assessing Cx32 expression**

### **Abstract**

This chapter describes the engineering of a new marker mouse that expresses the EGFP in place of Cx32 coding region. This new mouse line will allow us to determine more accurately when and where Cx32 is expressed at the protein level both *in vivo* and *in vitro*. In part, this will enable us to identify cells triggered to express Cx32 in the null mutant background and track their fate over the course of injury. The project was a collaborative effort between the Neural Regeneration Laboratory at the University of Ottawa, and Dr. David Paul's laboratory at Harvard Medical School (Boston, MA, USA).

**Statement of author contributions**

The stem cell transfections, blastocyst injections of the stem cells, and chimeric mouse generation were performed at the Gene Manipulation Core of the Children's Hospital for Mental Retardation Research Center (MRRC; Boston, MA). All other manipulations in the engineering of the mutant mouse, including screening of the ES cell transfectants, were performed by the author of this thesis.

## A1.1 Introduction

In Chapters 2 and 3, we provide evidence that downregulation of Cx32 promotes functional neuroregeneration in the CA3 pyramidal layer of the hippocampus following KA-induced injury. Specifically, NG2<sup>+</sup> progenitor cells activated by seizure-induced neuronal loss appear to be directed to a neuronal lineage more effectively in absence of Cx32. The potential role of Cx32 in regulating stem and progenitor cell has been reviewed in the previous chapters, but it must be emphasized that there is no means of directly tracking the expression of Cx32 *in vivo*. Colleagues in the Bennett laboratory have performed gain of function studies, overexpressing Cx32 in both primary progenitor cells cultured from postnatal hippocampus and teratocarcinoma cells, demonstrating that Cx32 expression inhibits neurogenesis. However, we have yet to establish the exact kinetics of Cx32 expression over the course of progenitor cell commitment following excitotoxic seizure. As discussed in Chapter 1 and demonstrated in Chapter 2, there is tremendous cross-reactivity among the Cx proteins. Despite the thorough screening of eight different antibodies for Cx32 antibodies in uninjured tissue (Chemicon, Zymed, 4 in-house generated reagents), cross-reactivity was detected by western blotting and immunofluorescence in some Cx32KO samples following kainate-induced injury (Figure 2.5 – D). To address this issue, a transgenic mouse line, expressing the EGFP in place of Cx32, was generated. This new transgenic mouse can be used not only to track the onset of Cx32 expression in adult hippocampal progenitor cell populations but will also be instrumental in settling many

controversies in the field, such as the developmental time at which Cx32 mRNA is expressed and in which cell lineages.

## **A1.2 Methods and Material**

### **A1.2.1 Generation of the 5' and 3' homologous fragments flanking the Cx32 coding region**

Homologous 129/SVJ genomic DNA flanking the 5' and 3' Cx32 coding region was amplified by polymerase chain reaction (PCR). Primer pairs and templates are indicated in Table A1. Primers were synthesized at the University of Ottawa BRI Core Facility or at the Harvard Medical School Biopolymers Facility. The primer set utilized to amplify a 987 base pair (bp) 5' fragment (5'Cx32) was synthesized with a 5' *Sac*II restriction site (CCGCGG) and a 3' *Pci*I restriction site (ACATGT). A longer 1457 bp amplicon (5'Cx32Scr) was also generated using a 5' primer further upstream of the targeting region to be able to optimize the genotyping primers. The 3' homology fragment (3'Cx32) was 630 bp.

For the 5'Cx32 and 5'Cx32Scr amplicons, PCR conditions were: 19.2 µl of nuclease free (NF) H<sub>2</sub>O, 2.5 µl of 10x Advantage 2 PCR buffer (Clontech, Mountain View, CA, USA), 0.5 µl of 10 mM deoxynucleotide triphosphate (dNTP; Fisher Scientific Ltd.), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 0.3 µl of 129SVJ1 genomic DNA (100 ng), and 0.5 µl of Advantage2 Taq Polymerase (Clontech). Cycles were as follows: 1 minute of denaturation at 95°C, 28 cycles of (25 seconds of denaturation at 95°C, 75 seconds of annealing at 64°C and 2 minutes of extension at 72°C), and a final extension time of 10 minutes at 72°C.

**Table A1: List of primer sets used for PCR amplification.** All primer sets were designed using the Primer Designer 4, version 4.20 computer software, and synthesized by the Ottawa BRI Core Facility or at the Harvard Medical School Biopolymers Facility.

<b>Amplicon</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>	<b>Template DNA</b>	<b>Product Length (bp)</b>
5'Cx32	5'Cx32Fw (GCGCCGCGGAC TCTTTTGTGCTTC GTCATTTTTAGC)	5'Cx32Rv (GTTACATGTGCC ATCCTGCCTCATT CACACCTGTG)	129SV Genomic	987
5'Cx32Scr	5'Cx32ScrFw (AACCGCGGTGG AGCAGGTATCTT CATTG)	5'Cx32Rv (GTTACATGTGCC ATCCTGCCTCATT CACACCTGTG)	129SV Genomic	1457
3'Cx32	3'Cx32Fw (CGCCTCTCACCT GAATACAA)	3'Cx32Rv (CATAACTCCCTC ACCCTAGATG)	129SV Genomic	630
Cx32KOEFGFP	Cx32KOEFGFPFw (GGTGGAGCAGG TATCTTCATTG)	Cx32KOEFGFPRv (GAAGTCGTGCT GCTTCATGT)	ES Cells or Screening Vector	1696
Cx32WTscr	Cx32WTScrFw (CCTTGCTCAGTG GCGTGAATCG)	Cx32WTScrRv (CACCTCCGCCA CGTTGAGGATA)	129SV Genomic	605
Sequencing	5'Cx32EGFPSeq (GCGAATGCTGG ATGTATGTG)	N/A	Targeting Vector	N/A
Sequencing	3'Cx32EGFPSeq (GAGCTCGGATC CACTAGTAA)	N/A	Targeting Vector	N/A

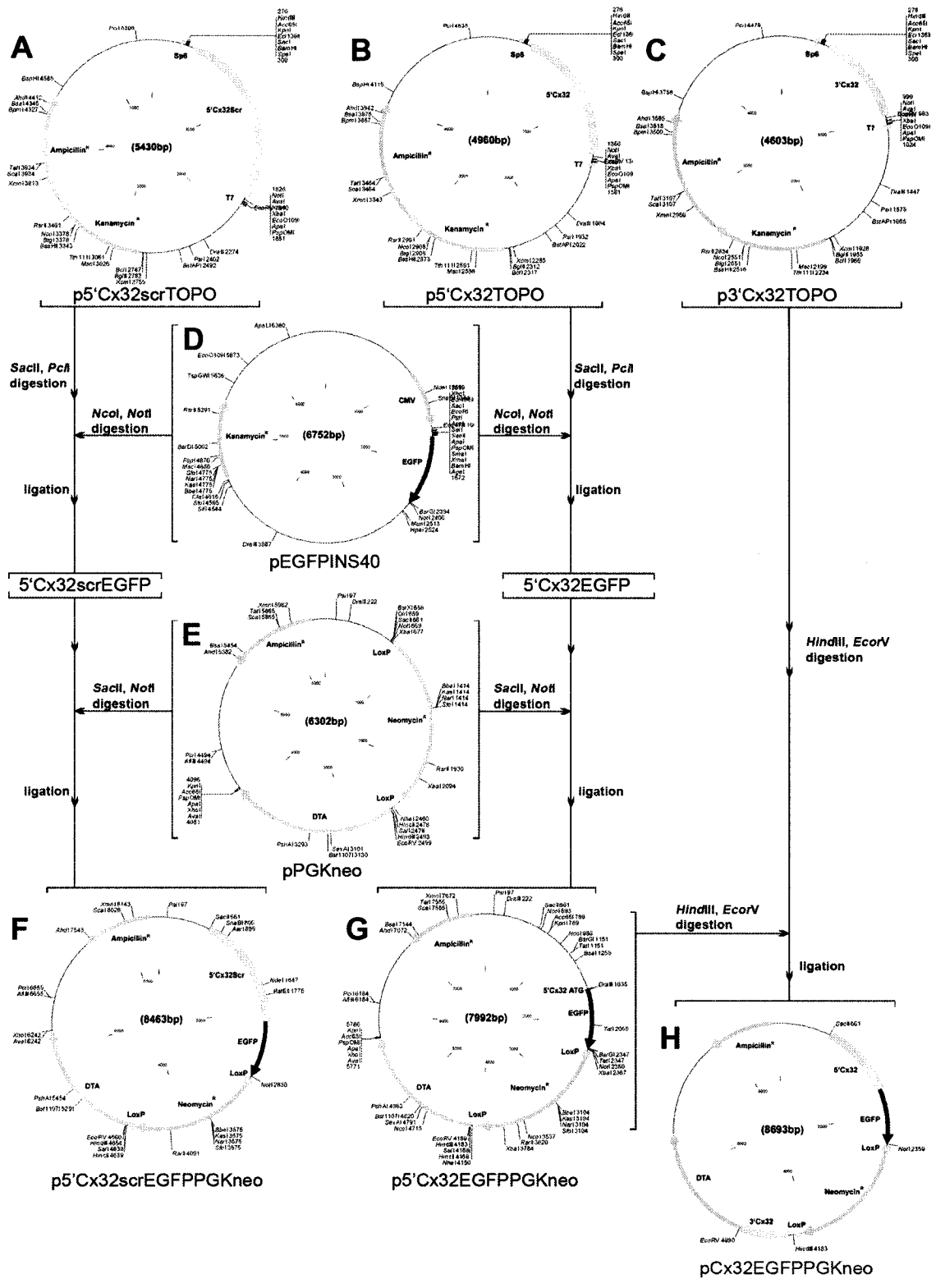
For the 3'Cx32 amplicon, PCR conditions were: 16  $\mu$ l of NF H<sub>2</sub>O, 2.5  $\mu$ l of 10x Advantage2 PCR buffer, 0.5  $\mu$ l of 10mM dNTP, 1  $\mu$ l of 10 $\mu$ M forward primer, 1  $\mu$ l of 10  $\mu$ M reverse primer, 3.5  $\mu$ l of 129SVJ1 genomic DNA (200 ng), and 0.5  $\mu$ l of Advantage2 Taq Polymerase. Cycling conditions were as follows: 90 seconds of denaturation at 95°C, 30 cycles of (25 seconds of denaturation at 95°C, 45 seconds of annealing at 60°C and 90 seconds of extension at 72°C), and a final extension time of 10 minutes at 72°C.

#### **A1.2.2 Construction of the Cx32<sup>EGFP</sup> targeting vector**

The 5'Cx32, 5'Cx32Scr, and the 3'Cx32 amplicons were separately inserted into the TA-cloning vector pCR<sup>®</sup>II-TOPO<sup>®</sup> (Invitrogen, San Diego, CA, USA). The EGFP coding region was obtained by restriction digest of the pEGFP INS40 plasmid, which was obtained from Dr. Charles Stout (Harvard Medical School). The targeting vector backbone, pPGKneo, was generously provided by Dr. David Paul (Harvard Medical School) (Deans et al., 2001). Figure A1 illustrates the main steps in the construction of the targeting vector used for homologous recombination with ES cells.

A 724 bp *Nco*I-EGFP-*Not*I fragment of the complete EGFP coding sequence from pEGFP INS40 was ligated in-frame replacing the ATG of the 972 bp 5'Cx32 *Sac*II-5'Cx32-*Pci*I fragment to generate a 1696 bp *Sac*II-5'Cx32EGFP-*Not*I fragment. The Cx32<sup>EGFP</sup> targeting vector consisted of the 1696 bp 5'Cx32EGFP and the 630 bp 3'Cx32 inserts ligated in pPGKneo. The EGFP coding DNA replaced 692 bp of the Cx32 coding region, and 158 bp of the Cx32

**Figure A1: Schematic representation of the targeting vector construction strategy.** The 5', 5'scr and 3' homology regions to the Cx32 coding region were amplified by PCR and inserted into a TA-cloning vector to generate p5'Cx32scrTOPO (A), p5'Cx32TOPO (B), and p3'Cx32TOPO (C). The pEGFPINS40 (D) plasmid was digested with NcoI and NotI to generate the EGFP fragment. Digestion of (A) and (B) with SacII and PciI generated the 5'Cx32scr and 5'Cx32 fragments. Ligation of EGFP and 5'Cx32scr or 5'Cx32 generated the 5'Cx32scrEGFP and 5'Cx32EGFP fragments respectively. The pPGKneo plasmid (E) was digested with SacII and NotI, and the product was ligated with 5'Cx32scrEGFP or 5'Cx32EGFP to generate the screening vector p5'Cx32scrEGFPPGKneo (F) and the p5'Cx32EGFPPGKneo plasmid (G) respectively. Digestion of (C) with HindIII and EcoRV generated the 3'Cx32 fragment. The plasmid (G) was digested with HindIII and EcoRV, and the product was ligated with the 3'Cx32 fragment to produce the targeting vector pCx32EGFPPGKneo (H).



**Figure A1**

coding region remained in the 3' homology insert. A neomycin resistance gene driven by the mouse phosphoglycerate kinase (PGK) promoter, flanked by LoxP sites for Cre-mediated excision, was cloned in front of the 3' homologous flanking region. A diphtheria toxin A chain (DTA) cassette was included downstream of the 3'Cx32 flanking sequence for negative selection of non-homologous random integration. Upon completion of the targeting vector, pCx32EGFPloxPGKneo (Figure A2), its identity was verified via a variety of restriction digestions and DNA sequencing. A second similar vector was constructed, p5'Cx32scrEGFPloxPGKneo with the addition of the 1443 bp 5'Cx32Scr fragment to be able to optimize genotyping fragments.

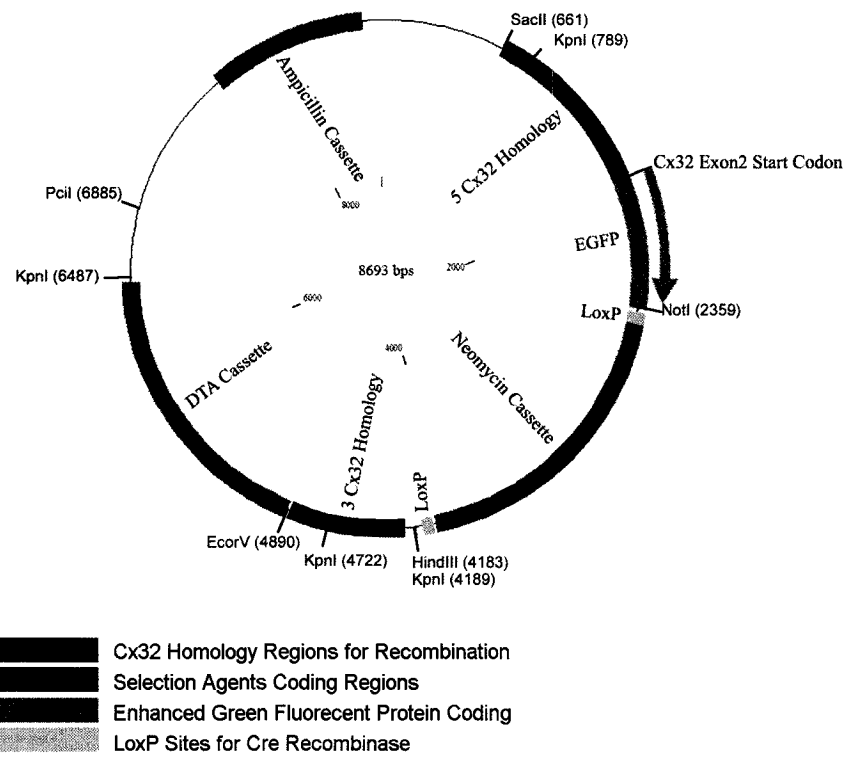
Upon completion of the targeting vector, pCx32EGFPloxPGKneo, its identity was verified via a variety of restriction digestions and DNA sequencing. The plasmid DNA of Cx32<sup>EGFP</sup> (120 µg) was linearized by *PciI* digestion, extracted using a phenol/chloroform solution, and precipitated using 0.1 volume of 3M sodium acetate pH 5.5 and 2 volumes of 100% EtOH. The mixture was kept at -20°C until shipment to the facility where ES cell transfection was performed.

### **A1.2.3 Embryonic stem Cell transfection, selection and genotyping**

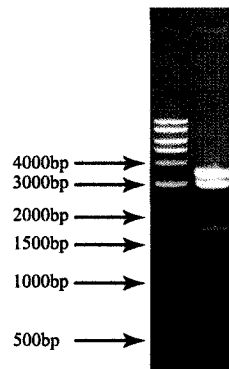
The linearized targeting vector was sent to the Gene Manipulation Core of the Children's Hospital for MRRC for ES cell transfection and homologous recombination (Figure A3). The ES cells were obtained by culturing the inner cell mass of 129SVJ1 mice blastocysts. Following transfection of the ES cells

**Figure A2: Plasmid map of the Cx32<sup>EGFP</sup> targeting vector pCx32EGFPloxPGKneo.** The plasmid was constructed as illustrated in Figure 4.1. The identity of the pCx32EGFPloxPGKneo targeting vector was verified by restriction digest with *KpnI* (B) and DNA sequencing. Plasmid DNA was linearized by restriction digest with *PciI*, and sent to a facility for ES cell transfection for homologous recombination and mutant ES cell generation. Plasmid contains the following selection cassettes: DTA for negative selection and neomycin for positive selection in mammalian cells, and ampicillin for positive selection in bacterial cells.

**A (Targeting vector)**



**B (KpnI restriction digest)**



**Figure A2**

with the targeting vector, the clones were positively selected with neomycin for incorporation of the targeting vector and negatively selected with DTA for random insertion of the targeting vector in the genomic DNA. The clones were plated on eight 24-well plates (N=192 wells total) in duplicates. One set of plates was frozen by the facility for future blastocyst procedure, while the other set was expanded until high confluence of the clones, and was returned to our laboratory for PCR screening of the clones.

Each plate was processed individually in order to minimize the risks of cross-contamination during the DNA extraction procedure. The ES clones were lysed in the plates by adding 0.5ml of lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg/ml fresh proteinase K) to each well in the plate. The plate was then incubated overnight at 37°C with shaking for complete lysis of the cells. DNA was treated with 2 µl of ribonuclease A (RNase A; Sigma; 10 mg/ml), precipitated, and resuspended in 200 µl of 1X TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH8.0).

The Cx32KOEgFP genotyping PCR primers were optimized using the p5'Cx32ScrEGFPloxPGKneo screening vector described above. PCR was performed with 10, 100, 1000, 10000, and 1000000 copies of the plasmid in equal volume of WT DNA obtained from mouse tail DNA. The Cx32WTScr genotyping primers were optimized using 129 SVJ1 WT DNA. Both the KO and WT reactions contained: 2 µl of 10x Advantage2 buffer, 14.5 µl of NF H<sub>2</sub>O, 0.4 µl of dNTP, 0.8 µl of 10 µM forward primer, 0.8 µl of 10 µM reverse primer, 1 µl of DNA, and 0.5µl of Advantage2 Taq Polymerase. Cycling conditions were: 2

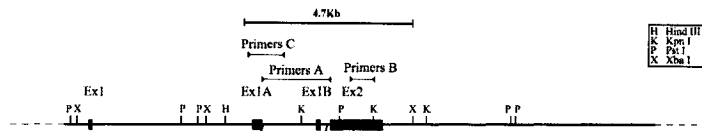
minutes of denaturation at 95°C, 37 cycles of (30 seconds of denaturation at 95°C and 3 minutes of annealing/extension at 65°C), and a final extension period of 5 minutes at 72°C.

#### **A1.2.4 Germline transmission and generation of chimeras**

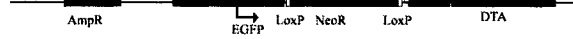
Positive clones were identified by PCR genotyping (Figure A3). These were separately injected into the inner cell mass of blastocysts that were previously obtained from 129SVJ1 pregnant female mice at the Children's Hospital MRRC in Boston, and implanted in pseudo-pregnant C57Bl/6 female mice.

**Figure A3: Homologous recombination of the targeting vector with 129SVJ1 ES cells.** (A) WT allele of the Cx32 gene. Primer pairs A and B were used to amplify the homology regions of Cx32 by PCR, and used to generate the targeting vector (B). 129SVJ1 mouse embryonic stem cells were stably transfected with the mutant DNA to generate an intermediate mutant allele by homologous recombination (C). The embryonic stem cells were positively selected with neomycin and negatively selected with diphtheria toxin, and expanded for genotyping. The DNA from 192 stem cells was isolated and genotyped by PCR with the PCR primer pairs C (A) and D (D), and 6 clones were identified for successful homologous recombination (E).

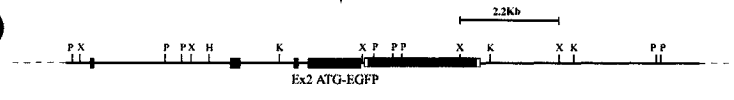
**A (WT Allele)**



**B (Targeting vector)**

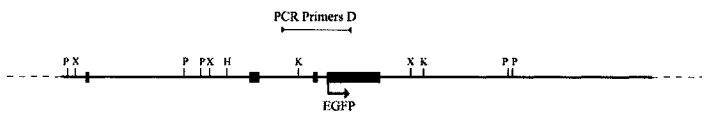


**C (Intermediate mutant allele)**

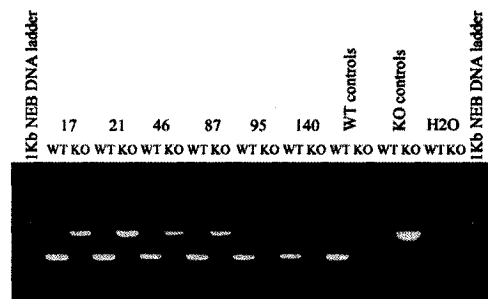


Cre-mediated excision of neo cassette

**D (Mutant allele)**



**E (PCR Genotyping of recombinant ES cells)**



**Figure A3**

## **A1.3 Results**

### **A1.3.1 Construction of the cloning vectors**

The p5'Cx32 CR<sup>®</sup>II-TOPO<sup>®</sup> plasmid, p3'Cx32 CR<sup>®</sup>II-TOPO<sup>®</sup> plasmid, and the p5'Cx32Scr CR<sup>®</sup>II-TOPO<sup>®</sup> plasmid were constructed by TA-cloning of the 5'Cx32 PCR fragment (982 bp), the 3'Cx32 PCR fragment (630 bp), and the 5'Cx32Scr PCR fragment (1457 bp) respectively. The identity of each plasmid was verified by multiple restriction digests.

The cloning procedures described here are schematically represented in Figure A1. The p5'Cx32 CR<sup>®</sup>II-TOPO<sup>®</sup> and the p5'Cx32Scr CR<sup>®</sup>II-TOPO<sup>®</sup> DNA were separately digested with the SacII and PciI restriction endonucleases to generate the 5'Cx32 fragment (972 bp) and the 5'Cx32Scr fragment (1443 bp) respectively, which were isolated via gel purification with the Qiaex II gel extraction kit (Qiagen, Mississauga, ON, Canada). The pEGFP INS40 plasmid was digested with the NcoI and NotI restriction endonuclease, and the EGFP fragment (724 bp) was isolated and purified. The pPGKneo plasmid was digested with the SacII and NotI restriction endonucleases, and the 6296 bp fragment was isolated and purified for subsequent ligation manipulations. Given the compatibility of the PciI restriction site with the NcoI restriction site, the 5'Cx32 and the 5'Cx32Scr fragments were separately ligated with the EGFP fragment to generate a the 5'Cx32EGFP fragment (1696 bp) or the 5'Cx32ScrEGFP fragment (2167 bp). These two fragments were independently ligated to the pGKneo fragment mentioned above to generate the partial targeting vector p5'Cx32EGFPloxPGKneo (Figure A2) and the screening vector

used for genotyping primer optimization p5'Cx32ScrEGFPloxPGKneo. The p3'Cx32 CR<sup>®</sup>II-TOPO<sup>®</sup> was digested with the HindIII and EcoRV restriction endonucleases to generate the 3'Cx32 fragment (709 bp). The partial targeting vector p5'Cx32EGFPloxPGKneo was digested with the same two restriction endonucleases to generate the 5'Cx32EGFPloxPGKneo fragment (7984 bp). The 3'Cx32 fragment and the 5'Cx32EGFPloxPGKneo fragment were ligated together to generate the targeting vector pCx32EGFPloxPGKneo. The identity of the targeting vector was verified by restriction digests and DNA sequencing.

### **A1.3.2 Transfection of ES cells, genotyping and blastocyst injections**

The pCx32EGFPloxPGKneo targeting vector was linearized with a single cutting restriction endonuclease and 120 µg was sent to the Children's Hospital MRRC for ES cell transfection and homologous recombination (Figure A3). Following transfection, the cells were positively selected with neomycin for incorporation of the targeting vector. The 192 transfected ES cells were then split in duplicates into 8 24 well plates. One of the sets was sent back to our laboratory for DNA extraction and PCR genotyping. Out of the 192 transfected ES cells, 6 were identified as successful recombinants (Figure A3 – E). From these six successful recombinants, two were chosen for blastocysts injection (17 and 21). The litter generated from the clone 17 blastocyst uterine injection generated 2 mice being 90-100% agouti, 1 mouse being 30% agouti and 4 black mice, while the litter generated from the clone-21 blastocyst uterine injection generated 6 mice 90-100% agouti, 1 mouse 55% agouti, and 5 black mice.

### **A1.3.3 Problems associated with ES cell injection**

As indicated in Figure A3, transfection of the ES cells with an expression vector that induces transient expression of Cre recombinase results in excision of the floxed neomycin cassette from the intermediate allele prior to blastocysts injection of the mutant ES cell. However, this was not performed due to an error by core facility in Boston. The presence of the neomycin promoter in proximity of the Cx32 promoter could result in lower transcription activity of the reporter gene EGFP in the mutant animals, as reported previously in the literature (McDevitt et al., 1997; Single et al., 2000; Filippov et al., 2003). In this case, detection EGFP might still be possible in regions of high expression such as the liver and highly myelinated areas in the brain. However, for the purpose of this research, sensitivity of the reporter system is preferable. To address this issue, mutant mice that are 90-100% Agouti are currently been bred to WT C57Bl/6 mice to assess germline transmission, and to mice constitutively expressing Cre recombinase in all cell types (kindly provided by Dr. David Paul) to excise the neomycin cassette.

## A1.4 Discussion

The green fluorescent protein (GFP) is a non-invasive marker used to assess a variety of cell properties both in vitro and in vivo. This genetic reporter system derived from the bioluminescent jellyfish *Aequorea victoria* in 1994 (Chalfie et al., 1994; Prasher, 1995) has contributed greatly to cell marking technologies by introducing a marker system with fewer disadvantages encountered with other systems, such as dilution during development with lysinated rhodamine dextran (LRD) or necessity for substrate reactions for visualization with the bacterial  $\beta$ -galactosidase gene (*lacZ*) (Li et al., 1997; Hadjantonakis et al., 1998).

Despite the numerous advantages of the system, GFP also has some disadvantages that render this reporter system not optimal for all use. Its use has been limited to a small scale due to its weak fluorescence and instability of the WT protein at high temperature. In order to resolve these problems, mutants for the GFP have been created. By substituting two amino acids (Phe64Leu, Ser65Thr) in proximity of the chromophore within the peptide sequence of the GFP protein, and by substituting 190 silent bases (Hadjantonakis et al., 1998), the resulting EGFP, exhibits a narrower spectrum and emits fluorescence at a higher intensity (Zernicka-Goetz et al., 1996; Okabe et al., 1997; Zernicka-Goetz et al., 1997).

The study of Cx-mediated communication relies highly on the availability and quality of research tools in the field. As it was shown in Chapter 2, Cxs are notoriously cross-reactive, following the expression pattern of Cx32 in vivo

following excitotoxic injury was very difficult, and unreliable using conventional immunolabelling techniques, given the poor specificity of the available antibodies. This chapter focused on the engineering of a marker mouse in which the Cx32 gene was knocked out and replaced by the EGFP coding region. This novel mouse line will allow us to determine more accurately the expression pattern of Cx32 following neuronal injury, and thereby have insight as to how Cx32 is implicated in progenitor cell specification in the brain.

A great deal of controversies related to the cell specificity in the Cx field has been resolved by the use of such reporter systems. For example, while some researchers argued that Cx45 localized to oligodendrocytes by immunohistochemical assays (Dermietzel et al., 1997; Kunzelmann et al., 1997), others have shown, using a lacZ reporter system, that it is not found in oligodendrocytes (Kruger et al., 2000). The confound was identified as cross-reactivity of the Cx45 antibody with the Cx47 protein (Teubner et al., 2001). Similarly, debate related to the cellular expression of Cx47 as being neuronal (Teubner et al., 2001) or oligodendrocytic (Menichella et al., 2001) has been successfully resolved by the use of an EGFP reporter system (Odermatt et al., 2003) similar to the one engineered during the work presented in this thesis. However, as discussed in the Results section, in our system the neomycin cassette was not excised from the mutant allele, which might result in lower transcriptional activity due to silencing of the Cx32 promoter by the neomycin transcription unit as previously reported in the Cx26 lacZ reporter mouse (Filippov et al., 2003), and thus yield lower sensitivity of the reporter system. In

the Phillipov *et al.* study, the lower expression of Cx26 resulted in embryonic lethality of the null mutant animals. However, given the postnatal expression of Cx32, this does not apply. Nonetheless, sensitivity of the system remains of great concern. Optimistically, mating of the mutant mice with mice constitutively expressing Cre recombinase in all cell types, kindly provided by Dr. David Paul, will resolve this potential problem.

As mentioned in the previous chapters, Cx32 appears to be a critical player in determining the fate of NPCs within the two neurogenic regions of the adult brain. However, the specific mechanisms through which Cx32 acts to influence fate determination remain elusive. By identifying cells that were predestined to express Cx32 by the presence of EGFP in the Cx32KO background, we will gain a great deal of mechanistic insight on the role of Cx32 in adult NPC specification.

## Appendix B: Curriculum Vitae

### MARIO MORIN

M.D.Candidate

Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada

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#### Education

<b>M.D. Candidate</b> University of Ottawa, Ottawa, ON	2006-2010
<b>M.Sc. (Biochemistry)</b> University of Ottawa, Ottawa, ON	2004-2006
<b>B. Sc. Honours, (Biochemistry)</b> University of Ottawa, Ottawa, ON	2000 - 2004
<b>Ontario Academic Courses (OAC) Diploma</b> École secondaire Château-Jeunesse, Longlac, ON	2000
<b>Ontario Secondary School diploma (OSSD)</b> École secondaire Château-Jeunesse, Longlac, ON	1996 - 2000

#### Certifications and Affiliations

<b>First Aid and Level C CPR</b> St. John Ambulance	2006 - present
<b>Member of the Multiple Sclerosis Society of Canada</b> Ottawa, ON	2004 - present
<b>Student member of the Society for Neuroscience</b> International	2004 - present
<b>Spanish Certification – Advanced/intermediate level</b> Zamora Academia Elemental de Español, Antigua, Guatemala	2006
<b>Assistant Instructor in Martial Arts</b> Fang Shen Do Kung Fu, Ottawa, ON	2003 - 2004

#### Honors, Awards and Scholarships

Faculty of Medicine entrance scholarship, Ottawa, ON	2006
Profiled in the Yearly Report of the University of Ottawa, Ottawa, ON	2006
Student profile in University of Ottawa Prospective Students, Ottawa, ON	2005
Student profile in the University Of Ottawa Gazette, Ottawa, ON	2005
Profiled in the newspaper La Gatineau, Maniwaki, QC	2005
Invited speaker for the University of Ottawa BIO-X Club, Ottawa, ON	2005
BMI Poster Day Award, 1 <sup>st</sup> Place (M.Sc. category), Ottawa, ON	2005
FDSRP Research Scholarship, Harvard Medical School, Boston, USA	2004
NSERC Undergraduate Summer Research Award, Ottawa, ON	2003
Dean's Honour list, Science, University of Ottawa, Ottawa, ON	2001
Admission scholarship, University of Ottawa, Ottawa, ON	2000 - 2002

## Work Experience

<b>CIHR Youth Engagement Program</b> Canadian Institutes for Health Research, Ottawa, ON <i>Inform and recruit young minds into health related science. Inform general public, attend various meetings and events, and report to supervisors.</i>	2004 - present
<b>Teaching Assistant (BCH4125)</b> Faculty of Sciences, University of Ottawa, Ottawa, ON <i>Evaluate and provide "scientific officer" feedback to grant proposals written by 4<sup>th</sup> year undergraduate biochemistry students.</i>	2005
<b>Teaching Assistant (BCH3756)</b> Faculty of Sciences, University of Ottawa, Ottawa, ON <i>Teach and supervise in a 3<sup>rd</sup> undergraduate molecular biology laboratory.</i>	2004
<b>NRL / FDSRP Summer Research Student</b> NRL, Faculty of Medicine, University of Ottawa, Ottawa, ON Neurobiology, Faculty of Medicine, Harvard University, Boston, USA <i>Design and engineer a targeting vector to produce a transgenic mouse model expressing EGFP in place of the Connexin32 protein.</i>	2004
<b>NSERC Summer Student</b> Faculty of Medicine, University of Ottawa, Ottawa, ON <i>Investigate neural regeneration potential in the CNS of Cx32KO mice.</i>	2003
<b>Teaching assistant</b> Ottawa-Carleton District School Board, Ottawa, ON <i>Provide academic help to elementary students in various sciences.</i>	2003
<b>Technical Library Publications Clerk</b> Transport Canada Aircraft Services Directorate, Ottawa, ON <i>Receive, assess, record, and file amendments to regulatory documents. Communicate with national branches to assure reception of material.</i>	2002
<b>Peer Animator and Social Worker in a Youth Centre</b> Mani-Jeunes, Maniwaki, QC <i>Organize and supervise various activities, organize local fundraisers, and provide support to the local youth with problems such as alcohol and drug abuse.</i>	2001- 2002

## List of Publications

- Morin M.Y., Vanderleest E., Midzic I., Toeg H., Messier C., Bennett S.A.L.. Behavioural assessment of spatial memory performance as an indicator of chronic neuronal impairment in the mouse hippocampus following excitotoxic injury (In preparation for Behavioral Brain Research).
- Weaver JGR., Tarze A, Moffat TC, LeBras M, Deniaud A, Brenner C, Bren GD, Morin MY, Phenix BN, Miller B, Dong L, Jiang SX, Sim VL, Zurakowski B, Lallier J, Hardin H, Wettstein P, van Heeswijk RPG, Douen A Kroemer R, Hou ST, Bennett SAL, Lynch DH, Kroemer G, Badley AD (2005) Inhibition of Adenine Nucleotide Translocator Pore Function and Protection Against Apoptosis in vivo by an HIV Protease Inhibitor. J. Clin. Invest. in press
- Melanson-Drapeau L. \*, Morin M.Y. \*, Haykal S., Pelletier L., Paul D.L., Bennett S.A.L. Altering connexin expression promotes regeneration of hippocampal pyramidal neurons following excitotoxic injury. (Submitted to J.Neuroscience). *Stared authors have contributed equally.*
- Hebb A.L.O, Boucher S., Davé S., Haykal S., Morin M.Y., Ryan S.D., Moffat T.C., Melanson-Drapeau L., and Bennett S.A.L. (May 2004). Connexin expression controls neural progenitor fate over the course of the estrous cycle. (Submitted to J. Neurosci, In preparation for resubmission).

## **List of Major Presentations**

### *Platform presentations*

- Morin M.Y.**, Melanson-Drapeau L., Vanderleest E., Messier C., Paul D.L., and Bennett S.A.L.. Enhancement of neural cell regeneration and survival by altering gap junctional communication following excitotoxic injury in the adult brain, Gap Junction Conference, Whistler (August 2005)
- Morin M.Y.**, Bennett S.A.L., Paul D.L.. Engineering of a marker mouse expressing EGFP in place of Cx32. Summer research presentation to representatives of the Faculty of Medicine at Harvard University, Boston, (MA, USA) (August, 2004).

### *Poster Presentations*

- Imbeault S., Lallier J., Hebb A.L.O., Haykal S., Davé S., **Morin M.Y.**, Hill J., Melanson-Drapeau L., and Bennett S.A.L.. Hormonally induced hyperproliferation in Cx32 null mutant females exhausts progenitor self-renewal in vivo, Gap Junction Conference 2005, Whistler, BC, Canada (August 2005)
- Morin M.Y.**, Melanson-Drapeau L., Bennett S.A.L. Kinetics of neurodegeneration and neuroregeneration in the Cx32 null mutant mouse following kainic acid induced seizure, BMI Poster Day, University of Ottawa, ON, Canada (March 2005)
- Melanson-Drapeau L., **Morin M.Y.**, Bennett S.A.L.. Connexin 32-mediated regulation of progenitor specification following brain injury, Keystone Symposia – Molecular Regulation of Stem Cells, Banff, AB, Canada (February 2005)
- Morin M.Y.**, Melanson-Drapeau L., Bennett S.A.L.. Kinetics of neurodegeneration and neuroregeneration in the Cx32 null mutant mouse following kainic acid induced seizure, Society for Neuroscience Conference, San Diego, CA, USA (October 2004)
- Melanson-Drapeau L., **Morin M.Y.**, Bennett S.A.L. Connexin32-mediated regulation of progenitor specification after brain injury, Society for Neuroscience Conference, San Diego (October 2004)
- Morin M.Y.**, Bennett S.A.L. Kinetics of neurodegeneration and neuroregeneration in the Cx32 null mutant mouse following kainic acid induced seizure, BMI Poster Day, Ottawa (April 2004)
- Morin M.Y.**, Melanson-Drapeau L., Bennett S.A.L. Cinétique de dégénérescence et de régénération neuronale dans le système nerveux central de souris mutantes par éjection de Cx32 après des convulsions induites par acide kainique, Conférence annuelle de l'Association francophone pour le savoir, Montreal (May 2004)

## **Extracurricular Activities**

- National Officer of Professional and Research Exchanges CFMS-IFMSA 2006 - present
- Constitution officer of Medical Students for Mental Health Awareness 2006 - present
- Class of 2010 Representative to the uGeePS curriculum committee 2006 - present
- Class of 2010 Representative to the Student affairs consultative committee 2006 - present
- Participant in the creation of the professional oath of the Class of 2010 2006
- Laboratory volunteer at S.F. Nacional Hospital, Antigua (Guatemala) 2006
- Help in organizing the symposium "The Future is Now, The Place is Ottawa" 2006
- Invited speaker for the University of Ottawa, Medicine Open House 2006
- Judge for the Medical Student Research presentations, Ottawa, ON 2005
- Host for the University of Ottawa, Faculty of Medicine Open House 2005
- Judge for the 2005 Ottawa Regional Science Fair 2005
- CIHR Youth Program representative, Ottawa Regional Science Fair 2005
- CIHR Youth Program representative, Expo-sciences Bell, 2005
- Research mentor for two 4<sup>th</sup> year undergraduate Biochemistry students 2004 - 2005
- Host for the University of Ottawa, Faculty of Science Open House 2004
- Assistant-Instructor in martial arts, Ottawa, ON 2003 - 2004
- Volunteer in the Emergency department of the Ottawa General Hospital 2002 - 2003