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IN VITRO CONNEXIN EXPRESSION AND FUNCTION IN POSTNATAL NEURAL PROGENITORS

Lianne G. Gauvin

A thesis submitted to
the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the degree of
Master of Science

Department of Biochemistry, Microbiology, and Immunology
University of Ottawa
Ottawa, ON, Canada

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Thesis Abstract

The studies presented in this thesis were performed to gain insight into the role of connexins (Cxs) and Cx-mediated communication in regulating postnatal neural precursor cell (NPC) fate. Cxs are protein subunits that oligomerize into hexamers to form hemichannels, permitting the passage of molecules between the cytoplasm and the extracellular space. Docking of two hemichannels elaborated by adjacent cells form a single gap junction channel allowing direct cell-cell communication. Here, I hypothesized that Cx-mediated gap junctions and open hemichannels in nonjunctional membranes represent two robust signalling mechanisms by which NPCs may communicate with neighbouring instructive cells and the extracellular environment. In Chapter 2 of this thesis, I show that Cx26, Cx29, Cx30, Cx37, Cx40, Cx43, Cx45 and Cx47 mRNA and protein but only Cx32 and Cx36 mRNA are expressed by primary postnatal hippocampal precursor cells grown as neurospheres. The specificity of immunodetection was confirmed using cultures derived from null-mutant mice controls. I also found that culture conditions affect Cx expression and that the repertoire of Cxs expressed by NPCs changes over the course of differentiation *in vitro*. NPCs grown as monolayers on laminin-coated coverslips expressed Cx26, Cx29, Cx30, Cx36, Cx37, Cx40, Cx43 and Cx45 protein but not Cx32 and Cx47. Every Cx protein examined except Cx32 was found in discrete cell lineages following differentiation to neuronal and glial lineages. In Chapter 3, a series of dye uptake assays were performed that established inducible hemichannel activity in NPCs cultured as adherent neurospheres. I found that this hemichannel activity was lost when neurosphere-derived cells were maintained as proliferating monolayers or induced to differentiate. Conversely, functional gap junction biochemical coupling, as assessed by passage of the anionic dye lucifer yellow, increased following differentiation. Finally, one Cx (Cx29) was chosen to assess the impact

of altering Cx expression on NPC fate. In Chapter 4, Cx29 null-mutation was found to reduce spontaneous astrocytic differentiation following growth factor withdrawal. Together, these observations provide further support for the hypothesis that the repertoire of Cx proteins dictates, in part, a progenitor cell's intrinsic capacity to respond to its microenvironment.

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List of Abbreviations

β-gal	beta-galactosidase
2D	two dimensions
3D	three dimensions
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
ATP	adenosine triphosphate
bps	base pairs
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
Cx	connexin
DCX	doublecortin
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DIV	days *in vitro*
DMEM/F12	Dulbecco's modified Eagle's medium F12
dNTP	deoxynucleotide triphosphate
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
FFA	flufenamic acid
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GJIC	gap junction intercellular communication
GRA	18α-glycyrrhetinic acid
GZA	glycyrrhizic acid
HRP	horseradish peroxidase
IP3	inositol 1,4,5-triphosphate
KDa	kilodalton
KO	knockout
LY	lucifer yellow
MANOVA	multiple analysis of variance
MAP2ab	microtubule associated protein 2ab
NAD^+	nicotinamide adenine dinucleotide
NCAM	neural cell adhesion molecule
NeuN	neuronal nuclei
NG2	NG2 chondroitin sulphate proteoglycan
NPC	neural precursor cell
OPC	oligodendrocyte precursor cell
P	postnatal day
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDGFαR	platelet-derived growth factor α receptor
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
PVDF	polyvinylidene difluoride
RA	retinoic acid
RAR	retinoid acid receptor
RD	rhodamine B isothiocyanate-dextran
RIP	rest in peace
RT-PCR	reverse transcriptase-polymerase chain reaction
RXR	retinoid X receptor
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGZ	subgranular zone
src	sarcoma
SVZ	subventricular zone
Tuj1	beta tubulin class III
WT	wild-type

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Chapter 1: General introduction

1.1 Connexin (Cx)-mediated communication

1.1.1 Brief history

The term gap junction is used to describe a clustered assembly of intercellular membrane channels that provides direct cell-to-cell communication between adjacent cells (104). Morphological and functional evidence for direct intercellular communication preceded the discovery of underlying structural determinants. Cell-to-cell communication and electrical coupling were first reported in invertebrate neurons (50). A series of studies followed in the 1970s identifying gap junction morphology by x-ray diffraction and freeze-fracture methodologies (21, 23, 60, 88). Subsequently, it was demonstrated that this unique type of cell-to-cell communication was also found in vertebrates including mammals (139). In the late 1980s, interest in gap junctions expanded due to technical advances in cellular imaging, molecular biology, electrophysiology, and knockout (KO) strategies facilitating mechanistic study (122).

1.1.2 Hemichannel, gap junction channel, and channel-independent signalling associated with gap junction intercellular channel assembly

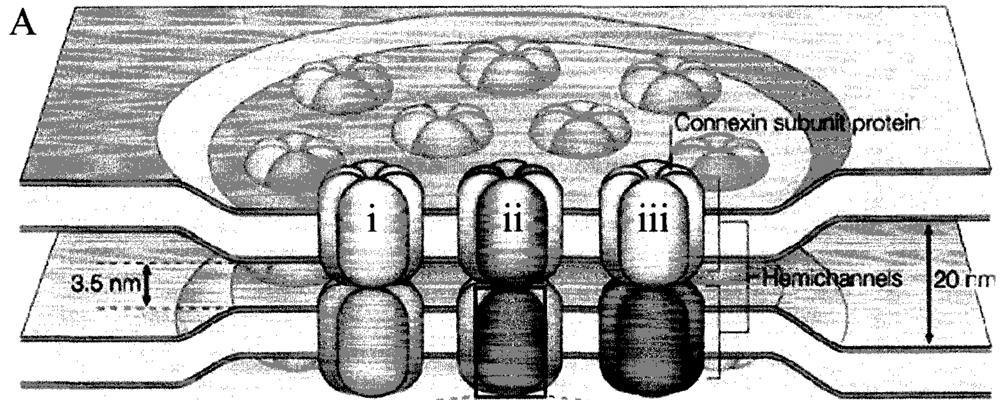
Gap junction channels connect the cytoplasmic compartments of two cells in close apposition providing a direct intercellular communication pathway. Morphologically defined gap junctional plaques may contain up to thousands of these individual intercellular channels. Gap junction channels are formed by docking of two hemichannels (connexons), each made up of oligomers of six Cx subunits, allowing passage of molecules <1 kilodalton (kDa) in size including ions, metabolites and second messengers (reviewed in reference 161). Cxs are a highly homologous family of >20 proteins (161). It is well established that most

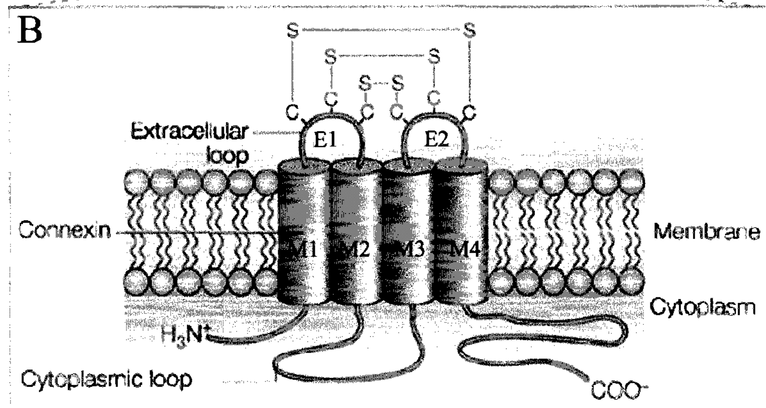
cells express more than one type of Cx and that different cell types may form gap junctions with one another (67). Experiments have also proven that hemichannels with different Cx composition can form functional gap junction channels (161). Thus, docking of two connexons consisting of the same Cx isoform forms a homotypic channel, whereas two homotypic connexons each consisting of a different Cx protein can form a heterotypic gap junction channel (161). Furthermore, alignment of two connexons consisting of multiple Cx proteins forms a heteromeric channel (Figure 1.1A) (161). Each Cx protein comprises four transmembrane domains, two extracellular loops, and three cytoplasmic components (i.e. N-terminus, cytoplasmic loop, and C-terminus) (Figure 1.1B) (136). Interestingly, gap junction channels may not be the only means of Cx-mediated communication. Unapposed hemichannel pores in non-junctional membranes are also likely to permit the passage of molecules between the cytoplasm and the extracellular milieu (reviewed in references 59, 143). Finally, there is accumulating evidence supporting Cx involvement in functions not directly associated with their channel-forming abilities indicating that protein-protein interactions at the C-terminal tail of Cx proteins may initiate intracellular signalling (reviewed in references 56, 143).

1.1.3 The Cx superfamily

Twenty Cx genes have been described in the mouse genome and 21 in the human genome (136). Cx diversity is largely attributed to the size of the C-terminal region and to a lesser extent to sequence variation in the cytoplasmic loops. All other topological regions of Cx proteins are conserved and exhibit a high degree of homology (161). The primary Cx nomenclature is based upon the predicted molecular mass (in kDa). For example, Cx26 refers to a Cx protein with predicted molecular weight of 26 kDa (161). Each of the different

Figure 1.1. Molecular organization of a gap-junctional plaque. A) Gap junction channels are formed by docking of two hemichannels in apposed plasma membrane of adjacent cells. Each hemichannel is composed of six Cx subunits. Three different types of intercellular channels have been reported depending on their molecular composition: homotypic (i), heteromeric (ii) and heterotypic (iii). B) Topological model of a Cx protein. Cx protein subunits are composed of three cytoplasmic domains, four transmembrane domains (M1-M4) and two extracellular loops (E1-E2). This figure was modified from Söhl *et al.*, 2005 using terminology defined by D. Paul (Harvard Medical School).


A
Connexin subunit protein
i
ii
iii
3.5 nm
Hemichannels
20 nm


B
S
S
S
S
S
S
C
C
C
C
C
C
E1
E2
Extracellular loop
Connexin
M1
M2
M3
M4
Membrane
Cytoplasm
H3N+
Cytoplasmic loop
COO-

Cx isoform produces homotypic channels with distinct unitary conductance, molecular permeability, ion selectivity and gating properties (67). This unique identity is modulated by different Cx assortments creating new channel properties that may not be evident in any of the parental homotypic channels (35, 68, 79, 160).

1.1.4 Gap junction intercellular communication (GJIC) and its importance in the mature mammalian brain

Most mammalian cells can communicate with their neighbours via a low-resistance intercellular pathway mediated by gap junctions. This form of communication is described as GJIC. GJIC serves a wide range of physiological roles mediating cell-cell communication through electrical transmission via exchange of ions (Ca^{2+}, K^{+}, Cl^{-} and Na^{+}) as well as biochemical coupling via exchange of small signalling molecules [i.e., cyclic nucleotides, adenosine triphosphate (ATP) and inositol 1,4,5-triphosphate (IP3)] (96). GJIC accounts for the synchronous activity and metabolic cooperation of cells. For example, gap junction channels in the heart play a critical role in maintaining tissue function. They allow electrical coupling between heart cells thereby permitting synchronized contractions of cardiac muscle cells (107, 140). GJIC also plays an important role in the mammalian brain. The majority of cell types in the brain express gap junctions that are regulated during development and differentiation (96). The subset of Cxs expressed and the level of Cx protein expression vary depending on the cell type and the stage of development [see Table 1.1 for details on cellular expression of Cx proteins in the central nervous system (CNS)] (104). Astrocytes are strongly coupled to one another and, to a lesser extent, with oligodendrocytes (3, 100). This heterotypic coupling between astrocytes and oligodendrocytes has been suggested to create a *panglial syncitium* that maintains ion homeostasis during axonal activity. The syncitium

Table 1.1: Cx subtypes and cellular expression in the CNS.

Cx subtype	Cell type	References
26[1]	neuronal precursors	(15)
	astrocyte	(38, 101)
	neuron	(98, 156)
29	oligodendrocyte	(2, 43)
30	astrocyte	(38, 102)
32	oligodendrocyte	(40, 85, 129)
36	neuron	(11, 28, 117, 135)
37[2]	neuron	(24)
	cerebral vasculature (endothelial cells)	(29)
40[2]	developing neuron	(24)
	cerebral vasculature (endothelial cells)	(29)
	astrocyte	(38)
43	neuronal precursors	(15)
	astrocytes	(39, 55, 163)
45[1]	neuron	(93)
	oligodendrocyte	(37, 80)
47	oligodendrocyte	(95, 106)

[1]There are conflicting reports regarding the types of cells that express Cx26 and Cx45
[2]Few studies have corroborated the pattern of expression of Cx37 and Cx40 in neurons and developing neurons, respectively

ensures spatial buffering by transfering ions entering oligodendrocytic cytoplasm from encased neurons to activated astrocytes thereby shuttling the ions from areas of high concentration to areas of lower concentration (3, 103, 151). Moreover, these glial gap junctions can cause a group of individual cells to function in synchrony in terms of metabolism and membrane potential (151). As to neurons, neuronal gap junctions primarily form electrical synapses with one another. Inter-neuronal communication is proposed to contribute to different cognitive processes, such as perception, memory and learning (136).

1.1.5 Hemichannel activity

Until recently it was assumed that extra-junctional connexons remained in a closed state, as their opening could result in membrane depolarization and the depletion of small molecules from the cytoplasm (59). Recent findings indicate that Cx-channels in non-junctional membranes can open under both physiological and pathological conditions, and that opening is physiological or deleterious depending on the situation (12). Basal hemichannel activity is likely very low to prevent non-specific cell lysis (31, 70), but channel opening can be provoked by several stimuli such as reduction in extracellular Ca^{2+} concentration, membrane depolarization, mechanical stress, and intercellular kinase activity (7, 33, 109, 144). Under physiological conditions, hemichannel opening accounts for the release of small molecules, such as ATP or nicotinamide adenine dinucleotide (NAD^{+}) into the extracellular space from Cx43 hemichannels in astrocytes (34, 46, 64, 157). It is also hypothesized that hemichannels serve as a means of uptake of small nutrients present in the extracellular milieu (127). The presence of functional extra-junctional connexons has been established in discrete cellular systems in the CNS such as astrocytes and neurons in the retina (59). Hemichannels can, however, also open under pathological conditions such as

metabolic inhibition contributing to cell loss (32). In a recent study, hemichannel opening in neurons was demonstrated to contribute to the pathological ionic dysregulation during stroke and may be a ubiquitous component of ischemic neuronal death (152).

1.1.6 Cx expression during murine CNS development

During the developmental process, gap junction-mediated communication has been shown to correlate with regional specification, axonal growth, migration, cellular differentiation, and circuit formation (49, 51, 65, 86, 165). Cx26, Cx43 and Cx45 are highly expressed in the embryonic brain, with unique expression patterns (15, 40, 42, 93). Peak Cx26 and Cx43 expression correlates with the onset of maximal neurogenesis in the embryonic period. Moreover, these two Cxs are expressed in both neuronal precursors and radial glial cells in the ventricular zone and their expression varies differentially through the cell cycle (15). These data have led researchers to infer that gap junction-mediated communication between ventricular zone cells regulates cell division (14, 15). Thus, gap junction coupling may play a role in fate determination of neuronal progenitor cells (146). In rodent brain, Cx32 and Cx36 are observed in the first postnatal week while Cx29 and Cx30 proteins are detected by the end of the second postnatal week of life (11, 40, 81, 98, 99). These Cxs appear to be important for postnatal brain development, yet their functional relevance remains unclear (115). In the postnatal period, Cx26 and Cx36 expression underlie neuronal GJIC (11, 98). Conversely, the increase in Cx32 and Cx43 expression correlates with oligodendrogenesis and astrogenesis, respectively (10, 38, 40, 98, 105). It is not clear whether these Cxs are expressed in postnatal progenitor cell populations and what is the impact of their expression on postnatal progenitor cell fate.

1.1.7 Cx gene mutations in disease and experimental investigation

Cx mutations are involved with a variety of CNS disorders, including X-linked Charcot-Marie-Tooth disease (13), a leucodystrophic disorder designated Pelizaeus-Merzbacher-like disease (153), sensorineural hearing loss (76), and congenital cataracts (134). Cx gene mutation analysis has contributed considerably to the current knowledge of the functional roles of Cx proteins.

To provide mechanistic insight, KO strategies have been employed by several investigators to better understand the role of specific Cxs in the CNS (reviewed in references 104, 137). These strategies involve generating Cx-deficient mouse lines in which the Cx gene is deleted ("knocked-out") or replaced by a reporter gene [i.e., beta-galactosidase (β-gal), enhanced green fluorescent protein (eGFP)]. Cx-specific function has also been addressed by replacing one Cx coding region with that of a different Cx cDNA (knock-in) (137). Many of these mice recapitulate disease states to different degrees. For instance, Cx43 null-mutant mice are neonatal lethal due to cardiac malformation (118) and have abnormal migration of neurons in the neocortex (51). Cx32KO mice are viable and fertile but exhibit mild dysmyelination in the peripheral nervous system (PNS) (4, 130). Conversely, Cx26 and Cx45 null-mutant mice are embryonic lethal, thus additional strategies must be followed to investigate the functional relevance of Cx26 and Cx45 in brain development (52, 78).

Cx-deficient mice have proven useful not only in establishing the functional roles of Cxs but also as unambiguous negative controls for the specificity of Cx antibodies, notoriously cross-reactive given the high homology between family members. Cx null-mutant mice with a reporter gene instead of the Cx gene have also proven valuable,

especially as confirmatory proof of cell-specific Cx expression (137). However, a major drawback to studying Cx null-mutant mice is the possibility of functional redundancy between Cxs therefore concealing, in part, the functional importance of the null-mutation as a result of Cx compensation. For instance, mice lacking both Cx32 and Cx47 die by postnatal week six from profound abnormalities in central myelin whereas Cx32 and Cx47 null-mutant mice are viable and do not exhibit any obvious CNS phenotype (95). Unpublished data from our laboratory also demonstrates that Cx32KO mice show compensatory increases in Cx47 mRNA and protein (Meena Na, Honours thesis).

1.2 Neural stem and progenitor cells

1.2.1 Neural stem and progenitor cells are regulated by their microenvironment: definition, function, and identification

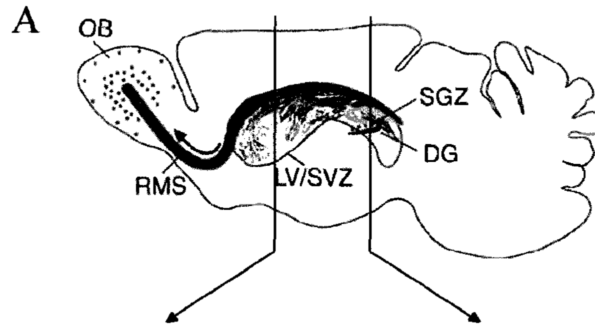
In this study, the Cx expression pattern in early postnatal progenitor cells was analyzed. Neural stem and progenitors cells persist throughout life in the adult brain that can self-renew and give rise to new neurons, astrocytes and oligodendrocytes. For the purpose of this thesis, I define "stem cells" as single cells with the capacity for unlimited self-renewal and multipotentiality; "progenitors" as cells that exhibit limited self-renewal capacity and unipotentiality or multipotentiality; and I use the term "precursor" to refer more generally to either a mixed or unknown proliferating population of neural stem cells and/or progenitors (159). In the adult brain, neural stem cell populations have been reported to reside in the subventricular zone (SVZ) of the lateral ventricles (120, 121) while putative progenitor populations are located in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (53, 108, 131). In the SVZ, commitment to a neuronal lineage involves at least three progenitor subtypes. Multipotential type B progenitor cells, immunopositive for the

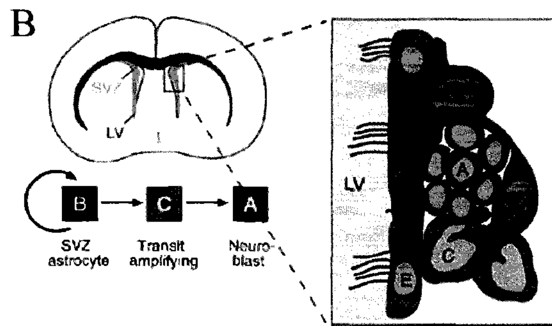
neural precursor intermediate filament nestin and the astrocytic marker glial fibrillary acidic protein (GFAP), produce transit amplifying nestin+/GFAP- type C daughter cells that expand the pool of neuronal progenitors before producing type A neuroblasts. Type A cells migrate along the rostral migratory stream to replenish olfactory bulb neurons (Figure 1.2A,B) (41). In the SGZ, commitment to a neuronal lineage involves only two progenitor subtypes. Multipotential type B cells produces a nestin+/GFAP-/neural cell adhesion molecule (NCAM)+ type D precursor cell which in turn generate granule neurons (Figure 1.2A,C) (48, 132). Neurogenesis, the process by which these neurons are born, is controlled by a combination of intrinsic properties exhibited by stem and progenitor cell populations and extrinsic control by the extracellular microenvironment (159). In this thesis, I begin to examine the role of Cx-mediated communication in the intrinsic capacity of postnatal stem and progenitor cells to respond to the external environment.

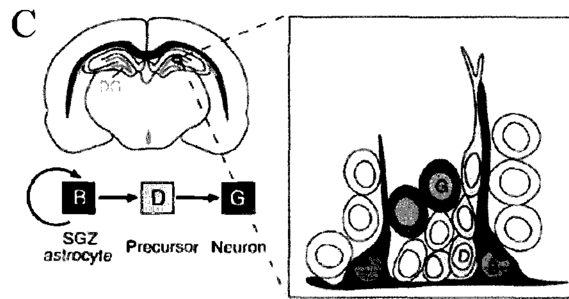
The discovery of neural stem and progenitor cells has raised new hope for neuroregenerative medicine. The ability of these multipotential cells to both self-renew and differentiate into multiple cell types suggests a capacity to repair damaged brain tissue following injury. Neural precursor cell (NPC)-based therapies for nervous system disorders such as multiple sclerosis, stroke, Huntington's disease, Parkinson's disease and spinal cord injury have achieved limited success in experimental models but are still in early stages (91). A comprehensive understanding of the molecular mechanisms regulating NPC fate is essential if we are to validate the potential of cell replacement therapies.

1.2.2 Neurospheres: an *in vitro* model of mouse NPCs suitable for the study of Cx expression and function

Figure 1.2. Neurogenesis in the adult mammalian brain. A) Schematic sagittal section through the adult mouse brain depicting the two sites of adult neurogenesis: the SVZ of the lateral ventricles (LV) and the SGZ of the dentate gyrus (DG). B) Organization and cell types in the adult SVZ. The SVZ is separated from the lateral ventricle by a layer or ependymal cell (E). Type B progenitor cells (SVZ astrocytes) produce transit amplifying type C precursor cells that expand the pool of neuronal progenitors before producing type A neuroblasts. C) Organization and cell types in the adult SGZ. Type B progenitor cells (SGZ astrocytes) produce type D precursor cells, which in turn generate granule neurons (G). OB, olfactory bulb; RMS, rostral migratory stream. This figure was modified from Doetsch, 2003.


A
OB
SGZ
DG
RMS
LV/SVZ

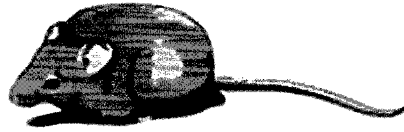

B
SVZ
LV
B
C
A
SVZ astrocyte
Transit amplifying
Neuro-blast
LV
A
C
E


C
DG
B
D
G
SGZ astrocyte
Precursor
Neuron
G
D

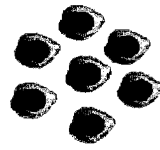
NPCs from postnatal SVZ or SGZ cultured *in vitro* can self-renew in the presence of epidermal growth factor (EGF) (119) and fibroblast growth factor (FGF) (27) forming floating spherical cell clusters termed neurospheres. Each neurosphere can be dissociated into single cells, replated in the presence of growth factors to give rise to new clonally derived neurospheres, thereby demonstrating self-renewal. Upon removal of growth factors and adhesion onto a coated support, cells migrate out of the neurosphere and form layers of differentiated neurons, astrocytes and oligodendrocytes, thereby demonstrating multipotentiality or, possibly, the presence of more than one NPC subtype in the primary neurosphere despite serial dilution (Figure 1.3) (97). Thus, neurospheres represent a simplified *in vitro* model of CNS differentiation from NPCs. The mitogen-responsive cells in the SVZ and SGZ that generate neurospheres are thought to be ultimately derived from type B precursor cells, although not necessarily directly, meaning that they can also be derived from the actively dividing transit-amplifying C cells and the type D intermediate precursor cells in the SVZ and SGZ, respectively (41).

Relevant to this thesis, cells located within embyronically-derived neurospheres are strongly coupled regardless of differentiation time and GJIC has been demonstrated to be essential for viability and differentiation of NPCs in these spherical cell clusters (25, 42). Cells within these neurospheres have also been shown to express Cx43 and possibly Cx45 (17, 25, 42), however expression of other Cx family members in postnatal neurospheres remains uncharacterized. In this study, NPCs were isolated from the hippocampus of postnatal day (P) 0 to P2 mice to explore the role of Cx-mediated communication in the control of NPC fate during early postnatal CNS development.

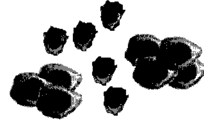
Figure 1.3. Expansion and differentiation of neural stem and progenitor cells as neurospheres *in vitro*. NPCs cultured *in vitro* in the presence of growth factors proliferate to form spherical cell clusters termed neurospheres. Each neurosphere can be dissociated into single cells, replated in the presence of mitogens to give rise to secondary neurospheres. Under differentiating conditions, multipotential cells will differentiate into all three CNS cell types; neurons, astrocytes and oligodendrocytes.



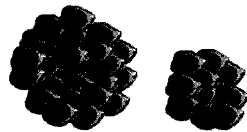
Cells are dissociated into a single-cell suspension and plated in serum-free media containing EGF and/or FGF



Upon dissociation and reseeding, a minority of cells will form secondary neurospheres

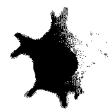


Only mitogen-responsive cells proliferate to form clusters of cells termed neurospheres

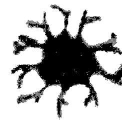


Upon removal of mitogens and adhesion onto a coated support, cells differentiate into CNS cell types

neuron



astrocyte



oligodendrocyte

1.2.3 Differentiation of NPCs in the neurosphere model

Specification of neural stem and progenitor cells during development is influenced by a range of extracellular signalling molecules that act through nuclear or cell surface receptors (147). For instance, the nuclear receptors for retinoids are known to affect many aspect of early CNS development (87). In the rodent brain, retinoic acid (RA), a retinoid, was demonstrated to stimulate neurogenesis of adult hippocampus and postnatal SVZ neural stem cell cultures (147, 158). Studies have shown that withdrawal of growth factors alone is sufficient to stimulate limited neuronal differentiation (108). However, treatment with RA potentiates this process two- to fourfold (108). Moreover, addition of 0.5% serum was shown to increase survival of cells following growth factor withdrawal (147). RA was used in this study to stimulate differentiation of P0-P2 NPCs expanded as neurospheres. Differentiation of NPCs can be monitored by a variety of neuronal and glial markers. *In vivo*, new neurons and glia (oligodendrocytes and astrocytes) are generated through a series of increasingly committed intermediate progeny. For example, neuronal specification can be monitored by the sequential acquisition of markers such as NCAM, beta tubulin class III (Tuj1), microtubule associated protein 2ab (MAP2ab), neuronal nuclei (NeuN) and ultimately high-molecular-weight neurofilament and tau (108). Tuj1, used in this study, is a marker of both committed late neuronal progenitors and newly born postmitotic neurons (108). Oligodendrocyte specification can be examined by the sequential acquisition of markers such as NG2 chondroitin sulphate proteoglycan (NG2), platelet-derived growth factor α receptor (PDGFαR), rest in peace (RIP) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (47, 71, 141). RIP was used in this study as a marker of committed late oligodendrocyte progenitors and mature oligodendrocytes while GFAP was used to identify differentiated astrocytes (47, 110).

1.3 Objectives

Cx-mediated gap junctions and open hemichannels in nonjunctional membranes represent two robust signalling mechanisms by which NPCs can communicate with neighbouring instructive cells and the extracellular environment. *I hypothesize that the repertoire of Cx proteins dictates, in part, a progenitor cell's capacity to respond to competing specification signals.* The repertoire of Cxs expressed by postnatal hippocampal progenitor cells has not been determined. Therefore, the studies presented in this thesis were aimed at identifying the Cxs implicated in regulating the differentiation of postnatal NPCs cultured *in vitro* and to test this hypothesis through null-mutation of one Cx identified in this screen. The overall objectives of this thesis are:

(1) To identify the Cx proteins expressed by actively proliferating wild-type (WT) neurospheres.

(2) To determine whether progenitor cell adhesion to a laminin substrate alters Cx expression.

(3) To establish how Cx expression changes over the course of differentiation to CNS cell types.

(4) To assess whether WT proliferating neurospheres and their progeny are capable of GJIC or functional hemichannel formation in non-junctional membranes.

(5) To determine whether Cx29 play a role in the differentiation of postnatal mouse NPCs.

Chapter 2: Cx expression in WT proliferating progenitor cells and their differentiated progeny

2.1 Introduction

Cxs 26, 43 and 45 are all expressed in embryonic brain, each with its own distinct pattern of expression (15, 40, 42, 93). A number of other Cxs are expressed early in the postnatal period and appear to be important for postnatal brain development (115). The subset and the expression of Cxs vary depending on the type of cell and the stage of development (104). For instance, within the mature neocortex, neurons communicate predominantly via chemical synapses and glial cells network mainly via gap junction channels. However, during early development, gap junctions enable neuronal precursors and immature neurons to communicate with each other (14). Once these developing neurons have adopted their final position within cortex, GJIC is reduce and communication is primarily through chemical synapses (165, 166). *In vitro* studies of embryonic neural progenitors expanded as neurospheres have shown cells within neurospheres to be strongly coupled (42). However, analysis of Cx expression in neurospheres has been limited to Cx43 and Cx45 (17, 25, 42). Characterization of the expression pattern of Cxs in postnatal NPCs when neurogenesis is, for the most part, complete is a necessary prerequisite for understanding the functional relevance of the corresponding Cx-channels in adult cell replacement therapies. Is the Cx expression pattern observed in embryonic NPCs recapitulated in postnatal brain when cells are cultured *in vitro* and is this pattern influenced by culture conditions? Thus, the aim of this study was to establish the full repertoire of Cxs expressed by postnatal NPCs expanded as neurospheres.

2.2 Objectives

The main objective of this chapter was to identify the Cx proteins expressed by WT proliferating neurospheres. Because some spontaneous differentiation occurs over the course of NPC expansion in neurospheres, antigenic assessment of cell lineage was performed to identify the cell type expressing each Cx subtype. NPCs are routinely expanded as neurospheres in suspension or as monolayer colonies following plating on a laminin substrate, thus I also established whether Cx expression differs between these two protocols. The secondary objective of this chapter was to determine whether Cx expression changes over the course of differentiation to CNS cell types.

2.3. Materials and methods

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and all cell culture reagents were obtained from Invitrogen (Burlington, Ontario, Canada) unless otherwise stated.

2.3.1 Animals

WT C57BL/6, Cx29KO, Cx30KO, Cx32KO, Cx36KO, Cx37KO, Cx40KO, Cx45 heterozygous, and Cx47KO mice were used as indicated. Cx37 and Cx40 null-mutant mice were generously donated by Dr. Alex Simon (University of Arizona, Phoenix, AZ), while Cx30KO and Cx32KO were kindly provided by Dr. Klaus Willecke (Universitat Bonn, Germany). Cx29, Cx36, and Cx47 null-mutant mice were originally obtained from Dr. David Paul (Harvard University, Boston, MA). All null-mutant mice were in a C57BL/6 genetic background.

2.3.2 Preparation, growth and differentiation of neurospheres

Adult and P0-P2 WT, Cx29, Cx30, Cx37, Cx40, and Cx47 null-mutant mice were sacrificed by lethal injection with sodium pentobarbital and decapitated. Whole brains were removed by dissection and cerebellums were blocked. The brains were placed rostral-side up and cut on a microtome (Leica VT1000 S, Leica Microsystems Inc., Richmond Hill, Ontario, Canada) into 500 μm coronal section in ice-cold artificial cerebrospinal fluid (aCSF; 26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2.2H_2O$, 1.3 mM $MgCl_2.6H_2O$, 10 mM D-glucose, 1% penicillin/streptomycin). Under a dissecting microscope, the hippocampi were precisely removed from appropriate sections with fine surgical instruments and cleaned free of meninges. Preliminary data using NPCs expanded from adult tissue indicated that yield was too low and growth rates too slow to screen comprehensively Cx expression within the time constraints of this thesis and subsequent studies were confined to analysis of NPCs derived from P0-P2 mice. Hippocampi from a litter of pups (n=3-6 pups) were pooled. Hippocampi were minced with scalpel and enzymatically dissociated in dissociation media (26 mM $NaHCO_3$, 124 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2.2H_2O$, 3.2 mM $MgCl_2.6H_2O$, 10 mM D-glucose, 1% penicillin/streptomycin, 0.1 % neural protease, 0.01 % papain, and 0.01% DNaseI) for 45 minutes at 37°C. A series of trituration and mild centrifugation steps followed to disperse the cells prior to resuspension in expansion medium [Dulbecco's modified Eagle's medium F12 (DMEM)/F12 containing 2 mM L-glutamine, 1% penicillin/streptomycin, 2% B27 supplement, 20 ng/ml human recombinant EGF and 10 ng/ml basic FGF2]. Cells were cultured at 37°C in a 5% CO_2/95% atmosphere, and fresh EGF and FGF2 were added every two days for the first eight days, during which single NPCs

expanded as floating neurospheres. For immunocytochemistry, RNA or protein extraction, neurospheres were allowed to grow for 12 days *in vitro* (DIV) after which they were utilized.

Spontaneous differentiation of NPCs was induced by transferring floating neurospheres grown for eight DIV on laminin (15 μg/ml)-coated glass coverslips in 10 cm Petri dishes and by withdrawal of growth factors. Differentiating NPCs were also cultured in maintenance medium (DMEM/F12 containing 1 mM sodium pyruvate, 200 mM D-glucose, 1% penicillin/streptomycin, 2% N2 supplement) in the presence of RA (0.5 μM) and fetal bovine serum (FBS; 0.5%) for six DIV to promote differentiation, during which medium was changed (half the volume) twice. As a control for cell adherence, neurospheres were plated on laminin in maintenance medium in the presence of EGF (20 ng/ml) and FGF2 (10 ng/ml). Cx expression was assessed by immunocytochemistry.

2.3.3 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of Cx expression in neurospheres

Total RNA was isolated from WT, Cx29KO, Cx30KO, and Cx37KO neurospheres grown for 12 DIV using Trizol® Reagent (Invitrogen) according to manufacturer's recommendations. Total RNA was also isolated from WT, Cx32KO, Cx36KO, Cx40KO, and Cx47KO mice brain. Total RNA was treated with DNaseI (Promega, Madison, WI) to eliminate residual genomic DNA. First-strand cDNA synthesis was performed using random primers (Promega) and Superscript™ II RT (BD Biosciences, San Jose, CA) according to manufacturer's recommendations. The resulting random-primed cDNA template was used to determine expression of several mouse Cx genes by PCR (Table 2.1). The following

Table 2.1: Mouse-specific Cx PCR primer pair sequences and amplicon sizes

Mouse Cx gene	Strand	Primer sequence (5'-3')	Amplicon Size (bps)
Cx26	Sense	GGATGTGGCAGTCAGTATCA	368
	Antisense	TCTTGGCAGGAAGAAGTGTC	
Cx29	Sense	GGTTTTCGGCAATGAT	278
	Antisense	AGAAGCTTGAGGCTTTTAGC	
Cx30	Sense	GCCAGGGTGCAAGAACGTCTGC	535
	Antisense	GGCATGGTTGGGTGGTTTCTC	
Cx32	Sense	GTGGCGTGAATCGGCACTCTAC	593
	Antisense	CTCCGCCACGTTGAGGATAATG	
Cx36	Sense	AGCGGAGGGAGCAAACGAGAAG	533
	Antisense	CTGCCGAAATTGGGAACACTGAC	
Cx37	Sense	AGAGCGGTTGCGGCAGAAAGAGG	551
	Antisense	TGGATGAGAGCCCGTTGTAGGTG	
Cx40	Sense	TTTGGCCAAGTCACGGCAGGG	311
	Antisense	TTGTCACTGTGGTAGCCCTGAGG	
Cx43	Sense	CCTGCCGCAATTACAACAAG	201
	Antisense	AAGGTCGCTGATCCACGATA	
Cx45	Sense	GAGGTGGGCTTTCTAATAGGGCAG	528
	Antisense	ATGGGGGTTGTTTTGGTGATGG	
Cx47	Sense	GCTGGAGGAGATCCACAATCATTC	233
	Antisense	GTGTGGAGATGACCACTATCTGGA	
GAPDH	Sense	TGGTGCTGAGTATGTCGTGGAGT	292

reagents were added to each PCR: for Cx26, 25 pmoles of both forward and reverse primers; for Cx29, Cx40 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 200 ng of both forward and reverse primers; and for all other reactions, 500 ng of both forward primer and reverse primer; 5 μl 10X PCR buffer, 4 μl 10mM deoxynucleotides triphosphates (dNTPs), and 1 mM $MgCl_2$. The PCR was brought up to a final volume of 50 μl with nuclease free water (Promega) and amplified using BD Advantage™ 2 Polymerase (Clonetech, Cambridge, UK) in the Whatman Biometra T-Gradient (Montreal-Biotech Inc., Kirkland, Quebec, Canada) using the following conditions: 94°C for 5 minutes, 35 cycles of 94°C for 25 sec, 59°C for 50 sec, and 72°C for 1 minute 45 sec, and a final incubation at 72°C for 7 minutes.

2.3.4 Protein isolation and Western analysis

NPCs expanded as neurospheres for 12 DIV were washed with 10 mM phosphate buffered saline (PBS; 10 mM sodium phosphate buffer, 154 mM NaCl, pH 7.5), pelleted and resuspended in RIPA buffer [1% Nonidet P-40, 0.5 % sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mM NaF, 50 μg/ml aproprotin, 1 mM sodium orthovanadate, 1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 10 mM PBS]. The lysed cells were incubated on ice for 30 minutes. Lysates were centrifuged for 30 minutes at 12,000 rpm. As positive controls, protein was isolated from WT adult and P0 mice brain, and as a negative control for Cx32 expression, protein from Cx32KO adult mouse brain was isolated. Protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Protein samples (30 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane (Fisher Scientific, Nepean, Ontario, Canada). Membranes were blocked

in 10 mM PBS containing 1% casein for one hour (blocking buffer). Primary antibodies were diluted in the same solution. Membranes were incubated with primary antibodies overnight at 4°C, rinsed twice with 10 mM PBS and twice in blocking buffer for ten minutes and incubated with the appropriate horseradish peroxidase (HRP)-tagged secondary antibody at room temperature for one hour. Following incubation, the membranes were rinsed four times in 10 mM PBS for ten minutes. Immunoreactivity was visualized by enhanced chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Inc., Rockford, IL). Dilutions of primary antibodies were as follows: rabbit anti-Cx26 (Zymed, San Francisco, Ca; 1:100), mouse anti-Cx32 (Zymed; 1:250), mouse anti-Cx43 (Chemicon, Mississauga, Ontario, Canada; 1:1,000) and mouse anti-actin (Sigma; 1:1,000). Secondary antibodies were peroxidase-conjugated anti-mouse IgG (Jackson Immunoresearch, West Grove, PA; 1:2,000) or peroxidase-conjugated anti-rabbit IgG (Jackson; 1:5,000).

2.3.5 Bromodeoxyuridine (BrdU) labelling

To label dividing NPCs, neurospheres grown for 11 DIV were pulsed with BrdU (Roche Diagnostics, Laval, Quebec, Canada; 20 μg/ml) for 24 hours prior to utilizing cells for immunocytochemistry.

2.3.6 Immunocytochemistry

WT, Cx29KO, Cx30KO, Cx37KO and Cx40KO proliferating neurospheres grown for 12 DIV were fixed, cryoprotected in 15% sucrose solution in 10 mM PBS with 0.001% sodium azide and serial sectioned (10 μm) using a cryostat (Leica Microsystems Inc.). For

analysis of WT proliferating monolayer of NPCs and differentiated progeny, neurospheres were differentiated as described above. Immunocytochemistry was performed on cryostat-cut neurospheres, monolayer of neural precursors and differentiated progeny. Cultures were fixed by adding formaldehyde directly in culture media to a final concentration of 3.7%, incubated for 20 minutes, and subsequently washed twice with 10 mM PBS. For BrdU detection, cryostat-cut sections were incubated in 2 N HCl for one hour and neutralized in 0.1 M borate buffer, pH 8.5 for 15 minutes. BrdU incorporation was detected using mouse anti-BrdU (Roche; 6 μg/ml) and a Cy3-conjugated anti-mouse IgG (Jackson, 1:800). For detection of Cxs and lineage analysis, primary antibodies were rabbit anti-GFAP (Sigma; 1:100), Cy3-tagged mouse anti-GFAP (Sigma; 1:800), mouse anti-nestin (Chemicon; 1:50), mouse anti-NCAM (Sigma; 1:400), rabbit anti-NG2 (Chemicon; 1:200), mouse anti-NeuN (Chemicon; 1:100), mouse anti-RIP (Chemicon; 1:1,000), mouse anti-Tuj1 (Research Diagnostics, Inc., Flanders, NJ; 1:250), rabbit anti-Cx26 (Zymed, 1:25), rabbit anti-Cx29 (provided by Dr. David Paul; 1:20), mouse anti-Cx30 (Zymed; 1:25), mouse anti-Cx32 (Zymed; 1 μg/ml), rabbit anti-Cx36 (Zymed; 1:100), rabbit anti-Cx37 (Alpha Diagnostics, San Antonio, Tx; 1:200), rabbit anti-Cx40 (Zymed; 1:50), mouse anti-Cx43 (Chemicon; 1:100), rabbit anti-Cx45 (Chemicon; 1:1,000), rabbit anti-Cx47 (provided by Dr. David Paul; 1:250), rabbit anti-β-gal (Chemicon; 1:2,500). Secondary antibodies were Cy3-conjugated anti-mouse (Jackson; 1:800), Cy3-conjugated anti-rabbit (Jackson; 1:600), fluorescein isothiocyanate (FITC)-conjugated anti-mouse (Jackson; 1:100) or FITC-conjugated anti-rabbit (Jackson; 1:100). Antibodies were diluted in antibody buffer [10 mM PBS, 0.3% Triton X-100, and 3% bovine serum albumin (BSA)]. Hoescht 33258 (Sigma; 2 μg/ml) staining was done according to standard histological procedure. All cell photos were taken

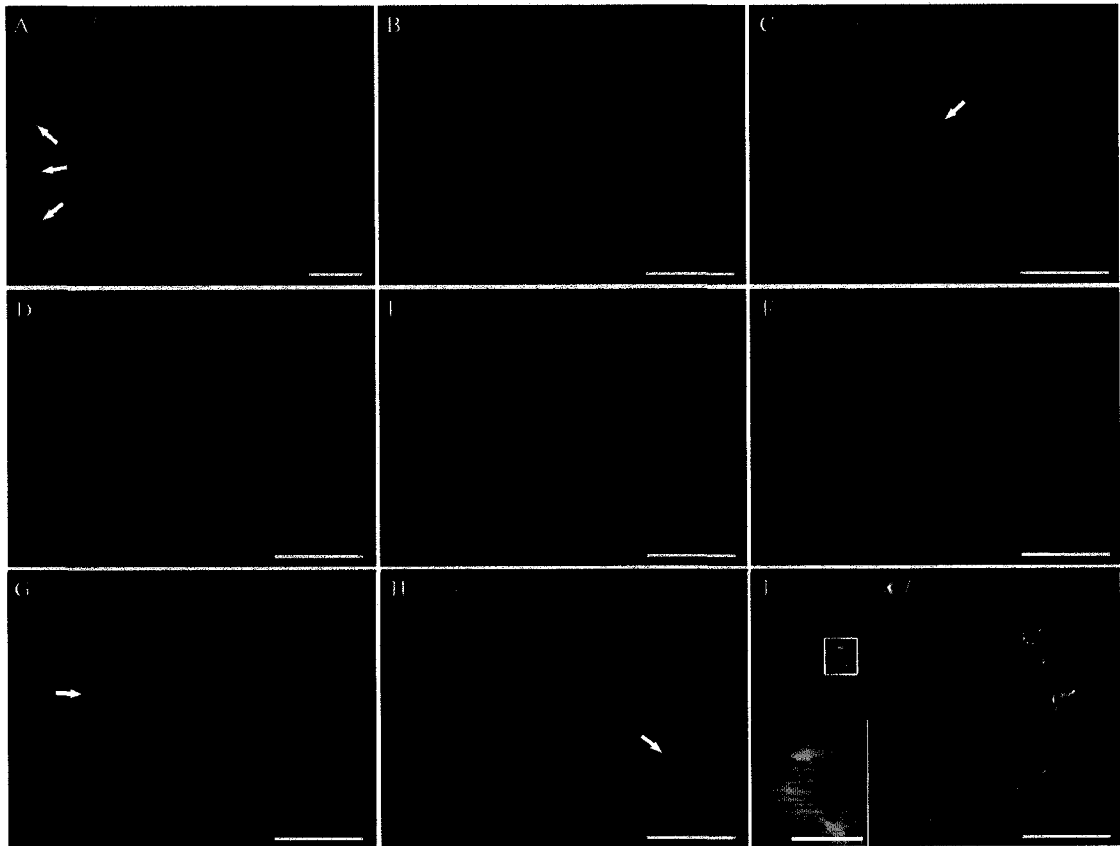
with a Leica DMXRA2 microscope equipped with a Hamamatsu ORCA-ER II camera (Quorum) and immunofluorescence was evaluated using OpenLab Software, version 3.17 (Improvision Ltd, Guelph, Ontario, Canada). Three dimensions (3D) reconstructions were performed as described in Results using Velocity v3.7 software (Improvision).

2.4. Results

2.4.1 Neurospheres contain a heterogeneous mix of NPCs

P0-P2 hippocampi were dissociated and plated as single cells in expansion medium containing 20 ng/ml of EGF and 10 ng/ml of FGF2 for 12 DIV. The EGF- and FGF2-responsive cells proliferated and generated neurospheres, which were cryostat cut and processed for immunocytochemistry. The cellular cytoarchitecture of the neurospheres cultured under the conditions used in this study is depicted in Figure 2.1. It should be noted that postnatal hippocampal NPCs themselves are recognized as a heterogeneous population composed of putatively multipotential nestin+/GFAP+ NPCs as well as more intermediate NG2+ and doublecortin (DCX)+ populations (reviewed in reference 41). In this study, we did not define the identity of each parental NPC prior to expansion but, instead, pooled hippocampi from a number of pups prior to single cell dissociation. Data are representative of the most prevalent morphology observed in at least 3 independent cultures (n=9-18 hippocampi). Sections were obtained from the approximate centres of the neurospheres to assess central core and periphery in two dimensions (2D). BrdU-pulsed neurospheres contain BrdU+ proliferating cells predominantly located at the edge of the spheres (Figure 2.1A, arrows). Cells expressing high levels of nestin were also more numerous at the periphery (Figure 2.1B). NCAM+ neuronal progenitors were present in the core of the spheres and more differentiated Tuj1+ and NeuN+ neurons were absent (Figure 2.1C, arrow;

Figure 2.1. Neurospheres contain a heterogeneous mix of NPCs. Neurospheres are composed of BrdU+ dividing cells located predominately at the periphery (A, arrows), nestin+ NPCs distributed more diffusely throughout the sphere (B), NCAM+ neuronal progenitor cells within the core (C, arrow), GFAP+ astroglia/progenitor cells throughout the sphere (F), and NG2+ progenitor cells and RIP+ early oligodendrocytes at the periphery of the sphere (G,H; arrows). Tuj1+ and mature NeuN+ neurons are not present in neurospheres (D,E). A subset of the GFAP+ population is also nestin+ defining early progenitor cells. These early NPCs are located primarily at the periphery of the sphere (I). Neurospheres were grown for 12 DIV in EGF and FGF2, cryoprotected, serial sectioned (10 μm) and processed by immunocytochemistry. Scale bars, 50 μm; inset in I, 10 μm.



D,E). GFAP+ astroglia were located ubiquitously in the neurosphere section (Figure 2.1F) whereas NG2+ and RIP+ oligodendrocyte progenitors were located at the edge of the spheres (Figure 2.1G,H; arrows). To investigate if any GFAP+ cells were also nestin+ indicative of type B multipotential early NPCs, GFAP and nestin double staining was performed. Results indicate that a subset of the GFAP+ population appears to also be nestin+ along the periphery of the neurosphere (Figure 2.1I).

Our 2D analysis provided initial spatial localization in sections processed from the approximate centre of the neurosphere. Next, we investigated whether this spatial organization remained consistent throughout the neurosphere. NG2+ progenitors were analyzed. Neurospheres were sectioned serially, stained with Hoescht and immunoreacted for NG2, imaged in OpenLab, and registered as serial stacks in Adobe Photoshop. These stacks were imported into Velocity v3.7, deconvolved, rendered, and reconstructed in 3D (Improvision). Data are presented in Supplemental Movie 1 (Appendix 1, appended on CD-ROM as an interactive QuickTime movie). Surprisingly, we found that the majority of NG2+ cells localized to the anterior section of the neurosphere above the midline with a second population enriched at the far posterior pole. Note in the interactive animation how the majority of NG2+ cells (green) are located along the anterior face of the sphere (i.e., in closest proximity to the media interface at the time of freezing).

2.4.2 EGF- and FGF2-responsive cells differentiate into neurons, astrocytes and oligodendrocytes

Neurospheres grown for eight DIV were plated on laminin-coated dishes in maintenance medium and in the presence of RA and FBS to promote differentiation. Neurospheres attached to the bottom of the plate and began to develop short processes.

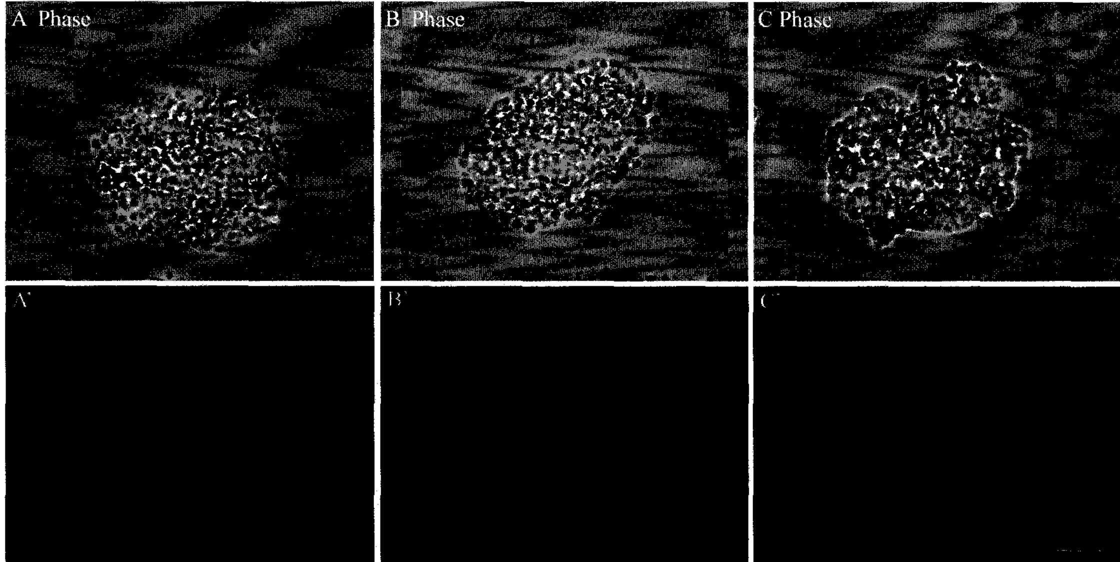
Within one day after plating, large numbers of cells had spread out from the centre of the sphere with diverse morphologies. Immunocytochemistry was performed six days after plating (for a total of 14 DIV) revealing the presence of all three CNS cell types, GFAP+ astrocytes (Figure 2.2D,D'), Tuj1+ neurons (Figure 2.2E,E') and RIP+ oligodendrocytes (Figure 2.2F,F'). To assess whether cell adherence to the laminin substrate alone promoted differentiation, neurospheres were plated in maintenance medium under proliferating conditions (20 ng/ml EGF and 10 ng/ml FGF2). Immunocytochemistry also revealed the presence of all three CNS cell types under these conditions (data not shown, see Chapter 4 for quantification). Based on this observation, subsequent experiments were performed using NPCs labelled with BrdU prior to plating to distinguish between NPC progeny that had spontaneously differentiated during expansion of floating neurospheres and the progeny of actively proliferating NPCs that differentiated following exposure to laminin (see Chapter 4).

2.4.3 WT Primary NPCs cultured as neurospheres express Cx26, Cx29, Cx30, Cx37, Cx40, Cx43, Cx45 and Cx47 protein but not Cx32 and Cx36

Cx26, Cx29, Cx30, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, and Cx47 protein and mRNA expression in WT NPCs, were assessed by immunocytochemistry and RT-PCR, respectively. In some cases, immunocytochemistry in transgenic NPCs, verifying comparable cellular expression of transgenic markers substituted for the gene of interest *or* Western blotting was performed to validate immunofluorescence analyses. Negative controls included relevant KO controls as described below.

We found that WT proliferating neurospheres express Cx26 and Cx43 protein and mRNA (Figure 2.3A,F). Cx26 and Cx43 null-mutant mice are embryonic lethal and neonatal lethal, respectively thus, appropriate KO negative controls were not available (52, 118).

Figure 2.2. NPCs differentiate into neurons, astrocytes and oligodendrocytes. Proliferating neurospheres (A, B, C) express GFAP (A'), very low levels or RIP (C') and do not express Tuj1 (B'). Upon adhesion to a laminin-coated substrate in the presence of RA and FBS, NPCs are induced to differentiate into the three main cell types of the CNS, astrocytes, neurons and oligodendrocytes demonstrated by GFAP+ (D'), Tuj1+ (E') and RIP+ (F') cells, respectively. Scale bars, 50 μm.



Laminin (RA + FBS)

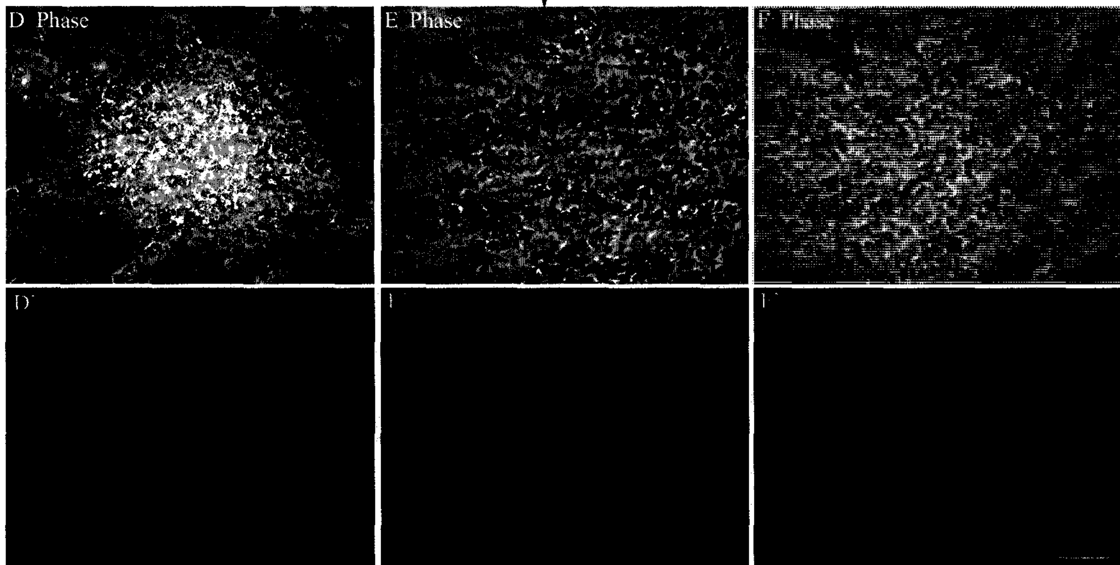
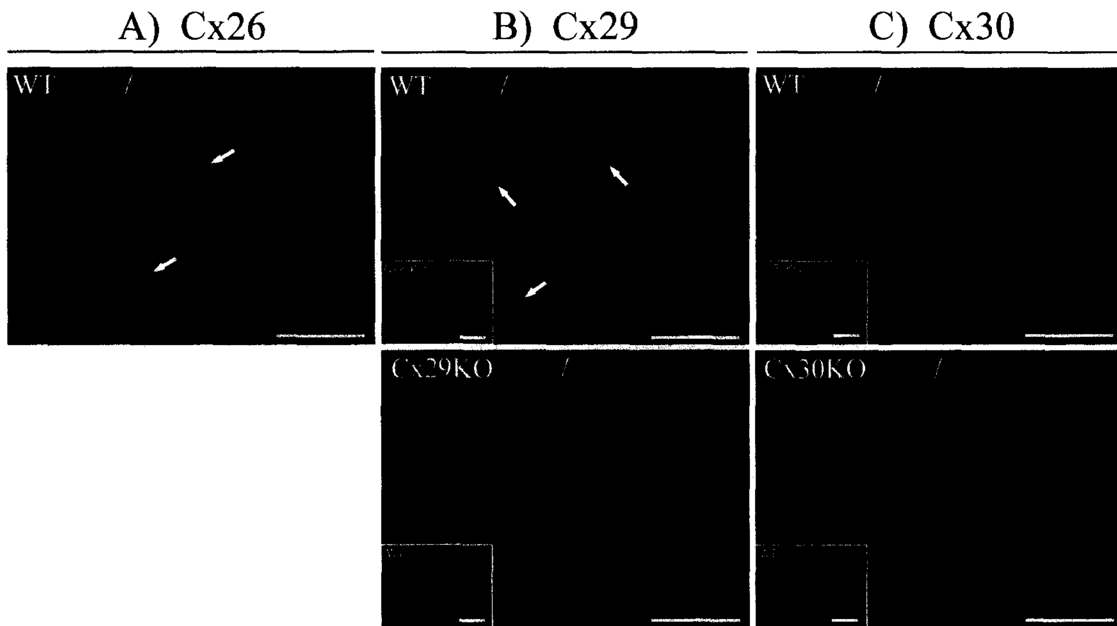
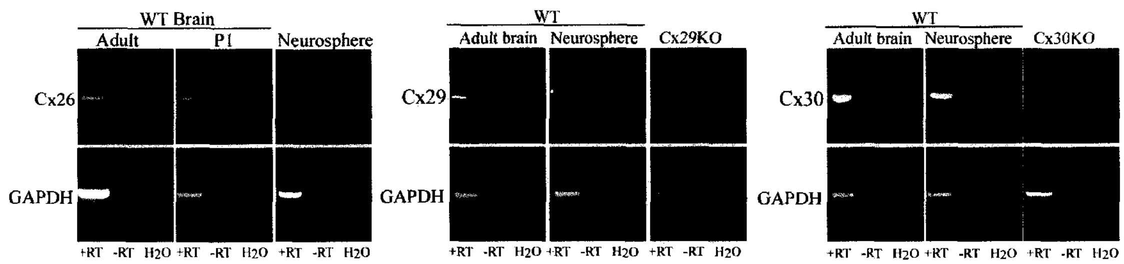


Figure 2.3. Primary NPCs cultured as neurospheres express Cx26 (A), Cx29 (B), Cx30 (C), Cx37 (D), Cx40 (E), Cx43 (F), Cx45 (G), and Cx47 (H) protein and mRNA. Neurospheres are grown for 12 DIV in EGF and FGF2, and processed for immunocytochemistry or RT-PCR. Cxs that show positive immunofluorescence in WT but not KO cultures (insets), verified by RT-PCR (bottom panels), and, where possible, by comparable cellular expression of transgenic markers substituted for the gene of interest (lower micrographs), were scored as positive. Arrows indicate positive cells. Scale bars, 50 μm. NPCs do not express Cx32 or Cx36 protein although mRNA is present (Appendix 2).



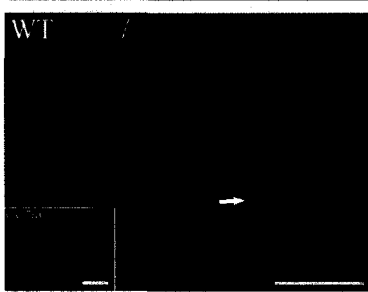
A) Cx26
B) Cx29
C) Cx30
WT
WT
WT
Cx29KO
Cx30KO

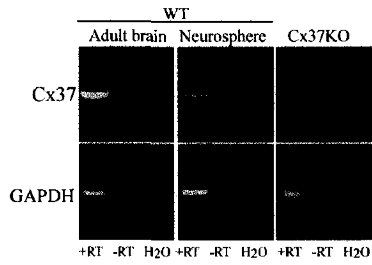


WT Brain
Adult
P1
Neurosphere
Cx26
GAPDH
+RT -RT H2O +RT -RT H2O +RT -RT H2O
WT
Adult brain
Neurosphere
Cx29KO
Cx29
GAPDH
+RT -RT H2O +RT -RT H2O +RT -RT H2O
WT
Adult brain
Neurosphere
Cx30KO
Cx30
GAPDH
+RT -RT H2O +RT -RT H2O +RT -RT H2O

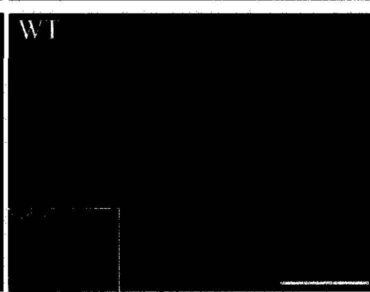
Figure 2.3 continued. Primary NPCs cultured as neurospheres express Cx26 (A), Cx29 (B), Cx30 (C), Cx37 (D), Cx40 (E), Cx43 (F), Cx45 (G), and Cx47 (H) protein and mRNA. Details are described on page 29. See Appendix 2 for evidence of mRNA but not protein expression of Cx32 and Cx36.

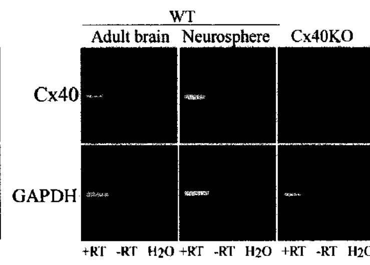
D) Cx37



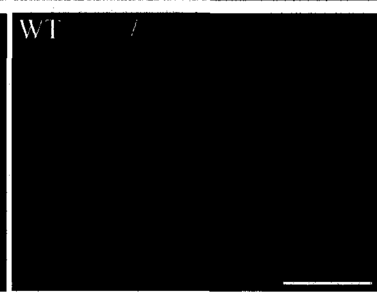


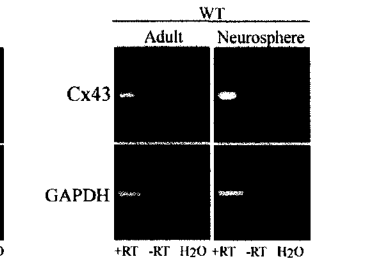
E) Cx40



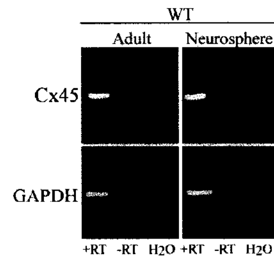


F) Cx43

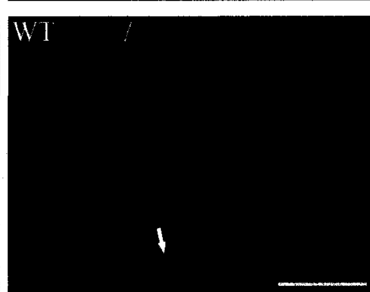


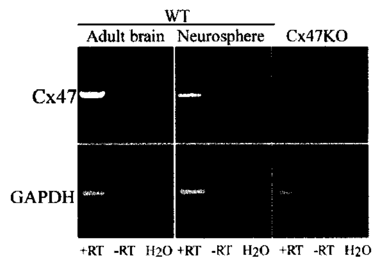


G) Cx45



H) Cx47





Positive results were instead, validated by Western blotting (Appendix 2C,D). Cx29, Cx30, Cx37 and Cx40 protein and mRNA are also expressed in WT neurospheres and appropriate KO controls were available to confirm antibody specificity (Figure 2.3B-E). In addition, comparable cellular expression of transgenic markers (β-gal) substituted for Cx29 and Cx30 was verified in Cx29KO and Cx30KO mice, respectively (Figure 2.3BC). Likewise, Cx45 and Cx47 protein and mRNA are expressed in our WT neurospheres, however negative controls were not available in time for this thesis but antibody specificity will be confirmed for upcoming publication (Figure 2.3G,H). Conversely, Cx32 and Cx36 mRNA but not protein was detected in neurospheres (Appendix 2A,B).

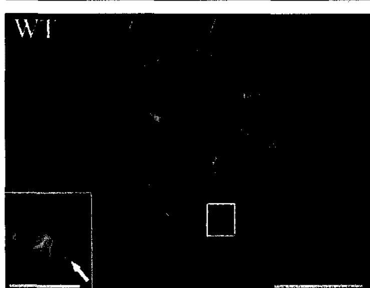
2.4.4 Cellular identity of Cxs in neurospheres

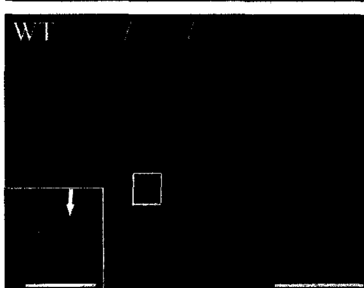
To establish the cellular identity of Cxs in NPCs, triple immunostaining was performed using Cx antibodies, lineage markers, and the nuclear marker Hoescht 33258. Cx26 was detected in a subset of nestin+ cells and a subset of GFAP+ cells (Figure 2.4A, arrows). On the other hand, Cx29 was localized to a subset of NG2+ oligodendrocyte precursors and some Cx29+ cells were detected adjacent to nestin+ cells (Figure 2.4B, arrows). Cx30 and Cx43 were found in GFAP+ astroglia (Figure 2.4C,F) and surprisingly Cx30, Cx37, Cx40 and Cx45 were all detected in a subset of nestin+ cells (Figure 2.4C-E,G). Finally, Cx47 was localized to RIP+ late oligodendrocyte progenitors (Figure 2.4H).

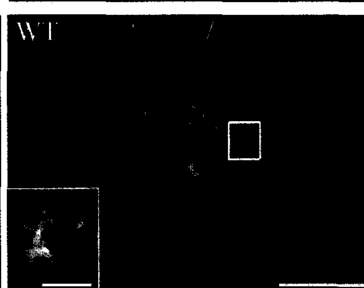
We used 3D reconstruction again to investigate the pattern of Cx29 and NG2 co-localization throughout the neurosphere (Appendix 1, Supplementary Movie 2). Note in the interactive animation how the majority of NG2+ cells (green) are Cx29+ (red) along the anterior face of the sphere (i.e., in closest proximity to the media interface at the time of

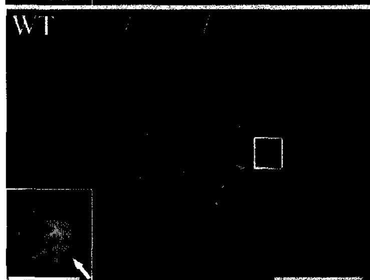
Figure 2.4. Cellular identity of Cxs in neurospheres. Neurospheres are grown for 12 DIV in EGF and FGF2, cryoprotected, serial sectioned (10 μm), and processed for immunocytochemistry. Cx26 localizes to nestin+ and GFAP+ cells (A, arrows), Cx29 localizes to a subset of NG2+ oligodendrocyte precursor cells and Cx29+ cells lie adjacent to nestin+ cells (B, arrows), Cx30 and Cx43 are detected in GFAP+ astrocytes (C, F), Cx30, Cx37, Cx40 and Cx45 all localize to a subset of nestin+ NPCs (C-E,G), and Cx47 localizes to RIP+ late oligodendrocyte progenitors (H). Scale bars, 50 μm; inset in photomicrographs, 10 μm.

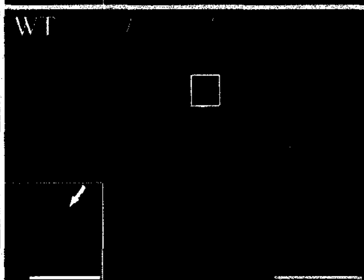
A) Cx26 B) Cx29 C) Cx30

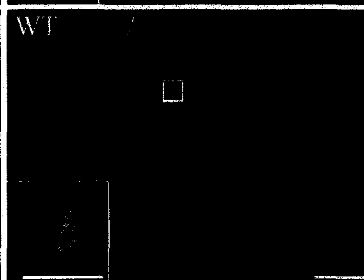




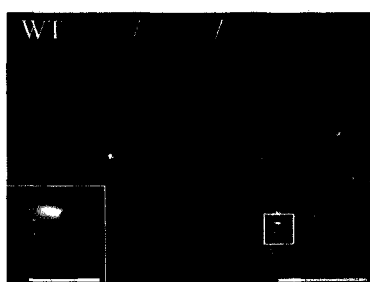


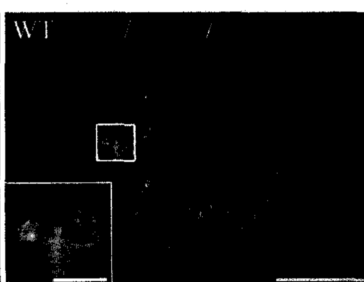


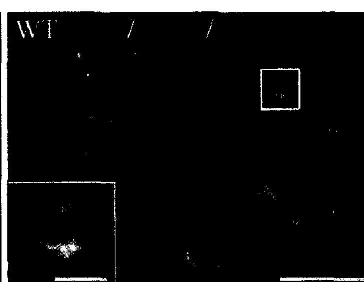




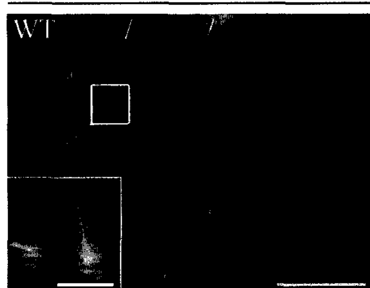
D) Cx37 E) Cx40 F) Cx43

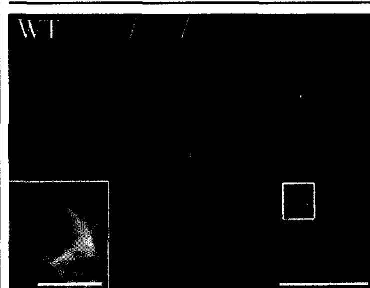






G) Cx45 H) Cx47





freezing) and at the posterior pole of the sphere (i.e., in closest proximity to the bottom of the tissue culture plate). Interestingly, the majority of cells located at the periphery of the sphere but in the posterior section of the neurosphere (i.e. from midline to just above the posterior pole along the Z-axis) are not co-localized rather Cx29+ cells lie adjacent to NG2+ cells below the midline.

2.4.5 Cx expression in WT NPCs' differentiated progeny

A different set of experiments was performed to investigate Cx expression following differentiation of neurospheres. In these experiments, hippocampal NPCs from P0-P2 WT mice were grown for eight DIV in non-adherent cell culture plates and plated on laminin-coated plates for six DIV in a defined medium free of serum in the presence of RA and FBS to yield differentiated cells. To determine whether plating cells on a laminin substrate alone alters Cx expression, neurospheres were plated on laminin in the presence of EGF and FGF2 hence, generating a monolayer of proliferating NPCs. Cx expression was assessed by immunocytochemistry. We found that expression of Cx26, Cx29, Cx30, Cx37, Cx40, Cx43 and Cx45 was maintained upon plating to a laminin substrate under proliferating conditions and differentiating conditions (Figure 2.5A-C,F-I; arrows). Conversely, Cx36 protein, absent in our WT proliferating neurospheres (Appendix 2), was detected upon exposure to laminin (Figure 2.5E, arrows). We were unable to maintain cells on glass coverslips in the absence of laminin to be able to perform immunocytochemistry to establish whether induction of Cx36 protein was triggered by laminin/integrin signalling or by adherence in and of itself. Cx32 protein expression remained undetected following monolayer culture (Figure 2.5D). Finally, Cx47 protein expression was absent in NPCs grown as monolayers and reappeared following differentiation (Figure 2.5J, arrows).

Figure 2.5. Proliferating NPCs (laminin, EGF + FGF2) and differentiated progeny (laminin, RA + FBS) cultured as monolayers express Cx26 (A), Cx29 (B), Cx30 (C), Cx36 (E), Cx37 (F) Cx40 (G), Cx43 (H), Cx45 (I) but not Cx32 (D) protein. Cx47 is detected in neurospheres (Figure 2.3) but not proliferating adherent NPCs. Cx47 is detected again upon differentiation (J). Neurospheres were grown for 8 DIV, plated on laminin-coated plates for six days in the presence of EGF and FGF2 *or* RA and FBS and processed for immunocytochemistry. Arrows indicate positive cells. Scale bars, 50 μm.

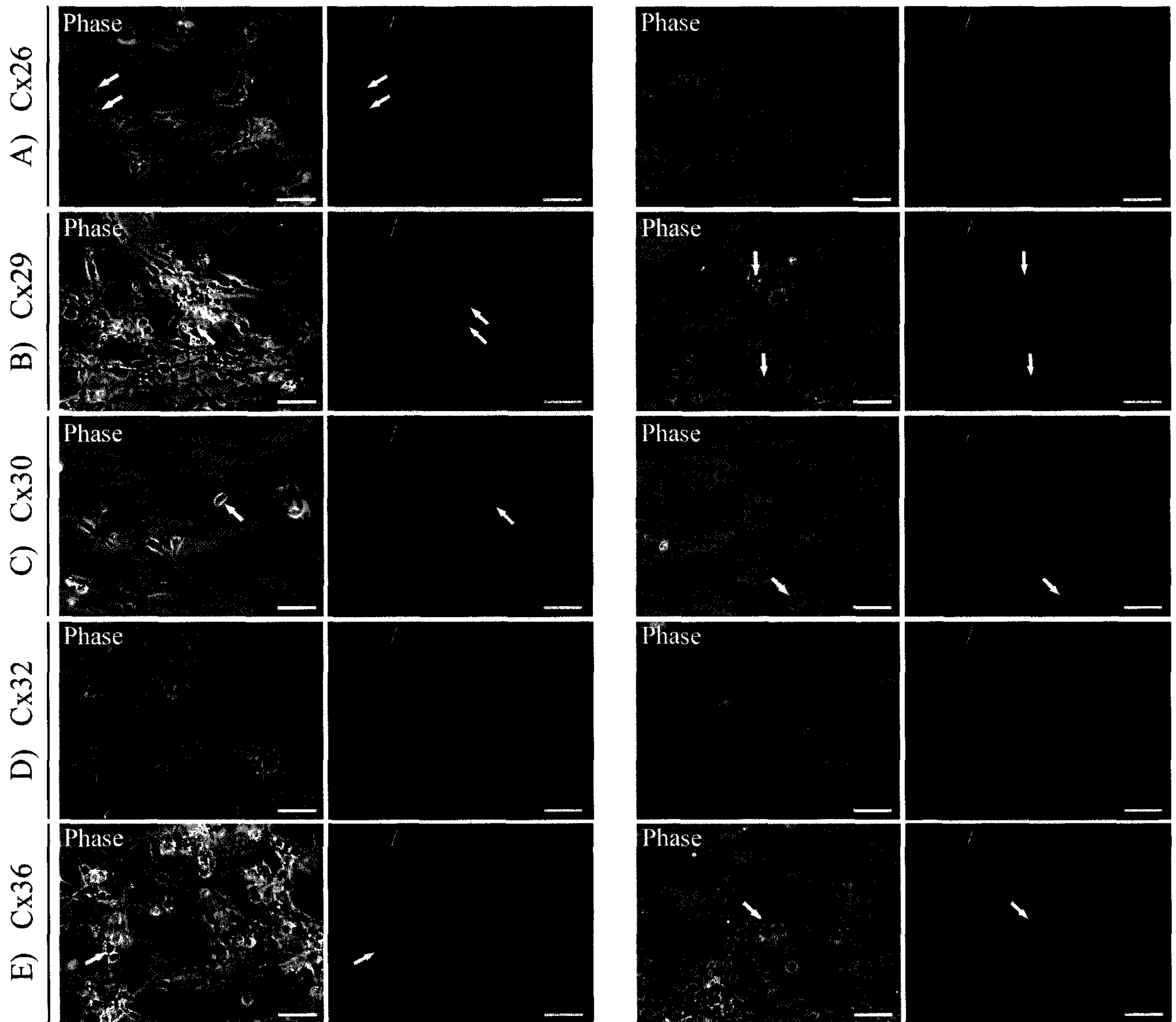
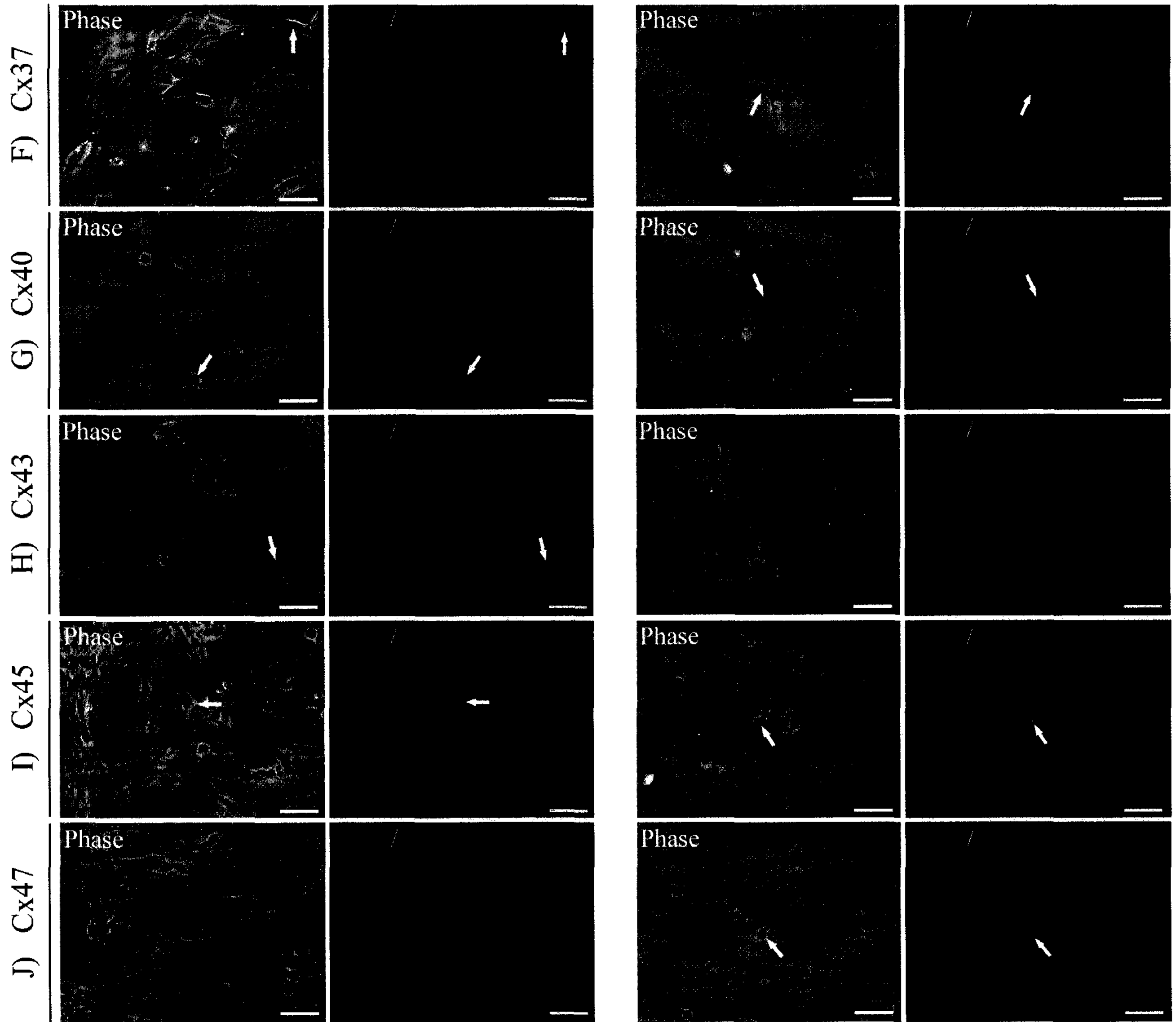

Laminin (EGF + FGF2)
Laminin (RA + FBS)
A) Cx26
B) Cx29
C) Cx30
D) Cx32
E) Cx36
Phase

Figure 2.5 continued. Proliferating NPCs (laminin, EGF + FGF2) and differentiated progeny (laminin, RA + FBS) cultured as monolayers express Cx26 (A), Cx29 (B), Cx30 (C), Cx36 (E), Cx37 (F) Cx40 (G), Cx43 (H), Cx45 (I) but not Cx32 (D) protein. Cx47 is detected in neurospheres (Figure 2.3) but not proliferating adherent NPCs. Cx47 is detected again upon differentiation (J). Details are described on page 34.



Laminin (EGF + FGF2)
Laminin (RA + FBS)
F) Cx37
G) Cx40
H) Cx43
I) Cx45
J) Cx47
Phase
Phase
Phase
Phase
Phase
Phase
Phase
Phase
Phase
Phase

2.5 Discussion

Cell-to-cell communication through Cx-channels provides a powerful means of intercellular signalling that participates in many aspects of mammalian development (reviewed in reference 146). In almost all regions of the brain, Cxs are already expressed at very early stages of development and they appear to be involved in many processes including neurogenesis, migration, cellular differentiation and synapse formation (49, 51, 65, 86, 165). To gain insight into the basic features of Cx expression in NPCs and differentiated progeny, I used a neurosphere system as a simplified *in vitro* model of CNS differentiation. The present data contributes further insight into the importance and complexity of Cx expression over the course of NPC *in vitro* culture and differentiation.

Under the conditions used in this study, our neurospheres contain a core of differentiating neuronal progenitors and astroglia surrounded by oligodenrocyte precursors and proliferating NPCs. Remarkably, a subset of these NPCs appear to be nestin+/GFAP+ type B progenitors. These results are in agreement with a recent study demonstrating the expression of more differentiated cells in the core of the sphere surrounded by proliferating undifferentiated NPCs on its outside edge (19). SGZ astrocytes (type B progenitor cells) are the primary precursors that give rise to granule neurons in the postnatal dentate gyrus of the hippocampus through the intermediate D cells (132). These GFAP+ type B progenitors cells were also shown to be immunopositive for nestin (48) hence, I propose that most of our neurospheres are generated from type B nestin+/GFAP+ NPCs residing in the postnatal hippocampus as this phenotype was detected in >95% of all neurospheres analyzed. It cannot be definitively ruled out, however, that the multiple cell types (astrocytes, neurons, and oligodendrocytes) are generated by different progenitor populations given the presence of NG2+ cells in our cultures, which have also been shown to display multipotent stem cell-

like properties (8). In a separate series of experiments performed by my colleagues, serial dilutions in 96 well plates were performed to ensure that each neurosphere was derived from a single cell. The same phenotypes were observed. Together, these data provide strong evidence that the putative nestin+/GFAP+ neurosphere generating NPCs are also shown to be multipotent and likely generate the intermediate progeny and, upon plating to a laminin substrate, the three terminally differentiated CNS cell types, astrocytes, oligodendrocytes and neurons.

When this hypothesis is placed in context with the 3D modelling of our neurospheres, a number of interesting speculations can be generated that will, of course, require further quantitative study. Surprisingly, we found that NG2+ progenitors, localizing primarily to the periphery of expanding neurospheres along the central axis in 2D analyses, demonstrated anterior/posterior polarity in 3D reconstructions. The majority of NG2+ cells were localized anterior to the midline, in closest proximity to the media/air interface at the time of freezing. We cannot state with certainty that this position was maintained throughout the 12 DIV but it is tempting to speculate that this initial observation reflects the influence of (a) the sheer force placed on a cell depending upon its position within the neurosphere and/or (b) the differential force exerted on the membranes of adjacent cells on the intrinsic capacity of a nestin+/GFAP+ hippocampal NPC to adopt an NG2+ lineage. Based on the protocol developed in this thesis, future studies will pursue an empirical assessment of spatial-structural determinants of NPC fate using 3D-modeling of neurosphere development over time.

Next, a collection of Cx genes, commonly expressed in the developing mammalian brain, was investigated for mRNA and protein expression in WT P0-P2 NPCs under a variety of culture conditions. Cx26 and Cx43 expression in our neurosphere model was not

surprising considering that these Cxs are expressed in rodent brain as early at embryonic day 12 and 14, respectively and are widely expressed in GFAP+ astroglia and progenitor cells (15, 40, 42, 104). In agreement with these studies, Cx26 localized to a subset of nestin+ NPCs and a subset of GFAP+ astrocytes whereas Cx43 expression localized to GFAP+ astrocytes in WT neurospheres. Further studies will aim at elucidating whether Cx26 and Cx43 are more specifically localized to the type B progenitors found in this model. Conversely, Cx29 and Cx30 protein expression in neurospheres was unexpected. It has been reported that significant levels of Cx29 and Cx30 protein are not expressed in rodent brain until P14 and P16, respectively (81, 99), although Cx29 mRNA has been detected in P1 cerebellum (2). Kunzelmann et al. proposed that the late onset of Cx30 expression in astrocytes, when compared to Cx26 and Cx43, indicates that this protein is required by mature not developing astrocytes (81). In support of this hypothesis, Cx30 was clearly localized to a subset of astrocytes in neurospheres. Thus, expression of Cx30 in neurospheres may represent spontaneous specification to a mature astrocytic phenotype. However, a subset of nestin+ cells were also shown to express Cx30. A colleague in the Bennett laboratory, Hadi Toeg, has demonstrated that Cx30 is expressed by proliferating nestin+ progenitor cells in the adult hippocampus suggesting a potential role for Cx30 not only in mature astrocytes but also in postnatal NPCs (Hadi Toeg, Honours thesis). Triple-labelling of neurospheres for GFAP, nestin, and Cx30 will be required to confirm the identity of Cx30 cells as astrocytes and/or Type B cells. Similarly, Cx29 expression in neurospheres may be explained by the array of cells expressed in these spherical cell clusters. For instance, our data suggests that Cx29 localizes to NG2+ oligodendrocyte precursor cells (OPCs). As to Cx32, this gap junctional protein is first observed towards the end of the first postnatal week closely corresponding with the time course of oligodendrocyte maturation

(40, 98) while Cx32 mRNA is expressed as early as P0 (115). The results presented here are consistent with these studies. Cx36 protein is widely expressed in mouse brain in the first postnatal week and is expressed only in mature neurons (11, 135). Thus, the absence of mature neurons in our neurosphere model explains the absence of this Cx. Very few studies have examined Cx37 and Cx40 expression in the developing CNS. Cx37 and Cx40 mRNA are present at P0 in rats and both peak at the end of the second week of postnatal life (115) and Cx37 is expressed in NT2 teratocarcinoma progenitor cell line (16). These studies corroborate Cx37 and Cx40 expression in our neurosphere model. Cx45 protein is detected in the embryonic mouse brain as early as E18.5 with its expression peaking at P1 and declining subsequently (93) therefore, it is not surprising to find Cx45 protein in our P0-P2 NPC derived neurospheres. As to Cx47, no studies to date have characterized the pattern of Cx47 expression through development hence, we are the first to report Cx47 expression in postnatal RIP+ late oligodendrocyte progenitors in postnatal neurospheres. Finally, this study also marks the first report showing Cx30, Cx37, Cx40 and Cx45 expression by nestin+ cells derived from postnatal tissue. To date, Cx37 and Cx40 have been observed in cerebral motor neurons and developing neurons of the spinal cord, respectively and both Cxs have been detected in cerebral endothelial cells (24, 29). Despite these studies, their pattern of expression in the brain remains poorly characterized. On the other hand, accumulating evidence suggest that Cx45 is expressed in neurons (92, 93). Further studies will be aimed at corroborating our findings *in vivo*.

To evaluate changes in Cx expression during differentiation of NPCs, Cx protein expression was evaluated in WT monolayer of proliferating NPCs, as a control for cell adherence, and WT differentiated progeny. According to our results, cell adhesion to a laminin substrate alters Cx36 expression. I propose that the emergence of Cx36 expression

in monolayer of neural precursors and differentiated progeny is due to the appearance of significant numbers of Tuj1+ neurons in these cultures (>3%) (see Chapter 4). Conversely, Cx32 protein expression remains absent upon adhesion to a laminin substrate and in differentiated progeny. Studies in the Bennett laboratory have shown that Cx32 protein is expressed by a subset of postnatal hippocampal NG2+ progenitor cells when they terminally commit to an oligodendrocyte lineage, specifically non-myelinating oligodendrocytes (94). Cx32 expression has also been reported in large myelinated fibers (77). Due to the absence of Cx32 protein in all our cultures, either this particular subset of cells is lacking from our cultures or that the NG2+ cells fail to commit to non-myelinating or a large fiber phenotype characteristic of mature Cx32+ oligodendrocytes. To pursue this hypothesis, other researchers in the Bennett laboratory are investigating whether Cx32 protein can be induced under culture conditions that specifically promote oligodendrogenesis. As to Cx47, this protein is not expressed upon adhesion to a laminin substrate but re-emerges under differentiating condition. This observation could reflect an inhibitory influence of laminin on Cx47 expression or, more likely, the failure of Cx47+/RIP+ cells in the neurospheres to survive the plating process.

Although the presence of Cx43 has been previously reported in neurospheres derived from embryonic NPCs, this study marks the first report demonstrating Cx26, Cx29, Cx30, Cx37, Cx40, Cx43, Cx45 and Cx47 expression in postnatal NPCs expanded as neurospheres and differentiated progeny. This study also highlights the cellular expression of these Cxs within proliferating neurospheres. Given that Cx identity underlies the permeability, ion selectivity, electrical conductance, and gating properties of Cx-channels, the finding that NPCs and differentiated progeny differentially express Cx proteins predicts that communication through gap junctions and/or hemichannels will differ between these two

cultures and thus be of functional significance during the process of differentiation. This hypothesis was tested in Chapter 3 of this thesis.

Chapter 3: Functional Cx-mediated hemichannel activity and GJIC changes over the course of NPC differentiation *in vitro*

3.1. Introduction

Cx-mediated communication can connect two adjacent cells or a cell and its extracellular environment through GJIC or functional hemichannels in non-junctional membranes, respectively. As reviewed in Chapter 1, hexameric assemblies of Cxs form connexons (hemichannels) anchored in the plasma membrane. These hemichannels are gated by several stimuli, including negative membrane potentials, high concentrations of extracellular Ca^{2+}, and intercellular H^{+} ions, various pharmacological agents and protein phosphorylation (12, 127). Hemichannel activity in the CNS has been recorded for many Cx proteins including Cx26, Cx30, Cx32, Cx43 and Cx45 thereby facilitating passage of metabolites and second messengers to and from the extracellular milieu (1, 31, 74, 154, 155). Cx-channels differ in their permeability to dye and tracer molecules with specificity dictated by the Cx making up each hemichannel. For instance, Cx26, Cx32, Cx43 and Cx45 channels are all permeable to a variety of fluorescent dyes including lucifer yellow (LY) whereas Cx30 channels are impermeable to LY (20, 90, 127). Very few studies have explored hemichannel activity in CNS development. A recent study demonstrated functional hemichannels in non-junctional membranes in NT2/D1 progenitor cells and a loss of hemichannel activity in differentiated cells (16). Human NT2/D1 cells are a clonal teratocarcinoma progenitor cell line that can be induced to differentiate terminally into functional neurons, astrocytes, oligodendrocytes and non-neuronal cells (6). Here, we sought to expand these findings to an analysis of primary postnatal NPCs.

When connexons of adjacent cells align, they give rise to a single axial channel linking two cells. Clusters of these intercellular channels form a gap junction. These

channels serve a wide range of physiological roles mediating cell-cell communication through electrical transmission as well as through chemical diffusion of small molecules (96). Electrical transmission by these channels is defined by the direct exchange of ions between cells, thereby synchronizing electrical activity between neurons, whereas biochemical coupling describes the exchange of metabolites and second messengers between cells (123). Generally, during development, there is a progressive decrease in GJIC, although gap junction coupled-glial and neuronal cells persist in many brain regions in adults (reviewed in reference 146). The correlation between changes in Cx expression and differentiation suggests that early GJIC permits signalling molecules to diffuse between NPCs and may play a role in the specification of NPCs to terminally differentiated CNS cell types (122). Recently, Duval and colleagues demonstrated GJIC in neurospheres expanded from mouse embryonic NPC by performing microinjection studies. They identified three distinct communication compartments in their neurosphere model: coupled proliferating NPCs, uncoupled cells undergoing neuronal and oligodendrocytic differentiation, and coupled differentiating astrocytes (42). It has yet to be established whether GJIC is present in postnatal NPCs and whether altering this communication will affect their *in vitro* differentiation.

3.2 Objectives

To assess whether primary postnatal NPCs are capable of functional Cx-mediated communication, hemichannel activity and GJIC in a) proliferating WT neurospheres, b) WT proliferating NPCs grown as monolayers, and c) NPCs differentiated to CNS lineages were determined. In this study, activity was confined to assessment of metabolic coupling (dye transfer) and specific passage of the anionic dye LY.

3.3 Materials and methods

All chemical reagents were obtained from Sigma-Aldrich. All dye uptake and dye transfer assays were performed with n=5 assays per experiment in duplicate for a total of n=10 experiments/condition. For quantification, three random fields were taken per experiment, for a total of 30 measurements/condition. All cell photos were taken with a Leica DMXRA2 epifluorescent microscope. Quantitative analysis for fluorescent dye uptake and dye transfer assays was performed using Openlab Software version 3.17 (Improvision).

3.3.1 Cell culture

Neurospheres were prepared, expanded, and differentiated as described in Chapter 2. Three groups were analyzed. To assess functional Cx-mediated activity in neurospheres, cultures were expanded in suspension for eight DIV and then plated on laminin-coated coverslips a) in maintenance media in the presence of EGF and FGF2 for 24 hours, b) maintained in a proliferative state in the presence of EGF and FGF2 for six days, or c) induced to differentiate by treatment with RA and FBS. All neurospheres were plated on round coverslips in 24 well plates.

3.3.2 Hemichannel activity fluorescent dye uptake assay

Transmembrane flux of LY (457.2 g/mol) but not Cx-channel impermeant rhodamine B isothiocyanate-dextran (RD; 10, 000 g/mol) into cells was used to identify the presence of open hemichannels in non-junctional membranes in cells (16). Cells were washed with 10 mM PBS and coated with LY (1 mg/ml)/RD (1mg/ml) in PBS solution in the presence and absence of 0.5 mg/ml of glass microbeads alone or in combination with 50 μM flufenamic

acid (FFA) or 100 μM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) as described by Stout *et al.* (2002) (144). Mechanical stimulation (i.e., the physical impact of the glass microbeads colliding with adherent cells) was used to trigger hemichannel opening (16, 144). Cells were incubated for three minutes at room temperature. After incubation cells were washed three times with 10 mM PBS and fixed in a 3.7% formaldehyde solution. The number of LY+/RD- cells was counted. Cells positive for LY and RD were excluded from the measurements to control for LY uptake resulting from loss of membrane integrity. For all assays, the number of LY+/RD- cells is expressed as a percentage of the total number of cells per microscopic field ± standard error of the mean (SEM). For statistical analyses, data were analyzed by multiple analysis of variance (MANOVA) followed by Tukey *post hoc* tests to identify conditions that were significantly different where applicable.

3.3.3 Gap junction fluorescent dye transfer

Biochemical coupling associated with GJIC was assessed by scrape loading. Cells were washed once with 10 mM PBS and incubated for one minute with LY/RD solution alone or in combination with the gap junction blocker 18α-glycyrrhetinic acid (GRA, 100 μM) or the inactive analog glycyrrhizic acid (GZA, 100 μM) as the GRA negative-control. Cells were scraped gently with a syringe needle and incubated at room temperature for an additional three minutes. After incubation, cells were washed twice with 10 mM PBS and fixed as described above. Dye transfer was quantified by counting the number of LY+/RD- cells in direct or in indirect contact with a LY+/RD+ cell adjacent to the scrape line. Cells positive for LY and RD were excluded from the measurements to control for LY uptake resulting from loss of membrane integrity. Data are expressed as the mean number of

LY+/RD- cells ± SEM. For statistical analyses, data were analyzed by an analysis of variance (ANOVA) followed by Tukey *post hoc* tests to identify conditions that were significantly different where applicable.

3.4 Results

3.4.1 Hemichannel coupling in WT adhered neurospheres but not in differentiated progeny

Transmembrane flux of LY but not RD into cells was used to identify the presence of open hemichannels in non-junctional membranes in WT adhered neurospheres, monolayer of neural precursors and differentiated progeny. Representative photomicrographs from dye uptake assays in WT adhered neurospheres are depicted in figure 3.1A. Spontaneous open hemichannel activity was extremely low in all three conditions (Figure 3.1A-D; untreated/-beads). A statistically significant increase in dye uptake was observed following mechanical stimulation with glass microbeads in WT neurospheres cultured on laminin for 24 hours (Figure 3.1A,B; untreated/+beads; $^{**}p<0.01$). The hemichannel activity was reduced by the addition of the dual-specificity chloride channel and Cx-channels blocker FFA (Figure 3.1A,B; +FFA/+beads; $^{**}p<0.01$). Dye uptake was statistically unaffected by the chloride channel inhibitor DIDS that does not impact upon Cx-mediated hemichannel communication (Figure 3.1A,B; +DIDS/+beads) although a partial non-specific inhibition was observed. Dye-uptake assays were also performed with FFA or DIDS in the absence of glass microbeads to eliminate possible effects of these inhibitors under non-inducing hemichannel conditions (Figure 3.1A,B; +FFA/-beads, +DIDS/-beads). Interestingly, significant

Figure 3.1. Dye uptake through open Cx-mediated hemichannels was detected in neurospheres within 24 hours of plating on laminin but not in NPCs maintained on laminin for six days either in a proliferative state or following differentiation. WT neurospheres were grown for eight DIV in EGF and FGF2 and plated on laminin in a defined medium free of serum in the presence of EGF and FGF2 for one day (A,B), or six days (C), or in presence of RA and FBS for six days (D). Spontaneous hemichannel activity was observed at low levels in neurosphere-derived NPCs one day after plating (A, untreated/-beads, arrows; B). LY influx could be further induced by mechanical stimulation with glass microbeads (A, untreated/+ beads, arrows; B). Dye uptake was significantly reduced by the Cx-hemichannel and chloride channel blocker FFA (A, +FFA/+beads, arrows; B). The chloride channel inhibitor DIDS was used as a control and showed moderate inhibition that did not reach statistical significance (A, +DIDS/+beads, arrows; B). Hemichannel activity was not detected in NPCs cultured for six days on laminin or differentiated with RA and FBS (C,D). Arrows indicate representative LY+/RD- cells. Scale bars, 50 μm.

A. 1 day (EGF + FGF2)

Phase

untreated

- beads

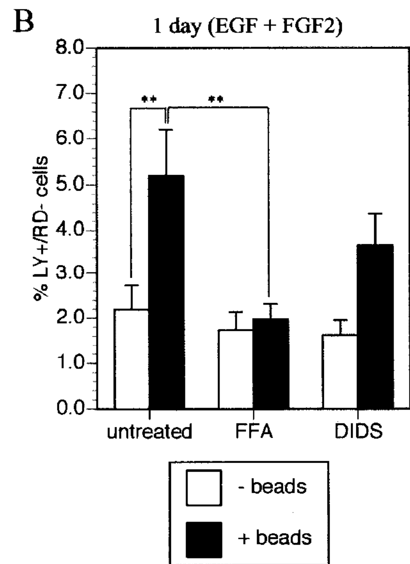
+ beads

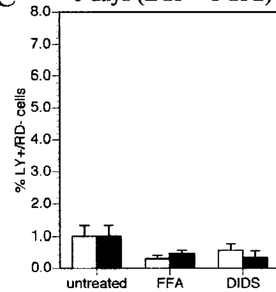
+ FFA

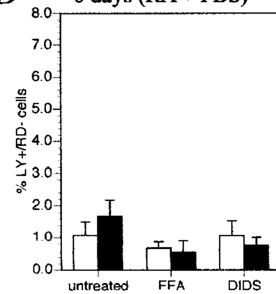
+ beads

+ DIDS

+ beads







spontaneous or inducible hemichannel activity was not detected when NPCs were maintained on laminin whether as proliferating cultures or following differentiation (Figure 3.1C,D).

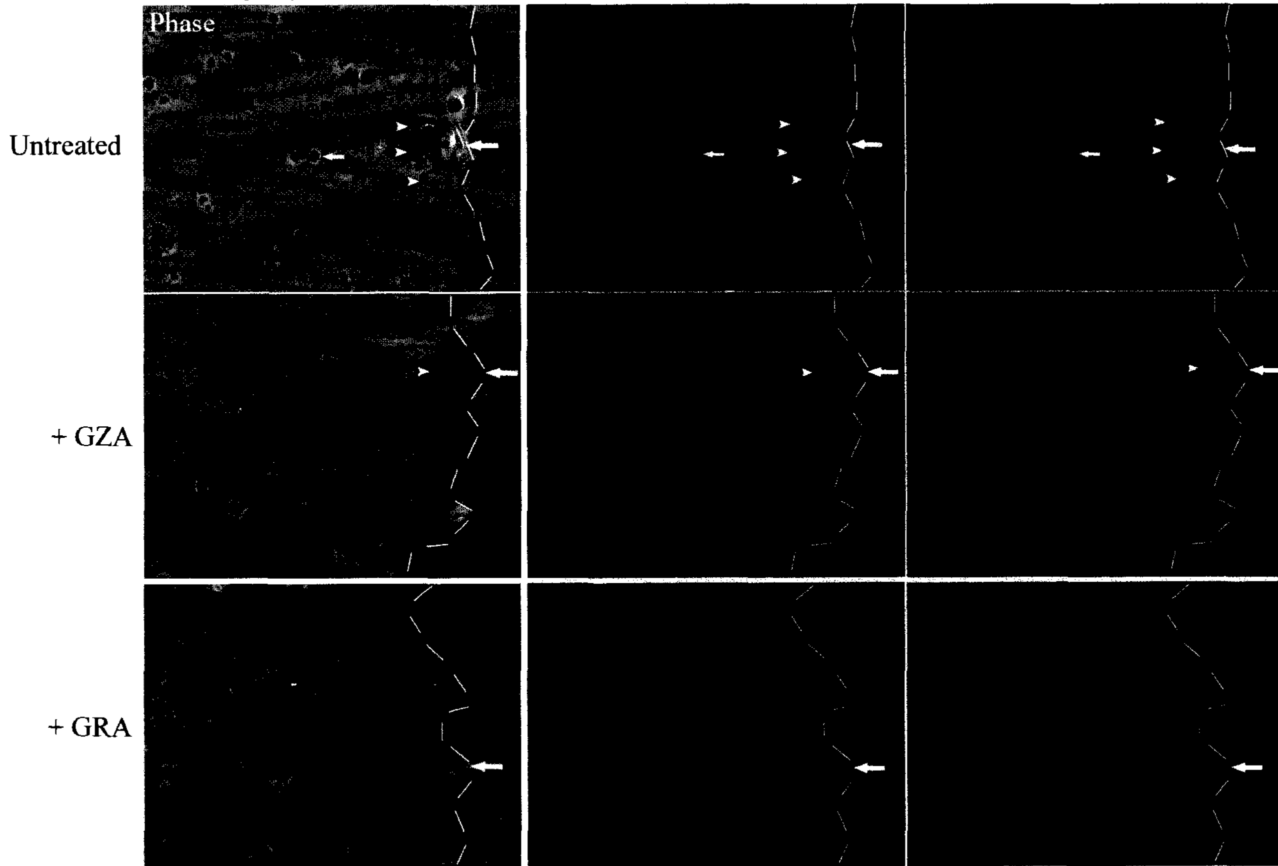
3.4.2 GJIC in differentiated progeny of neurospheres but not in adhered neurospheres

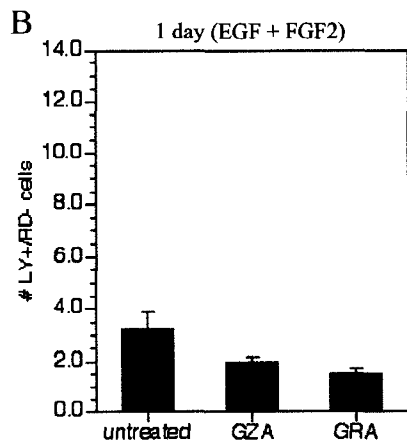
Biochemical coupling associated with GJIC was assessed by scrape loading. Representative photomicrographs from dye transfer assays in neurospheres plated on laminin for six days in the presence of RA and FBS (WT differentiated progeny) are depicted in figure 3.2A. Biochemical coupling indicated by intercellular dye was detected in WT differentiated progeny (Figure 3.2A,D; untreated). Specificity was confirmed by the average 80% reduction in LY+/RD- dye transfer observed overall in the presence of the gap junction channel blocker GRA (Figure 3.2A,D; +GRA; ** $p<0.01$). GJIC was affected only minimally by the GRA inactive analog GZA (Figure 3.2A,D; +GZA). Little or no dye transfer was observed in neurospheres plated on a laminin substrate in the presence of EGF and FGF2 for 24 hours and six days (Figure 3.2B,C).

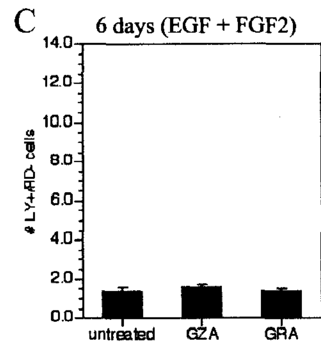
3.5 Discussion

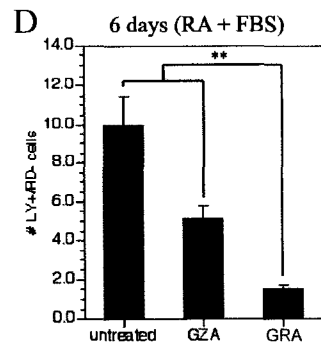
Cx-mediated communication was initially thought to involve only gap junction channels. Hemichannels found at non-junctional membranes were assumed to remain permanently closed until docking with another hemichannel to avoid cell death (126). Accumulating evidence over the last decade support the existence of regulated hemichannel opening *in vitro* (126). The *in vivo* functions of hemichannels are still controversial but their activity has been linked to a surprising number of physiological and pathological conditions (143). In this study, a dye transfer methodology, initially described by Stout *et al.* (144) was

Figure 3.2. Biochemical coupling indicated by intercellular dye was detected in WT neurospheres' differentiated progeny. WT neurospheres were grown for eight days in EGF and FGF2 and plated on laminin in a defined medium free of serum in the presence of EGF and FGF2 for one day (B), or six days (C), or in the presence of RA and FBS for six days (A,D). GJIC in all three conditions was assessed by scrape-loading LY/RD dye transfer assays. Differentiated NPC progeny readily pass LY to adjacent cells (A,D). Note RD+ scrape-loaded cells (A, large arrows) in direct apposition to LY+/RD- cells (A, untreated and +GZA, arrowheads). Dye transfer was inhibited by the gap junction blocker GRA (A, +GRA; D) but not by the control compound GZA (A, +GZA; D). Significant LY+/RD- dye transfer was not detected in proliferating NPCs one or six days after plating (B,C). The small arrow depicts a LY+/RD+ cell (A, top panels). These LY+/RD+ cells indicate LY uptake resulting from loss of cell membrane integrity and were excluded from all measurements. Scale bars, 50 μm.


A. 6 days (RA + FBS)
Phase
Untreated
+ GZA
+ GRA


B
1 day (EGF + FGF2)
LY+/RD- cells
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
untreated
GZA
GRA


C
6 days (EGF + FGF2)
LY+/RD- cells
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
untreated
GZA
GRA


D
6 days (RA + FBS)
**
LY+/RD- cells
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
untreated
GZA
GRA

used to assess functional hemichannel formation in non-junctional membranes and GJIC in WT proliferating neurospheres and their progeny. The experiments presented in this chapter consist of the first study evaluating hemichannel activity and biochemical coupling in a simplified *in vitro* model of postnatal CNS differentiation.

This study provides evidence of inducible hemichannels in non-junctional membranes of NPCs cultured as neurospheres in contact with laminin for a short period of time (24 hours) but not in conditions of sustained proliferation in the presence of EGF and FGF2 and over the course of differentiation. Spontaneous hemichannel activity was very low in neurospheres plated on a laminin substrate for 24 hours, suggesting that the majority of channels are closed in the absence of stimulation. Moreover, the bead-induced hemichannel activity was significantly reduced by FFA but not DIDS indicating that the detected increase in LY uptake in the presence of beads is the result of hemichannel activity and not the result of dye uptake by chloride channels. These results are in agreement with a recent study showing a decrease in hemichannel activity over the course of neural precursor differentiation in a teratocarcinoma neural progenitor cell line (16) suggesting that hemichannel activity progressively decreases over the course of differentiation.

Identification of the specific Cxs comprising the extra-junctional connexons participating in the observed hemichannel activity in WT adhered neurospheres will be necessary to understand their functional relevance. This is important given that the structural diversity of Cxs is reflected by their diversity in function meaning that Cx type dictates the permeability, molecule size selectivity, and gating properties of Cx-channels (161). In this study, only LY was used to establish hemichannel function. LY is an anionic dye capable of traversing Cx26, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45 and Cx47 containing channels but not Cx30 channels (20, 44, 90, 142, 150). Moreover, permeability of Cx29 channels to LY

remains uncharacterized. Thus, we cannot rule out the possibility that further Cx-specific hemichannel activity is present in our cultures. Future studies will require expanding the charge and size of dyes used to assess activity. In this study, it is likely that the observed hemichannel activity is mediated mostly by Cx43. Accumulating evidence suggests that Cx43 definitely forms open hemichannels and is possibly the predominant Cx involved in hemichannel formation (12, 128). Other important Cxs involved in hemichannel activity include Cx26 and Cx32 (22, 74). Cx32 is evidently not implicated in the hemichannel activity reported here given that it is not expressed by our P0-P2 cultures. It is also unlikely that Cx26 plays a significant role in the decrease in hemichannel activity because its expression appears to increase over the course of differentiation in our neurosphere model (see Chapter 2). Conversely, Cx43 expression in our NPCs decreases upon adhesion to a laminin substrate and under differentiating conditions (see Chapter 2) correlating with the decrease in hemichannel activity observed over time in these experiments. Consistent with these findings, Cx43 levels have also been reported to be at their highest in proliferating NPCs in the developing rodent brain (15) which then decrease as NPCs differentiate into neurons *in vivo* (83) and *in vitro* (124).

We cannot, however, rule out the possibility that some of the observed hemichannel activity is Cx-independent. A moderate inhibition of dye influx was observed in NPC cultures treated with DIDS although this decrease did not reach statistical significance. DIDS is routinely used as a negative control for FFA to rule out the involvement of chloride channel-mediated dye uptake (16, 144). Thus, it may be that a small percentage of dye uptake is the result of chloride channel activity. Interestingly, a recent study by Suadicani showed that FFA and a number of other Cx-channel blockers are also $P2X_7$ receptor antagonists demonstrating the lack of specificity of these compounds (145). $P2X_7$ receptors

are permeable to cations and small molecules, including LY (5). Thus, it remains a possibility that the decreased observed hemichannel activity upon exposure to FFA is in fact a decrease in $P2X_7$ receptor activity. This hypothesis will be assessed in future studies by testing whether dye uptake in proliferating neurospheres is affected by oxidized-ATP, an irreversible blocker of $P2X_7$ receptors (32).

Several lines of evidence indicate that GJIC is widespread and functionally important in early cortical development. Dye transfer studies indicative of gap junctional communication has been demonstrated in the embryonic and postnatal brain and in several areas of the mammalian CNS including the cortex (112, 166), hippocampus (124, 125), and ventricular zone (14, 86). It has also been reported that GJIC progressively declines during differentiation of neural progenitor cells in the hippocampus and ventricular zone (14, 124). Intriguingly, our data show functional gap junction coupling in differentiated progeny of NPCs but not in undifferentiated proliferating NPCs. These results were especially surprising in light of a recent study demonstrating that GJIC is widespread among *embryonic* CNS NPCs expanded as neurospheres (42). Absence of statistically significant biochemical coupling in our neurosphere model could reflect an intrinsic difference between embryonic and postnatal NPCs. Second, the failure of LY to transfer in our proliferating neurospheres may be explained by their repertoire of Cx expression. Intracellular domains of mouse Cxs were demonstrated to affect the diffusional properties of gap junction channels and their permeability to various dyes including LY, even between closely related Cxs (90) hence, as with the hemichannel activity described above, a different repertoire of Cx expression could alter GJIC significantly. Third, recent studies have suggested gap junction- and hemichannel- independent mechanisms of Cxs on cell growth and differentiation (73). For instance, in primary cultures of chick lens, overexpression of Cx45.6 stimulated lens cell

differentiation without concomitant increase in GJIC (61). It was also demonstrated that the C-terminus of Cx45.6 alone is sufficient to promote lens cell differentiation to a similar extent as WT Cx45.6 further reinforcing the GJIC- and hemichannel-independent role of Cx45.6 (61, 164). Although the mechanisms underlying this activity remain unclear, interaction between the C-terminus of Cx proteins and Cx binding partners may represent one means of channel-independent communication and a means to alter intracellular signal transduction. A number of Cx binding partners have already been identified; these include zonula occludens 1, cadherins, catenins, sarcoma (src), tight junction proteins and a variety of kinases (56, 143). This novel role for Cxs may explain the absence of significant biochemical coupling in adhered neurospheres and suggests that Cx-mediated growth and differentiation of postnatal NPCs could be channel-independent. Finally, a recent study demonstrated that laminin matrices enhanced mouse neural stem cells migration, expansion, and differentiation into neurons and astrocytes when compared to other substrates (45). Laminins, a family of trimeric glycoproteins present in the extracellular matrix, signal via integrin receptors anchored in cell membranes which function in cellular signalling via the integrin's cytoplasmic domain (9). Given that laminin signalling enhance NPC differentiation (45) and that GJIC decrease during differentiation, it may be that laminin signalling, even for a short period of time (i.e., 24 hours), is likely to reduce GJIC considerably explaining the absence of statistically significant biochemical coupling in WT adhered neurospheres. In support of this hypothesis, Guo and colleagues demonstrated that Cx expression is altered in alveolar type II epithelial cells (downregulation of Cx43 and upregulation of Cx26) upon plating onto laminin matrices (63). Conversely, a report showed that adhesion of keratinocytes to laminin promotes GJIC when compared to other

extracellular matrix substrates (82). These results provide evidence that laminin signalling can modulate GJIC.

This thesis does not specifically address changes in electrical coupling in primary postnatal NPCs and following differentiation. Given that Cx36 is mainly responsible for gap junction-mediated electrical coupling between neurons during the early postnatal development (reviewed in reference 146), electrical coupling between NPCs in our neurospheres model is likely to be minimal given that Cx36 is not expressed (see Chapter 2). However, it cannot be excluded that other neuronal Cxs (i.e., Cx45 or Cx57) or even pannexins could be involved. Over the course of CNS development a decrease in synchronized electrical activity is observed. Decreased electrical coupling has been reported over the course of hippocampal progenitors differentiation *in vitro* (124) and cortical neuron development *in vivo* (111). Peinado demonstrated that the ability of cortex to sustain wave electrical activity ends around P12, when a major reduction in neuronal gap junction takes place in the neocortex (111). In recent investigations, electrical coupling mediated by GJIC was shown to persist in GABAergic inhibitory interneurons in all neocortical layers in the adult brain (54, 156). It is also speculated that if Cx36 is not the exclusive Cx involved in coupling of these neurons, it is at least a necessary constituent (30). In fact, evidence suggest that Cx36 participates exclusively in electrical coupling, not biochemical coupling (149). Hence, emergence of Cx36 expression in our monolayer of neural precursor and differentiated progeny (see Chapter 2) indicates that electrical coupling between adjacent neurons is likely to occur in these cultures.

Altogether the data presented in this chapter corroborate the increasing evidence that functional hemichannel activity in non-junctional membranes is a significant function of Cx

proteins and that these unpaired connexon channels may play important physiological roles in postnatal NPC function.

Chapter 4: Cx29 null-mutation alters spontaneous specification of mouse NPCs

4.1 Introduction

In Chapters 2 and 3, the repertoire and channel-mediated function of Cxs expressed in postnatal NPCs cultured *in vitro* were assessed. Expression and function was found to change over the course of differentiation and to be influenced by culture conditions. What role do these Cxs play in directing NPC fate *in vitro*?

Cx-mediated communication plays an important role during the development of the mammalian brain (reviewed in reference 146). Cxs 26, 32, 36, 43 and 45 are each developmentally regulated in the CNS (reviewed in reference 96). NPCs and radial glial in the murine embryonic brain both express Cx26 and Cx43 and both cell types are found among the clusters of coupled ventricular zone cells (15). GJIC between ventricular zone cells appears to regulate cell division (14), and thereby seems to be important for fate determination (proliferation and specification) of NPCs in embryonic tissue (146). The function of Cx-mediated communication in postnatal brain is not well understood. In a recent review, it was suggested that Cx26, Cx36 and Cx45 may be involved in early postnatal coupling during the maturation of the neuronal circuitry (96). Cx32 and Cx43 expression progressively increases in the first postnatal month likely related to the specification of glial progenitors to an oligodendrocyte lineage and to the proliferation of astrocytes, respectively (96). It has yet to be demonstrated directly whether these or the other Cxs identified in postnatal NPCs in Chapter 1 influence NPC proliferation or differentiation *in vitro*. To this end, I and a colleague in the Bennett laboratory, Sophie Imbeault, have begun to assess the role of Cx null-mutation on NPC proliferation and differentiation. My studies focused on Cx-mediated control of NPC differentiation to a

neuronal or glial lineage. Studies conducted in parallel by Sophie Imbeault address Cx-mediated control of proliferation and are not included in this thesis.

Here, the effect of Cx29 null-mutation on NPC fate was assessed. Cx29 was chosen as it is predicted to form predominantly hemichannels and not participate significantly in GJIC although this hypothesis has yet to be proven directly [(3) and David Paul, Harvard Medical School, personal communication)]. This function is consistent with the putative hemichannel activity and lack of GJIC observed in Chapter 3. As a result, Cx29 was chosen as the first candidate Cx to assess impact of null-mutation on NPC fate *in vitro*. In adult tissue, Cx29 is expressed at high levels in oligodendrocytes (2). Two other Cxs, Cx32 and Cx47, are also oligodendrocyte-specific and have been shown by our lab to be expressed either by NPC subtypes (94) or by NPCs *in vitro* (Chapter 2 of this thesis). Data from our lab provides converging evidence that Cx32 expression inhibits neurogenesis and promotes gliogenesis in specific NPC subtypes resident in neurogenic regions of the brain (Lysanne Melanson-Drapeau, Ph.D thesis, 2006; Mario Morin, M.Sc. thesis, 2006). Cx47 expression has also been shown to be crucial for normal CNS myelination (95). However, the role of Cx29 in oligodendrogenesis, the other important Cx expressed in oligodendrocytes, remains elusive. Cx29 was demonstrated to localize to NG2+ OPCs in our neurosphere model. NG2+ cells can be multipotential with subsets capable of both glial and neuronal differentiation (8). To investigate whether Cx29 expression influences NG2+ cell fate, neuronal, oligodendrocyte and astrocyte differentiation was compared in WT and Cx29KO NPCs expanded as neurospheres.

During CNS development, differentiation is influenced by a variety of extracellular signalling molecules that act through nuclear or cell surface receptors (147). One of these extracellular signalling molecules, RA, plays an important role in the developing nervous

system. Several studies suggest that RA is essential for initial anteroposterior neural patterning and the subsequent development of spinal cord and hindbrain structures (87). In the rodent brain, RA exposure stimulates neurogenesis of adult hippocampus and postnatal SVZ neural stem cell cultures (147, 158). RA, a metabolite of vitamin A, works via ligand-activated transcription factors known as RA receptors (RARs) and retinoid X receptors (RXRs) (57, 89, 113). RAR can bind and become activated by two RA stereoisomers, all-trans RA and 9-cis RA, while RXR can only be activated by 9-cis RA (69, 84). In this study, all-trans RA and FBS were used to trigger differentiation of P0-P2 NPCs expanded as neurospheres.

4.2 Objectives

An *in vitro* protocol of NPC differentiation was established to elucidate whether Cxs play a role in differentiation of primary neural progenitor cultures. Commitment of WT and Cx29KO NPCs to an oligodendrocyte, astrocyte and neuronal lineage was then evaluated under a) proliferating conditions [laminin (EGF + FGF2)], b) spontaneous [laminin alone] and c) directed [laminin (RA + FBS)] differentiation.

4.3 Materials and methods

All differentiation assays were performed with n=5-8 experiments/condition. For quantification, five random fields were taken per experiment for a total of 25-40 measurements/condition.

4.3.1 Cell culture

WT and Cx29KO NPCs were expanded as neurospheres for eight DIV prior to plating onto laminin-coated plates as described in Chapter 2.3.2. NPCs were pulsed with BrdU (20 μg/ml) for 24 hours prior to plating. BrdU-labelled neurospheres were washed twice with 10 mM PBS and plated on maintenance medium free of serum on laminin alone or in the presence of EGF and FGF2 *or* RA and FBS as described in Chapter 2.3.2. Six days later, specification of BrdU+ NPCs was assessed by immunocytochemistry.

4.3.2 Immunocytochemistry

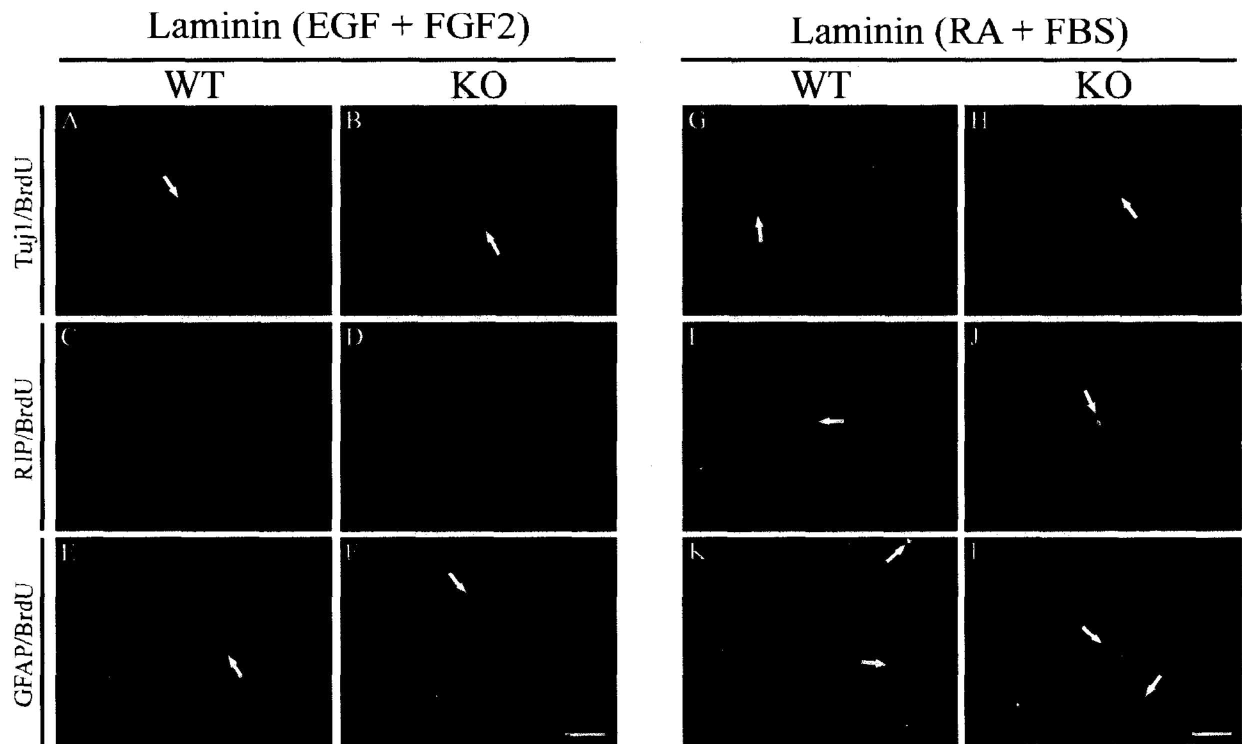
Cells were fixed and processed for BrdU detection as described in Chapter 2.3.6. BrdU incorporation was detected by immunofluorescence using FITC-tagged rat anti-BrdU (Cedarlane Laboratories Limited, Hornby, Ontario, Canada; 1:50). Primary antibodies used for lineage analysis were mouse anti-Tuj1, Cy3-tagged mouse anti-GFAP and mouse anti-RIP and secondary antibody was Cy3-conjugated anti-mouse as described in Chapter 2.3.6. Data are expressed as the mean percentage of BrdU+ NPCs that specified to a neuronal lineage (BrdU+/Tuj1+), to an oligodendrocyte lineage (BrdU+/RIP+), and to an astrocyte lineage (BrdU+/GFAP+) ± SEM. For statistical analyses, data were analyzed by a MANOVA followed by Tukey *post hoc* tests to identify conditions that were significantly different where applicable. All cell photos were taken with a Leica DMXRA2 epifluorescent microscope. Quantitative analysis was performed using Openlab Software version 3.17 (Improvision).

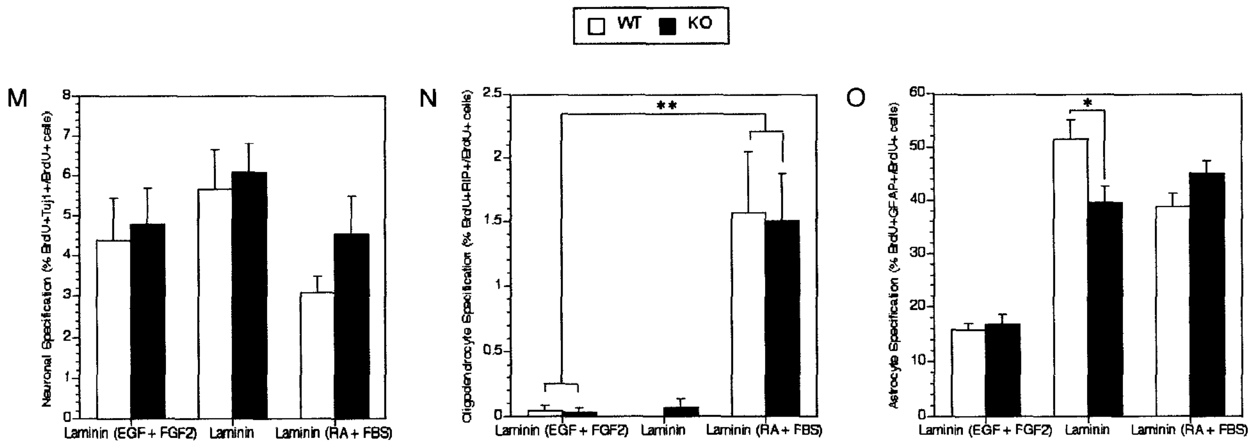
4.4. Results

4.4.1 A working model to test Cx involvement in differentiation of NPCs

WT murine hippocampal NPCs, expanded as neurospheres, were pulsed with BrdU prior to plating. BrdU labelling was performed to ensure that differentiated cells quantitated represented the progeny of actively proliferating NPCs at the time of plating and not terminally differentiated cells that may be present in cultures prior to plating. Neurosphere-derived NPCs were plated in a defined medium free of serum on laminin in the presence of EGF and FGF2 (proliferating cultures), on laminin in the absence of growth factors (spontaneous differentiation), *or* on laminin in the presence of RA and FBS (induced differentiation). Six days later, differentiation was assessed by counting the percentage of BrdU+ cells that expressed antigenic markers of a neuronal, oligodendrocyte, and astrocyte lineage. Representative photomicrographs showing WT and Cx29KO NPC differentiation under proliferative conditions [laminin (EGF + FGF2)] and induced differentiation [laminin (RA + FBS)] conditions are depicted in Figure 4.1 (A-L; arrows). Following growth factor removal, no change in oligodendrocyte lineage commitment, a small but statistically insignificant increase in neuronal differentiation, and an average 30% increase in astrocytic differentiation (**$p<0.01$) was detected in WT neurospheres compared to NPCs plated on laminin in the presence of growth factors (Figure 4.1M-O; laminin). To induce differentiation, neurospheres were plated in the presence of 0.5 μM RA and 0.5% FBS. Under these conditions, WT NPCs commit to an oligodendrocyte and astrocyte lineage (**$p<0.01$) but do not increase neuronal number [Figure 4.1M-O; laminin (RA + FBS)]. Astrocytic commitment was comparable to that observed following growth factor withdrawal. Only oligodendrocyte differentiation was significantly increased by RA and FBS and only to a limited extent (~ 2% of the population). We found the refractivity of NPCs to RA and FBS induction surprising given previous reports using rat NPCs (108, 138).

Figure 4.1. Cx29 null-mutation alters spontaneous differentiation of mouse NPCs. WT and Cx29KO neurospheres were grown for eight days in EGF and FGF2, labelled with BrdU on the seventh day, and 24 hours later, plated on a defined medium free of serum on laminin alone (spontaneous differentiation), or in the presence of EGF and FGF2 (A-F, proliferative conditions) *or* RA and FBS (G-L, induced differentiation). Six days after plating, differentiation was assessed by counting the percentage of BrdU+ NPCs that committed to a neuronal (BrdU+/Tuj1+) (M), oligodendrocyte (BrdU+/RIP+) (N), and astrocyte (BrdU+/GFAP+) lineage (O). Scale bars, 50 μm. Spontaneous but not directed differentiation to an astrocyte lineage was reduced in Cx29 null-mutant NPCs.


Laminin (EGF + FGF2)
WT
KO
Laminin (RA + FBS)
WT
KO
Tuj1/BrdU
RIP/BrdU
GFAP/BrdU


WT
KO
M
N
O
Neuronal Specification (% BrdU+Tuj1+/BrdU+ cells)
Oligodendrocyte Specification (% BrdU+RIP+/BrdU+ cells)
Astrocyte Specification (% BrdU+GFAP+/BrdU+ cells)
Laminin (EGF + FGF2)
Laminin
Laminin (RA + FBS)
**
*

Our initial intent was to evaluate changes in neuronal, astrocyte, and oligodendrocyte differentiation in the presence and absence of Cx29. In an attempt to promote neuronal differentiation, higher concentrations of RA in the presence FBS were tested (5 μM RA and 0.5% FBS). No significant change in neuronal differentiation was observed under these conditions (data not shown).

4.4.2 Cx29 null-mutation alters spontaneous but not directed differentiation of mouse NPCs to a glial lineage

To assess whether Cx29 null-mutation alters the fate of postnatal mouse NPCs, Cx29KO NPCs expanded as neurospheres were induced to differentiate as described above. We found that Cx29 null-mutation did not affect neuronal or oligodendrocyte differentiation under the three conditions examined but reduced astrocytic differentiation under conditions of spontaneous differentiation (Figure 4.1O; *$p<0.05$).

4.5 Discussion

Numerous studies show that GJIC between NPCs and adjacent "instructive" cells regulates NPC fate (17, 25, 42, 94). For example, between E13 and E18, proliferating NPCs depend upon Cx26 and Cx43 to couple to adjacent radial glia (14, 15, 86, 125). This GJIC is thought to be an important determinant of entry in G0 associated with neuronal commitment and terminal differentiation (25). Moreover, studies demonstrate that neurogenesis is associated with a loss of Cx43 and Cx26 and an increase in Cx36 (11, 40, 62, 98), while oligodendrogenesis is associated with de novo induction of Cx32 and an increase in Cx47 expression (94, 95). Thus, temporal changes in Cx expression over the course of mammalian

development are likely to contribute to specification. The aims of this study were to apply an *in vitro* protocol used to elicit differentiation in immortalized rat NPCs (138) to postnatal murine NPC cultures and to directly test whether Cx29 contributes to the differentiation of these cultures.

Our findings indicate that withdrawal of growth factors from our plated WT cultures triggered some neuronal differentiation and considerable astrocytic differentiation. Furthermore, treatment of our primary cultures with RA and FBS enhanced oligodendrocyte differentiation but did not induce significant neuronal differentiation, even when NPCs were exposed to higher amounts of RA. These results were surprising in light of previously published results. Song *et al* have used this technique with success to enrich NPC cultures for neurons (138). Moreover, the presence of endogenous RA and RAR mRNA in the newborn rat hippocampus has been demonstrated (167) suggesting that P0-P2 rat NPCs are RA-responsive. Absence of significant neuronal differentiation of our NPCs under RA stimulation could reflect an intrinsic difference between rat and mouse hippocampal NPCs. I propose that our mouse EGF- and FGF2-responsive NPCs lack the RAR and are therefore unresponsive to all-trans RA stimulation although this hypothesis was not directly tested. Second, the increase in oligodendrocyte differentiation under these conditions is possibly due to the removal of mitogens and the addition of FBS. Addition of 0.5% FBS was shown to increase survival of cells upon withdrawal of growth factors (147). Future studies will be aimed at identifying conditions that significantly induce neuronal specification in these cultures.

Using this gliogenic protocol, this study demonstrates that Cx29 null-mutation does not affect differentiation of early postnatal NPCs to a neuronal or oligodendrocyte lineage but does reduce astrocytic differentiation when cells are allowed to spontaneously

differentiate in response to withdrawal of growth factors. Interestingly, this defect is overcome by the addition of RA and FBS. Why does Cx29, a Cx solely expressed in oligodendrocytes, affect commitment of NPCs to an astrocyte lineage? Although assumed primarily to form hemichannels in non-junctional membranes, (David Paul, personal communication), Cx29 has been shown to colocalize with Cx43 in astrocytes but not Cx26 or Cx30 in adult mice (3, 100). If Cx29 and Cx43 can form functional gap junctions at the oligodendrocyte/astrocyte junction, then it may be Cx29/Cx43 coupling between Cx29+/NG2+ progenitors observed in our cultures (Chapter 2) and adjacent Cx43+ NPCs directs one or more of these populations to astrocytes when cells are allowed to spontaneously differentiate. This hypothesis implies that differentiation signals to commit to an astrocyte lineage can be shuttled from NG2+ progenitors to NPCs through Cx29/Cx43 heterotypic gap junctions. In the absence of Cx29, this subset of precursors may stay uncommited explaining the decrease in GFAP+ astrocytes in Cx29KO cultures with no concomitant increase in oligodendrocytes and neurons. Furthermore, this hypothesis may also explain why a difference in differentiation is not observed in the proliferating monolayer of NPCs because the presence growth factors maintains this subset of Cx43+ cells in a proliferative state. Finally, the driving effect of RA and FBS is likely Cx29-independent and/or increases the necessary intracellular second messenger concentrations through other signalling pathways therefore overriding the inhibition observed in cultures that spontaneously differentiate in the absence of mitogens. However, this explanation is difficult to reconcile with the lack of GJIC detected in our cultures in Chapter 3 emphasizing the need to extend our panel of dyes used to assess hemichannel activity and GJIC to establish whether specific channels, impermeant to LY, are forming between Cx29+/NG2+ and Cx43+ NPCs *in vitro*. It will also be important to test these hypotheses in a system

optimized for more extensive neuronal and oligodendrocyte commitment to be able to characterize the role of Cx29 and other Cxs on NPC fate in more detail.

This study provides a model to directly assess whether Cxs play a role in differentiation in primary cultures *in vitro*. Given that Cx26 and Cx43 were shown to be expressed in NPCs and radial glial (15), and that Cx43 present a continuous increase in expression in the postnatal period *in vivo* whereas Cx26 expression increases only in the first 2 weeks (98), it would be of special interest to determine whether Cx26 and Cx43 null-mutation impacts upon lineage commitment of postnatal mouse NPCs. Finally, this study marks the first report demonstrating that Cx29 null-mutation reduces astrocytic differentiation when NPCs are allowed to spontaneously differentiate *in vitro*. These observations provide additional support for the hypothesis that the repertoire of Cx proteins influences, in part, the intrinsic capacity of NPCs to commit to a given CNS lineage.

Chapter 5: General discussion and conclusions

The role of Cx-channels in mammalian brain development is still unclear and remains an important research question. The studies presented in this thesis aim to gain insight into the role of Cxs and Cx-mediated communication during postnatal CNS neurogenesis and gliogenesis. New terminally differentiated neurons and oligodendrocytes in postnatal brain are derived through a series of intermediate progenitors generated from a single cell source. In postnatal hippocampus, the plasticity of NPCs and their progeny is partially defined intrinsically by epigenetic control, transcription, and post-transcriptional regulation (reviewed in reference 26). This "epigenetic memory" determines whether a NPC progeny maintains their unspecialized identity when exposed to signals that direct progenitors to adopt a different fate or initiate specification. The intrinsic capacity of NPCs to respond to extracellular signals balances extrinsic control of NPC fate in which the paracrine factors and neighbouring "instructive" cells direct NPCs to proliferate or adopt a different lineage. Identifying the (a) genes active in postnatal NPCs that are gradually silenced in their increasingly committed progeny and (b) cell lineage-specific genes that are sequentially turned on in their place is crucial to our understanding of how multipotential progenitor cell number is regulated in adult brain (26).

In this thesis, I hypothesize that the repertoire of Cxs expressed by multipotential NPCs and their intermediate progeny dictates, in part, their intrinsic ability to respond to their microenvironment. Changes in Cx expression are thus predicted to alter NPCs capacity to communicate with neighbouring cells and/or respond to changes in their microenvironment and impact upon their ability to differentiate to neuronal or glial subtypes. To test this hypothesis, I used a simple *in vitro* model system to establish the pattern of Cx expression in postnatal multipotential NPCs defined by nestin and GFAP immunoreactivity

and more committed progenitor cells defined by multiple antigenic lineage markers. Next, I established whether Cx expression is influenced by changing the microenvironment from suspension culture to monolayer culture and through the course of differentiation *in vitro*. Finally, one Cx (Cx29) was chosen to test the hypothesis that altering Cx expression impacts upon the capacity of NPCs to differentiate to a neuronal or glial lineage.

Cx expression by postnatal progenitor cells expanded as neurospheres

To date, analysis of Cx expression in neurospheres has been limited to the study of Cx43 and Cx45 in embryonic neural stem cells (17, 25, 42). The repertoire of Cxs expressed by postnatal hippocampal NPCs remains largely uncharacterized. Here, I show that P0-P2 NPCs expanded as neurospheres express Cx26, Cx29, Cx30, Cx37, Cx40, Cx43, Cx45 and Cx47. Expression of Cx26, Cx30, Cx37, Cx43 and Cx45 was somewhat expected given that other researchers had already located these Cxs in immortalized progenitor cells *in vitro* (16, 125) and postnatal NPCs *in vivo* (40, 93). Interestingly, Cx30, Cx37, Cx40 and Cx45 were all detected in subsets of nestin+ progenitor cells. Cx37 and Cx40 expression in postnatal nestin+ NPCs was most surprising given that these Cxs are most commonly associated with expression in the vascular endothelium (29, 36, 66). Here, I also show, for the first time, that Cx30 is expressed by nestin+ cells. Cx30+/nestin+ cells have also been identified *in vivo* in the adult hippocampus by a colleague in the Bennett laboratory corroborating our *in vitro* results (Hadi Toeg, Honours thesis). Given co-localization with GFAP+ astrocytes, it will be interesting to examine whether Cx30 localizes to nestin+/GFAP+ type B multipotential progenitors in triple-labelling experiments. As for Cx45, immortalized mouse embryonic hippocampal neuronal progenitor cells (MK31) immunoreactive for nestin were shown to also express Cx45 at the mRNA level, thus supporting our data (125).

I found that Cx expression by NPCs can be altered by extrinsic changes in the microenvironment. The pattern of Cxs expressed by NPCs changed when proliferating neurospheres were cultured as monolayers on laminin even when maintained with the same growth factors (EGF and FGF2) as the suspension cultures. As demonstrated in other studies (63, 82), plating cells onto laminin matrices appears to modulate Cx expression. Given that laminin effects on mouse NPCs have been linked to activation of β1 integrin on the cell surface, it can be speculated that activation of β1 integrin by laminin and downstream activation of the MAP kinase pathway underlies the observed changes in Cx expression (19, 72, 148). In support of this hypothesis, cells expressing high levels of β1 integrin were detected only on the edge of neurospheres expanded from P0-P2 mouse and rat forebrain (19). Moreover, in a recent study, laminin signalling was demonstrated to instruct cell fate and alter functional specification of neurons thereby clearly showing that environmental interactions alone can modify the development of NPCs (58).

This study also explores for the first time the idea that spontaneous changes in the intrinsic identity of progenitor cell progeny are dictated, in part, by their three-dimensional location in the expanding neurosphere. This concept has been addressed by others with respect to the position of cells in the core or periphery of the neurosphere. Proliferating NPCs can be found mainly at the edge of the neurosphere demonstrated by expression of markers such as nestin, Notch1 and BrdU while differentiated GFAP+ and Tuj1+ cells are located in its core (reviewed in reference 18). Interestingly, NPCs located on the edge of neurospheres also express β1 integrins that have proven to be important for fate determination as discussed above (19, 72, 148). However, these studies examine the two-dimensional impact of proximity to the core of the neurosphere or periphery. By 3D

reconstructions, I have been able to identify anterior/posterior localization of NG2+/Cx29+ NPC progeny at the perimeter of the sphere suggesting not only core/periphery influences but also anterior/posterior polarity. While underlying mechanisms were not explored in this thesis due to time constraints, this observation lays the foundation for future analysis of the neurospheres' three-dimensional structure and special-structural determinants of NPC fate.

Cx-mediated communication is altered over the course of NPC differentiation

The differential pattern of Cx expression observed between NPCs and differentiated progeny led to the hypothesis that communication through hemichannels and/or gap junctions will differ between these two cultures that may be of functional significance during the process of differentiation. The data presented in Chapter 3 validated this hypothesis through dye uptake experiments and fluorescent dye transfer designed to address hemichannel activity and metabolic coupling mediated by GJIC in proliferating NPCs and differentiated progeny (as discussed in Chapter 3, electrical coupling was not investigated). Hemichannel activity was observed in proliferating NPCs but not in conditions of sustained proliferation and differentiation. Conversely, functional gap junction coupling was detected in differentiated progeny of NPCs but not in undifferentiated proliferating NPCs. Taken together, the results presented in Chapter 2 and 3 show that Cx expression and function change over the course of differentiation and are influenced by culture conditions. Chapter 2 highlights more specifically the diversity of Cx expression in proliferating NPCs and differentiated progeny. Chapter 3 suggests that Cx-mediated hemichannels are active in NPCs. However, future studies will be necessary to determine whether this activity influences NPC differentiation (causal) or whether the changes observed are the result of differentiation (coincidental).

Lastly, the role of specific Cxs in determining NPC fate was addressed. Chapter 2 and 3 suggested that Cx-mediated communication is likely important for neural progenitor proliferation and/or differentiation but did not directly address the impact of altering Cx expression on NPC proliferation or differentiation. To test these hypotheses, a colleague in the Bennett laboratory, Sophie Imbeault, has begun investigating Cx-mediated control of proliferation. My work, presented in Chapter 4 of this thesis, has begun to address the hypothesis that Cxs play a direct role in differentiation. Initially, my goal was to evaluate changes in neuronal and glial differentiation *in vitro* in response to Cx null-mutation. To this end, an *in vitro* protocol of NPC differentiation was established but failed to significantly induce neuronal differentiation. Nevertheless, this gliogenic protocol was used to investigate the effect of Cx29 null-mutation on NPC fate. Cx29 was chosen as it is predicted to form primarily hemichannels and not participate significantly in GJIC [(3) and David Paul, personal communication]. Moreover, this function is consistent with the putative hemichannel activity and lack of functional gap junctions observed in proliferating NPCs (Chapter 3). Results demonstrated that Cx29 null-mutation reduced astrocytic differentiation when NPCs were allowed to spontaneously differentiate *in vitro* but did not alter glial differentiation when NPCs were exposed to "instructive" concentrations of external paracrine factors directing differentiation. This finding validates the hypothesis that Cx29 plays a direct role in the intrinsic control of NPC fate but also suggests that this control does not override other extrinsic signals that direct NPC differentiation. These data are consistent with other studies from our lab indicating that Cx32 affects NPC specification in neurogenic regions of the brain [(94), Lysanne Melanson-Drapeau, Ph.D thesis, 2006; Mario Morin, M.Sc. thesis, 2006] and are in keeping with the observation of other laboratories indicating that Cx47 expression is important for normal CNS myelination (95). Together these results

support the hypothesis that Cx-mediated communication acts, in part, to regulate differentiation of NPCs to a glial lineage.

Advantages and limitations of the neurosphere model

How physiologically relevant are the findings reported in this thesis? In our protocol, NPCs isolated from the postnatal mouse brain were exposed to high levels of growth factors to generate neurospheres. A recent study reports that the exposure of explanted CNS cells to high levels of growth factors leads to embryonic stem cell-like changes in gene expression (116). The potential for growth factors to alter NPC behavior and intrinsic characteristics should not be disregarded. Ideally NPCs should be studied *in vivo* in their complex neurogenic environments where they reside. However, the use of neurospheres allows a detailed analysis of signalling events in a complex niche where neural progenitors remain in contact with supporting cells and are readily accessible to biochemical or genetic manipulation enabling researchers to distinguish between intrinsic (NPC) or extrinsic (supporting cells) events (18). Nevertheless, our *in vitro* findings will need to be corroborated *in vivo*.

In this study, we did not address the impact of co-culture with instructive cells on NPC Cx expression or fate. This highlights another important drawback to growing NPCs in culture is the absence of the instructive niche present in the mammalian brain. The microenvironment where the NPCs reside appears to play a fundamental instructive role in NPC development (162). For instance, recent studies show that neural stem cells interact with endothelial cells and astrocytes in the neurogenic niche in the adult brain (133, 138). Both endothelial cells and astrocytes secrete signalling molecules that influence neighbouring neural stem cells to proliferate and to differentiate into neurons (133, 138).

Even though investigators attempt to duplicate these specific microenvironments by refinement of the media conditions used to culture NPCs, these complex neurogenic niches remain difficult to duplicate *in vitro*. Nevertheless, studying NPCs expanded as neurospheres offers important advantages. First, neurospheres are easy to prepare and to maintain in large quantities. Second, neurosphere cultures are especially useful for the analysis of time-dependent changes in NPC populations in a context-dependent manner. Third, neurospheres have proven valuable in investigating the role of cell-cell communication and cell-extracellular matrix adhesion during neural development and differentiation (reviewed in reference 18).

Neurospheres not only provide a useful model for neurobiology research but also possess medical advantages for use in cell replacement therapies. Transplantation of intact neurospheres in healthy rat brains facilitate cell survival, proliferation and differentiation however with poor integration into the surrounding brain parenchyma (75). Many studies have investigated the use of whole or dissociated neurospheres as possible cell replacement therapies with limited success (reviewed in reference 114). Targeted manipulation of Cx expression represents a possible means of directing NPC activation, specification, and survival following brain injury directly or following transplantation of NPCs as dissociated or whole neurospheres. These strategies could be exploited to improve the outcome of future clinical therapy involving NPCs.

Summary

In conclusion, this thesis expands our knowledge of Cx expression and Cx-mediated communication in the context of proliferating NPCs expanded as neurospheres and differentiated progeny. It also provides insight into the role of Cx29 in the control of NPC

fate. Overall, the data presented in this thesis support the hypothesis that the repertoire of Cx protein dictates, in part, a progenitor cell's capacity to respond to competing differentiation signals present in its microenvironment.

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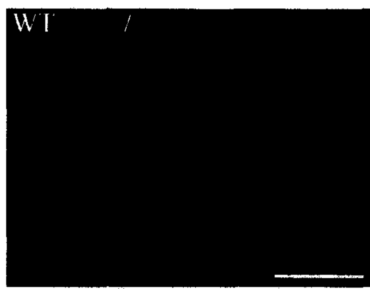
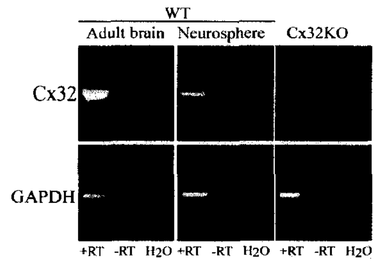
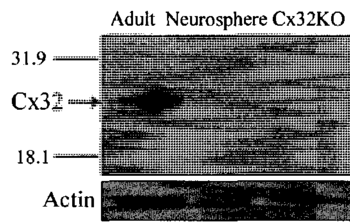
Appendix 1. Supplemental Movie 1 and 2 (CD-ROM)

Appendix 2. Primary NPCs cultured as neurospheres express Cx26 and Cx43 but do not express Cx32 and Cx36. Neurospheres are grown for 12 DIV in EGF and FGF2 and processed for immunocytochemistry, RT-PCR or Western blotting. Data reveal that neurospheres express Cx32 (A) and Cx36 (B) mRNA but not protein. Western blotting results confirm Cx26 (C) and Cx43 (D) expression in neurospheres. Scale bars, 50 μm.

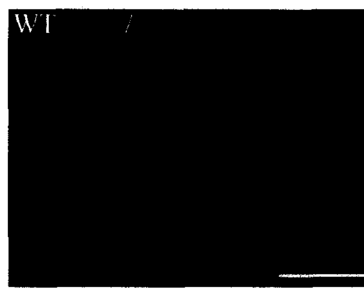
A) Cx32

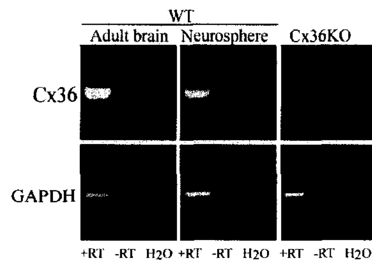




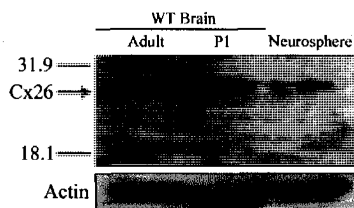


B) Cx36

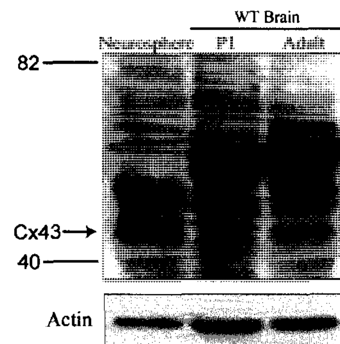




C) Cx26



D) Cx43



Appendix 3. Curriculum Vitae

Lianne Gauvin

EDUCATION

2005 – present	MD PROGRAM Northern Ontario School of Medicine, Sudbury, Ontario
2004 – present	MASTERS OF SCIENCE Department of Biochemistry, Microbiology and Immunology University of Ottawa, Ottawa, Ontario Thesis: Characterization of Connexin Expression, Gap Junction Intercellular Coupling and Hemichannel Formation in Neurospheres and Differentiated Progeny Supervisor: Dr. Steffany A. L. Bennett
2000 - 2004	BACHELOR OF SCIENCE (HONOURS) Dept. of Biochemistry, University of Ottawa

HONOURS AND AWARDS

2004	Canada Graduate Scholarship - Master's, awarded by NSERC
2004	University of Ottawa National Excellence Scholarship
2004	Biochemistry Program Excellence Award, awarded by the Graduate Studies Committee of the University of Ottawa
2004	Ontario Graduate Scholarship - declined
2002 - 2003	NSERC Undergraduate Student Research Awards (2)
2000 - 2004	University of Ottawa Admission Scholarship
2000 - 2004	Recipient of the Queen Elizabeth II Aiming for the Top Scholarship Award
2000 - 2004	Dean's Honour List

APPOINTMENTS AND RESEARCH POSTIONS

Winter 2005	TEACHING ASSISTANT (part-time) University of Ottawa
Summer 2004	NRC-FUNDED STUDENT RESEARCHER (full-time) Institute for Biological Sciences National Research Council, Ottawa, Ontario Supervisor: Dr. Sheng T. Hou

Summer 2003	NSERC-FUNDED STUDENT RESEARCHER (full-time) Department of Biochemistry, Microbiology and Immunology University of Ottawa Supervisors: Dr. Steffany A.L. Bennett and Dr. Earl G. Brown
Summer 2002	NSERC-FUNDED STUDENT RESEARCHER (full-time) Department of Chemistry University of Ottawa Supervisor: Dr. René Roy

SKILLS

Lab Techniques

Mammalian Tissue Culture	*In vivo* manipulations	Plaque Assay
Protein/DNA electrophoresis	RT-PCR	Immunohistochemistry
Fluorescence microscopy	Western Blotting	Tissue cutting
Experimental design	Immunoprecipitation	(Cryostat and Vibratome)
Enzyme assays		

Related Skills

WHIMIS Certified	Laboratory Safety Certified	Biohazard Safety Certified

Computer skills

Mac OS / Windows NT	Microsoft Excel	WordPerfect / Word
DeltaGraph spreadsheet	Adobe Photoshop	Internet: NCBI database
Microsoft Power Point	Systat	

Language skills

Excellent oral and writing skills in both French and English

SCHOLARLY AND PROFESSIONNAL ACTIVTIES

University committees

2005 – present	Northern Ontario School of Medicine Admissions Committee
2005 – 2006	Northern Ontario School of Medicine Student Society

Memberships

2006 – present	Canadian Doctors for Medicare
2005 – present	Canadian Medical Association
2005 – present	Ontario Medical Association
2004 – present	Society for Neuroscience
2001 – present	Golden Key International Honour Society

PUBLICATIONS

Papers submitted for publications

Gauvin LG, Hill JL, Brown EG, Bennett SAL. Type 1 Lang reovirus transduces proliferating neural progenitor cells in vitro and in vivo (*submitted to J. Neurosci, reviews received, in preparation for resubmission*)

Hu YW, **Gauvin LG**, Bennett SAL, Liu H, Franks D, Gardel C, Brown EG. Reovirus infection of the murine respiratory tract induces systemic infection with age and strain dependent disease (*submitted*)

Abstract

Gauvin LG, Melanson-Drapeau L, Morin M, Bennet SAL (2004) Connexin32-mediated regulation of progenitor specification following brain injury. Society for Neuroscience 34th Annual Meeting. October 2004

ACADEMIC INVOLVEMENT

2006	Involvement in the 2006 Northern Ontario School of Medicine interview process (presentations)
2004	Speaker at the Bio-X Honours Info Session