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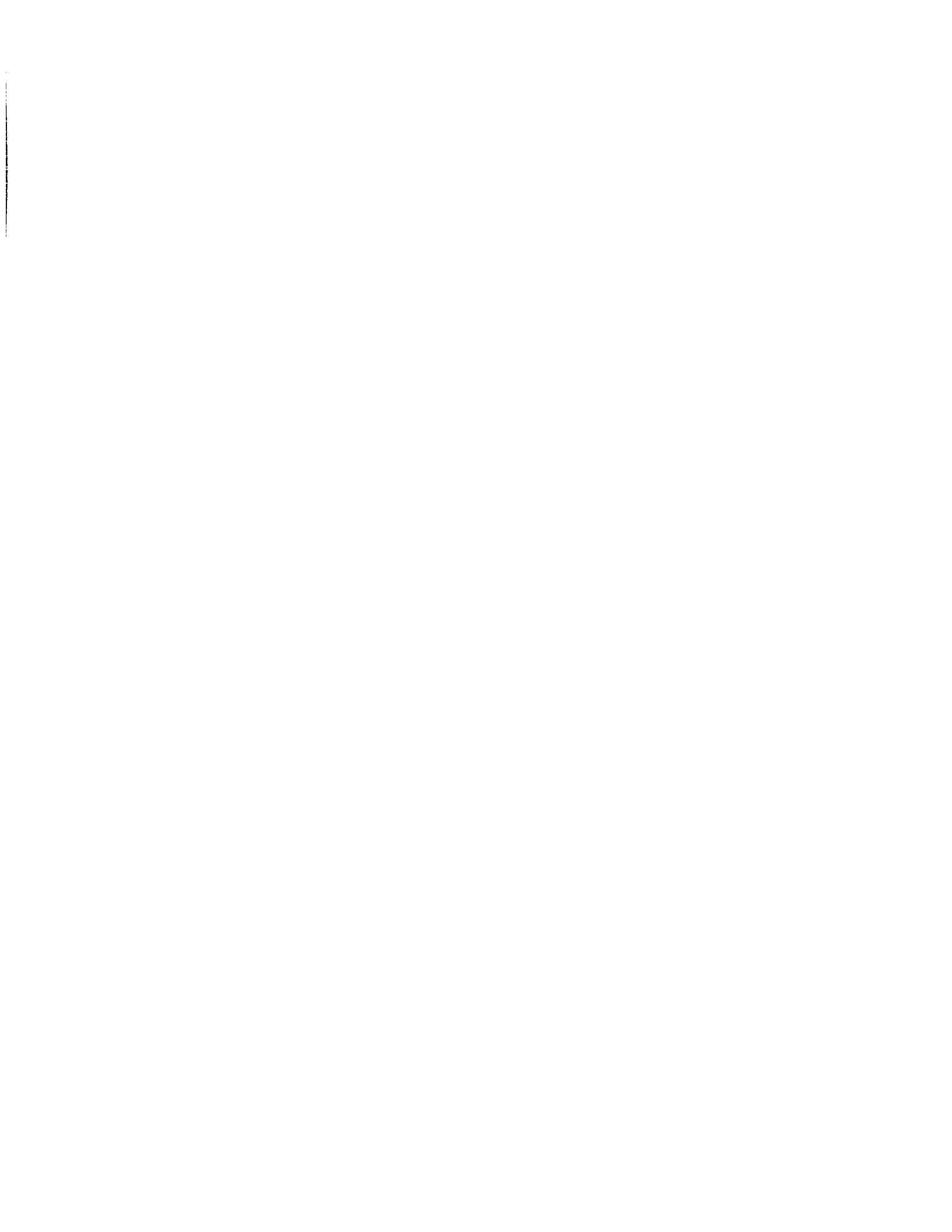
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**CONNEXIN EXPRESSION, CONNEXIN-MEDIATED COMMUNICATION, AND
THE EFFECT OF CONNEXIN OVEREXPRESSION IN AN *IN VITRO* MODEL
OF HUMAN NEURONAL DIFFERENTIATION**

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the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the degree of
Master of Science**

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Thesis Abstract

The connexin (Cx) family of transmembrane proteins oligomerize to form hemichannels, permitting communication with the extracellular environment, and gap junctions, forming the basis for intercellular communication between adjacent cells. Although hemichannel communication remains unexplored, it is well established that gap junction intercellular communication is maximal in the developing central nervous system prior to formation of functional chemical synapses. The NT2/D1 (NT2) *in vitro* system, which can be terminally differentiated to hNT cultures containing human central nervous system neurons, was used as a starting point to further our understanding of the functional significance of Cx proteins and Cx-mediated communication in influencing neural progenitor fate. In Chapter 1, several Cxs, typically expressed by the mammalian central nervous system, were analyzed for expression in NT2 and hNT cultures. Cx30, Cx36, Cx37, and Cx43 were detected in undifferentiated NT2 cultures. Cx36, Cx43, and Cx46.6 were detected in all hNT cultures tested while Cx30 and Cx37 were detected in only a subset of hNT cultures. In Chapter 3, fluorescent dye transfer assays validated previous reports of decreased gap junction intercellular communication in hNT cultures compared to NT2 cultures. A series of dye uptake assays revealed inducible hemichannel activity in NT2s but not hNTs suggesting that like gap junction intercellular communication, hemichannel activity decreases following terminal neuronal differentiation. This marks the first report of hemichannel activity in neural progenitors. In Chapter 4, the specific role of Cx32 in neuronal differentiation was addressed through

transfection of NT2s with Cx32. Cx32 expression blocked terminal neuronal differentiation without affecting oligodendrocyte differentiation as determined by lineage analysis of NT2s and hNTs. We propose a model where Cx32 expression by neural progenitors prevents differentiation to neurons and promotes differentiation to oligodendrocytes in NT2 cell through activation of hemichannels.

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List of Abbreviations

ANOVA	analysis of variance
bp	base pair
BSA	bovine serum albumin
Ca²⁺	calcium
cAMP	adenosine 3',5' - cyclic monophosphate
CMTX	X-linked Charcot-Marie Tooth disease
CMV	cytomegalovirus
CNS	central nervous system
Cx	connexin
DES	DNA extraction solution
DIDS	4,4' – diisothiocyanatostilbene- 2, 2'- disulfonic acid
DMEM/F-12	Dulbecco's modified Eagle's medium F-12
DMSO	dimethylsulfoxide
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetra-acetic acid
ER	endoplasmic reticulum
EST	expressed sequence tag
EtOH	ethanol
FBS	fetal bovine serum
FFA	flufenamic acid
G418	Geneticin® G-418 sulfate
GAP43	growth associated protein 43
GJIC	gap junction intercellular communication
GRA	18α- glycyrrhetic acid
HA	hemichannel activity
HCx32	human connexin 32
HRP	horseradish peroxidase
HSP	heat shock protein
IC PBS	immunocytochemistry PBS
kD	kilodalton
LY	lucifer yellow CH dilithium salt
MCS	multiple cloning site
mRNA	messenger RNA
Myelin PLP	myelin proteolipid protein
NF-68	neurofilament 68
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
RA	retinoic acid
RD	rhodamine B isothiocyanate-dextran
RE	restriction enzyme
RT-PCR	reverse transcription-polymerase chain reaction

SDS-PAGE sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SEM standard error of the mean
TC PBS tissue culture PBS
TGN *trans* Golgi network
WB PBS Western blot PBS

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Chapter 1: General Introduction

1.1 Intercellular Communication

1.1.1 Brief History

Multicellular organisms have developed multiple strategies for coordinating cellular activities. One such strategy involves the use of channels spanning the plasma membranes of two neighbouring cells separated by a 2-4 nm space. These channels were first identified in 1959 through electrical coupling experiments in crayfish [1] followed by freeze-fracture analysis and thin-section electron microscopy experiments during the 1970s [2-6]. It was from these studies that the term "gap junction" was coined. Although the existence of gap junction channels in many cell types was documented during the 1970s, it wasn't until the late 1980s that the protein constituents of gap junctions (connexins) were identified.

1.1.2 The connexin superfamily

The major protein constituent of rat liver gap junctions was successfully cloned in 1986 [7]. The predicted molecular weight of this protein is 32 kD. Current nomenclature for connexin (Cx) proteins is based on their predicted molecular weight [8]. Therefore, the 32 kD liver Cx protein is termed Cx32. To date more than 23 Cx proteins have been cloned [9]. The members of this family show high sequence homology within and between vertebrate species and are characterized by the lack of introns in their coding sequences (with the exception of Cx36) and a common protein structure: three cytoplasmic domains (amino-

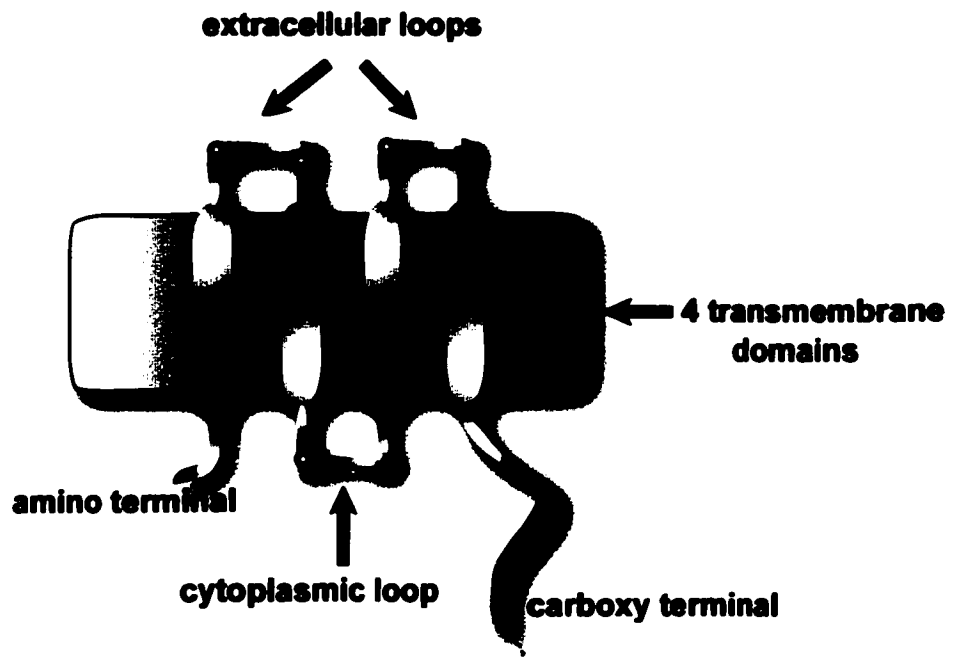
terminal, carboxyl-terminal, and loop domain), two extracellular loop domains, and four transmembrane domains (Figure 1.1). The diversity between connexin proteins is restricted to the carboxyl terminus and, to a lesser extent, the cytoplasmic loop domains (for review see [10-12]).

1.1.3 Hemichannel and Gap Junction Intercellular Channel Assembly

The majority of connexin proteins are co-translationally inserted into the endoplasmic reticulum (ER) membrane and subsequently undergo hexameric oligomerization near the ER/Golgi-apparatus transition to form what is termed a connexon [13, 14]. A connexon is designated homomeric when it is composed of six identical Cxs or heteromeric when it is composed of more than one Cx type [10]. Following oligomerization, the connexon is trafficked through the *trans* Golgi network (TGN) secretory pathway and inserted into the plasma membrane where it forms a hemichannel linking the cell cytoplasm to the extracellular environment¹. Following insertion into the plasma membrane hemichannels from adjacent cells align and dock to form an aqueous intercellular pore termed a gap junction intercellular channel. The regulation of connexon-connexon interaction is believed to be mediated by the extracellular domains of the Cx proteins comprising the connexon [16]. Gap junction channels are termed homotypic when the Cx composition of the contributing connexons are identical or heterotypic when the Cx composition of the contributing connexons differ [10]. A

¹ As an exception, Cx26, in addition to co-translational insertion into the ER, also undergoes post-translational integration into the plasma membrane independent of membrane components of the ER/Golgi secretory pathway [15].

Figure 1.1: Graphic representation of generic Cx protein structure. Members of Cx superfamily share high sequence homology and a common protein structure consisting of 3 cytoplasmic domains (amino-terminal, carboxy-terminal, and loop domain), 2 extracellular loop domains, and four transmembrane domains. The diversity of Cx proteins is restricted to variability in the cytoplasmic loop domain and the carboxy terminus.



simplified graphical representation of connexon, hemichannel and gap junction formation is presented in Figure 1.2.

1.1.4 Gap Junction Intercellular Communication

Gap junction channels link the cytoplasm of adjacent cells allowing the direct exchange of ions (Ca^{2+} , K^+ , Cl^- and Na^+) forming the basis of electrical coupling and the transfer of second messengers and metabolites (such as cyclic nucleotides, inositol 1,4,5-triphosphate (IP_3), and ATP) forming the basis of biochemical coupling. The permeability and charge selectivity of gap junction channels are dictated by the Cx composition, however it is widely accepted that molecules >1.2 kD in size cannot be transferred through gap junction channels. Gap junctions composed of Cx32 have been shown to be 12-fold more permeable to adenosine than gap junctions composed of Cx43 [17] and homomeric Cx32 gap junction channels have been shown to pass cAMP more readily than heteromeric Cx32/Cx26 gap junction channels [18]. In addition, recent studies have shown that gap junctions composed of Cx36 preferentially allow the transfer of positively charged inorganic ions [19, 20].

Intercellular communication mediated by gap junctions accounts for the synchronous activity and metabolic cooperation of cells. For example gap junctions participate in synchronized contraction of cardiac and smooth muscle [21, 22], coordinate transmission of neuronal action potentials at electrotonic synapses [23], and are required for proper pattern formation during development [24]. These latter studies implicate Cxs in the control of neurogenesis and neural progenitor commitment.

Figure 1.2: Assembly of Cx proteins into connexons, hemichannels, and gap junctions.

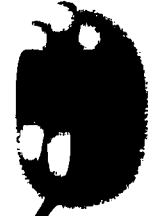
Cx proteins undergo hexameric oligomerization in the ER/Golgi apparatus forming connexons. The connexons are trafficked through the TGN secretory pathway and inserted into the plasma membrane to form hemichannels linking a cells cytoplasm with the extracellular environment. Successful alignment and docking of connexons from adjacent cells leads to the formation of intercellular channels. Groups or plaques of these intercellular channels are termed gap junctions.

Connexin



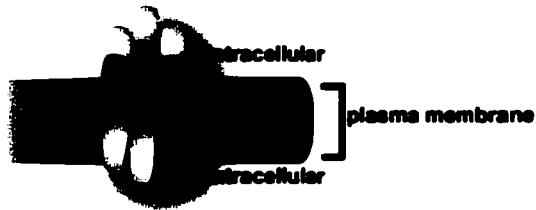
hexameric
oligomerization

Connexon



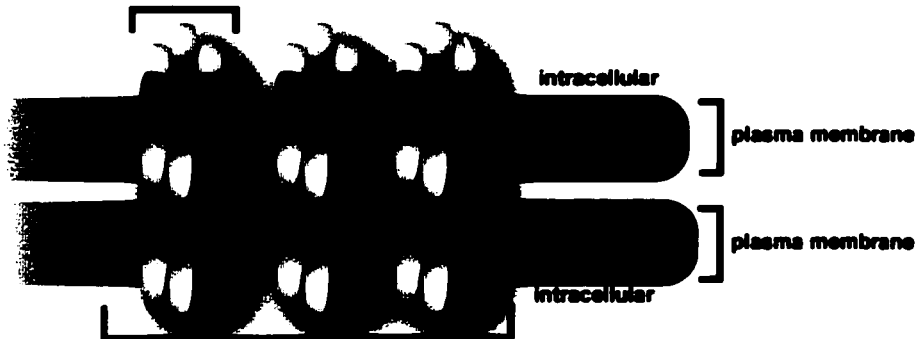
plasma membrane
insertion

Hemichannel



alignment of adjacent cell
plasma membranes

**Intercellular
Channel**



Gap Junction

1.1.5 Hemichannel Activity (HA)

As described above, hemichannels connect cell cytoplasm to the extracellular environment. Several members of the connexin family are known to form hemichannels that can be induced to open *in vitro* following current application [25-32]. However, reports of biologically relevant HA are limited. In cultured astrocytes Cx43 hemichannels have been shown to control Ca²⁺ signaling through the release of ATP [33], while in osteocytes, Cx43 hemichannels have been shown to mediate bisphosphate-induced anti-apoptotic signaling [34]. In the outer retina, Cx26 hemichannels have been shown to act as current sinks modulating Ca²⁺ channel activity and subsequent glutamate release [48]. Although functional roles for Cx43 and Cx26 hemichannels have been identified to some degree through these studies, the functional significance of hemichannel formation by the other connexin proteins, including Cx32, remains to be demonstrated. For the most part, HA remains an unexplored function of Cx proteins.

1.1.6 Cx expression during central nervous system (CNS) development

Of the more than 23 Cx genes identified, 11 are known to be expressed in the mammalian CNS: Cx26, Cx29, Cx30, Cx31.9, Cx32, Cx36, Cx37, Cx43, Cx45, and Cx46.6. Table 1.1 summarizes the CNS localization of these Cxs. Differential expression of several of the CNS Cxs during development indicates they are not functionally redundant. Although HA remains unexplored, it is well-established that electrical and biochemical coupling mediated by GJIC is

Table 1.1: Connexin gene expression in the mammalian CNS.

Connexin	CNS cell and/or tissue expression
Cx26	Pinealocytes, ependyma, leptomeninges [47], neuroepithelium, astrocytes [38], radial glia [64], retinal horizontal cells [48]
Cx29	Oligodendrocytes [52]
Cx30	Ependymal cells, leptomeningeal cells, astrocytes [41]
Cx31.9	Cerebral cortex, hippocampus [58]
Cx32	oligodendrocytes, inhibitory CA1 hippocampal interneurons and basket cells [51,53]
Cx36	Spinal motor neurons and cerebral neurons [20,57]
Cx37	Spinal motor neurons [57], blood vessel endothelium [55], olfactory ensheathing cells [56]
Cx43	Neural progenitors, differentiating neuroblasts, radial glia [64], astrocytes, ependyma, leptomeninges [47]
Cx45	Oligodendrocytes [51]
Cx47	Spinal motor neurons and cerebral neurons [19]

maximal in the developing nervous system prior to the formation of functional synapses and progressively decreases over the course of brain embryogenesis and neuronal differentiation [35-37].

Studies in rodents and mice have given considerable insight to the developmental regulation of Cx expression in the mammalian CNS. Cx43 is expressed at the earliest developmental stages and its expression remains high throughout adulthood [38]. The highest levels of Cx26 expression occur during the first two postnatal weeks and decline to almost undetectable levels at the end of the third postnatal week in rodents [39]. This pattern of expression closely matches the development of neuronal coupling [40] and has been suggested to be involved in functional coupling during neural circuit formation [39]. The down regulation of Cx26 expression coincides with the upregulation of Cx30, Cx32, Cx36, and Cx47. Both Cx30 and Cx47 expression are not detected until the second postnatal week in mice [19, 41]. Maximal Cx36 expression occurs during the second postnatal week followed by a rapid decrease in expression to low levels in adult murine neurons [42]. In rodents, maximal Cx32 expression is observed through postnatal weeks 3 to 6 [41]. The expression kinetics of both Cx32 and Cx36 coincide with the later stages of differentiation, neurogenesis, cell migration, and neural circuit formation [39, 43]. Despite these extensive studies investigating developmental regulation of Cx expression in the mammalian brain, reports of the functional significance of individual Cxs in the context of neuronal differentiation are limited. Global GJIC in two *in vitro* models of neuronal differentiation, the NT2 and P19 cell models, has been shown to

significantly decrease neuronal yield following retinoic acid (RA)-induced differentiation [44, 45]. To our knowledge, Cx43 has been the only Cx identified in these cells lines [44, 45] prior to this thesis. Similarly, it has been shown that conditionally immortalized murine embryonic hippocampal neuronal progenitors are strongly coupled by Cx43 gap junctions and that coupling strength declines over the course of neuronal differentiation [35, 36]. Interestingly, expression of Cx26, Cx36, Cx40, and Cx45 was concurrent with decreased coupling with Cx36 expression reaching maximal levels following terminal differentiation to postmitotic neurons [36]. Taken together, these studies provide strong evidence that GJIC is implicated in successful neuronal differentiation, however the functional significance of individual Cxs remains unclear.

1.1.7 Cx expression in the adult CNS

Although GJIC is decreased dramatically following completion of CNS development, electrical and biochemical coupling mediated by GJIC has been reported in the adult brain [19, 46]. As discussed above, Cx proteins show distinct temporal patterns of expression during CNS development. Similarly Cx proteins show distinct localization patterns of expression in the mature CNS. Both Cx26 and Cx43 are expressed in pinealocytes, ependyma, and leptomeninges [47]. Cx26 has also been localized to horizontal cell dendrites in the outer retina [48]. Cx30 and Cx43 are the predominant astrocytic Cxs [49, 50] while Cx29, Cx32, and Cx45 are the predominant oligodendrocytic Cxs [51, 52]. Cx29 expression is localized to the inner aspects of small myelin sheaths while Cx32 expression is localized to the outer aspects of large myelin sheaths [52].

This differential cellular localization further supports Cxs as functionally independent of each other even when expressed in the same cell. In addition to its expression in oligodendrocytes, Cx32 has also been localized to inhibitory CA1 hippocampal interneurons and basket cells [51, 53]. To date only two Cxs, Cx36 and Cx47, have been identified whose expression is exclusively neuronal. Interestingly, Cx47 and Cx36 expression patterns are distinct from, although partially overlapping with, one another *in vivo* suggesting Cx expression varies in neuronal subtypes [19, 20, 54]. For example, spinal motor neurons and CA3/CA4 hippocampal neurons express both Cx47 and Cx36 while the ganglion cell layer and inner plexiform layer of the retina express only Cx36 [20]. In the mature brain Cx37 is expressed in the endothelium of large blood vessels [55], astrocytes and olfactory ensheathing cells [56]. Spinal motor neurons are the only CNS neuron type known to express Cx37 [57]. Finally, Cx31.9 has recently been detected in both the cerebral cortex and hippocampus of adult humans, although not localized to a specific cell type [58].

Upregulation of GJIC has been reported in the mature CNS following CNS injury. Increased gap junctional coupling in spinal motor neurons has been shown to be recapitulated following nerve damage [59]. Interestingly, the increase in GJIC observed in this report did not correlate with changes in Cx expression indicating that coupling is not regulated at the level of mRNA or protein expression but may be modulated by mechanisms affecting gap junction channel assembly, permeability, and/or open state in the adult CNS [59].

Altogether, the divergent localization of Cx expression in the adult CNS and the developmental regulation of Cx expression in the developing CNS indicates that Cx expression is relevant to neurogenesis. In support of this hypothesis, GJIC has been shown to influence neural progenitor activation, axonal growth and guidance, regional specification, and synaptogenesis during CNS development [24, 60]. Upregulation of Cx43-mediated GJIC has been observed following treatment of neural and glial progenitors with basic fibroblast growth factor (a known neural stem cell mitogen) [61, 62] and pharmacological inhibition of GJIC has been observed to decrease neural progenitor proliferation [63]. Specifically, Cx43 and Cx26 have been implicated in the coordination of progenitor proliferation and self-renewal based on the following observations: (1) Cx43-mediated GJIC is maximal as progenitors exit S-phase and enter G2; (2) GJIC is not observed in M-phase; (3) Increased Cx26 expression and recapitulation of GJIC is observed during G1 and (4) Cx43 expression and GJIC ceases when progenitors become postmitotic and morphologically differentiate to neurons [35, 36, 64].

1.2 Cx gene mutation

Cx gene mutation analysis has contributed significantly to the current understanding of the functional roles of Cxs. Targeted disruption of Cx genes in mice have given insight into the developmental functions of several Cx genes (for reviews see [65, 66]. In addition, mutations in specific Cx genes have been associated directly with human disease conditions. Specifically, Cx26 mutations

are associated with genetic nonsyndromic deafness while Cx50 mutations are associated with congenital cataracts (for review see [65]). The most recognized Cx gene associated with human disease is Cx32. Mutations in the Cx32 gene are the genetic determinant of the X-linked dominant form of Charcot-Marie-Tooth disease (CMTX).

1.2.1 CMTX

CMTX is a motor and sensory peripheral neuropathy characterized by slowly progressive distal muscle weakness and atrophy of the lower limbs [67, 68]. There is currently no cure or preventative treatments for CMTX. More than 160 distinct CMTX causing Cx32 mutations have been identified [69], the majority of which are missense mutations spread throughout the coding region. However deletion of the entire coding sequence [70] and two instances of non-coding regulatory region mutations [71] have been identified in CMTX patients. Cx32 is predominantly expressed by myelinating Schwann cells in the peripheral nervous system (PNS) and is located in the paranodal loops of the non-compact myelin and in the Schmidt-Lanterman incisures [72]. Based on localization Cx32 has been proposed to form gap junctions between different membrane layers of the myelin sheath generated by the same Schwann cell resulting in a shortcut pathway for radial diffusion of nutrients between the distal and adaxonal Schwann cell cytoplasm [73]. Although the severe clinical CMTX phenotypes manifest in the PNS, a subset of CMTX patients have recently been reported to display some CNS abnormalities: specifically these patients show decreased

visually evoked potentials, decreased brainstem auditory evoked potentials, and decreased central motor nerve conduction [74, 75].

1.2.2 Experimental models of Cx disruption

In addition to studies of human genetic disease, the diverse functions of Cxs have been underscored through studies of mice with targeted Cx gene disruption. Cx40 and Cx43 are the prominent Cxs expressed in the mammalian heart [76], however, Cx43 deficient mice die shortly after birth [65] while Cx40 deficient mice survive but display aberrant cardiac conduction [77]. Deletion of Cx26 is embryonic lethal while [65] deletion of Cx37 leads to female infertility [65]. Similar to humans, Cx32 null mice develop a peripheral neuropathy similar to CMTX [78], but unlike humans display a high incidence of hepatic tumours [79]. Work in the Bennett laboratory with the Cx32 null mouse has shown that the loss of Cx32 results in an increased population of neural progenitors highlighting a direct role for Cx32 in the control of neural cell fate (Melanson-Drapeau et al, submitted to *J Neurosci* 07/19/02). Cx36 null mice develop normally but show impaired electrical coupling in the brain and retina [80, 81]. Altogether, studies of Cx null mice have been paramount to the current understanding of the complex roles of Cx gene function. However, the inherent complexity and difficulties of *in vivo* animal studies necessitates the use of *in vitro* cell models to further establish the functional significance of Cx expression and Cx-mediated communication in the context of specific cellular processes such as neuronal differentiation.

1.3 The Ntera2/D1 (NT2) cell line: an *in vitro* model of human neuronal differentiation suitable for the study of Cx function.

The NT2 cell line was derived by injecting the Tera2 heterogeneous cell line (derived from a lung metastasis of a Caucasian man with a primary testicular germ cell tumour) into nude mice and cloning the resultant tumours [82]. The NT2/D1 line is immortalized human cell line of neural progenitors that have been shown to consistently differentiate to cultures containing postmitotic CNS neurons (referred to as hNT cultures) following 4 weeks of RA treatment and 3 weeks of mitotic inhibition [83]. In addition to neuronal differentiation, NT2 neural progenitors have recently been successfully differentiated to astrocytes under specific cell culture conditions [84]. Expression of Cx43 and biochemical coupling mediated by GJIC has been identified in NT2 neural progenitors and has been shown to progressively decrease over the course of RA-induced neuronal differentiation to hNT cultures [85]. In addition, global GJIC blockage is known to decrease the capability of NT2s to successfully differentiate to a neuronal phenotype [44]. Together, these studies solidify the NT2 cell line as a model system for the study of Cx expression and Cx-mediated communication in the context of neuronal differentiation. However, it will be important to identify the additional Cxs, if any, expressed by NT2 neural progenitors and hNT cultures to fully understand the significance of Cx gene expression and Cx-mediated communication. The importance of utilizing human *in vitro* models to study the role of Cx expression during neuronal differentiation was highlighted through a statement made by Bani-Yaghoub *et al* [85] who pointed out that the

developmental appearance of Cx40 and Cx45 is different in the human heart than that of the rodent heart [86-88]. Thus, it is necessary to utilize human cell models in order to fully understand the functional significance of individual Cx proteins and Cx-mediated communication as it relates to the human neuronal differentiation.

In addition to being a model system for human neurobiology research, the NT2 also has medical relevance. Following ischemic injury, hNT neurons implanted into the brains of rodents have been shown to remain postmitotic, retain neuronal phenotype, and improve motor and cognitive impairments [89-91]. Phase I clinical trials have been completed in humans using grafted hNT neurons for stroke therapy. The first postmortem brain findings of a phase I clinical stroke trial patient implanted with hNT neurons more than 2 years prior to death report the survival of hNT neurons with no evidence of neoplasm [92]. As discussed by Nelson *et al* [92], the NT2 cell line has several distinctive medical advantages for use in human brain therapies. Specifically they do not pose ethical or legal problems because they are not derived from human embryos, they do not harbour known human pathogens or potentially infectious agents present in xenografts, they are available in unlimited quantities, and they are amenable to genetic engineering [93]. Therefore, the medical relevance of the NT2 cell line to therapies for brain damage as a result of neurodegenerative disease and brain injury necessitates a comprehensive understanding of the molecular mechanisms, specifically the role of Cx-mediated GJIC and HA, underlying NT2 progenitor cell fate and differentiation.

1.4 Objectives

The functional significance of Cx expression and Cx-mediated communication in the context of human neuronal differentiation remains unclear. The studies presented in this thesis were aimed at furthering our understanding of the functional significance of Cx protein expression and Cx-mediated communication in the context of neuronal differentiation. In addition, studies were also aimed at determining the direct effects of Cx32 expression on neuronal differentiation. The human NT2 cell line was the *in vitro* system of choice to facilitate these studies. The overall objectives of this thesis are:

- (1) To identify the repertoire of Cx gene expression in NT2 neural progenitors and terminally differentiated hNT neurons.
- (2) To determine the type of Cx-mediated communication in NT2 neural progenitors and hNT neurons.
- (3) To investigate the direct effect of Cx32 overexpression on NT2 neuronal differentiation.

Chapter 2: The NT2 *in vitro* model of neuronal differentiation expresses several Cxs commonly found in the mammalian CNS *in vivo*.

2.1 Introduction

As reviewed in Chapter 1, GJIC is maximal in the developing CNS and is the principal means of cell communication prior to the formation of functional chemical synapses (for review see [23, 35, 47]). At least 11 Cx genes are known to be expressed in the mammalian CNS: Cx26, Cx29, Cx30, Cx31.9, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, and Cx47 (see Table 1.1). The array of Cxs expressed in a given cell determines molecular size permeability, ionic selectivity, and gating properties of gap junction intercellular channels and/or hemichannels, indicating that expression of one Cx type is not functionally equivalent to another [10, 12]. *In vitro* studies of the human NT2 cell line and other cell models have shown that global gap junction blockage results in a marked decrease in neuronal differentiation [36, 44]. However, analysis of Cx expression by NT2 cells has been limited to Cx43 [44, 85]. The contributions of other CNS Cxs have yet to be assessed. Thus, the aim of this study was to establish the full repertoire of Cxs expressed by the NT2 cell line.

2.2 Objectives

In order to gain a greater understanding of the role of Cx gene expression during neuronal differentiation, we analyzed the pattern of Cx gene expression in the NT2 neural precursors and in hNT cultures terminally differentiated by

retinoic acid (RA) *in vitro*. Cx expression patterns were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) to detect mRNA and Western blot and/or immunocytochemistry to detect protein.

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, ON, Canada) unless otherwise stated.

Cell Culture

NT2 cultures were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2mM L-glutamine at 37°C in a 5% CO₂/95% air atmosphere. To differentiate to neuronal hNT cultures, 2 x 10⁶ cells were seeded in a 75 cm² flask and treated with 10 μM RA three times a week for 4 weeks. Following RA treatment, the cells were replated 1:3 and incubated for two days. The conditioned media was removed and the cells were replated at 1 x 10⁷ cells per 10 cm² plate. Plates were coated with poly-D-lysine (100 μg/mL)/laminin (20 μg/mL). Cells were incubated for 24 hours in a 1:1 mix of DMEM/F-12 and conditioned media. Cultures were then supplemented with 1 μM cytosine β-D-arabinofuranoside and 10 μM 5-fluoro-2'-deoxyuridine mitotic inhibitors for 4 days. Cells were subsequently fed every 4 days for a minimum of 3 weeks with DMEM/F-12 containing the following: 1 μM cytosine arabinoside, 10 μM

fluorodexoyuridine, and 10 μ M uridine. Mitotic inhibitors were removed 24 hours prior to experimentation.

Reverse Transcription

Total RNA was isolated from cultured cells using the TRIzol® Reagent (Invitrogen) according to manufacturer's recommendations. Total RNA was treated with DNaseI (Promega Corporation, Madison, WI, USA) to eliminate residual genomic DNA. First strand cDNA synthesis was performed using random hexamers (Promega) and Superscript II RT (Invitrogen) according to manufacturer's recommendations. The resulting cDNA template was used to determine expression of several human Cx genes by PCR.

PCR

All reagents were obtained from Invitrogen unless otherwise stated. The following reagents were added to each PCR reaction: 25 pmoles forward primer, 25 pmoles of reverse primer, 5 μ L 10X PCR Buffer, 4 μ L of 10 mM deoxynucleotide triphosphates (dNTPs), and 1mM MgCl₂. The PCR reaction was brought up to a final volume of 50 μ L with nuclease-free water (Promega) and 30-35 PCR cycles of the following standard PCR program were performed in the GeneAmp® PCR System 2400 (Applied Biosystems, Foster City, CA, USA): predwell 94 °C for 5 minutes, 30-35 cycles of 94 °C for 30 seconds, 55 °C for 60 seconds, and 72 °C for 2 minutes, and a postdwell at 72 °C for 7 minutes.

Protein Isolation

Cells were washed with 10 mM tissue culture phosphate buffered saline (TC PBS, 10 mM sodium phosphate buffer, pH 7.5, 154 mM NaCl) and scraped from the plate following the addition of RIPA buffer (1% Nonidet P-40 , 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) in extraction PBS (10 mM sodium phosphate, 150 mM sodium chloride). Protease inhibitors were added prior to extraction (1 mM NaF, 50 µg/mL aprotinin, and 0.1 mM sodium orthovanadate). The lysed cells were incubated on ice for 30 minutes. Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mg/mL. Lysates were centrifuged for 30 minutes at 12 000 rpm. Protein concentration was determined using the Bio-Rad DC protein assay kit (Hercules, CA, USA). In cases where extraction of both RNA and protein from the same cell cultures was required, Trizol® reagent was used to isolate protein according to the manufacturer's protocol.

Western Blot Analysis

Protein samples (30 µg) were submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane (Fisher Scientific, Nepean, ON, Canada), the membrane was rinsed in methanol for 2 minutes, washed three times in 10mM Western blot (WB) PBS (9.5mM sodium phosphate dibasic, 0.1mM sodium phosphate monobasic, 2.7mM potassium chloride, 4.3mM sodium chloride, pH 7.5), saturated in blocking buffer (1% casein in 10 mM WB PBS) for 1 hour at room temperature, and incubated with primary antibody diluted in blocking buffer

overnight at 4°C. The membrane was rinsed twice in 10 mM WB PBS and twice in blocking buffer for ten minutes and incubated with the appropriate horseradish peroxidase (HRP)-tagged secondary antibody at room temperature for 1 hour. Following incubation, the membrane was rinsed four times in 10 mM WB PBS for ten minutes. Detection for all Western blot analyses was performed on BioMax film (Eastman Kodak Company, Rochester, NY, USA) using the SuperSignal West Pico Chemiluminescent Substrate kit (MJS BioLynx Inc., Brockville, ON, Canada). The manufacturer and working dilution for all antibodies used in Western blot analysis are provided in Appendix A.

Immunocytochemistry

Undifferentiated NT2 cells were maintained as described above. For analysis of hNT cultures, cells were differentiated as described above with the exception that cells were plated into dishes containing glass coverslips coated with poly-D-lysine (100 µg/mL)/laminin (20 µg/mL) prior to commencing mitotic inhibition.

For immunocytochemical analysis, media was removed. Cells were washed with 10mM TC PBS and fixed by incubation in cold 3.7% formaldehyde freshly prepared in 10mM immunocytochemical (IC) PBS (2.5 mM monobasic sodium phosphate, 7.5 mM dibasic sodium phosphate, 154 mM NaCl, pH 7.2) for ten minutes followed by incubation in 100% methanol for 3 minutes. The cells were washed with 10 mM TC PBS and reacted with primary antibody diluted in Ab buffer (10 mM IC PBS, 3% bovine serum albumin (BSA), and 0.3% Triton-X 100, pH 7.2) overnight at 37°C in a humid chamber. Following incubation with

primary antibody, the cells were washed three times in 10mM IC PBS for five minutes and incubated with the appropriate anti-mouse or anti-rabbit IgG Cy3 fluorophore-tagged secondary antibody diluted in Ab buffer for 35 minutes at 37°C in a humid chamber. In order to remove unbound secondary antibody, cells were washed three times in 10 mM IC PBS for five minutes. Cells were then coverslipped with Vectashield (Vector Laboratories Inc., Burlingame, CA) sealed, and visualized using the Leica DM RXA2 epifluorescent microscope (Leica, Longueuil, QC, Canada). The manufacturer and working dilution for all antibodies used in immunocytochemical analysis are provided in appendix A.

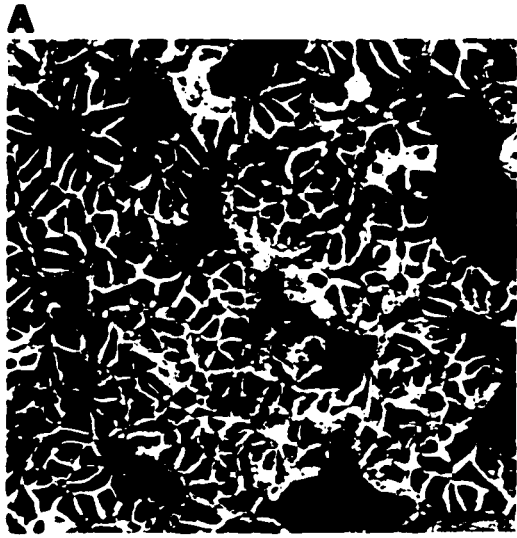
2.4 Results

2.4.1 Retinoic acid treatment differentiates NT2 cells to postmitotic human neurons (hNTs).

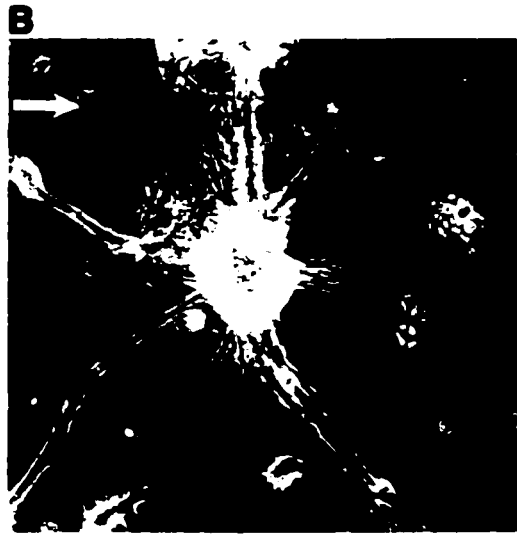
The NT2 cell line has recently become one of the most widely used *in vitro* models of human neuronal differentiation. Undifferentiated NT2 cells (Figure 2.1A) cultured in complete media supplemented with 10 μ M RA for four weeks followed by mitotic inhibition for a minimum of three weeks differentiate to hNT cultures (Figure 2.1B). Hallmark features of neuronal differentiation include the development of typical neuronal morphology (Figure 2.1B) characterized by migration into large aggregates (Figure 2.1B, black arrow) and extensive process outgrowth (Figure 2.1B, arrowhead). The hNT cultures also contain non-neuronal cells (Figure 2.1B, white arrow).

Figure 2.1: NT2 cells differentiate to hNT cultures containing postmitotic neurons and non-neuronal cells following retinoic acid and mitotic inhibitor treatments.

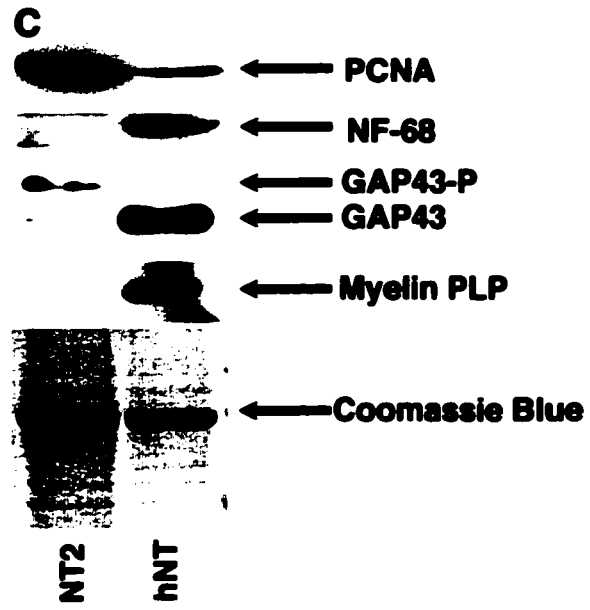
Undifferentiated NT2 cells (A) cultured in complete media supplemented with 10 μ M RA for four weeks followed by mitotic inhibition (see cell culture methods) for three weeks differentiate to hNT cultures (B). Note the development of typical neuronal morphology (B) characterized by aggregation of cell bodies into bundles (B, black arrow) and extensive process outgrowth (B, arrowhead). The hNT cultures also contain non-neuronal cells (B, white arrow). Western blot analysis comparing hNT cultures to NT2 cultures demonstrate decreased levels of proliferating nuclear cell antigen (C, PCNA) indicative of growth arrest, the appearance of the neuron-specific marker neural filament 68 (C, NF-68), the appearance of the oligodendrocyte marker myelin proteolipid protein (myelin PLP), and the prevalence of growth-associated protein 43 (C, GAP43). Coomassie blue stained gels (C, Coomassie blue) are shown as the protein loading control. Scale bars, 50 μ m.



NT2



hNT



To further characterize the differentiation of NT2 cultures to hNT cultures at the molecular level, Western blot analyses were performed with antibodies raised against specific cell lineage markers. The following antibodies were used: proliferating cell nuclear antigen (PCNA; a 36kD marker of actively dividing cells [94-96]), neurofilament 68 (NF-68; a 68kDa marker for differentiated CNS neurons, [97, 98]), growth associated protein 43 (GAP43; a 43kD marker for proliferating neuronal precursors [99], neurons undergoing axonal outgrowth [100] as well as oligodendrocyte pre-progenitors and progenitors [101, 102]), and myelin proteolipid protein (myelin PLP, a 33.4kD marker for oligodendrocyte progenitors as well as developing and mature oligodendrocytes [103, 104]).

As expected, decreased levels of PCNA (Figure 2.1C, PCNA) were observed in hNT cultures when compared to NT2 cultures. This decrease in PCNA is indicative of the relative growth arrest associated with differentiation to postmitotic hNT cultures following RA and mitotic inhibitor treatments. The development of a neuronal phenotype in hNT cultures was confirmed by the appearance of the neuron-specific marker neurofilament 68 (Figure 2.1C, NF-68) absent in NT2 cultures. Interestingly, myelin proteolipid protein was detected in hNT cultures but not NT2 cultures (Figure 2.1C, myelin PLP) indicating the presence of oligodendrocyte progenitors and/or mature oligodendrocytes following RA and mitotic inhibitor treatments. GAP43 protein expression was observed for both NT2 and hNT cultures (Figure 2.1C, GAP43, GAP43-P). GAP43 can exist in two forms: unphosphorylated (GAP43) and serine-phosphorylated (GAP43-P). GAP43 is continuously cycled between intracellular

compartments with the unphosphorylated form being associated with the plasma membrane and the phosphorylated form being associated with the cytoskeleton [105]. Both GAP43 and GAP43-P were detected in NT2 cultures while only GAP43 was present in hNT cultures (Figure 2.1C, GAP43, GAP43-P). However, upon higher exposure (data not shown) the phosphorylated form of GAP-43 was detected at low levels in hNT cultures. The presence of GAP43/GAP43-P in NT2 cultures further confirms these cells as neuronal precursors while the presence of GAP43/GAP43-P in hNT cultures is a marker of active axonal outgrowth which is consistent with the extensive process outgrowth observed in culture (Figure 2.1B, arrowhead). Coomassie blue stained gels (Figure 2.1C, Coomassie blue) are shown for equal protein loading control². Altogether, morphological and Western blot analyses show that retinoic acid-induced differentiation of NT2 cultures gives rise to differentiated hNT cultures containing postmitotic human neurons.

2.4.2 Design and optimization of human-specific Cx PCR primer pairs.

The first step in establishing the pattern of Cx expression in the NT2 cell line was to determine which Cx mRNAs are expressed. This required the design and optimization of human-specific Cx PCR primer pairs for subsequent use in RT-PCR analysis of NT2 and hNT cultures. PCR primer pairs were designed for the following human Cx genes: Cx26, Cx30, Cx36, Cx37, Cx43, and Cx46.6.

Existing Cx32 PCR primers in the laboratory, originally designed for the mouse

² As a technical note, standard protein loading controls such as actin or tubulin could not be used because levels of these cytoskeletal proteins are known to change over the course of NT2 differentiation to hNT cultures (see Figure 4.6). As a result Coomassie Blue was the only possible standard and is currently used in the literature in similar NT2 studies.

Cx32 gene, were compatible with human Cx32 as determined by BLAST (NCBI, Bethesda, MD, USA) sequence analysis against the human genome. Several considerations were taken into account in the primer design process. Firstly, primer pairs were designed such that they specifically recognized only their respective Cx gene and did not cross-react with other members of the human Cx gene family (as determined by BLAST sequence analysis against the human genome). Primer pairs were designed on a single exon to allow optimization on human genomic DNA instead of human cDNA thereby reducing labour and cost. In addition, the primers were designed such that the amplicon could be used to generate a specific probe for Northern blot analysis in future experiments (as determined by BLAST sequence analysis against the human genome). All primer pairs were subject to analysis by Primer Designer software (Scientific & Educational Software, Durham, NC) to determine their quality and compatibility for PCR. The human Cx primer pair sequences and amplicon sizes are provided in Table 2.1. Table 2.2 provides information for primer pairs whose optimized conditions for PCR amplification differ from standard conditions described in the PCR methods. The resulting Cx PCR amplicon for each Cx primer pair is provided in Figure 2.2 (+) along with parallel reactions containing no template DNA to control for reagent contamination (Figure 2.2, -).

2.4.3 The NT2 cell line expresses mRNA for several Cx genes

RT-PCR analysis of total RNA extracted from NT2 cultures revealed mRNA expression of Cx30, Cx36, Cx37, and Cx43 in all cultures tested (Figure 2.3A, NT2 +RT, 2.3C, NT2). Expression of mRNAs for Cx26 and Cx32 were not

Table 2.1: Human-specific connexin PCR primer pair sequences and amplicon sizes.

Human Cx gene	Strand	Primer Sequence (5'-3')	Amplicon size (bp)
Cx26	Sense	CTGCAGCTGATCTTCGTGTC	308
	Antisense	AAGCAGTCCACAGTGTTG	
Cx30	Sense	GTGACGAGCAAGAGGACTTC	512
	Antisense	CAGCAGCAGGTAGCACAAC	
Cx32	Sense	CTGCTCTACCCGGGCTATGC	750
	Antisense	ACGGCTGAGCATCGGTCGCTCTT	
Cx36	Sense	AAGCCGCTACTCTACAGTC	596
	Antisense	CCTGGCAGGTCCTTGTTAC	
Cx37	Sense	ATCTGGCTGACGGTGCTCTT	619
	Antisense	GCACCAACTCCAGCAGGTTA	
Cx43	Sense	CTCAGCAACCTGGTTGTGAA	709
	Antisense	TCGCCAGTAACCAGCTTGTA	
Cx46.6	Sense	GACGAGCAGGCCAAGTTCAC	572
	Antisense	ACCTCGAAGCCGTACAGCAG	

Table 2.2: Optimized PCR conditions for human-specific connexin primer pairs deviating from the standard PCR program described in PCR methods.

Human Cx gene	Annealing temperature (C°) and time (seconds)	Extension temperature (C°) and time (seconds)	PCR reagents
Cx30	53, 30	72, 45	10 pmoles of each primer, 1.5mM MgCl ₂ , 7.5% DMSO, 1.5 μL of Taq DNA polymerase
Cx36	50, 60	standard	standard
Cx46.6	61, 30	72, 45	7.5% DMSO, 1.5 μL of Taq DNA polymerase

Figure 2.2: Optimization of human connexin-specific PCR primers .
Human connexin-specific primer pairs were optimized for PCR using human NT2 genomic DNA template (+). Reactions containing no template DNA were performed to control for reagent contamination (-). PCR Amplicon sizes are 308 bp, 512 bp, 750 bp, 596 bp, 619 bp, 709 bp, 572 bp for Cx26, Cx30, Cx32, Cx36, Cx37, Cx43, and Cx46.6 respectively.

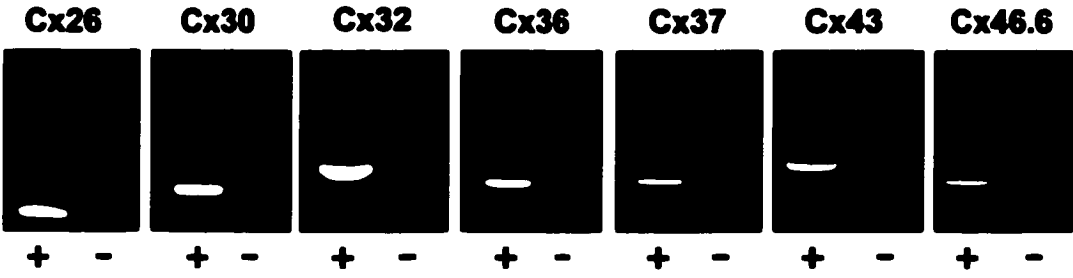
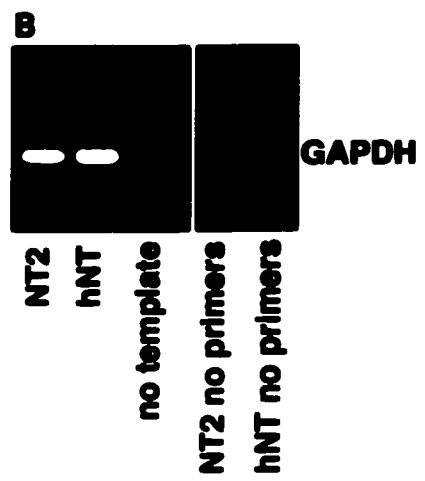
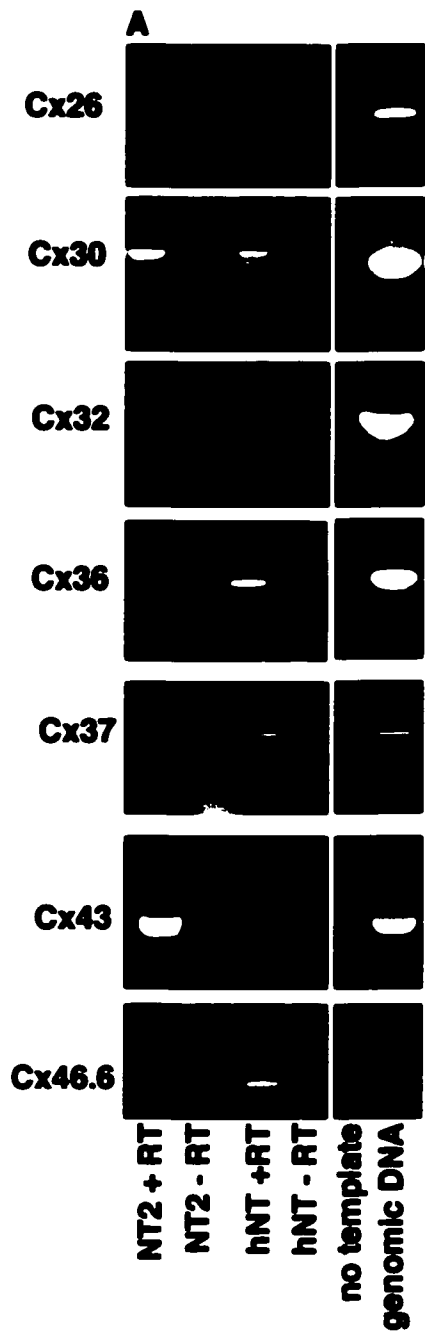


Figure 2.3: NT2 and hNT cultures express mRNAs for connexins present in the mammalian central nervous system.

RT-PCR analysis of random-primed total RNA from NT2 and hNT cultures using primers optimized for human connexin genes revealed expression of mRNAs for several connexins typical of cells in the mammalian CNS (A). The number of cultures positive for connexin mRNA expression relative to the total number of cultures tested is provided in C. Cx26, Cx32, and Cx46.6 mRNAs were absent while Cx30, Cx36, Cx37 and Cx43 mRNAs were present in all NT2 cultures tested (A, NT2 +RT). Cx26 and Cx32 mRNAs were absent while Cx36, Cx43, and Cx46.6 mRNA were present in all hNT cultures tested (A, hNT +RT). Cx30 and Cx37 mRNAs were present in 1/3 and 2/3 of hNT cultures tested (A, hNT +RT). The integrity of the cDNA templates was confirmed by amplification of GAPDH mRNA (B, NT2, hNT). RT reactions were performed without the addition of reverse transcriptase enzyme to control for genomic DNA contamination in the sample following DNaseI treatment (A, -RT). Reactions were also performed without the addition of cDNA template to control for contamination PCR reagents (A,B, no template). NT2 genomic DNA was used as a positive control for PCR (A, genomic DNA). Finally, reactions were carried out in the absence of primers as a control for artifact PCR (B, no primers).



C

Connexin Gene	NT2	hNT
Cx26	0:2	0:3
Cx30	2:2	1:3
Cx32	0:2	0:2
Cx36	2:2	2:2
Cx37	2:2	2:3
Cx43	2:2	2:2
Cx46.6	0:2	1:1

detected in any of the NT2 cultures tested (Figure 2.3A, NT2 +RT, 2.3C, NT2). In the case of hNT cultures, the pattern of Cx mRNA expression was more complex. Total RNA from at least two independent hNT cultures was subjected to RT-PCR analysis for each Cx, (Cx46.6 data is provided in section 2.4.5). All hNT cultures tested displayed mRNA expression of Cx43 and Cx36. However, only 1/3 and 2 /3 of cultures tested displayed mRNA expression of Cx30 and Cx37 respectively (Figure 2.3A, hNT +RT, 2.3C, hNT). This suggests that the pattern of Cx expression at the mRNA level is sensitive to inherent variation in differentiation conditions between independent experiments. Similar to the NT2 cultures, Cx26 and Cx32 mRNA expression was not detected in any of the hNT cultures tested (Figure 2.3A, hNT +RT, 2.3C, hNT).

Several control reactions were performed in parallel to exclude false positive and false negative results for each of the Cxs analyzed. PCR amplification of mRNA encoding for the glycolytic enzyme glyceraldehyde-3'-phosphate dehydrogenase (GAPDH), present in all cell types, was used to control for the quality of the cDNA template (Figure 2.3B, NT2, hNT). Reactions were performed without the addition of reverse transcriptase enzyme during the reverse transcription of total RNA to control for genomic DNA contamination in the sample following DNaseI treatment and to eliminate false positive results during PCR (Figure 2.3A, -RT). Reactions were also performed without the addition of cDNA template to control for DNA contamination in PCR reagents (Figure 2.3A, B, no template). Reactions were performed with NT2 genomic DNA (same template used for primer optimization) to serve as a positive control

for successful PCR and eliminate false negative results (Figure 2.3A, genomic DNA). Finally, reactions were carried out in the absence of primers to detect any artifacts of PCR and again eliminate false positive results (Figure 2.3B, no primers).

2.4.4 The NT2 cell line expresses protein for several Cx genes

In order to determine the pattern of Cx protein expression in the NT2 cell line immunocytochemical and/or immunoblot analyses were performed on protein extracted from NT2 and hNT cultures. It should be noted that, with the exception of the Cx43 antibody, the commercially available Cx antibodies have not previously been tested experimentally for reactivity with their human proteins (see Appendix A for antibody supplier and working dilutions). Therefore, total protein from the human hippocampus was used to determine and optimize Cx antibody reactivity prior to testing on NT2 and hNT lysates. Immunocytochemical and Western blot analyses were performed only for those Cxs whose mRNA transcript was present in NT2 cultures and/or hNT cultures. Antibody dilutions for immunocytochemical analyses of Cx expression were optimized on paraffin-embedded and/or cryostat brain sections from wild type C57BL/CJ mice available in our laboratory (data not shown) .

Cx43 protein expression was successfully detected by both immunocytochemical and Western blot analysis (Figure 2.4). Typical staining for Cx proteins in gap junctions is punctate and localized to plasma membranes between adjacent cells. This pattern of staining was observed in both NT2 cultures and hNT cultures (Figure 2.4A, arrows). Cx43 protein expression was

restricted to the non-neuronal cells in the differentiated hNT cultures as determined by the morphology of the Cx43 positive cells (Figure 2.4A, hNT). For Western blot analysis, human hippocampus protein was used to confirm reactivity of the Cx43 antibody with the human Cx43 protein (Figure 2.4B). Two Cx43 protein bands representing different phosphorylation states, migrating at approximately 42kD and 44kD, were detected in the human hippocampus protein sample confirming successful reactivity (Figure 2.4B, Cx43, Cx43-P). A single Cx43 protein band (44kD) was detected in NT2 cultures but not in hNT cultures. The inability to detect Cx43 protein by Western blot analysis in hNT cultures likely reflects the high ratio of neurons (Cx43 negative, Figure 2.4A) to non-neuronal cells (Cx43 positive, Figure 2.4A) in culture. Coomassie blue stained gels (Figure 2.4 B, Coomassie blue) are shown as the protein loading control.

Cx36 protein was detected in NT2 and hNT cultures by immunocytochemical analysis (Figure 2.5). Cx36 protein was present in subpopulations of both neurons (Figure 2.5, hNT top panel) and non-neuronal cells (Figure 2.5, hNT bottom panel). The high fluorescent intensity of Cx36 staining of cells in both the NT2 and hNT cultures may explain the inability to resolve a punctate pattern of staining typical of Cxs forming gap junctions. Positive reactivity of the Cx36 antibody with human Cx36 was not obtained with Western blot analysis of human hippocampus protein suggesting an inability of this antibody to recognize the denatured form of human Cx36. Therefore, Cx36 protein expression could not be confirmed by Western blot analysis.

Figure 2.4: NT2 and hNT cultures express Cx43 protein.

Expression of Cx43 protein in NT2 and hNT cultures was confirmed by immunocytochemical (A) and Western blot analysis (B). Punctate staining localized to plasma membrane contacts between adjacent cells was observed in both NT2 and hNT cultures (A, arrows) as was cytoplasmic staining. Cx43 protein expression was restricted to non-neuronal cells in hNT cultures (determined by cell morphology of Cx43 + cells in A, hNT). Reactivity of the anti-Cx43 antibody with human Cx43 was confirmed by Western blot analysis of human hippocampus protein (B, human hippocampus). Two protein bands 42kD (Cx43) and 44kD (Cx43-P) in size corresponding to the different phosphorylation states of Cx43 were detected in human hippocampus. Cx43 protein (44kD) was observed in NT2 cultures but was not detected in hNT cultures (B, NT2 and hNT respectively). Scale bars, 50 μ m.

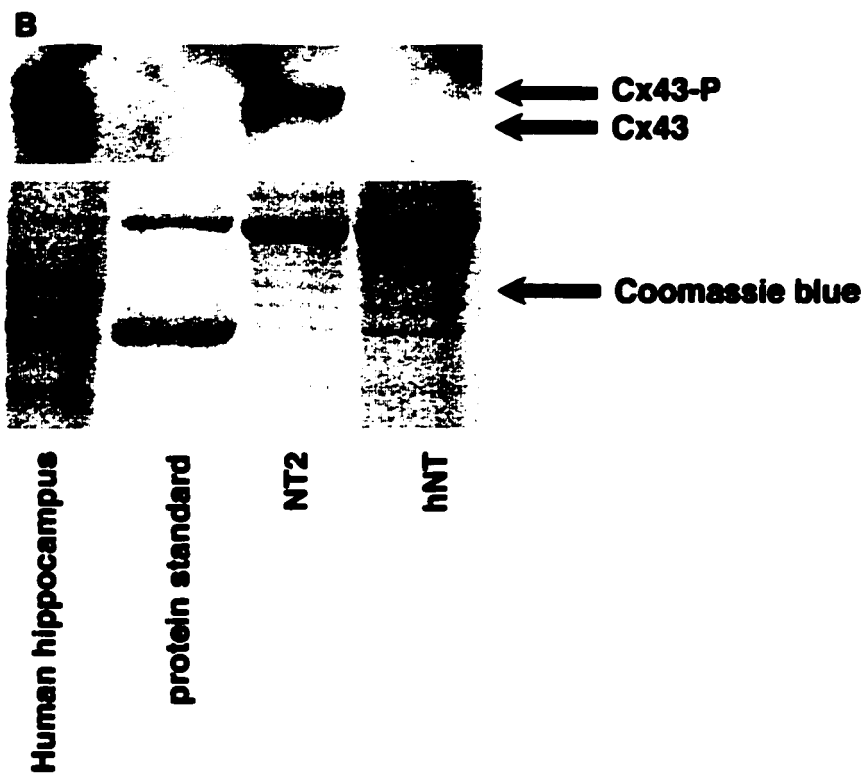
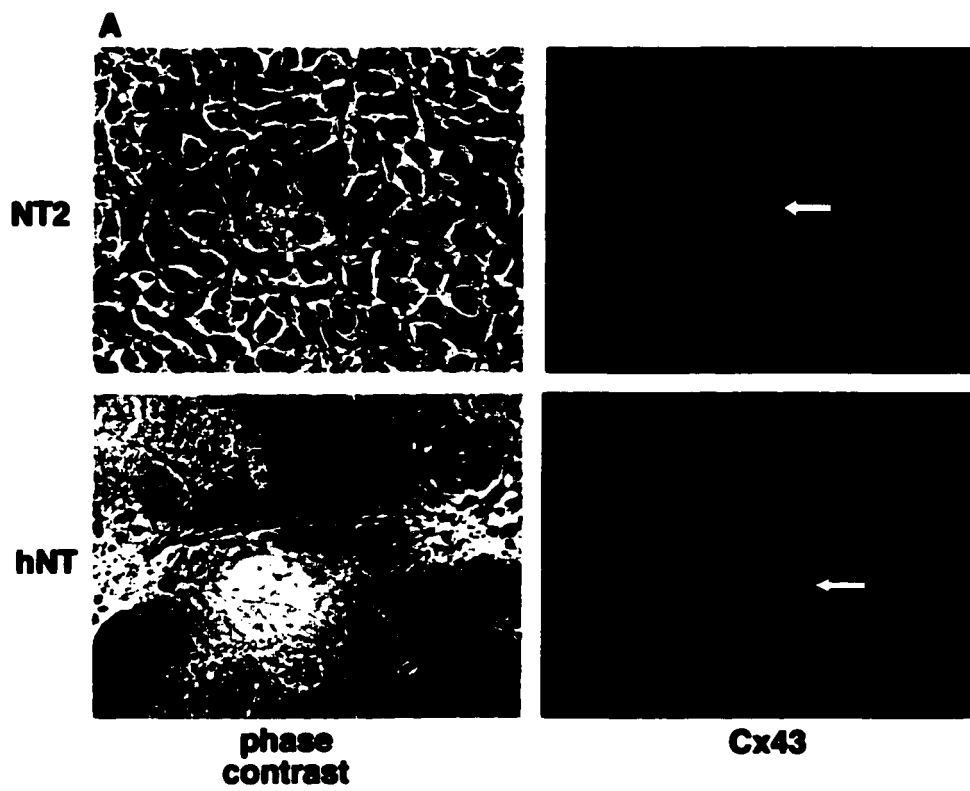
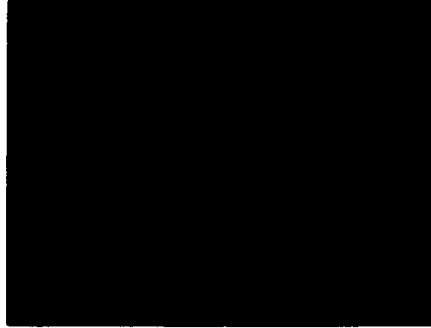
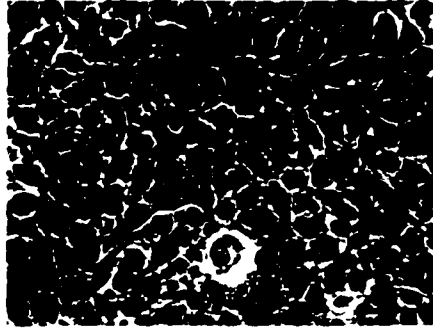
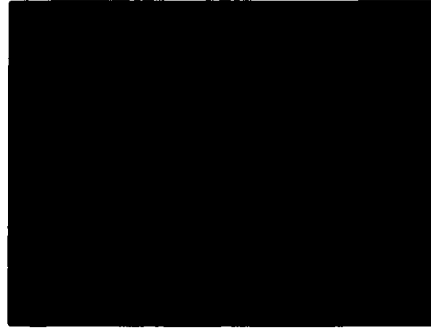
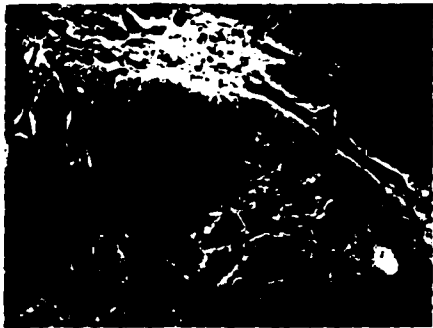
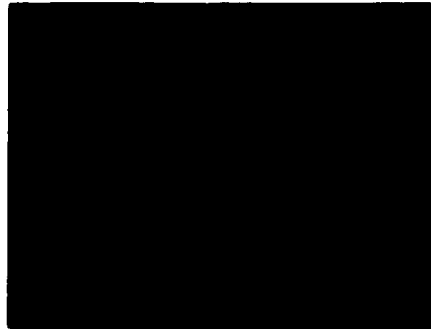
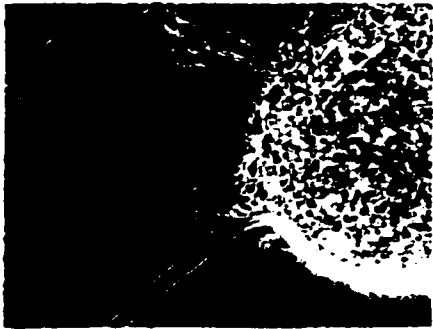


Figure 2.5: NT2 and hNT cultures express Cx36 protein.
Expression of Cx36 protein in NT2 and hNT cultures was confirmed by immunocytochemical analysis. Cx36 protein was present in subpopulations of neurons (hNT, top panel) and non-neuronal cells (hNT, bottom panel) in hNT cultures. Scale bars, 50 μm .

NT2



hNT



**phase
contrast**

Cx36

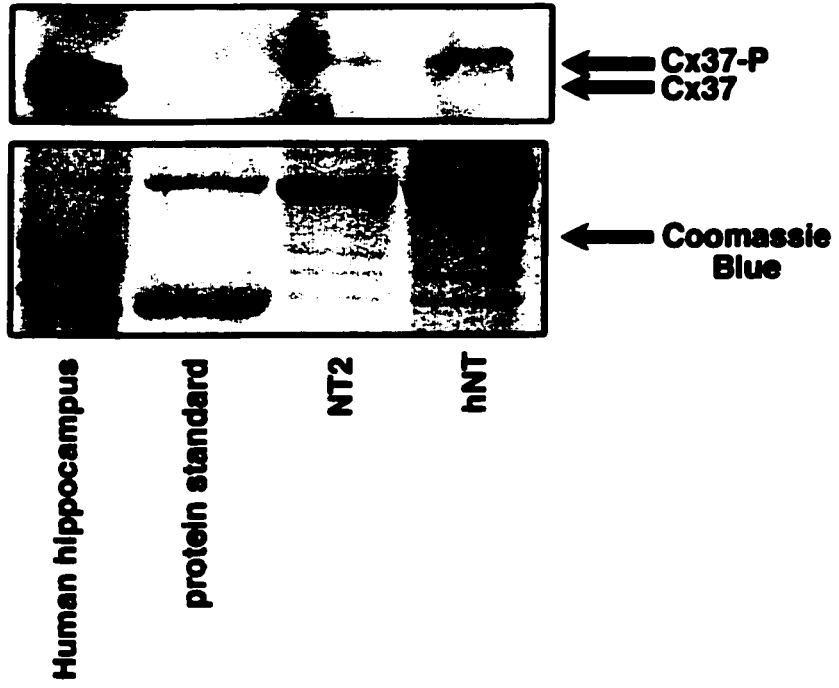
Conversely, Cx37 protein was detected by Western blot analysis but not by immunocytochemical analysis. Reactivity of the Cx37 antibody with human Cx37 was confirmed by Western blot analysis of human hippocampus protein (Figure 2.6), which displayed two reactive protein bands migrating at approximately 36kD and 38kD corresponding to the unphosphorylated (Cx37) and phosphorylated (Cx37-P) forms of Cx37 respectively. A single Cx37 protein band (38kD) was observed in both NT2 and hNT cultures (Figure 2.6).

Results for Cx30 protein expression were inconclusive. Positive reactivity of the Cx30 antibody with human Cx30 was not obtained with Western blot analysis of human hippocampus protein suggesting an inability of this antibody to recognize the denatured form of human Cx30. Immunocytochemical analyses of NT2 and hNT cultures were also negative for expression of Cx30 protein. However, a positive control for reactivity with the human Cx30 protein for immunocytochemical analysis was not performed. Therefore, it cannot be concluded if Cx30 protein is or is not expressed by NT2 and/or hNT cultures.

2.4.5 Validation of a virtual Cx: Cx46.6 mRNA expression in hNTs but not NT2s.

Cx46.6 exists as a virtual human Cx gene within the GenBank database at the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). It was originally predicted from NCBI contig NT 004908 by automated computational analysis using the gene prediction method BLAST, supported by mRNA and human EST evidence (see Genbank GI: 11427877 submitted by

Figure 2.6: NT2 and hNT cultures express Cx37 protein. Expression of Cx37 protein in NT2 and hNT cultures was confirmed by Western blot analysis. Reactivity of the anti-Cx37 antibody with human Cx37 was confirmed by immunoblot of human hippocampus protein. Two protein bands 37kD and 38kD in size corresponding to the unphosphorylated (Cx37) and phosphorylated (Cx37-P) states of Cx37 were detected in human hippocampus. Cx37 protein (38kD) was detected in NT2 and hNT cultures.



Bloemker, BK, Swaroop, A, and WJ Kimberling). The predicted protein sequence for Cx46.6 is 436 amino acids in length (GI: 27385777). In 2001, Teubner *et al* reported functional expression of a new mouse Cx gene designated Cx47 in several types of neurons within the mouse CNS [19]. Interestingly, they used the sequence of the Cx46.6 virtual human Cx gene in combination with rat EST data to generate the PCR probe used to screen and eventually clone the Cx47 gene from a mouse genomic DNA library. Together, the cloning approach and expression pattern of the mouse Cx47 gene suggests that Cx46.6 may be a true human Cx gene and that it is likely to be expressed in human CNS neurons. Therefore, Cx46.6 was included in the battery of Cx genes whose expression was investigated in the NT2 cell line.

PCR primer pairs were designed (see Table 2.1 for primer sequences) and optimized based on the virtual mRNA sequence of Cx46.6 (Figure 2.2, Cx46.6, +). The optimized conditions for PCR amplification that differ from the standard conditions described in the PCR methods are provided in Table 2.2. The 572 bp Cx46.6 PCR amplicon was subject to direct sequencing in both the forward and reverse orientations. Alignment of the two sequences produced a high quality sequence fragment 468 bp in length from a possible 572 bp. Alignment of this sequence with the virtual Cx46.6 mRNA sequence (Figure 2.7A) shows 99% sequence homology over the 468 bp sequence fragment with only two bp mismatches (Figure 2.7B). Therefore, it can be concluded that the fragment amplified by the Cx46.6 PCR primer pair is the virtual Cx46.6 gene. RT-PCR analysis for Cx46.6 expression revealed mRNA expression for Cx46.6

in hNT cultures and not NT2 cultures (Figure 2.3A, Cx46.6 hNT +RT and NT2 +RT respectively) suggesting that, like its mouse counterpart Cx47, Cx46.6 is expressed by CNS neurons. RT-PCR control reactions for false positive and false negative were performed in parallel as described in 2.4.3.

Figure 2.7: Alignment of virtual Cx46.6 mRNA sequence and Cx46.6 PCR product.

The PCR amplicon generated by the Cx46.6 primer pair was sequenced in both the forward and reverse directions and a 468 bp sequence fragment was generated from a possible 572 bp. Alignment of this fragment with the virtual mRNA sequence of Cx46.6 (GI: 11427877) (A) resulted in a 99% sequence homology over the entire 468 bp of the sequence fragment with only two bp mismatches (B).

2.5 Discussion

Prior to the formation of functional chemical synapses, the principal means of communication in the mammalian CNS is mediated by gap junctions formed by connexin proteins (for review see [23, 35, 47]). The importance of connexin-mediated communication on neural progenitor commitment is underlined by evidence that global blockage of GJIC results in a marked decrease in neuronal differentiation of several *in vitro* cell systems including the NT2 cell model [36, 44]. The role of individual Cxs in the process of neuronal differentiation remains poorly understood. The present data contributes further insight into the importance and complexity of Cx gene expression during human neuronal differentiation.

A battery of Cx genes, commonly expressed by cells of the mammalian CNS, were investigated for mRNA and protein expression in the NT2 cell line. Confirming previous reports, Cx26 and Cx32 expression were not detected in NT2 or hNT cultures [85]. RT-PCR analysis revealed mRNA expression of Cx30, Cx36, Cx37, and Cx43 in both undifferentiated NT2 and differentiated hNT cultures. Protein expression was confirmed by immunoblot and/or immunocytochemical analysis for Cx36, Cx37, and Cx43. Consistent with previous reports, Cx43 protein expression was restricted to non-neuronal cells in hNT cultures [85]. Cx43 has been shown to be expressed in ependymal cells, leptomeningeal cells, and astrocytes in the mature CNS [47, 49, 50] but is also predominantly expressed by neural progenitors in the developing brain [64, 85]. Therefore, the localization of Cx43 to non-neuronal cells in hNT cultures

suggests the non-neuronal cells present in hNT cultures are likely glia and/or immature neurons blocked in differentiation.

The complexity of Cx gene expression is underscored by the observed pattern of Cx36 expression and the inconsistency of Cx30 and Cx37 expression in hNT cultures. Cx30 mRNA was detected in 1/3 hNT cultures. Cx30 is expressed predominantly in astrocytes [41, 106]. The ability to differentiate NT2s to a mixed population of neurons and astrocytes by slightly decreasing the concentrations of mitotic inhibitors following RA treatment has recently been reported [84]. Therefore the expression of Cx30 in hNT cultures likely reflects the degree of astrocytic differentiation relative to neuronal differentiation in hNT cultures. The differentiation protocol used for our studies promotes neuronal differentiation; the single hNT culture expressing Cx30 mRNA likely reflects variable tissue cultures conditions promoting glial differentiation relative to the other two cultures that tested negative for Cx30 mRNA expression. Further experiments will examine Cx30 expression in NT2s differentiated under astrocyte-promoting conditions.

Cx36 was detected in 3/3 hNT cultures while Cx37 was detected in only 2/3 hNT cultures tested. In the mature brain Cx37 is expressed in the endothelium of large blood vessels [55], astrocytes and olfactory ensheathing cells [56]. However, to date, spinal motor neurons are the only CNS neuron type known to express Cx37 [57]. Although Cx36 mRNA was detected in all hNT cultures tested, immunocytochemical analysis localized Cx36 protein expression in some but not all neurons and some non-neuronal cells in hNT cultures. Cx36

has been shown to be expressed in several types of neurons in the adult brain including spinal motor neurons [57], CA3/CA4 hippocampal neurons [54], interneurons of the somatosensory cortex [46], the ganglion cell layer and inner plexiform layer of the retina, inferior olive neurons, glomeruli of the olfactory bulb, granule cell layer of the dentate gyrus, and Purkinje cells of the cerebellar cortex [20]. Therefore, the presence of Cx37 in 2/3 hNT cultures and the localization of Cx36 to only a subpopulation of neurons may reflect heterogeneity of neuron subtypes in hNT cultures. In support of previous reports [85], Cx26 and Cx32 were not detected in hNT cultures. Although predominantly expressed in oligodendrocytes, Cx32 has been localized to inhibitory CA1 hippocampal interneurons and basket cells [51, 53]. Therefore the absence of Cx32 suggests that these neuron subtypes are likely not present in hNT cultures. In addition to expression in pinealocytes, ependyma, and leptomeninges [47], Cx26 is localized to horizontal cell dendrites in the outer retina [48]. The absence of Cx26 indicates that horizontal cells are not a likely candidate neuron subtype in hNT cultures and that the culture does not model the retinal CNS. Future studies will focus on determining the identity of connexin-positive cells by antigenic detection of different neuronal subtypes in hNT cultures.

In addition to investigating expression of Cxs known to be expressed in the mammalian CNS, expression of a virtual human connexin (Cx46.6) was also examined by RT-PCR analysis. As discussed in Results (2.4.5), Cx46.6 exists as a virtual Cx gene within the GenBank database at NCBI. In 2001 Willecke's group reported functional expression of a new gap junction gene, Cx47,

transcribed in mouse brain and spinal cord neurons [19]. Of specific interest was the approach used to clone the Cx47 mouse gene; the virtual human Cx46.6 gene sequence was used in combination with rat EST data to generate a PCR probe used to screen and subsequently clone the Cx47 gene from a mouse genomic DNA library. Together, the cloning approach and the CNS expression of Cx47 advocates Cx46.6 as true human Cx gene likely to be expressed in CNS neurons. This hypothesis was empirically tested in the present study. RT-PCR analysis with primers designed for the virtual mRNA sequence, revealed mRNA expression for Cx46.6 in hNT cultures but not NT2 cultures. This marks the first report of biological expression of the Cx46.6 virtual connexin gene. The presence of Cx46.6 in hNT cultures and absence in NT2 cultures implies that Cx46.6 expression is restricted to neurons similar to its mouse homologue Cx47. Interestingly, neuronal Cx47 and Cx36 expression patterns are distinct from, although partially overlapping with, one another *in vivo* suggesting specific Cx expression is dependent on neuronal subtype [19, 20, 54]. Cx47, like Cx36, is expressed in spinal motor neurons, CA3/CA4 hippocampal neurons, Purkinje cells of the cerebellar cortex, and the granule cell layer of the dentate gyrus [19]. However, unlike Cx36, Cx47 is not expressed in the retina but is expressed in hippocampal interneurons, pyramidal neurons of the neocortex, and Golgi neurons [19]. Together, the distinct neuronal expression patterns of Cx36 and Cx47 further support the hypothesis that hNT cultures may contain more than one neuron subtype. Interestingly, spinal motor neurons are the only neuron subtype known to simultaneously express Cx36, Cx37, and Cx47 [19, 57]. Thus

the detection of all three of these Cxs in hNT cultures, suggests that this *in vitro* model may recapitulate characteristics of the spinal cord CNS environment. The lack of commercial anti-Cx47 antibodies and the unsuccessful immunolocalization of Cx37 with available anti-Cx37 antibodies necessitate the need for future *in situ* hybridization experiments to localize Cx36, Cx37, and Cx46.6 in hNT cultures.

Although the presence of Cx43 and the absence of Cx26 and Cx32 expression have been previously reported for the NT2 cell line [85], this study marks the first report of Cx30, Cx36, Cx37 and Cx46.6 expression in this *in vitro* human model. Given that Cx identity underlies the ion selectivity, molecular size permeability, and gating properties of gap junction channels and hemichannels, the finding that NT2 and hNT cultures differentially express multiple connexin genes predicts that communication through gap junctions and/or hemichannels will differ between NT2 neural progenitors and neurons in hNT cultures and thus be of functional significance during the process of neuronal differentiation. This prediction was tested in Chapter 3 of this thesis.

Chapter 3: NT2 neural progenitors but not differentiated hNT neurons are capable of biochemical coupling through gap junctions and hemichannels

3.1 Introduction

As reviewed in Chapter 1, Cx proteins undergo hexameric oligomerization to form connexons that are trafficked to the plasma membrane where they form hemichannels and/or intercellular gap junction channels. Changes in GJIC associated with neuronal differentiation have been studied extensively (for reviews see [23, 47, 107]) and it has been established that GJIC inhibition reduces the capacity of the NT2 cell line to differentiate to postmitotic neurons [44]. All Cxs permit passage of small ions between cells forming the basis of electrical coupling and it is well-established that developing and terminally differentiated cells in the CNS are electrically coupled albeit with Cx-specific variations in gating properties [36, 46, 57, 59]. However, passage of larger second messengers and dyes between cells (biochemical coupling) is Cx-specific. For example, Cx43 homotypic gap junction channels have been shown to preferentially pass ATP, Ca^{2+} , and to a lesser extent adenosine while Cx32 homotypic gap junction channels mediate passage of adenosine and, to a lesser extent ATP, AMP, and ADP [17, 33]. Furthermore, Cx32 homotypic gap junction channels pass cGMP more readily than Cx32/Cx26 heterotypic channels [18].

The ability of most Cxs to form permeable hemichannels has been reported *in vitro*. However published reports on the functional significance of communication mediated by hemichannels are limited. Hemichannels comprised of Cx43 have been shown to (1) control Ca^{2+} intercellular signaling in astrocytes

through the release of ATP [33] and (2) mediate bisphosphate-induced anti-apoptotic signaling in osteocytes [34]. In the outer retina, Cx26 hemichannels have been shown to act as current sinks modulating Ca^{2+} channel activity and subsequent glutamate release [48]. Hemichannels have not been documented in the NT2 model or, for that matter, in any *in vitro* model of neuronal differentiation. Therefore, hemichannel activity (HA) remains an, as of yet, unexplored function for Cxs in differentiating neurons. In addition to confirming reports of decreased GJIC biochemical coupling in NT2s following neuronal differentiation [85], the aim of this study was to establish the levels of endogenous HA in NT2 neural precursors and terminally differentiated hNT neurons.

3.2 Objectives

Decreased GJIC following neuronal differentiation of NT2s to hNTs was confirmed by a series of fluorescent dye transfer assays in the presence and absence of the gap junction blocker 18 α - glycyrrhetic acid (GRA) [108]. To identify HA in neural precursors and differentiated neurons, a series of fluorescent dye uptake assays in the presence and absence of glass microbeads (hemichannel activators [33]) were performed in the presence or absence of flufenamic acid (FFA, a hemichannel/chloride channel inhibitor, [109]) or 4,4' – diisothiocyanatostilbene- 2, 2'- disulfonic acid (DIDS, a chloride channel inhibitor that does not inhibit hemichannels [109]).

3.3 Materials and Methods

All chemical reagents were obtained from Sigma-Aldrich. All cell photos were taken with a Leica DM RXA2 epifluorescent microscope. Quantitative analysis for fluorescent dye transfer and dye uptake assays were performed using Openlab 3.0.8 software using Ratio and Measurement modules (Improvision Ltd, Guelph, ON, Canada).

Cell Culture

NT2 cultures were maintained and differentiated to hNT cultures as described in Chapter 2 Cell Culture Methods with the exception that cells were plated into dishes containing glass coverslips coated with poly-D-lysine (100 µg/mL)/laminin (20 µg/mL) prior to mitotic inhibition.

GJIC Fluorescent Dye Transfer Assay

NT2 cells were grown to 100% confluence. hNT cultures were plated at saturation densities described in Chapter 2 Methods. Cells were washed twice with 10mM TC PBS and incubated with lucifer yellow (LY) (1 mg/ml)/ rhodamine B isothiocyanate-dextran (RD) (1 mg/ml) solution or LY/RD solution containing 100 µM GRA. Cells were scraped gently with a 26^{3/8}-gauge syringe needle and incubated at room temperature for 3 minutes. Following incubation, cells were washed twice with 10mM IC PBS and fixed as described in Chapter 2 Immunocytochemistry Methods. All dye transfer assays were performed in triplicate. In NT2 assays, dye transfer was quantified by measuring the distance of LY transfer from the scrape line. Cells positive for both LY and RD were

excluded from measurements to control for LY uptake due to loss of cell membrane integrity. The mean LY transfer distance was set as 100% LY transfer and dye transfer in the presence of the GJIC inhibitor was standardized to this value. In hNT assays, the number of LY+/RD- cells along the scrape line was established using serial photos taken along the entire length of the scrape (9-14 photos/coverslip) to permit accurate assessment of GJIC in both the hNT neuronal bundles and monolayer non neuronal cells. For statistical analyses, results were subject to one-way analysis of variance (ANOVA) followed by a *post hoc* Dunnetts *t* test with a confidence level of 95%.

HA Fluorescent Dye Uptake Assay

For NT2 culture analyses, NT2 cells were grown at low density sufficient to visualize independent colonies. Cells were washed with 10mM TC PBS and coated with LY/RD solution in the presence and absence of glass microbeads alone or in combination with 50 μ M FFA or 100 μ M DIDS essentially as described in [33]. Cells were incubated for 3 minutes at room temperature. Following incubation, cells were washed twice with 10 mM IC PBS and fixed as described in Chapter 2 Immunocytochemistry Methods. Two replicate experiments were performed for each condition. For quantitation, 16-20 random photos were taken per condition and the number of +LY/-RD cell colonies not in contact with adjacent colonies was counted. Cells positive for both LY and RD were excluded from quantitation to control for LY uptake due to loss of cell membrane integrity. For NT2 assays, the number of +LY/-RD cells is expressed as a percentage of the total number of cells/colony per microscopic field. Since

subconfluent cultures cannot be obtained for hNT cultures, absolute values for the number of +LY/-RD cells were used as quantitative measurements of HA. For statistical analyses, results were subject to univariant ANOVA followed by a *post hoc* Tukey test with a confidence level of 95%.

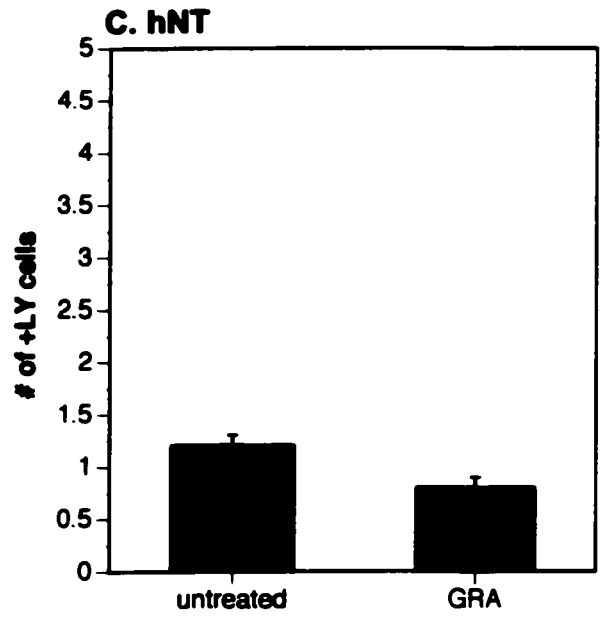
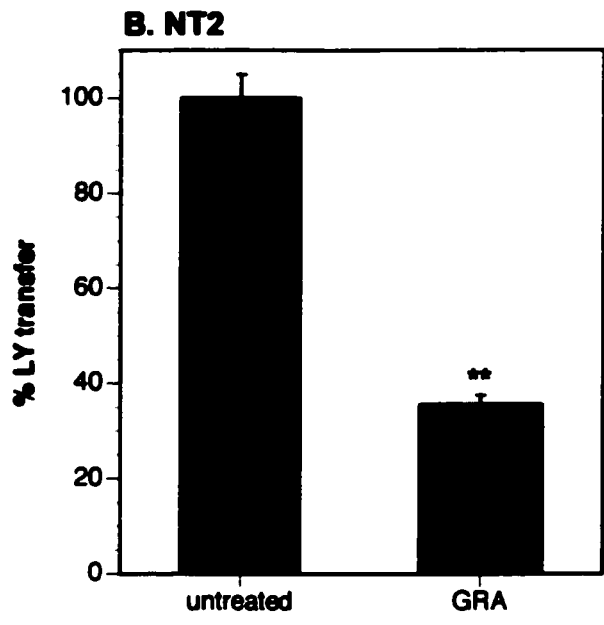
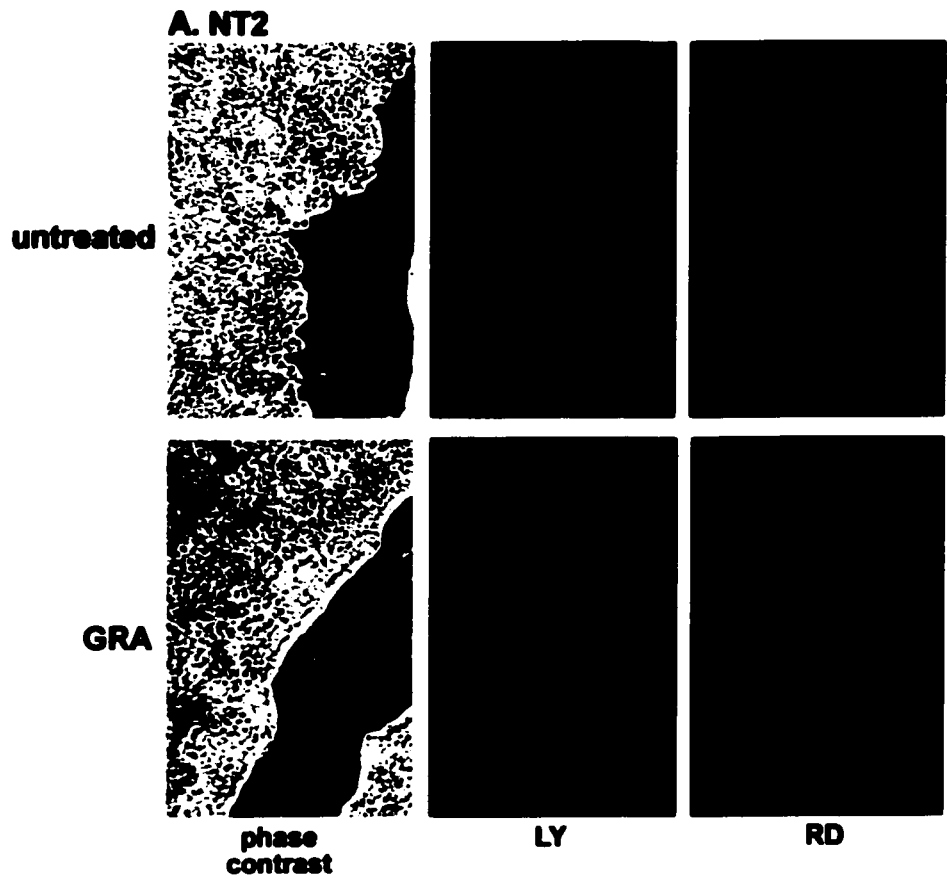
3.4 Results

3.4.1 Endogenous GJIC in NT2 but not hNTs

GJIC (biochemical coupling) in NT2 and hNT cultures was assessed by LY/RD dye transfer assays in the presence or absence of the established gap junction blocker GRA [108]. As reviewed in Chapter 1, gap junctions allow the passage of molecules < 1.0 kD in size; LY at a size of 0.457 kD readily diffuses through gap junctions while RD at a size of 10.2kD is unable to diffuse through gap junctions. Thus, RD transfer indicates a loss of membrane integrity and was used to control for false positive LY transfer results. Representative photomicrographs of gap junction-mediated LY transfer, RD transfer controls, and successful inhibition of LY transfer by the GJIC inhibitor GRA in NT2 cultures are provided in Figure 3.1A. Quantitative analysis of LY dye transfer shows substantial GJIC activity in untreated NT2 cells that is reduced 70% by the GJIC inhibitor GRA (Figure 3.1B, **p<0.01). Conversely, little to no LY dye transfer was observed for hNT cultures; the mean number of cells receiving LY was 1.2 (SEM = 0.1) for untreated cultures and 0.8 (SEM= 0.1) for GRA treated cultures (Figure 3.2C). There was no statistically significant difference between untreated and GRA treated hNT cultures.

Figure 3.1: Endogenous GJIC in NT2s but not hNTs.

GJIC was assessed by LY/RD dye transfer assays in NT2 and hNT cultures. Results of quantitative analysis for GJIC dye transfer assays (n=3 per condition) in NT2 and hNT cultures are provided in B and C respectively. Scrape-loaded untreated NT2 cultures but not hNT cultures readily pass LY to adjacent cells (A. untreated and C. untreated respectively). RD transfer was not observed in NT2 cultures indicating LY transfer is mediated by gap junctions and is not the result of damage to the cell membrane (A. RD). NT2 GJIC-mediated LY transfer is reduced 70% by the gap junction blocker GRA (A. GRA, B. GRA, ** p <0.01). Scale bars, 50µm.



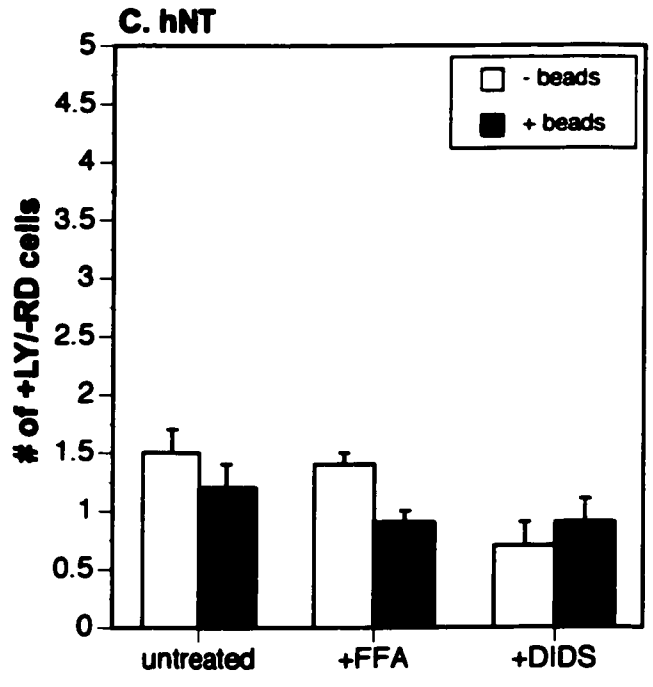
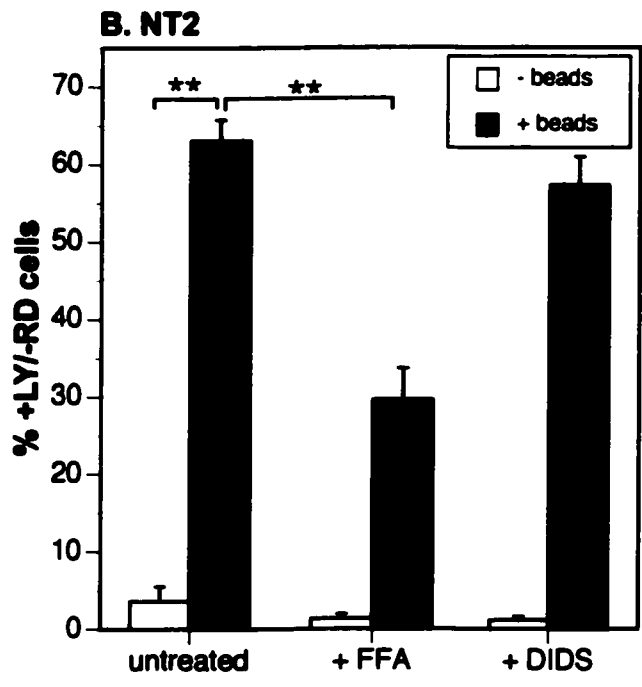
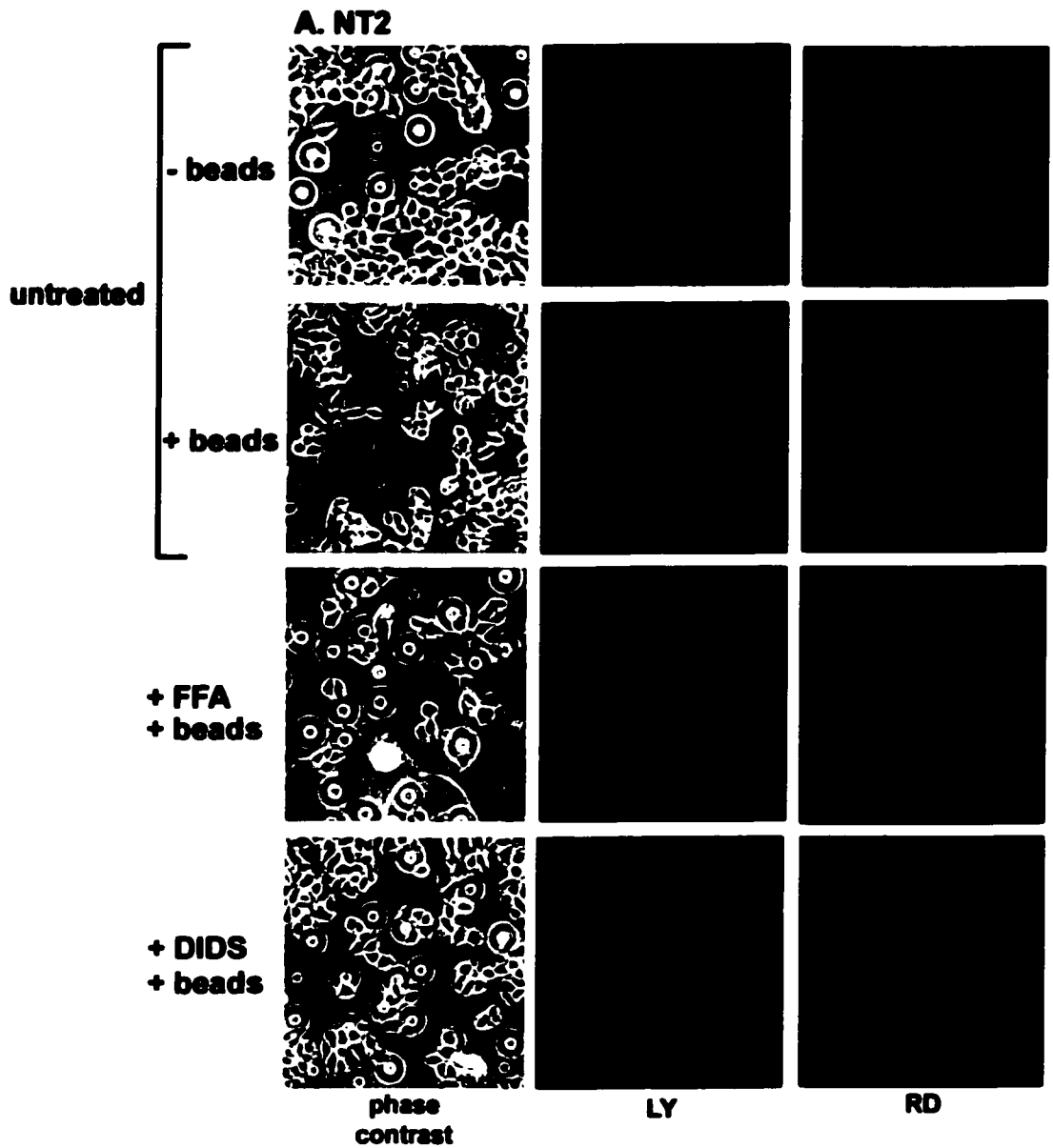
3.4.2 Inducible HA in NT2s but not hNTs

To identify HA in NT2 and hNT cultures, a series of LY/RD dye uptake experiments in the presence and absence of glass microbeads alone or in combination with the hemichannel/chloride channel blocker FFA or the chloride channel blocker DIDS were performed. Representative photomicrographs from HA dye uptake assays in NT2 cultures are provided in Figure 3.2A. Although spontaneous HA was very low (Figure 3.2A, B, untreated/-beads), HA was induced 17-fold in NT2 cultures by glass microbead activators (Figure 3.2A,B, untreated/+beads, $p < 0.01$). Bead-induced NT2 HA was reduced 50% by the addition of FFA (Figure 3.2A,B, FFA/+beads, $p < 0.01$) but remained unaffected by the addition of DIDS (Figure 3.2A,B DIDS/+beads). Significant HA was not observed for hNT cultures in either the absence or presence of bead activators (Figure 3.2C). HA assays were performed with FFA or DIDS in the absence of beads to eliminate possible effects of these inhibitors under non-inducing HA conditions (Figure 3.2B,C, FFA/-beads, DIDS/-beads).

Figure 3.2: Endogenous HA in NT2s but not hNTs.

HA was assessed by LY/RD dye uptake assays in NT2 and hNT cultures.

Results of quantitative analysis of replicate HA dye uptake assays in NT2 and hNT cultures are provided in B and C respectively. HA was induced in untreated NT2 cultures but not hNT cultures following the addition of bead activators (A, B untreated/+beads vs. -beads, ** $p < 0.01$ and C. untreated/+beads vs. -beads respectively). The bead-induced HA in NT2s was reduced 50% by the addition of the hemichannel/chloride channel inhibitor FFA (A, B, FFA/+beads, ** $p < 0.01$). The addition of DIDS, a chloride channel blocker that does not inhibit hemichannels, had no effect on bead-induced HA in NT2s (A, B, DIDS/+beads). Scale bars, 50 μ m.



3.5 Discussion

Cx-mediated communication occurs via two mechanisms: (1) through the formation of gap junctions that allow intercellular communication between adjacent cells and (2) through the formation of unopposed hemichannels that permit the passage of ions and second messengers to and from the extracellular milieu. Gap junction coupling forms the basis for both electrical and biochemical coupling; HA can affect both membrane potential and intracellular signaling. In this study, we used dye transfer methodology to address biochemical signaling mediated by GJIC and HA in NT2 progenitors and terminally differentiated hNT neurons. It has been reported that GJIC is markedly decreased in the NT2 cell line following differentiation and that GJIC inhibition reduces the capacity of NT2s to differentiate to hNT neurons [44, 85]. However, HA remains an unexplored function for Cxs during the process of neuronal differentiation. The experiments presented in this chapter confirm these previous reports and mark the first study to identify HA in the context of neuronal differentiation.

GJIC has been studied extensively over the course of mammalian CNS development. Dye transfer indicative of biochemical coupling has been shown to be widespread among CNS neural progenitors during development and reduced dramatically following progressive differentiation in several regions of the mammalian CNS including but not limited to the cortex [63], hippocampus [35, 36], and spinal cord [57]. Therefore, as expected and previously reported [44, 85], significant biochemical coupling, mediated by GJIC (assessed by LY dye

transfer) and inhibited by the gap junction blocker GRA, was observed in NT2 neural progenitors but not in terminally differentiated hNT cultures.

Similarly, transmembrane dye influx mediated by HA, assessed by LY uptake, was observed in NT2 neural progenitors stimulated with glass microbeads but not in terminally differentiated hNT cultures. The bead-induced HA was significantly reduced by FFA but not by DIDS indicating that the observed increase in LY uptake in the presence of bead activators is the result of HA and not the result of dye uptake by chloride channels. This marks the first report of HA in neural progenitors. The presence of HA in NT2s together with the lack of HA in hNT cultures suggests that like GJIC, HA is progressively decreased over the course of neuronal differentiation. Clearly, this conclusion must be verified empirically by assessing dye uptake at several time-points during differentiation of NT2s to hNT neurons. Given that Cx type dictates the ion permeability, molecular size selectivity, and gating properties of gap junction channels and hemichannels, identification of the specific Cxs comprising the hemichannels participating in the observed HA in NT2s will be required as a first step in understanding their functional relevance. Recent studies have reported that Cx43 hemichannels (1) control Ca^{2+} intercellular signaling in cultured astrocytes through the release of ATP [33] and (2) mediate bisphosphate-induced anti-apoptotic signaling in osteocytes [34]. In addition Cx26 hemichannels have been shown to act as current sinks modulating of Ca^{2+} channel activity and subsequent glutamate release in the outer retina [48]. Intracellular concentrations of both Ca^{2+} and ATP along with other small

molecules such as cAMP are important in signaling neuronal differentiation [110, 111]. Therefore, biochemical coupling mediated by HA may be one means by which neural precursors interact with their environment and regulate their activation and differentiation. Future experiments will be aimed at investigating the effects of HA inhibition on neuronal differentiation and at identifying the important molecular signals being exchanged via hemichannels.

While this thesis did not address changes in electrical coupling, a decrease in synchronized electrical activity is observed over the course of CNS development. Specifically decreased electrical coupling has been reported over the course of spinal motor neuron development [57], cortical neuron development [112], and neuronal differentiation of hippocampal progenitors *in vitro* [36]. However, in recent studies electrical coupling mediated by GJIC has been shown to persist in subsets of adult neurons from the hippocampus and cortex of rodents [46]. As previously discussed, the Cx composition of a given gap junction channel dictates its molecular size selectivity and ion permeability and will therefore dictate its ability to transfer dyes such as LY. Gap junctions formed by Cx36 have been shown to be incapable of transferring LY *in vitro* [20]. Together with the identification of Cx36 gap junctions in adult neurons [20, 113, 114], it has been speculated that Cx36 participates exclusively in electrical coupling, not biochemical coupling [19, 20, 54]. Therefore, the continued expression of Cx36 in hNT cultures (refer to Chapter 2) but lack of biochemical coupling mediated by GJIC or functional HA supports the proposed exclusive role

of Cx36 in control of electrical coupling in mature neurons. Future experiments will be aimed at verifying electrical coupling mediated by Cx36 in hNT cultures.

In addition to Cx36, gap junctions composed of Cx47 (the mouse homologue of human Cx46.6) have also been identified in adult neurons [19]. However, unlike Cx36 gap junctions, Cx47 gap junctions have been shown to readily pass several dyes including LY *in vitro* [19]. Thus it has been speculated that Cx47 gap junctions, in contrast to Cx36 gap junctions, participate in the biochemical not electrical coupling of mature neurons. Despite Cx46.6 expression in hNT cultures (refer to Chapter 2) biochemical coupling mediated by GJIC or HA was not observed. This appears to be in direct conflict with the proposed role of Cx47 in biochemical coupling of adult neurons. However, the expression of several Cxs, including Cx47, has been shown to persist in mature spinal motor neurons deficient in biochemical coupling [19, 57]. Interestingly biochemical coupling was shown to be recapitulated in spinal motor neurons following nerve injury without obvious differences in Cx expression [57]. Therefore, it has been proposed that biochemical coupling (and likely electrical coupling) is not regulated at the level of mRNA or protein expression but is modulated by mechanisms that affect gap junction assembly, permeability, and/or open state [57]. This hypothesis is consistent with the lack of GJIC and HA in light of persistent expression of Cx46.6 in hNT cultures. Thus it is tempting to speculate that the persistence of certain Cxs, specifically Cx46.6, in biochemically uncoupled mature neurons serves as a readily accessible intracellular pool that can be called upon quickly to recapitulate transient

biochemical coupling mediated by GJIC and/or HA under specific conditions such as nerve cell injury. Alternatively, Cx47 may form heterotypic or heteromeric connexons with Cx36 thereby altering capacity of LY transfer. Connexin partners with Cx36 have yet to be identified in mature neurons.

Altogether the present data support the increasing evidence that HA is a significant function of Cx proteins. Therefore, when addressing the roles of individual Cxs, it is now necessary to interpret results in the context of both HA and GJIC. Overall, the studies presented in this thesis have set the stage for a new direction in Cx research focusing on a possible role for HA in neuronal differentiation and CNS development.

Chapter 4: RA-induced differentiation of the NT2 cell line to CNS neurons is blocked by transfection with Cx32

4.1 Introduction

Expression of Cx32 in the adult mammalian CNS is restricted to oligodendrocytes [115] and some neurons [38, 53, 116-118]. In rodents, maximal Cx32 expression is observed through postnatal weeks 3 to 6 [38]. These expression kinetics coincide with the later stages of differentiation, neurogenesis, cell migration, and neural circuit formation in the rodent CNS [39, 43]. As reviewed in Chapter 1, mutations in the Cx32 gene result in the human peripheral neuropathy CMTX and recent studies have reported neuronal dysfunction in cerebral motor, visual, and acoustic pathways in the CNS of a subset of CMTX patients [74, 75]. Together these reports implicate a role of Cx32 in neural cell development in the mammalian CNS.

Previous work in the Bennett laboratory with Cx32 null mutant mice has shown that altering connexin expression influences neural progenitor number (Melanson-Drapeau *et al*, submitted to *J Neurosci*, 07/19/02). Two mechanisms are proposed: the loss of Cx32 enhances retention of subpopulations of neural progenitors or, alternatively, the loss of Cx32 prevents terminal differentiation of neural progenitors *in vivo*. However, a direct effect of Cx32 expression on neuronal differentiation has yet to be empirically determined. Given the inherent complexity of *in vivo* studies, the NT2 *in vitro* model was chosen to study the effect of ectopic Cx32 expression on neuronal differentiation.

4.2 Objectives

To elucidate possible roles of Cx32 in neuronal differentiation, we sought to generate clonal NT2 lines stably transfected with human Cx32 (HCx32). Two mammalian expression vectors expressing HCx32 were constructed: pcDNAHCx32 (expressing HCx32 constitutively as a *Myc/His* fusion protein) and pINDHCx32 (expressing HCx32 following ponasterone induction). Although attempts at generating clonal NT2 lines stably transfected with the dual-vector pVgRXR/pINDHCx32 inducible system were unsuccessful, clonal NT2 lines stably transfected with pcDNAHCx32 or empty vector (pcDNA3.1 *Myc/His A*) control were obtained. The effect of constitutive Cx32 expression on RA-induced differentiation of the NT2 cell line was assessed by morphological analysis and Western blot analysis using cell lineage markers.

4.3 Materials and Methods

All chemicals reagents were obtained from Sigma-Aldrich.

Plasmids

All plasmid vectors were obtained from Invitrogen. All restriction enzymes (REs) and ligation reagents were obtained from Promega.

The coding region of HCx32 was PCR amplified (see PCR methods, Chapter 2) from the pSPHCx32 template provided by Roberto Bruzzone (Institut Louis Pasteur, France) using primers designed with RE sites *Xho*I (primer HCx32F 5'-CTCGAGTGAGGCAGGATGAACTGG-3') and *Hind*III (primer HCx324R 5'- TAATAAAGCTTGCAGGCCGAGCAGC-3') compatible for insertion

into the pcDNA3.1 *Myc/His A* vector. The HCx32 PCR amplicon and pcDNA3.1 *Myc/His A* vector were RE digested with *XhoI* and *HindIII* according to manufacturer's recommendations. The HCx32 gene was excised from the pSPHCx32 template by RE digest with *HindIII* and *BamHI* according to manufacturer's recommendations for subsequent ligation into the pIND vector.

All RE digests were agarose gel purified and combined in standard ligation reactions containing T4 DNA ligase enzyme with varying vector:insert ratios (including a control reaction for vector recircularization) according to manufacturer's recommendations and incubated overnight at 16°C. The ligation reactions were diluted and transformed into *Escherichia coli* (*E. coli*) JM109 competent cells (Promega). Antibiotic resistant colonies were selected and their plasmids purified. RE digest analysis of purified plasmids were performed according to manufacturer's recommendations followed by direct DNA sequencing at the University of Ottawa Biotechnology Research Institute (Ottawa, ON, Canada).

Cell Culture

NT2 cultures were maintained and differentiated as described in Chapter 2, Methods.

Transfection

For constitutive expression experiments, undifferentiated NT2 cells were transfected at passage 57 with pcDNA3.1 *Myc/His A* (5 µg) or pcDNAHCx32 *Myc/His* (5 µg) by lipofection using Transfast Transfection Reagent (Promega)

with an optimized charge ratio of 1:1. After two days in DMEM/F-12 complete medium, transfectants were selected with 300 µg/mL of Geneticin® G-418 sulfate (G418, a neomycin analogue, GibcoBRL) for a minimum of 14 days. Clonal NT2 lines resistant to G418 were expanded to passage 61 and frozen stocks prepared. For inducible Cx32 expression experiments, NT2s were co-transfected with pVgRXR and pINDCx32 or pINDLacZ (control) as described above. Repeated attempts to generate stable zeocin/G418-resistant co-transfectants were unsuccessful.

Genomic DNA Extraction

Genomic DNA was extracted from NT2 stable transfectants as described in Chapter 2 Methods.

RT-PCR

RT of total RNA extracted from NT2 transfectants and subsequent PCR analysis was performed as described in Chapter 2 Methods. Primer sequences used for verification of plasmid insertion in to the genome are as follows: pcDNA3.1 forward primer 5'-CCACTGCTTACTGGCTTATC-3' and pcDNA3.1 reverse primer 5'-AACTAGAAGGCACAGTCGAG-3.

Protein Isolation and Western Blot Analysis

Protein was isolated and subjected to Western blot analysis as described in Chapter 2 Methods. Refer to Appendix A for antibody suppliers and working dilutions.

4.4 Results

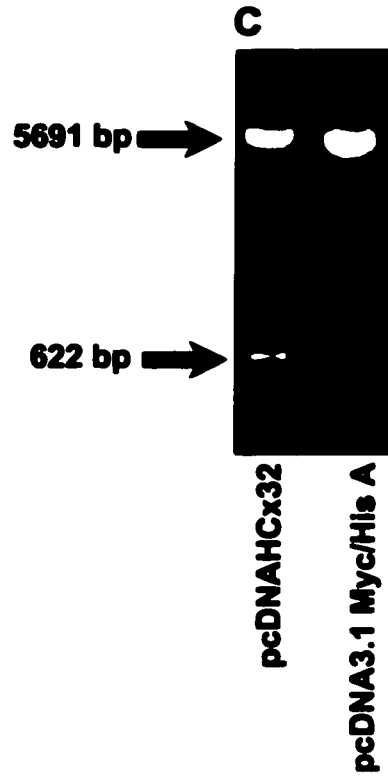
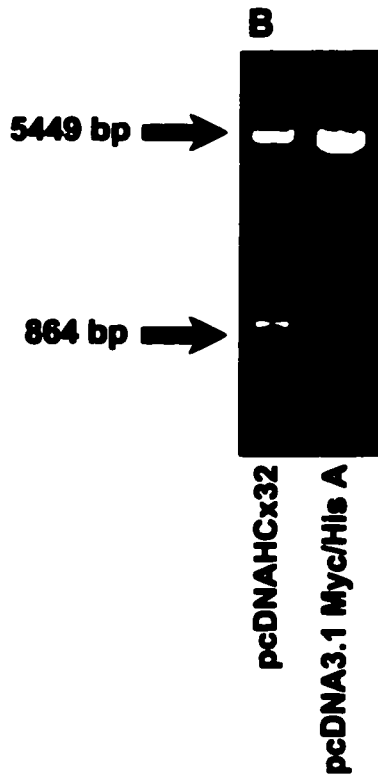
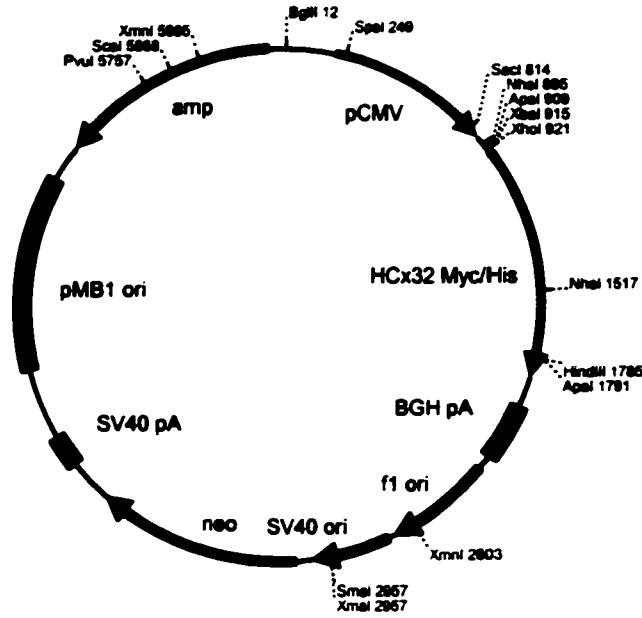
4.4.1 Creation of a constitutive human Cx32 mammalian expression vector

Primers were designed and optimized for PCR with RE sites (*Xho*I and *Hind*III) compatible for insertion of HCx32 into the pcDNA3.1 *Myc/His A* (Invitrogen) expression vector to produce an HCx32 *Myc/His* fusion protein. This vector is referred to as pcDNAHCx32. A map of pcDNAHCx32 is provided in Figure 4.1A. RE digests confirmed insertion of HCx32 as well as the correct orientation of HCx32 with respect to the cytomegalovirus (CMV) promoter. The pcDNAHCx32 plasmid contains an 864 bp fragment corresponding to the HCx32 insert and a 5449 bp fragment corresponding to the pcDNA3.1 *Myc/His A* vector sequence when double-digested with *Xho*I and *Hind*III REs (Figure 4.1B). Correct orientation of HCx32 with respect to the CMV promoter was confirmed by *Nhe*I RE digest. A single *Nhe*I site is present within the HCx32 sequence (position 1517 on pcDNAHCx32 map, Figure 4.1A) as well as within the pcDNA3.1 *Myc/His A* multiple cloning site (MCS) (position 895 on pcDNAHCx32 map, Figure 4.1A). The pcDNAHCx32 vector contains a 622 bp fragment confirming correct orientation of HCx32 and a 5691 bp fragment corresponding to the pcDNA3.1 *Myc/His A* vector sequence. Integrity of pcDNAHCx32 vector was further confirmed by direct sequencing in both the forward and reverse directions using commercial priming sites on either side of the HCx32 insert.

Figure 4.1: Generation of a Cx32 constitutive expression vector.

The coding region of the human Cx32 (HCx32) gene was PCR amplified from a template provided by Roberto Bruzzone (Institut Louis Pasteur, France) and inserted into the pcDNA3.1 *Myc/His A* expression vector (Invitrogen). The resulting plasmid is referred to as pcDNAHCx32 (A) and expresses HCx32 as a *Myc/His* fusion protein. Successful insertion of the HCx32 gene was confirmed by RE digest analysis: *XhoI/HindIII* double digests for insert confirmation (B) and *NheI* digest to confirm correct gene orientation (C). Restriction digests were performed in parallel with the pcDNA3.1 *Myc/His A* empty vector (B, C,) as a control for restriction enzyme function.

A. pcDNAHCx32 (6313 bp)



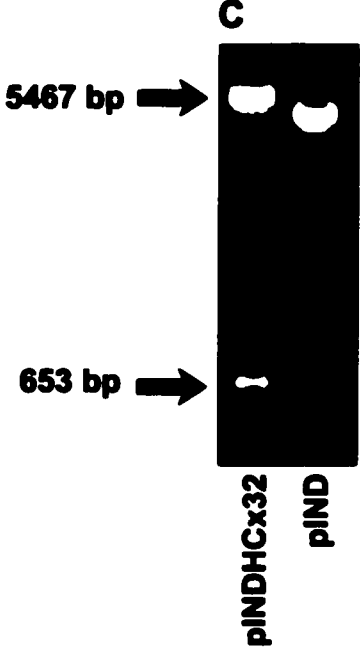
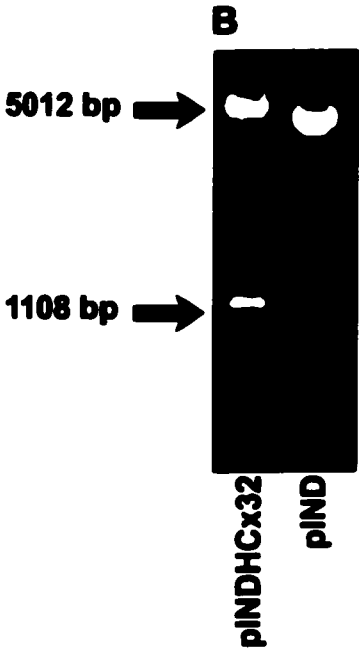
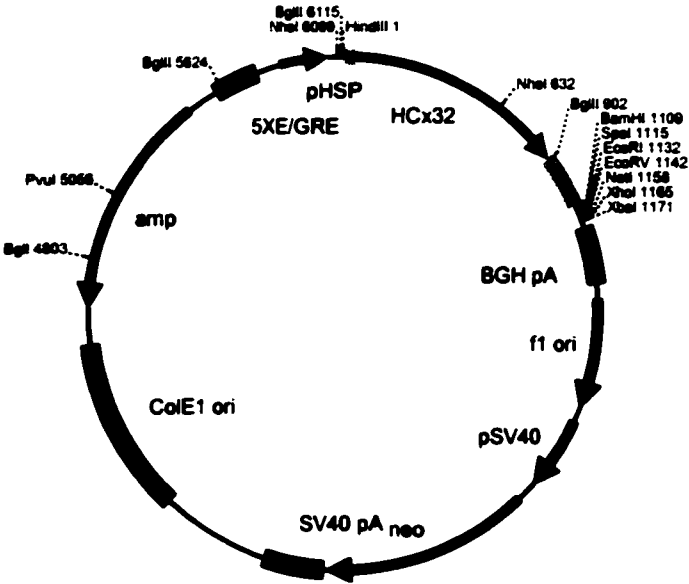
4.4.2 Creation of an inducible human Cx32 mammalian expression vector

The HCx32 coding region was subcloned from pSPHCx32 (provided by Roberto Bruzzone, Institut Louis Pasteur, France) into the pIND (Invitrogen) expression vector using *HindIII* and *BamHI* REs. The resulting plasmid is referred to as pINDHCx32. A map of pINDHCx32 is provided in Figure 4.2A. RE digests confirmed insertion of HCx32 as well as correct orientation of HCx32 with respect to the heat shock protein (HSP) promoter within the pIND vector. The pINDHCx32 vector contains an 1108 bp fragment corresponding to HCx32 and a 5012 bp fragment corresponding to the pIND vector sequence when digested with *HindIII* and *BamHI* (Figure 4.2B). Correct orientation of HCx32 with respect to the HSP promoter was confirmed by *NheI* RE digest (Figure 4.2C). A single *NheI* site is present within the HCx32 sequence (position 632 on pINDHCx32 map, Figure 4.2A) as well as within the pIND MCS (position 6099 on pINDHCx32 map, Figure 4.2A). The pINDHCx32 vector contains a 653 bp fragment confirming correct orientation of HCx32 and a 5467 bp fragment corresponding to the pIND vector sequence when digested with *NheI* (Figure 4.2C). The integrity of the pINDHCx32 plasmid was further confirmed by direct sequencing in the forward direction using a primer specific to the HCx32 sequence (available in the Bennett laboratory) and confirmed to be pIND with an HCx32 insertion in the correct orientation. The pINDHCx32 expresses HCx32 only in the presence of both the pVgRXR (Invitrogen) vector and the inducing agent ponasterone.

Figure 4.2: Generation of a Cx32 inducible expression vector for use in the Ecdysone Mammalian Expression System (Invitrogen).

The coding region of HCx32 gene was subcloned from a template provided Roberto Bruzzone (Insitut Louis Pasteur, France) into the pIND expression vector (Invitrogen) using existing *Hind*III and *Bam*HI RE sites. The resulting plasmid is referred to as pINDHCx32 (A) and expresses HCx32 only in the presence of both the pVgRXR (Invitrogen) vector and the inducing agent ponasterone. Successful insertion of the HCx32 was confirmed by restriction digest analysis; *Hind*III/*Bam*HI double digests confirmed HCx32 insertion (B) and *Nhe*I digest to confirm correct gene orientation (C). Restriction digests were performed in parallel with pIND empty vector (B, C) to control for restriction enzyme function.

A. pINDHCx32 (6120 bp)



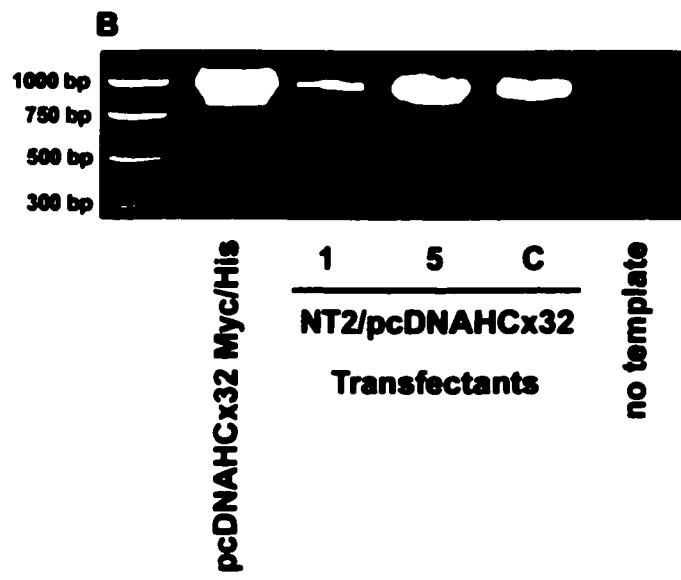
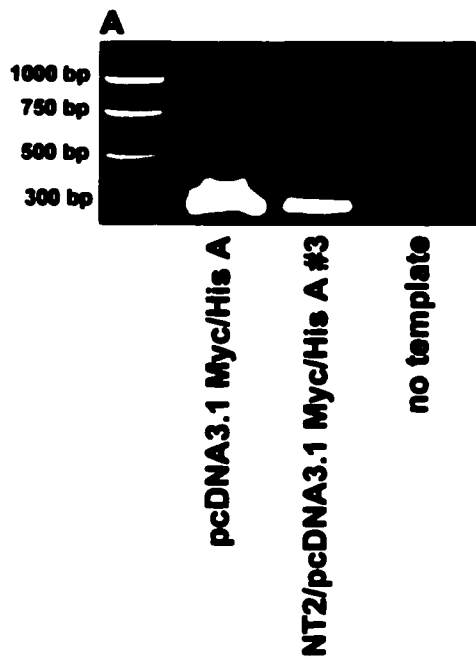
4.4.3 Successful transfection of the NT2s with pcDNAHCx32 but not pINDHCx32

Undifferentiated NT2 cells were transfected at passage 57 with pcDNAHCx32 or pcDNA3.1 *Myc/His A* (empty vector control) to generate stable clonal lines expressing the HCx32 *Myc/His* fusion protein or empty vector respectively. Stable clonal lines transfected with pcDNAHCx32 are referred to as NT2/pcDNAHCx32 while those transfected with the empty vector are referred to as NT2/pcDNA3.1 *Myc/His A*. A total of 16 NT2/pcDNAHCX32 transfectants and 17 NT2/pcDNA3.1 *Myc/His A* transfectants were generated. Attempts at both co-transfection and sequential transfections with pINDHCx32/pVgRXR or pINDlacZ/pVgRXR control plasmids were unsuccessful.

Plasmid insertion into the NT2 genome was confirmed by PCR (Figure 4.3). The primers used to detect plasmid insertion are located within the MCS of pcDNA3.1 *Myc/His A* vector and on either side of HCx32 in pcDNAHCx32 (see PCR methods for primer sequences). Plasmid insertion into the NT2 genome was confirmed for five NT2/pcDNA3.1 *Myc/His A* transfectants (Figure 4.3A) and five NT2/pcDNAHCx32 transfectants (Figure 4.3B). It should be noted that data is shown for a representative NT2/pcDNA3.1 *Myc/His A* transfectant and three representative NT2/pcDNAHCx32 transfectants. These representative transfectants were used for all subsequent experiments. Parallel PCR reactions were performed with pcDNA3.1 *Myc/His A* and pcDNAHCx32 plasmid templates to serve as positive controls for successful PCR and eliminate false negative results (Figure 4.3A, pcDNA3.1 *Myc/His A*, 4.3B, pcDNAHCx32). Parallel PCR

Figure 4.3: PCR confirmation of plasmid insertion into the NT2 genome following transfection.

Plasmid insertion into the genome of NT2s transfected with pcDNA3.1 *Myc/His A* (A) or pcDNAHCx32 (B) were confirmed by PCR amplification of genomic DNA with primers located within the MCS of pcDNA3.1 *Myc/His A* on either side of HCx32 in pcDNAHCx32. Reactions containing plasmid templates were used as a positive control for PCR (A, pcDNA3.1 *Myc/His A*, B, pcDNAHCx32). Reactions were also performed without template to control for PCR reagent contamination (no template).



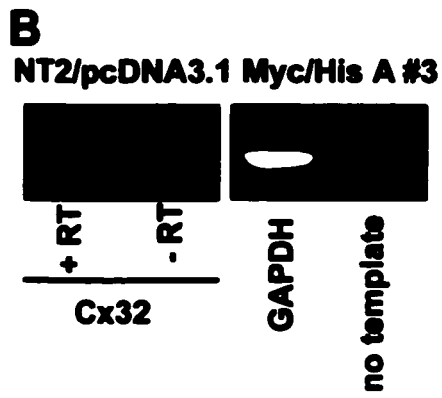
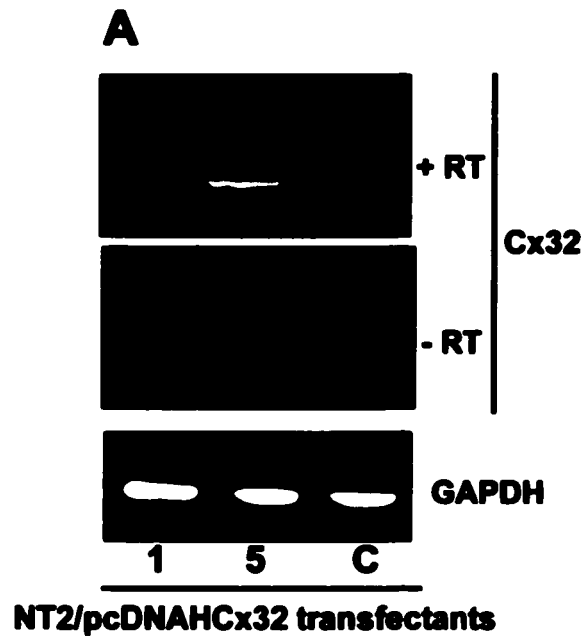
reactions were also performed without the addition of template DNA to eliminate false positive results due to PCR reagent contamination (Figure 4.3A and B, no template).

Following confirmation of plasmid insertion into the genome, RT-PCR analysis was performed on total RNA extracts to detect HCx32 mRNA expression. Initial RT-PCR analyses were negative for HCx32 expression in all NT2/pcDNAHCx32 transfectants tested (data not shown). However, NT2s are known to be inherently genetically unstable at passage numbers >63 (Stratagene and observations from our laboratory). Since the initial RT-PCR analyses were performed on total RNA extracts from transfectants following repeated passage (~8) for a total passage number of (~65), the RT-PCR analyses were repeated on total RNA extracted from the lowest available passage number (61) for each transfectant. RT-PCR of passage 61 total RNA revealed HCx32 mRNA expression in one of the three NT2/pcDNAHCx32 transfectants (Figure 4.4A, +RT). It should be noted that an additional NT2/pcDNAHCx32 transfectant positive for HCx32 mRNA expression was later identified (data not shown). As expected RT-PCR analysis of total RNA extracted from the representative NT2/pcDNA3.1 Myc/His A transfectant was negative for HCx32 mRNA expression (Figure 4.4B, +RT).

Several control reactions were performed in parallel to exclude false positive and false negative results during RT-PCR. PCR amplification of GAPDH mRNA, present in all cell types, was used to control for the quality of the cDNA template (Figure 4.4A and B, GAPDH). Reactions were performed without the

Figure 4.4: HCx32 mRNA expression in NT2/pcDNAHCx32 transfectants but not NT2/pcDNA3.1 Myc/His A transfectants.

RT-PCR analysis of random-primed total RNA from undifferentiated NT2/pcDNAHCx32 cultures (passage 61) revealed HCx32 mRNA expression in 1 of 3 cultures (A, +RT, data shown for 3/5 cultures tested). HCx32 expression was not detected in the representative NT2/pcDNA3.1 Myc/His A culture (B, +RT). The integrity of the cDNA templates was confirmed by amplification of the internal control GAPDH (A, B, GAPDH). RT reactions were performed without the addition of reverse transcriptase enzyme to control for genomic DNA contamination (A, B, -RT). Reactions were also performed without the addition of cDNA template to control for contamination PCR reagents (C, no template). The pcDNAHCx32 plasmid template was used as a positive control for PCR (C).



addition of reverse transcriptase enzyme during the RT of total RNA to control for genomic DNA contamination in the sample following DNaseI treatment and eliminate false positive results during PCR (Figure 4.4A and B, –RT). Reactions were also performed without the addition of cDNA template to control for DNA contamination in PCR reagents (Figure 4.4B and C, no template). Reactions were performed with pcDNAHCx32 plasmid template to serve as a positive control for successful PCR and eliminate false negative results (Figure 4.4C).

All attempts to detect HCx32 *Myc/His* fusion protein expression by immunocytochemical or immunoblot analysis with anti-*Myc*, anti-*His*, and anti-*Cx32* antibodies in NT2/pcDNAHCx32 transfectants were unsuccessful. Anti-*myc* immunoblot and immunocytochemical analyses were confounded by the fact that pcDNA3.1 *myc* epitope tag is of human origin and thus antibodies cross-react with endogenous *myc* expressed by human NT2 cells. The anti-*His* antibody is specific for tandem *His* epitopes but failed to reliably detect protein expression in transfectants.

4.4.4 RA-induced differentiation of NT2s to CNS neurons is blocked by HCx32 transfection

A total of five NT2/pcDNAHCx32 transfectants (all positive for plasmid insertion into the genome and of which two were positive for HCx32 mRNA expression) underwent RA and mitotic inhibitor treatments to determine the effect of HCx32 on differentiation. To control for artefacts of stable transfection, five NT2/pcDNA3.1 *Myc/His A* transfectants (all positive for plasmid insertion into the

genome) also underwent RA and mitotic inhibitor treatments. Note that undifferentiated NT2/pcDNAHCx32 and NT2/pcDNA3.1 *Myc/His A* transfectants appear normal in culture based on cell morphology compared to untransfected NT2 cultures (compare Figure 2.1A to Figure 4.5A). Following RA and mitotic inhibitor treatments, four of the five empty vector transfectants successfully differentiated to neurons while none of the five HCx32 transfectants successfully differentiated to neurons (Figure 4.5B, data is shown for representative transfectants). Neuronal differentiation was assessed by morphological analysis (Figure 4.5) and Western blot analysis (Figure 4.6).

As reviewed in Chapter 2, the hallmark features of neuronal differentiation exhibited by RA-treated NT2 cells are migration of small refractile hNT cell soma into large aggregates (Figure 2.1B, black arrow) and extensive neuritic outgrowth (Figure 2.1B, arrowhead). Each of these morphological hallmarks was exhibited by four out of five empty vector cultures. Thus, transfection of NT2s with plasmid DNA does not significantly affect their ability to morphologically differentiate to a neuronal phenotype (Figure 4.5B, NT2/pcDNA3.1 *Myc/His A* #3). The single NT2/pcDNA3.1 *Myc/His A* transfectant that failed to differentiate to neurons likely represents a clone whose plasmid insertion site was within a region of the genome critical for neuronal differentiation. In the case of the five HCx32 transfectants, cultures failed to develop the morphological hallmarks of differentiation to a neuronal phenotype (Figure 4.5 B, NT2/pcDNAHCx32

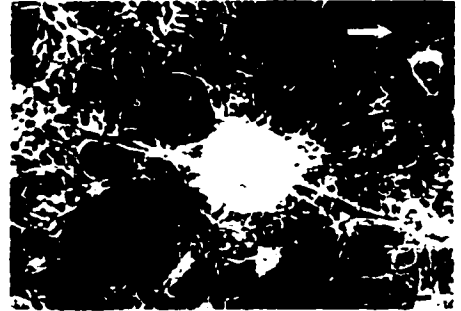
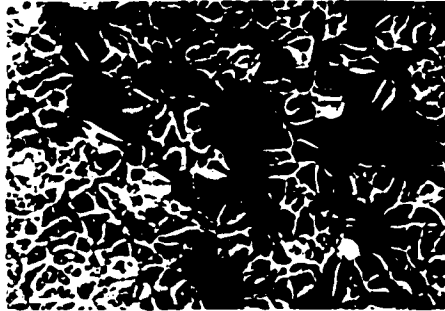
Figure 4.5: NT2/pcDNAHCx32 transfectants fail to differentiate to postmitotic neurons.

Undifferentiated NT2s transfected with HCx32 (A, NT2/pcDNAHCx32) cultured in complete media supplemented with 10 μ M RA for four weeks followed by mitotic inhibition for three weeks failed to differentiate to neurons (B, NT2/pcDNAHCx32). Note that the empty vector control culture successfully differentiated to postmitotic neurons (B, NT2/pcDNA3.1 *Myc/His A #3*) characterized by aggregation of cell bodies into bundles (B, black arrow) and extensive neuritic outgrowth (B, arrowhead). The differentiated NT2/pcDNAHCx32 cultures contain cells similar in morphology to the non-neuronal cells in the NT2/pcDNA3.1 *Myc/His A #3* culture (B, white arrow). Scale bars, 50 μ m.

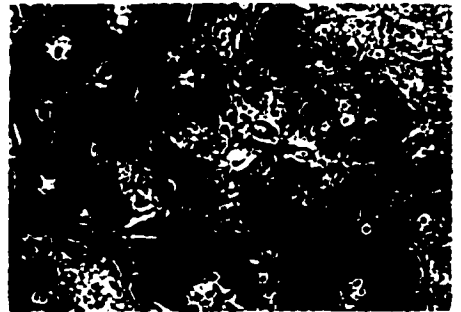
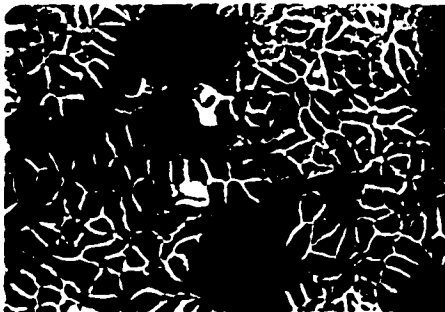
A. Undifferentiated

B. RA Differentiated

NT2/pcDNA3.1 Myc/His A #3

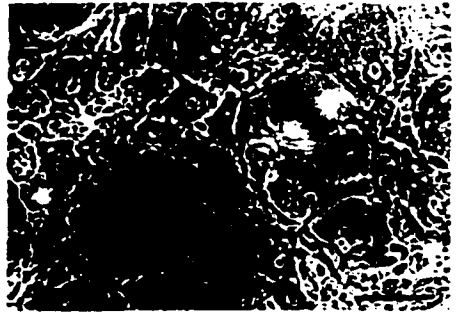
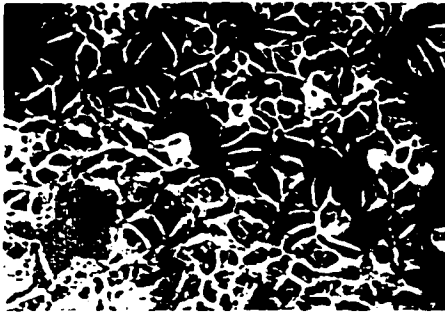


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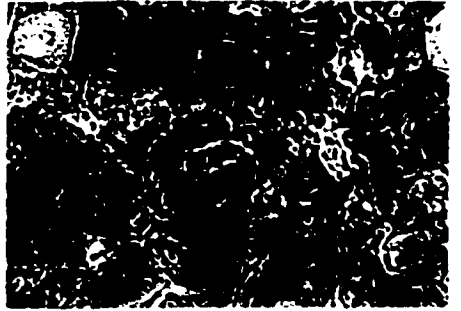
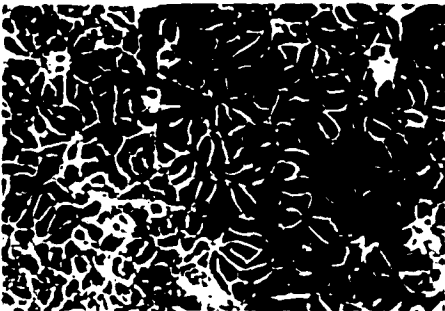


**NT2/pcDNAHCx32
Transfectants**

5



C



transfectants). The cells present in the differentiated NT2/pcDNAHCx32 cultures appeared similar in morphology to the non-neuronal cells observed in hNT cultures (Figure 2.1B, white arrow) and NT2/pcDNA3.1 *Myc/His A* cultures (Figure 4.5B, white arrow).

To further support morphological analyses, Western blotting was performed with antibodies raised against cell lineage markers to confirm the inability of HCx32-transfected NT2s to differentiate to neurons. The following antibodies were used: (1) proliferating cell nuclear antigen (PCNA; a 36kD marker of actively dividing cells [94-96], (2) neurofilament 68 (NF-68; a 68kDa marker for differentiated CNS neurons,[97, 98]), (3) growth associated protein 43 (GAP43; a 43kD marker for proliferating neuronal precursors [99], neurons undergoing axonal outgrowth [100] as well as oligodendrocyte pre-progenitors and progenitors [101, 102], (4) myelin proteolipid protein (myelin PLP, a 33.4kD marker for oligodendrocyte progenitors as well as developing and mature oligodendrocytes [103, 104], and (5) class III β -tubulin (Beta-Tubulin III) a neural differentiation-dependent marker [119-122]

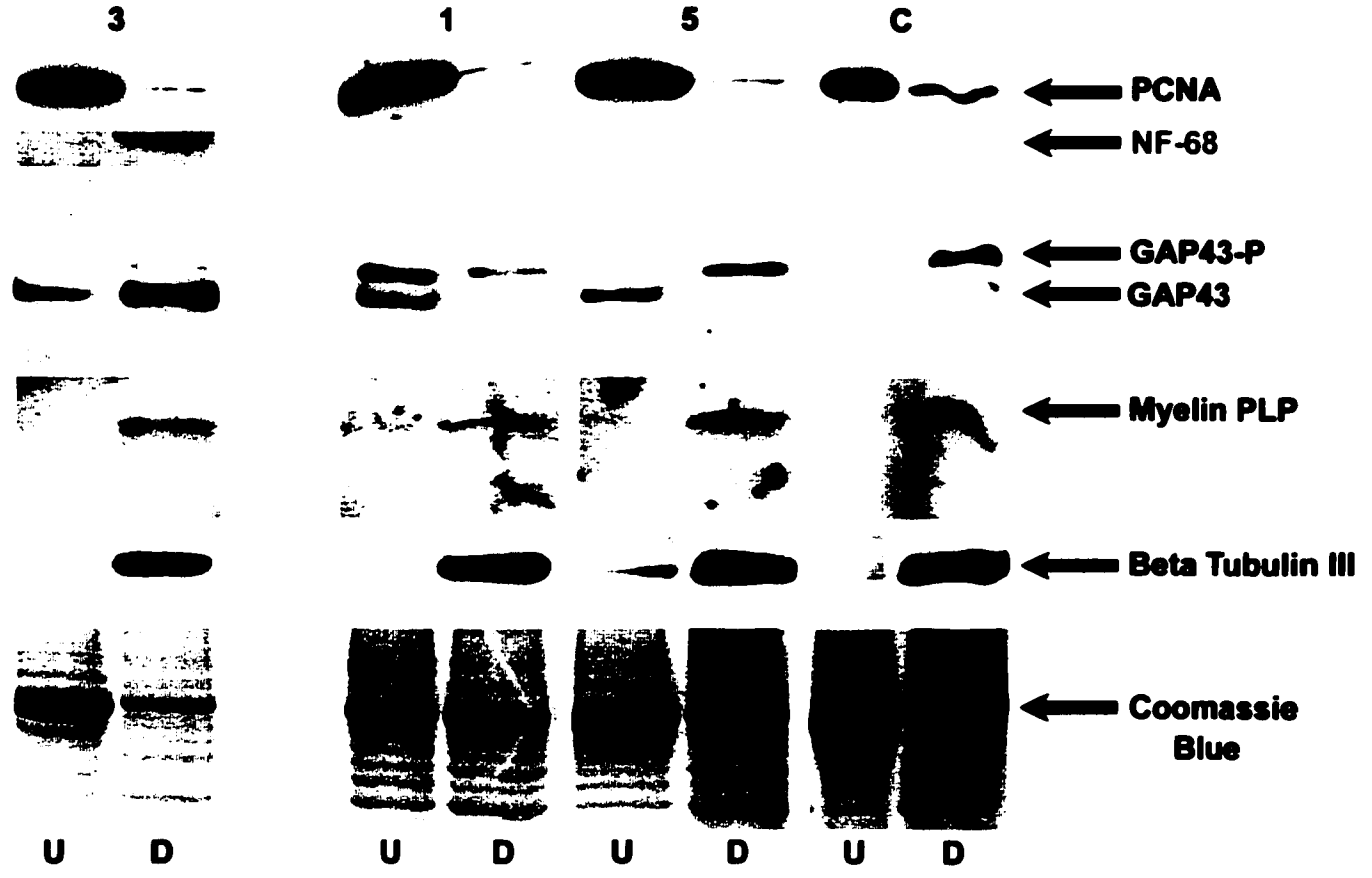
Decreased levels of PCNA were observed for RA-differentiated NT2/pcDNA3.1 *Myc/His A* #3 and NT2/pcDNAHCx32 cultures when compared to the respective undifferentiated cultures (Figure 4.6, PCNA). This decrease in PCNA is indicative of the relative growth arrest associated with differentiation to postmitotic cultures following RA and mitotic inhibitor treatments. Consistent with morphological analysis, NF-68 was detected in the differentiated NT2/pcDNA3.1 *Myc/His A* #3 culture but not the differentiated NT2/pcDNAHCx32 cultures

Figure 4.6: Western blot analysis confirms inability of NT2s transfected with HCx32 to differentiate to neurons.

Immunoblotting of NT2 transfectants confirms absence of the neuron-specific marker NF-68 in differentiated HCx32 transfected cultures (NF-68, NT2/pcDNAHCx32, D) present in the differentiated empty vector transfectants (NF-68, NT2/pcDNA3.1 Myc/His A #3, D). In all transfectants, decreased levels of PCNA were observed following RA and mitotic inhibitor treatments indicative of growth arrest (PCNA, U vs. D); the oligodendrocyte marker myelin PLP was observed for differentiated cultures only (myelin PLP, U vs. D); the expression of GAP43 persisted through differentiation (GAP43/GAP43-P, U vs. D); and high levels of β -tubulin were observed in differentiated cultures compared to undifferentiated cultures (Beta-Tubulin III, U vs. D). Coomassie blue stained gels (Coomassie blue) are shown as protein loading controls.

NT2/pcDNA3.1 Myc/His A

NT2/pcDNAHCx32 Transfectants



(Figure 4.6, NF-68). The absence of NF-68 in differentiated NT2/pcDNAHCx32 cultures substantiates the morphological analysis demonstrating the inability of HCx32 transfected NT2s to differentiate to postmitotic neurons.

Comparable to untransfected NT2s, GAP43 protein expression was observed in the undifferentiated and differentiated empty vector and HCx32 transfected cultures (Figure 4.6, GAP43, GAP43-P). In all transfected NT2 lines, low levels of both the unphosphorylated and phosphorylated (GAP43 and GAP43-P respectively) were detected at higher exposures (data not shown). Similar to hNT cultures, NT2/pcDNA3.1 Myc/His A #3 and NT2/pcDNAHCx32 cultures displayed myelin PLP expression following differentiation (Figure 4.6, myelin PLP). Class III β -tubulin was detected in differentiated NT2/pcDNA3.1 Myc/His A #3 and all NT2/pcDNAHCx32 cultures (Figure 4.6, Beta Tubulin III) indicating initiation of a differentiation program. Low levels of β -tubulin were also detected in all undifferentiated cultures at higher exposure (data not shown). Coomassie blue stained gels (Figure 2.1C, Coomassie blue) are shown as the protein loading control.

4.5 Discussion

During CNS development, Cx32 expression coincides with the later stages of differentiation, neurogenesis, cell migration, and neural circuit formation [39, 43]. In addition, neuronal dysfunctions within the CNS of some patients with CMTX have been reported [74, 75]. Together, these observations provide circumstantial evidence that Cx32 influences neural cell development in the mammalian CNS. This thesis marks the first study to test empirically the effect of Cx32 expression on neuronal differentiation.

Stable HCx32 transfectants were generated to evaluate the effect of HCx32 expression on neuronal differentiation of the human NT2 cell line. Data are presented demonstrating that NT2s transfected with HCx32 were unable to differentiate to neurons following RA and mitotic inhibitor treatment. Failure to differentiate was confirmed by the absence of the neuron-specific marker NF-68 in immunoblot analyses and the absence of morphological features typical of differentiated neurons in culture. The observation that empty vector transfectants retained the capacity to terminally differentiate into neurons discounts possible artifacts associated with transfection. Successful differentiation of empty vector transfectants to neurons was confirmed by the presence of the neuron-specific marker NF-68 in immunoblot analysis and the presence morphological features indicative of neurons. These results implicate Cx32 in the control of neuronal differentiation.

The majority of Cx32 present in the adult CNS is expressed by oligodendrocytes [51, 115]. Therefore it is tempting to speculate that HCx32

expression in NT2s has blocked differentiation to neurons but promoted differentiation to oligodendrocytes. It is well established that oligodendrocytes share a common progenitor with astrocytes (for review see [123]). The ability to differentiate NT2s to astrocytes under specific culture conditions has recently been reported [84]. Therefore, it is reasonable to conceive that NT2s also have the potential to differentiate to oligodendrocytes. The myelin PLP immunoblots presented in this thesis begin to address this hypothesis. Myelin PLP is a marker of mature and developing oligodendrocytes as well as oligodendrocyte progenitors [103, 104]. The untransfected hNT cultures as well as the empty vector transfectants express myelin PLP following differentiation as determined by Western blot analysis. These data suggest that at least a subset of the non-neuronal cells in the differentiated cultures belong to the oligodendrocyte lineage and represent the first demonstration that NT2 cells can differentiate into glia other than astrocytes. Overexpression of HCx32 in NT2s blocked neuronal differentiation without affecting oligodendrocyte differentiation given that myelin PLP expression was detected in the HCx32 transfected cultures. Together these data indicate that undifferentiated NT2s, in addition to being capable of differentiation to neurons and astrocytes [84], may also be capable of differentiation to oligodendrocytes. Therefore, the NT2 cell line likely represents a multipotential pool of neural progenitors capable of becoming each of the major CNS cell types (neurons, oligodendrocytes, and astrocytes). Future experiments will directly assess the capability of NT2s to differentiate to oligodendrocytes in

the presence of specific oligodendroglia inducers and the effect of Cx32 on the types oligodendrocytes generated.

As reviewed in Chapter 1, individual Cxs oligomerize to form hemichannels allowing intracellular communication with the extracellular environment and gap junction channels allowing intercellular communication between adjacent cells. Cx32 is known to form both active hemichannels and gap junction channels [25, 124]. Therefore, it is likely that transfection of NT2s with HCx32 has increased communication via gap junctions and/or hemichannels. Since the kinetics of Cx32 expression in the developing CNS coincide with a marked decrease in GJIC [43, 125], Cx32-mediated hemichannel formation may be more relevant *in vivo* than Cx32-mediated GJIC. In support of this hypothesis, Jennifer Morin (a 4th year student in the Bennett laboratory under my mentorship) has demonstrated that overexpression of Cx32 in the PC12 cell line elicits functional hemichannels. Salient to this study, low numbers of open hemichannels blocked nerve-growth factor-induced differentiation to a neuronal phenotype while high numbers of open hemichannels elicited lytic cell death (Morin, Boucher, and Bennett, in preparation). Thus, in two different *in vitro* models, using the same Cx32 constructs, expression of Cx32 was found to influence neural progenitor survival and block differentiation to a neuronal phenotype. These data permit speculation as to the nature of transjunctional communication in mediating progenitor fate. Intracellular concentrations of small molecules such as Ca²⁺, cAMP, ATP, and adenosine are important in signaling neuronal and oligodendrocyte differentiation [110, 111, 126]. An increase in

active hemichannels could result in the disruption of intracellular concentrations of pivotal second messengers preventing progression through a neuronal differentiation program. In support of this hypothesis, overexpression of Cx43 has been shown to elicit transjunctional passage of ATP, Ca²⁺, and to a lesser extent adenosine release while overexpression of Cx32 mediates adenosine and, to a lesser extent ATP, AMP, and ADP passage [17, 33]. Furthermore, Cx32 homotypic gap junction channels pass cGMP more readily than Cx32/Cx26 heterotypic gap junction channels [18]. Relative intracellular concentrations of these molecules have been shown to influence differentiation to neuronal or glial lineage. For example, increased activation of A1 adenosine receptors blocks neuritic extension in PC12 and primary cultures [127] while an increase in intracellular adenosine concentration is associated with glial differentiation [111]. Future experiments will be aimed at measuring GJIC and HA in HCx32 transfected NT2s and determining transjunctional passage of second messengers during differentiation.

Previous work in our laboratory with Cx32 null mutant mice has shown that the loss of Cx32 results in an increased population of neural progenitors (Melanson-Drapeau et al, submitted to *J Neurosci* 07/19/02). However, the CNS of the Cx32 null mouse develops normally indicating that Cx32 is not an absolute requirement for differentiation of neural progenitors to neurons, oligodendrocytes, and astrocytes. Although the studies performed in the current chapter did not address directly the mechanism of Cx32's effects on neuronal differentiation, a model can be proposed where expression of Cx32 by neural progenitors

prevents differentiation to neurons and may promote differentiation to oligodendrocytes. Conversely, in the absence of Cx32, neural progenitors retain multipotential capacity. Upregulation of Cx32 has been demonstrated following damage to oligodendrocyte populations during stroke and epileptiform seizure [53, 128]. Under the proposed mechanism, the observed increase in Cx32 following these CNS injuries could play a role in directing the differentiation of activated neural progenitors down an oligodendrocyte lineage to re-populate damaged white matter. Although the proposed mechanism requires experimental verification, the data presented in the current chapter confirms that Cx32 can directly influence neural progenitor differentiation and that overexpression blocks differentiation to a neuronal phenotype. Future experiments will address whether this effect is Cx-specific and relevant to oligodendrocyte differentiation.

Chapter 5: General Discussion and Conclusions

The studies presented in this thesis were designed to add to the existing understanding of the functional significance of Cx proteins during the process of human neuronal differentiation. To facilitate this purpose, the NT2 human *in vitro* model was used to determine Cx expression and Cx-mediated communication in neural progenitors and terminally differentiated CNS neurons as well as to investigate the direct effects of Cx32 on neuronal differentiation.

Several Cx genes normally expressed in the mammalian CNS were investigated for mRNA expression by RT-PCR and protein expression by immunoblot analysis and/or immunocytochemistry in the NT2 cell line. In the literature, Cx43 had been the only Cx identified in the NT2 cell line [85]. In this thesis, undifferentiated NT2 cultures were found to express four out of seven Cxs tested: Cx30, Cx36, Cx37, and Cx43. Following RA-induced neuronal differentiation, hNT cultures were found to consistently express Cx36, Cx43, and Cx46.6.

The differential pattern of Cx expression observed between NT2 and hNT cultures in Chapter 2 led to the hypothesis that Cx-mediated communication would differ and thus be of functional significance between NT2 neural progenitors and hNT neurons. The data presented in Chapter 3 validated this hypothesis experimentally through a series of fluorescent dye transfer and dye uptake experiments designed to address biochemical coupling mediated by GJIC and HA in NT2 progenitors and terminally differentiated hNT cultures (as discussed in Chapter 3 electrical coupling through gap junctions was not

assessed). Biochemical coupling mediated by GJIC and HA was observed in NT2 but not hNT cultures. Although decreased GJIC over the course of neuronal differentiation has been previously confirmed *in vitro* and *in vivo* [36, 57, 63, 85], this study marks the first report of HA in neural progenitors and the absence of HA in terminally differentiated neurons. Reports of functionally significant HA are extremely limited. Interestingly, the two of the three published studies to date showing functionally significant HA both involved cells from the mammalian CNS, specifically cultured astrocytes and cells of the outer retina [33, 48]. Thus identification of biochemical coupling mediated by HA in NT2 neural progenitors further strengthens the increasing evidence of HA as a significant function of Cxs and a specific function in the mammalian CNS. Taken together, the diversity of Cx expression in the NT2 cell line and the subsequent identification of HA in NT2 progenitors highlights a complex function for Cxs in CNS development where expression of multiple Cxs at different time points dictates the functional properties of and molecular signals exchanged by both hemichannels and gap junctions likely determining the survival of neural progenitors and their differentiation to CNS neurons.

To provide more insight into a potential role for Cxs in progenitor activation and commitment, the role of Cx32 in the process of neuronal differentiation was assessed in Chapter 4 through creation of an HCx32 expression vector subsequently used to generate stable HCx32 NT2 transfectants. The HCx32-transfected NT2s proved to be a pertinent model to investigate the role of Cx32 in the process of neuronal differentiation. Morphological and immunoblot

analyses revealed that NT2s transfected with HCx32 were incapable of differentiation to a neuronal phenotype implicating a direct role of Cx32 in the control of neural cell fate. Given that the majority of Cx32 in the CNS is expressed by oligodendrocytes and the finding that myelin PLP, a marker of developing and mature oligodendrocytes, was expressed by differentiated HCx32-transfected cultures, it was hypothesized that expression of Cx32 may promote oligodendrocytic differentiation of NT2 neural progenitors. It is reasonable to conceive that NT2s have the potential to differentiate to oligodendrocytes in light of the fact that NT2s have been differentiated to astrocytes [33] and that oligodendrocytes and astrocytes share a common progenitor (for review see [123]). However, this must be validated experimentally by assessing oligodendrocytic differentiation of untransfected and HCx32-transfected NT2 cells in the presence of specific oligodendroglia inducers.

In the rodent CNS maximal Cx32 expression coincides with a drastic decrease in GJIC [43, 125]. Solitary Cx32 hemichannels have been shown to open following depolarization in *Xenopus* oocytes [129]. This led to the hypothesis that Cx32 mediates its effects on the differentiation of neural progenitors through HA not GJIC. As mentioned in Chapter 4, this hypothesis is supported through data collected by a student under my mentorship showing overexpression of Cx32 in PC12 cells results in increased biochemical coupling mediated by HA and decreased nerve-growth factor-induced differentiation to a neuronal phenotype in Cx32-transfected PC12 cells (Morin, Boucher, and Bennett, in preparation). The data presented in this thesis together with the

observed increased population of neural progenitors in Cx32 null mice (Melanson-Drapeau *et al*, submitted to J Neurosci 07/19/02) lead to a proposed mechanism whereby Cx32 expression by neural progenitors prevents differentiation to neurons and promotes differentiation to oligodendrocytes. In addition we speculate that the effects of Cx32 are mediated through HA not GJIC.

In conclusion, this thesis expands our knowledge of Cx expression and Cx-mediated communication in the context of human neuronal differentiation and gives direct insight into the role of Cx32 in the control neural cell fate. As discussed in Chapter 1, the NT2 cell line, in addition to being a model system for human neurobiology research, possesses distinct medical advantages for use in human brain therapies and is currently being used in clinical transplantation therapy. Understanding the molecular mechanisms underlying the control and regulation of neural progenitor survival and differentiation is a necessary prerequisite to future studies aimed at developing therapeutics to counter CNS damage associated with neurodegenerative disease, stroke and CNS injury. Targeted manipulation of Cx expression and/or Cx-mediated communication may be one means of controlling NT2 progenitor fate and improve the outcome of clinical therapy involving these cells and neural stem cells in general.

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129. **Castro, C., et al., *Altered formation of hemichannels and gap junction channels caused by C-terminal connexin-32 mutations.* J Neurosci, 1999. 19(10): p. 3752-60.**

Appendix A: Antibody suppliers and working concentration/dilutions used for Western blot and immunocytochemical analysis.

Antibody Name	Type	Supplier	Dilution for immunocytochemistry	Dilution for Western blot analysis
Rabbit Anti-GAP43	polyclonal	Chemicon International, Temecula, CA, USA	1:100	1:2000
Mouse Anti-PCNA	monoclonal	Oncogene Research, Cambridge, MA, USA	n/a	1:100
Mouse Anti-NF68	monoclonal	Sigma-Aldrich, St. Louis, MO USA	n/a	1:400
Mouse Anti-Myelin PLP	monoclonal	Biogenesis Inc., Kingston, ON	1:100	1:200
Mouse Anti- β -tubulin isotype III	monoclonal	Sigma-Aldrich	n/a	1:400
Mouse Anti-Cx30	monoclonal	Zymed Laboratories Inc., San Francisco, CA	1 μ g/mL	5 μ g/mL
Rabbit Anti-Cx36	polyclonal	Zymed Laboratories Inc.	5 μ g/mL	1 μ g/mL
Mouse Anti-Cx37	monoclonal	Alpha Diagnostics, San Antonio, TX, USA	10 μ g/mL	1 μ g/mL
Mouse Anti-Cx43	monoclonal	Chemicon	1:300	1:1000
Anti-mouse IgG HRP	polyclonal	Jackson Immunolabs	n/a	1:2000
Anti-rabbit IgG HRP	polyclonal	Jackson Immuno Laboratories	n/a	1:5000
Anti-mouse IgG Cy3	polyclonal	Jackson Immuno Laboratories	1:800	n/a
Anti-rabbit IgG Cy3		Jackson Immuno Laboratories	1:600	n/a

*n/a: not applicable

Appendix B: Curriculum Vitae

Sherri Boucher

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EDUCATION

B.Sc. Highest Honours Biology and Biotechnology **2000**
CARLETON UNIVERSITY OTTAWA, ONTARIO

Thesis Title: Regulation of the *nfsA* gene in *Escherichia coli* by SoxS (published)
(Awarded Carleton University prize for best thesis in the molecular biology division)

M.Sc. Biochemistry **2002**
UNIVERSITY OF OTTAWA OTTAWA, ONTARIO

Thesis Title: Connexin expression, connexin-mediated communication, and the effect of connexin overexpression in an in vitro model of human neuronal differentiation.

EMPLOYMENT

Research Assistant, Wheat Molecular Genetics **05/2000-09/2000**
NRC PLANT BIOTECHNOLOGY INSTITUTE, CEREAL GROUP SASKATOON, SASKATCHEWAN

Gained experience in Northern blot analysis, radioisotope handling, SDS-PAGE, RNA and DNA isolation from plant tissues, Western blot analysis, enzyme assay, plasmid manipulation, plant cell culture, plant cell transformation (biolistics), and PCR.

Research Assistant, Molecular Genetics **05/1999-09/1999**
CARLETON UNIVERSITY OTTAWA, ONTARIO

Gained experience with techniques in DNA isolation, DNA purification, DNA sequencing, primer design, radioisotope handling, enzyme assays, PCR, gel electrophoresis, plasmid manipulation, cloning, and manipulation of *E. coli*. Experience working with nucleotide sequence databases, protein sequence databases, and multiple sequence alignment software.

TEACHING EXPERIENCE

Laboratory Demonstrator, University of Ottawa
Course Code BCH3356 (2001/2002)
Responsible for supervising and marking ten students in a 6 hour/week 3rd year molecular biology laboratory.

**Tutor/Lecturer, University of Ottawa
Course Code BCH2336 (2002)**

Responsible for the preparation and presentation of a series of four 3 hour lectures on manipulation of solutions, calculating dilutions, analysis of standard curves, and statistical analysis of experimental results including errors evaluation, significance tests, and regression analysis for 2nd year biochemistry students.

**Guest Lecturer, University of Ottawa
Course Code BCH4125 (2001)**

Responsible for the preparation and presentation of a 20 minute lecture covering laboratory techniques for assessing necrotic and apoptotic cell death to 4th year biochemistry students.

AWARDS/SCHOLARSHIPS

- NSERC PGS B : 2002-2004 (\$38 200)
 - Strategic Areas of Development Admission Scholarship : 2002 (\$6000)
 - Ontario Graduate Scholarship: 2002-2003 (\$15 000, declined)
 - NSERC PGS A : 2000-2002 (\$34 600)
 - University of Ottawa Excellence Award: 2000-2004 (tuition + \$4000)
 - EWR Steacie Scholarship : 1999-2000 (\$1500)
 - Dow Chemical Continuing Education Scholarship: 1996-2000 (\$12 000)
 - David A. Golden Scholarship: 1998-1999 (\$1500)
 - NSERC Undergrad Summer Res Scholarship: 1999 (\$5000), 2000 (\$5000,declined)
 - Carleton University Dean's Honor List: 1996-2000
 - William E. Beckel Scholarship: 1997-1998 (\$1500)
 - Carleton University Entrance Scholarship: 1996-1997 (\$1500)
-

PUBLICATIONS

Paterson S, Boucher S, and IB Lambert (2002). Regulation of the *nfsA* gene in *Escherichia coli* by SoxS. *Journal of Bacteriology* **184** (1): 51-58.

Boucher S and SAL Bennett (2002). Differential connexin expression, gap junction intercellular coupling, and hemichannel formation in nNT2/D1 neural progenitors and terminally differentiated hNT neurons. *Journal of Neuroscience Reviews* (submitted 09/20/02).

Abstracts

Boucher S and Bennett SAL (2001) Transfection of PC12 and Ntera2 cells implicates connexin 32 in the control of neural development . Proceedings of the Canadian Federation of Biological Sciences (Ottawa, Canada, poster presentation by S Boucher).

Boucher S and Bennett SAL (2001) Connexin 32 plays a role in the regulation of neural cell fate in an *in vitro* model of neuronal differentiation. Soc Neurosci Abstracts (San Diego, USA, poster presentation by S Boucher)