OLIGODENDROCYTES and MOTONEURONS: TWO CHOLINERGIC CELL TYPES DERIVED FROM MULTIPOTENT SPINAL NEUROEPITHELIAL PRECURSOR CELLS

BY

STEPHEN CHRISTOPHER MACDONALD

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Abstract

Cholinergic neurons have been implicated in various neural behaviours including motor control, autonomic function and propriospinal communication. Neuroepithelial precursors have recently emerged in the field of regenerative neurobiology and have been shown to proliferate in vitro under the influence of mitogenic growth factors. Upon removal of these mitogens, the precursor cells differentiate down neuronal and glial lineage pathways. Neural precursors offer new avenues in the study of cholinergic developmental biology and cell replacement therapies. In pursuing cholinergic neurons derived from neuroepithelial precursors, two discoveries were made that are described in this thesis.

The first study reports that some ChAT expressing cells in differentiated neurosphere cultures express oligodendrocyte and not neuron markers. Neurospheres were cultured in the presence of trophic factors and processed immunohistochemically for oligodendroglial and cholinergic markers. Almost all ChAT expressing cells in treated cultures expressed oligodendrocyte markers and displayed dramatic growth responses to the cytokine CNTF. Treatment with the cholinergic antagonist atropine during differentiation significantly decreased the amount of oligodendroglial differentiation, indicating a role for acetylcholine in oligodendrocyte differentiation.

In differentiated neuroepithelial precursor cultures, a small fraction of ChAT expressing cells differentiated not into oligodendrocytes, but neurons

expressing MAP-2. The multipolar morphology and large size of these cells could suggest a motoneuron identity. Further immunostaining revealed cells expressing the motoneuron markers Islet-1 and REG2. When co-cultured with dissociated skeletal myocytes, functional neuromuscular junctions were established as indicated by immunohistochemistry, drug perfusion and electrophysiology. After transplantation into the transected sciatic nerve, precursors differentiated into neurons expressing ChAT and the α-1c calcium channel subunit found on spinal motoneurons. Precursor-derived neurons also projected axons towards the distal musculature and formed cholinergic terminals. These results suggest that functional motoneurons can differentiate from proliferative neural precursors.

Together these two projects demonstrate that cholinergic cell types differentiate from neuroepithelial precursors of a common origin. These findings support the hypothesis that functional motoneurons can differentiate from proliferative precursors and serendipitously demonstrate a traditional neurotransmitter synthesis enzyme being expressed in developing oligodendrocytes. This thesis also contains the most comprehensive study to date characterizing neural precursor-derived motoneurons which has relevance to cell replacement strategies for neurodegenerative conditions such as Amytrophic Lateral Sclerosis (ALS).

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List of Abbreviations

ACh	Acetylcholine
AChE	Acetycholinestrase
AHP	After Hyperpolarization
ALS	Amyotrophic Lateral Sclerosis
ARIA	Acetycholine Receptor Inducing Agent
BDNF	Brain-Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
cAMP	cyclic Adenosine Mono Phosphate
ChAT	Choline Acetyltransferase
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
DβH	Dopamine β Hydroxylase
DMEM/ F12	Dulbecco's Modified Eagle's Medium/ Ham's F12
DRG	Dorsal Root Ganglion
EGF	Epidermal Growth Factor
En-1	Engrailed-1
ES cell	Embryonic Stem Cell
GABA	γ- Amino Butyric Acid
GalC	Galactocerebroside
GDNF	Glial Cell-Derived Neurotrophic Factor
IGF	Insulin-like Growth Factor
MBP	Myelin Basic Protein
NIH	National Institutes of Health
NT-3	Neurotrophin 3
NT-4	Neurotrophin 4
O-2A	Oligodendrocyte- Type 2 Astrocyte
PDGF	Platelet-Derived Growth Factor
PDGFRα	Platelet-Derived Growth Factor Receptor Alpha
PLP	Proteolipid Protein
PNS	Peripheral Nervous System
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
Shh	Sonic Hedgehog
SVZ	Sub Ventricular Zone
TH	Tyrosine Hydroxylase
VAChT	Vesicular Acetylcholine Transporter

Introduction

This thesis will describe the differentiation and characterization of two cholinergic cell types (oligodendrocytes and motoneurons) that differentiate from proliferative neuroepithelial precursor cells. Principles of stem cell biology will be discussed followed by a review of the normal pathways of oligodendrocyte and motoneuron differentiation. The discovery of cholinergic oligodendrocytes is novel and the role of acetylcholine with respect to oligodendrocytes will also be addressed.

Neural Stem Cells

Recently there has been a surge of interest in the identification of neural stem cells. It has been known for some time now that cells in the adult brain are capable of generating new neurons. This knowledge was the basis for the search for a stem cell that can generate all the different cell types of the central nervous system.

Definitions and terminology:

Most of the principles for stem cell identification have been adapted from hematology and the study of the hematopoietic stem cell. The term stem cell has inadvertently been misused to describe many mitotically active central nervous system (CNS) cell types, and a solid definition of the term 'stem cell' remains elusive. Common features, however, have been agreed upon by researchers in the field, and these make an operational definition available (reviewed by Snyder

1994, Weiss 1999). It has been proposed that a stem cell is a proliferative cell that undergoes further specialization with each successive cell division to generate progressively more restricted cell types until final cell differentiation occurs. That is, the cell can proliferate to produce an identical cell capable of further division and a partially committed progenitor cell with limited mitogenic potential (asymmetric division) or else two identical cells (symmetric division). There are certain characteristics that define a stem cell. The current operational definition of a stem cell states that a stem cell should: 1) exhibit self renewal for the life of the organism 2) generate large numbers of progeny through transient amplification of a progenitor population 3) retain multilineage potential over time and 4) generate new cells in response to injury or disease.

There is some terminology that needs to be defined to clarify the study of stem cell biology (National Institutes of Health website, see reference list). The earliest stem cell is the fertilized unicellular zygote. This is termed a *totipotent* stem cell and has the capacity to specialize into all extra-embryonic membranes and tissues, as well as the total embryo itself. The next specialized stem cell is termed *pleuripotent* and is capable of giving rise to most tissues of an organism. A pleuripotent cell can not differentiate into embryonic membranes such as the chorion, amnion, etc. but only tissues of the organism itself. The classic "embryonic stem" (ES) cell used in transgenic applications is an example of a pleuripotent stem cell. The pleuripotent stem cell further specializes to different classes of tissue-specific stem cells termed *multipotent*. Examples of multipotent stem cells are the hematopoietic stem cell or the central nervous system stem

cell. Multipotent stem cells undergo further asymmetric cell division to generate more restricted *progenitor* cells which are more committed down particular cell lineage pathways. In the case of CNS stem cells, the O-2A glial progenitor (which can yield oligodendrocytes and type-2 astrocytes but not neurons) would be a good example (Raff et al 1983). CNS progenitor cells then form terminally differentiated neurons and glial cells and acquire mature cell characteristics. The term *stem-like* refers to a cell that satisfies the operational definition of a stem cell (i.e. being multipotent and capable of self renewal and self maintenance), but not satisfying all stem cell criteria. A *precursor* is a general term applied to any undifferentiated stem cell or progenitor cell.

Identification of Neural Stem Cells

During neuronal development cell number is determined by an active proliferation phase before differentiation and a subsequent cell death phase afterwards. The first cells in the neural tube consist only of columnar epithelial cells. From this primordial epithelium, the first neurons and radial glia differentiate and migrate outwards to appropriate locations. The last cells to differentiate from the epithelium are the astrocytes and oligodendrocytes (for review see McKay 1988). Neuroepithelial cells proliferate under precise direction such that neurons differentiate at specific developmental stages (Sidman et al 1959). Mitotically active neuroepithelial precursor cells express the intermediate filament protein nestin and make up 98% of the undifferentiated cells before neuronal differentiation (Lendahl et al 1990). Upon onset of differentiation, nestin is

dramatically down-regulated along with two other proteins associated with cell division, cyclin and CDC2 (Hayes et al 1991). This knowledge prompted further study of the CNS neuroepithelium in the search for a CNS stem cell. Adult CNS neurogenesis was known at the time but was thought to be restricted to discreet brain regions such as the subventricular zone, the dentate gyrus and the olfactory bulb (Kaplan and Hinds, 1977; Morshead et al 1992; Altman and Das 1965; Luskin et al 1993). The possible applications of neural stem cells appeared endless and included tissue culture studies to study cell development and ultimately led to transplantation experiments to replace damaged cells after CNS lesions. One of the original goals was to generate a catalogue of homogeneous cell type precursors for use after selective CNS injuries (Snyder 1994).

It has been said that there are as many versions of history as there are historians. Similarly there is a great diversity in the field of neural precursors, and different groups appear to have isolated and described precursor cell populations that express different characteristics, and different growth properties after being isolated in different manners. Each population is described as stem cells or progenitor cells. These include adherent neuroepithelial precursors (Cattaneo and McKay 1990, Kalyani et al 1997), neurally restricted embryoid bodies from ES cells (Strubing et al 1995), and neurospheres (Reynolds and Weiss 1992)

Initial experiments to isolate and identify rodent CNS stem cells involved taking undifferentiated embryonic cells and attempting to immortalize the cells in

a proliferative state using genetic or neurotrophin-induced means. Several groups have immortalized nestin expressing CNS cells by incorporating the SV40 large T- antigen using a temperature sensitive promoter with the ultimate aim being use in cell replacement therapies (Whittemore and White 1993, Giordano et al 1993).

Probably the most popular method of generating CNS precursors is to harvest tissue from a particular CNS region and perpetuate the cells with the mitogenic factors, EGF or bFGF. The resulting clones consistently develop into free floating clusters of precursor cells called *neurospheres*, a term coined by the Canadian group to first isolate them in this fashion. These authors reported that the adult rat striatum could yield neurospheres in response to EGF (Reynolds and Weiss 1992). These cells were multipotent (i.e. capable of generating neurons, astrocytes and oligodendrocytes) and capable of self-renewal and self-maintenance. Upon removal of the mitogens the neurospheres differentiated into mature CNS cell types including neurons expressing substance P and GABA.

While many of the initial studies were done in mouse or rat, it was subsequently reported that rat cells had a limited potential for proliferation and after a few weeks in culture, there was a dramatic death of proliferating cells (Svendsen 1997). Surprisingly, neurospheres generated from mouse tissue did not display the same cell death as rat neurospheres. This would suggest that differences in stem cell characteristics exist between species. Further examination of neuroepithelial stem cells by van der Kooy's group demonstrated that in the brain the first mitogen responsive cells arise at approximately E8.5,

are responsive only to bFGF, and divide symmetrically until E14. Then the cell cycle increases, and they divide asymmetrically to E17 (Tropepe et al 1999). EGF has no action on cell proliferation at this stage, but between E11 and E13, a population of EGF-responsive precursor cells arises from the bFGF-responsive population. It was shown by this group that while the adult ependyma is capable of proliferation, only the subependyma could yield self-renewing neurospheres (Chiasson et al 1999). However, the subependymal zone arises from the ependymal zone during development, so the stem cells must exist in the ependymal zone at an earlier time point and subsequently migrate to the subependymal layer.

Until recently, the neuroepithelial stem cell was not demonstrated in the adult human. Recent work has shown that embryonic human brain stem cells do exist but require both EGF and bFGF to proliferate (Vescovi et al 1999). These cells satisfy the definitions of stem cells and demonstrate that the human CNS possesses definitive *stem cells* not simply "proliferative precursors". These cells were subsequently transplanted into the rat to yield neurons and glial elements (Vescovi et al 1999). A similar study demonstrated that neurospheres could arise from adult human brain subependymal zone and hippocampus (Kukekov et al 1999). Adult stem cells are generally more difficult to study because they are often present in such small quantities and are difficult to isolate and purify. It has also been suggested that adult stem cells from human or rodent lose some of their multipotency with age and yield more glial elements than neurons (Y. Arsenijevic personal communication, Zhu 1999).

Neuroepithelial stem cells have been localized throughout the CNS neuroepithelium and in the murine spinal cord (Weiss et al 1996). Interestingly the spinal cord neurospheres also required both EGF and bFGF for proliferation. It was originally thought that spinal neurospheres were more primitive that those found in the brain. The co-dependence of spinal precursors on both bFGF and EGF is similar to spheres derived from the human embryo (Vescovi et al 1999). This may imply that spinal cord neurospheres are of a more complex nature than the brain-derived counterparts or perhaps they are as evolutionarily advanced as human CNS neurospheres.

The Role of Stem Cells in Transplantation

There have also been reports of neurosphere transplantation in the CNS. One of the initial reports of neurospheres transplanted into the brain showed the formation of a thin band of transplanted cells unlike the large mass generated by a fetal tissue graft (Svendsen et al 1996). In this study, transplanted neurospheres remained in an undifferentiated state or differentiated and migrated very little from the graft site. EGF-generated neurospheres transplanted into a myelin-deficient rat spinal cord were reported to differentiate into oligodendrocytes and contribute to remyelination, suggesting that neurospheres respond to the needs of the host environment (Hammang 1997). More recently, Vescovi et al transplanted embryonic human neuroepithelial stem cells into the rat striatum and reported differentiation of astrocytes and neurons but no oligodendrocytes (Vescovi et al 1999). Still, grafted neurospheres displayed more noticeable differentiation than those transplanted by Svendsen et al (1996) and

could survive in the host animal for up to one year. It appears as though the utility of neurospheres for cell transplantation is becoming more evident.

There appears to be more and more evidence arising to suggest that stem-like cells respond to local cues after transplantation into a host organism to differentiate into cells of that CNS region. Alone in culture however, it has been suggested that differentiation results in cells of the region that the tissue was harvested from (Gotz et al 1995). However it was demonstrated that precursors in co-culture with mesencephalon developed tyrosine hydroxylase immunoreactivity similar to those cells in the explant (Ptak et al 1995). It was concluded that in a peer pressure fashion, the precursors responded to environmental cues in the culture environment to guide differentiation into a particular phenotype. It was also reported that striatal ventricular zone precursors, when transplanted to the striatum, differentiated into striatal neurons as demonstrated by morphology and axon trajectory (Fishell 1995). The same precursors transplanted into the cortex adopted morphology and trajectories similar to cortical neurons. This suggests that within the forebrain, there exists position specific factors that guide differentiation. Snyder's group has been successful using a neural stem cell clone (C17.2) that possesses stem-like features and also appears to respond to local differentiation cues. The C17.2 clone is an immortalized clone derived from the cerebellum and has been transplanted into many CNS regions. The most dramatic result comes from a study where the C17.2 clone was transplanted into the *mea* mutant mouse in which the anterior lobe of the cerebellum does not form due to absence of

granule cell differentiation (Rosario et al 1997). Cell transplantation resulted in almost a total repopulation of granule cells in the anterior lobe. Transplantation into a normal mouse results in some minor granule cell differentiation. When transplanted into a normal subventricular zone (SVZ), C17.2 cells integrate into the host SVZ and travel along the rostral migratory stream to become neurons of the olfactory bulb (Snyder 1994). This group concluded that when transplanted into regions where neurogenesis was still active, the stem cells differentiated into neurons appropriate for that area. When transplanted into areas where neurogenesis was complete, the precursors yielded only glial cells.

Can Motoneurons Differentiate from Stem Cells?

There have been several preliminary findings to suggest that motoneurons are capable of differentiation from stem cells; however, in no case have definitive markers been used. Preliminary work in transplanting C17.2 cells into the spinal cord resulted in some larger neurons migrating to the spinal ventral horn and expressing acetylcholine receptor inducing agent (ARIA) (Park et al 1999). The authors suggest that stem cells can migrate to the ventral horn and choose a motoneuronal developmental path. Precursors produced using bFGF were generated by Gage's group and allowed to differentiate in culture (Ray and Gage 1994). These cells were identified as motoneurons by immunostaining with choline acetyltransferase (ChAT) or the p75 neurotrophin receptor. These authors also transplanted their stem cells into the spinal cord and identified subsequently differentiated cells as motoneurons by virtue of large size and placement in the ventral horn. ChAT and p75 are not motoneuron specific as

there are several cholinergic populations of neurons in the spinal cord, and p75 is also expressed in some cholinergic partition neurons as well as in oligodendrocytes (Cassacia-Bonnefil et al 1996, Michael et al 1997). Adherent neural restricted precursors have also differentiated into neurons expressing ChAT and p75, which the authors identified as motoneurons (Kalyani et al 1996). In all cases, no report of cell number was indicated, and it is assumed that motoneuron differentiation occurred at low frequencies. There was also no previous functional characterization of precursor-derived motoneurons. While immunohistochemistry for protein markers is a valid starting point, it is essential to use more specific motoneuronal markers such as islet-1, REG2, or the α1c L-type calcium channel subunit (Ericson et al 1992, Livesey et al 1997, Jiang et al 1999). The use of more specific markers combined with demonstrating functional properties such as projections to muscle, cholinergic terminals and neuromuscular junction formation to definitively identify a cell as a motoneuron.

The Role of Neurotrophins on Stem Cells

Neurotrophins play a role in precursor cell proliferation and differentiation as well. The first member of the modern neurotrophin family was nerve growth factor discovered in 1952 by Rita Levi-Montalcini et al (reviewed in Levi-Montacini 1987). Later isolation of brain-derived neurotrophic factor (BDNF) followed by neurotrophin-3 and neurotrophin-4 have established the current neurotrophin family. These growth factors act via receptor tyrosine kinases (trk receptors) to exert physiological actions. The ligands and receptors are paired as follows: NGF-trkA; BDNF, NT4-trkB; NT3-trkC (for review see Lindsay et al

1994). The p75 neurotrophin receptor is a low affinity neurotrophin receptor that binds all neurotrophins with equal affinity, and it is suggested to play a role in differentiating different actions of the same neurotrophins.

The stem cell literature is also quite variable depending on the precursor isolation and tissue type studied. The neurotrophin BDNF has been shown to promote neuronal differentiation of EGF-generated neurospheres from the brain (Ahmed et al 1995). In this study, BDNF had no action of promoting cell survival, however, and without further trophic support, the newly generated neurons did not survive after 28 days. It was subsequently reported that BDNF promotes the survival of neurons differentiating from ependymal and subependymal zones (Kirschenbaum and Goldman 1995). These authors speculated that BDNF might function as a permissive factor for neuronal recruitment in adulthood. Recently, another view on the role of neurotrophins has been suggested. It has been shown that retinoic acid (a classical differentiation factor) induces adult rat hippocampal stem cells to differentiate into neurons by inducing the adoption of a neuronal fate (Takahashi et al 1998). After committing to a neuronal lineage, expression of neurotrophin receptors occurs. Further treatment with NT-3 and BDNF then enhanced maturation of differentiated neurons into phenotypes expression GABA, AChE, and TH. These authors conclude that neurotrophins support survival after neuronal differentiation has occurred but do not contribute to the differentiation process.

Two concepts have recently emerged in this field demonstrating variations of differentiation. Classical differentiation is unidirectional with a cell becoming

increasingly more restricted. Some schools of thought believe that a proliferating cell may be capable of de-differentiation into a prior less restricted progenitor cell under specific growth cues (e.g. Oligodendrocytes ⇒ O-2A cells; Grinspan et al 1993). Another concept is that of trans-differentiation wherein a cell can alter it's commitment to a particular lineage thus differentiating into cell types other than those determined by its initial asymmetric division. The most popular example of trans-differentiation was recently reported where these authors isolated CNS stem cells from the adult rat and transplanted them into an irradiated host to generate cells of the hematopoietic lineage effectively turning "brain into blood" (Bjornson et al 1999).

Cholinergic development

Acetylcholine (ACh) was the first neurotransmitter to be discovered by Loewi in 1921. Today, the synthesis and mechanisms of ACh action are well known, and there is renewed interest in what is described as the oldest neurotransmitter. It wasn't until 22 years later that ChAT, the enzyme synthesizing ACh, was discovered (Nachmansohn and Machado 1943). There was the subsequent discovery of hydrolytic inactivation of ACh by AchE, but the study of ACh was very slow moving due to technical limitations (Augustinsson and Nachmansohn 1949). Further study of ChAT was virtually impossible at the time because the protein was present in such low concentrations. The development of anti-ChAT anti-sera in the early 1980's permitted the isolation of purified ChAT protein and revolutionized the field of ACh research (Levey et al

1981, Eckenstein and Thoenen 1982). The purification of ChAT ultimately permitted the cloning of both the cDNA and ultimately the mammalian ChAT gene (Berrard et al 1987, Brice et al 1989, Ishii et al 1990). Relatively recently, the vesicular acetylcholine transporter (VAChT) and it's gene have been cloned and characterized (Erickson et al 1994, Usdin et al 1995). The VAChT was initially found in *C. elegans* and named unc-17 (Alfonso et al 1993). It functions to package ACh into synaptic vesicles. The gene for VAChT was localized to the first intron of the ChAT gene and codes for a 77KDa protein. It is now known that both ChAT and VAChT arise from a common cholinergic gene locus under the control of common cis-acting elements (reviewed in Eiden et al 1998). Both ChAT and the VAChT have been localized to cholinergic cells, and it is now accepted that both are protein markers of cholinergic cells (Gilmor et al 1996, Weihe 1996). It has been reported that while expression of both markers occurs throughout the cholinergic cell, the VAChT is more highly expressed in the terminals and cell bodies than in the cholinergic fibres (Weihe et al 1996). Conversely, ChAT is expressed more highly in cholinergic fibres. This suggests that these two proteins are complementary entities for visualizing cholinergic cells.

The first cholinergic cells to differentiate in the mouse spinal cord are spinal motoneurons that differentiate at embryonic day 10 (Wentworth 1984). Development in the rat occurs in similar fashion with all time points being one gestational day later. Following a defined rostro-caudal developmental gradient, cervical motoneurons develop prior to sacral groups by approximately 2

developmental days (Barber et al 1991). After motoneurons differentiate, these groups subsequently segregate into autonomic and somatic motoneuron groups (Markham and Vaughn 1991). The most dorsal of the autonomic motoneurons differentiate before more ventral autonomic motoneurons, contrary to classic ventro-dorsal progression. Motoneuron differentiation is complete by E13 (E12 in mouse) with E11 being the peak day of cholinergic differentiation.

Immediately after the onset of motoneuron differentiation, cholinergic interneurons also develop around E11-E12 in the rat (E10-E11 in mouse). Interneuron differentiation follows the classic ventro-dorsal pattern with dorsal horn cholinergic neurons being the last to differentiate. Differentiation of cholinergic central canal groups is completed by E15 (E14 in mouse). There exists at least 5 types of cholinergic neurons in the spinal cord, and these are subgrouped by location: 1) small dorsal horn neurons 2) central canal neurons 3) partition cells 4) sympathetic preganglionic neurons 5) somatic motoneurons (Barber et al 1984). Cholinergic spinal neuron differentiation is completed by E16 in time for differentiation of myelinating glial cells.

Oligodendrocyte Development

Oligodendrocytes are the myelin producing cells of the central nervous system. Oligodendrocyte progenitors first develop from localized foci of the neuroepithelium at E12.5 in the mouse (E14 in the rat) after motoneuron differentiation is complete (Pringle et al 1998). In the ventricular neuroepithelium, the diffusible factor sonic hedgehog (Shh) is released from the floor plate and promotes oligodendrocyte differentiation from the ventral most zone of the pax6

domain of the spinal ventricular zone much like in motoneuron differentiation (Pringle et al 1996, Sun et al 1998). The classical oligodendrocyte progenitor cell is the O-2A cell described by Raff et al (1983). This bipotential O-2A progenitor cell is isolated in culture and is labeled with the A2B5 antibody and can differentiate in culture to yield oligodendrocytes or type-2 astrocytes (lineage described in Sontheimer et al 1989). The next more restricted progenitor cell is labeled with the O4 antibody and still has the potential to generate oligodendrocytes or type-2 astrocytes. The next step in the lineage is the first committed oligodendrocyte and is labeled with the O1 antibody that targets the myelin constituent galactocerebroside (GalC). This period occurs around the time of birth with active myelination occurring at P4. Fully mature oligodendrocytes are the last step in oligodendrocyte differentiation and express the marker O10 as myelination is completed. O-2A progenitors have also been identified by the expression the platelet-derived growth factor receptor-α (PDGFR-α) (Yu et al 1994). Oligodendrocyte precursors form two longitudinally oriented bands of cells on either side of the spinal cord central canal near the ventral apex of the canal (Pringle et al 1996).

There exists a major component of CNS myelin, proteolipid protein (plp) that also expresses a shorter slice variant called DM-20 (Timsit et al 1995). It has been speculated that plp/DM-20 is also localized to oligodendrocyte progenitors. Interestingly, the population of plp/DM-20+ neuroepithelial cells does not co-localize PDGFR-α which is found in O-2A cells, and it was originally thought that these two groups of cells represented two separate populations of

oligodendrocyte progenitors (Spassky et al 1998). The plp/DM-20+ group is expressed earlier and more ventrally than the PDGFR-α+ population, closely abutting the floor plate. It was not originally thought to be an oligodendrocyte precursor population because no significant amount of plp/DM-20 could be detected in purified O-2A cultures (Spassky et al 1998). However, this did not exclude the possibility that this cell group was another non-O-2A cell oligodendrocyte progenitor.

The oligodendrocyte progenitor cell withdraws from the cell cycle and differentiates after a limited number of cell divisions (Tokumoto et al 1999). These events are set by an internal cellular timer which dictates two separate components. The first of these components is the timing component which records total elapsed time. The second effector component arrests mitosis and promotes terminal differentiation. This effector component can be triggered by mitogen withdrawal, or treatment with inducing agents such as thyroid hormone or retinoic acid (Tokumoto et al 1999). At this point all three treatments result in differential gene expression causing differentiation. These authors reported that regardless of the treatment, there has been no common gene expression altered as of yet and it appears that these factors all cause differentiation by different mechanisms.

It has been demonstrated in tissue culture that at E14 (the onset of oligodendrocyte precursor differentiation), only the ventral half of the spinal cord yields oligodendrocytes (Warf 1991). This group also demonstrated that all spinal segments have the capacity to yield oligodendrocytes. After E16, the dorsal half

of the spinal cord acquires the potential to yield oligodendrocytes. These authors concluded that oligodendrocyte progenitors migrated from the ventral to the dorsal region of the spinal cord enabling the dorsal most regions to yield oligodendrocytes. It was subsequently demonstrated in the chick spinal cord that the dorsal spinal cord is also capable of generating oligodendrocytes (Cameron-Curry and LeDouarin 1995). These authors point out that Warf et al did not directly demonstrate migration of oligodendrocyte progenitors. It was later reported using antibody mediated complement lysis that oligodendrocytes are not generated in the dorsal spinal cord until E16 (Hall 1996). This group supported the findings of Warf et al (1991) but again did not show direct evidence of migration. Recently, Chandran et al demonstrated that the dorsal spinal cord epithelium in fact has the potential to generate oligodendrocytes as well but only after treatment with EGF and bFGF (Chandran et al 1998). This suggests that oligodendrocyte progenitors do not migrate as previously thought (Warf et al 1991) but differentiate in their own proper location.

After oligodendrocyte differentiation about 50% of oligodendrocytes die due to competition for limited survival factors (Barres et al 1992). This phenomenon is analogous to the developmental cell death seen in neuronal cell types such as motoneurons (Hamburger and Oppenheim 1982). PDGF and insulin-like growth factors (IGFs) are survival factors for new oligodendrocytes, and increasing the concentration of PDGF in the developing optic nerve decreases death by 90% (Barres et al 1992). PDGF normally is derived from astrocytes in vivo which differentiate before oligodendrocytes and promote

oligodendrocyte survival by activation of PDGF receptors. PDGF normally drives oligodendrocyte progenitor division, and an eventual lowering of PDGF concentration ultimately slows down the cell cycle and promotes differentiation (Calver 1998). Interestingly, after oligodendrocytes differentiate and begin expressing GalC, PDGF receptor expression is dramatically downregulated (Hart et al 1989). After overexpression of PDGF in the optic nerve, hyperproliferation of oligodendrocytes occurs with concomitant ectopic oligodendrocyte differentiation (Calver et al 1998). These authors show that despite this excessive oligodendrocyte differentiation, the excess cells die before reaching maturity leaving a normal number of myelinating oligodendrocytes. This suggests that PDGF plays a role in the differentiation but not the survival of oligodendrocytes. It has been reported that a host of trophic factors are implicated in the survival of oligodendrocytes. These include CNTF, IGF, NT-3, LIF and IL6 (Barres et al 1993). CNTF has demonstrated one of the most profound effects on oligodendrocyte survival by decreasing the developmental cell death in the optic nerve by 80% (Barres et al 1993). The same study showed that BDNF acts in synergy with CNTF to increase oligodendrocyte survival. Interestingly, the concentration of CNTF/LIF needed for survival are similar or identical to the concentrations needed for motoneuron survival.

Mature oligodendrocytes are responsible for synthesizing the multilamellar myelin sheath around CNS axons. Myelination of the spinal cord begins prior to birth in a rostro-caudal gradient with expression of myelin proteins such as GalC and myelin basic protein (MBP) at the ventral funiculus (Schwab and Schnell

1989). Expression of myelin proteins then occurs in lateral and then dorsal regions of the spinal white matter until myelination is complete. These authors noted also that only a subpopulation of oligodendrocytes expressed myelin proteins in each given location. Myelination of the rat optic nerve does not occur until post-natal day 5. It was recently reported that large diameter axons are among the first to become myelinated followed in turn by smaller diameter axons (Fanarraga et al 1998). These authors speculated that this was likely due to larger axons expressing the recognition molecules for the initiation of myelination before smaller diameter axons. It was also reported that a single oligodendrocyte myelinates similar size axons as the first axon that it comes in contact with (Fanarraga et al 1998). Interestingly, only one or a small number of internodes from large diameter axons are myelinated by a single oligodendrocyte while many smaller axon internodes can be myelinated by a single oligodendrocyte (Remahl and Hildebrand 1990)

The phenomenon of dedifferentiation has been demonstrated in oligodendrocytes. In this instance, mature oligodendrocytes adopt more immature characteristics similar to the O-2A progenitor cell in response to bFGF (Grinspan et al 1996). bFGF is expressed by neurons and is upregulated after injury. Treatment of mature oligodendrocytes with bFGF results in a decrease in the number of GalC+ oligodendrocytes and increases the number of A2B5+ oligodendrocyte precursors. It is thought that proliferation of oligodendrocyte progenitors ultimately contributes to remyelination. There is evidence that some remyelination occurs after injury or lesion (Ludwin 1987). During the period of

early remyelination post lesion, oligodendrocyte precursors that also express PDGFRα begin to proliferate near the lesion (Redwine and Armstrong 1998). Local PDGF-A expression by astrocytes is also upregulated after injury. Perhaps the combination of bFGF induced dedifferentiation (Grinspan et al 1996) of mature oligodendrocytes to O-2A progenitors and reexpression of PDGFRα results in the proliferation of oligodendrocyte progenitors needed for remyelination.

Acetylcholine and oligodendrocytes

One of the first studies demonstrating the developmental role of ACh was by Hohmann et al (1991). These authors noted that lesions of the basal forebrain cholinergic system resulted in abnormal development of the cortex and pyramidal cell distribution (Hohmann et al 1991). It was then reported that oligodendrocyte O-2A cells are responsive to ACh (Kastritsis and McCarthy 1993). Treatment with the stable ACh analogue, carbachol, induced an increase in intracellular Ca^{2+} in cultured oligodendrocytes. It was also observed in this study that immature oligodendrocytes were much more responsive than mature oligodendrocytes. The authors concluded that the cellular effector systems were either downregulated or uncoupled with differentiation. This was one of the first demonstrations of a developmental role of ACh in oligodendrocytes. It was subsequently reported that rat oligodendrocytes in culture expressed both M1 and M2 muscarinic acetylcholine receptors. Carbachol treatment induced inositol phosphate accumulation which resulted in mobilization of intracellular calcium

and an attenuation of the increase in cAMP which could be elicited by β-adrenergic stimulation (Cohen and Almazan 1994).

The M3 muscarinic acetylcholine receptor has more recently been implicated in mediating intracellular Ca^{2+} waves in oligodendrocytes, and it is suggested that the M3 receptor is the main receptor subtype on oligodendrocyte progenitors and mature oligodendrocytes. (Larroca and Almazan 1997). It has been suggested that the Ca^{2+} waves mediated by muscarinic stimulation play a role in regulating O-2A cell proliferation and differentiation (Simpson and Russell 1998). Carbachol has also been reported to increase the expression of c-fos mRNA and $[H]^3$ thymidine incorporation suggesting new DNA synthesis and cell proliferation. These actions were all blocked by an atropine-sensitive mechanism implying muscarinic receptor mediation.

It has been generally assumed that synthesis of ACh was of neuronal in origin. There have been previous reports of synthesis of ACh by cultured astrocytes and microglia (Wessler et al 1997). Reports of some nervous functions depending on diffuse cholinergic activation imply a possible autocrine/ paracrine mechanism of ACh (Zaidi and Matthews 1997, Bjorklund and Dunnett 1995). This is contrary to the classical role of ACh acting as a fast neurotransmitter via synaptic release and rapid degradation by acetylcholinestrases. Non-neuronal ACh has been localized to various tissues including airways, alimentary and urogenital smooth muscle, skeletal muscle and the pericardium (Reviewed in Wessler et al 1999). Non neuronal ACh is believed to act in an autocrine or paracrine fashion to regulate cellular processes such as

mitosis, cell differentiation, secretion and absorption. These authors have coined the term "The Trophic Property of ACh" to describe these phenomena.

The release of non-neuronal neurotransmitters is quite variable, and there are several mechanisms that have been reported. It was reported by Dennis and Miledi (1974) that electrical stimulation of Schwann cells resulted in a non-quantal release of ACh likely through membrane breakdown. There have also been reports of exocytotic release of neurotransmitters as well from astrocytes and neuronal membrane locations lacking synaptic specialization (Bezzi et al 1998, Zaidi and Matthews 1997). These mechanisms may indicate that there are direct signaling mechanisms of glial cells back to neurons.

It has been suggested that the responsiveness of oligodendrocytes to neuroligands is developmentally regulated (Kastritsis et al 1993). It was subsequently reported that neuronal contact is essential to maintain the coupling of muscarinic ACh receptors in mature oligodendrocytes (He et al 1996). It has already been mentioned that oligodendrocytes in culture possess neurotransmitter receptors to a variety of ligands, some of which become downregulated or uncoupled after differentiation (Kastritsis and McCarthy 1997). Neuronal contact with oligodendrocytes maintains this responsiveness, but if neuronal contact is disrupted the receptor becomes uncoupled to its effector systems and the responsiveness can not be restored after reestablishing neuronal connections (Kastritsis and McCarthy 1997). This would now imply that the responsiveness of oligodendrocytes to neurotransmitters is transiently independent of axonal contact, but as the oligodendrocyte matures, there

develops a requirement of the oligodendrocyte for a neuronal-derived factor to maintain neuroligand responsiveness.

Motoneuron Development

The differentiation of motoneurons has been the subject of much attention in the last ten years, and there have been many findings regarding the differentiation of motoneurons from the spinal neuroepithelium. Motoneurons are among the first cells to differentiate in the spinal cord (Langman and Haden 1970). Most of the recent work on motoneuron differentiation was performed on chick or quail embryos. One of the first examinations of motoneuron progenitors used recombinant retrovirus vectors to study the lineage of motoneurons differentiating from the chick neuroepithelium (Leber et al 1990). Labeled progenitors resulted in the differentiation of many labeled cell types including glia and other neuronal types. These authors concluded that motoneuron progenitors remain multipotent even up to a point of terminal differentiation. It was discovered by Yamada et al (1991) that dorso-ventral cell differentiation was patterned by the floor plate and notochord. They demonstrated that grafting ectopic floor plate or notochord to more dorsal positions induced the differentiation of motoneurons in corresponding areas of the neural tube. This work provided evidence that motoneuron development was directed by the floor plate and notochord.

The discovery of Islet-1 in the nervous system dramatically enhanced the study of motoneuron differentiation. Islet-1 is a protein first described in pancreatic Islet cells and is also localized to neuronal subtypes in the dorsal root

ganglia, intermediolateral nuclei and somatic motoneuron pools of the adult rat (Thor et al 1991). The expression of Islet-1 was explored in the chick model of motoneuron differentiation, and it was concluded that Islet-1 was the first molecular marker of motoneuron differentiation and now offered a more specific marker of all motoneurons in the spinal cord. (Ericson et al 1992). Islet-1 is expressed immediately after the final cell division of motoneurons and before the expression of mature motoneuronal properties. Islet-1 belongs to a family of transcription factors possessing a LIM homeodomain, and initially it was thought that Islet-1 functioned to maintain the differentiated phenotype of the cell in which it was expressed. The expression of Islet-1 is instructed by floor plate and notochord derived signals and is seen in both visceral and somatic motoneurons.

The actions of the floor plate and notochord on motoneuron differentiation resulted in the discovery by Yamada et al (1993) that motoneuron differentiation is induced by a diffusible factor released from the floor plate and notochord. It was also noted that floor plate differentiation was dependent on contact with the notochord suggesting another contact-mediated signal was required for floor plate differentiation. The nature of this diffusible factor remained elusive until 1994 when another report by Jessell's group revealed that the diffusible motoneuron-inducing factor released by the floor plate and notochord was a protein encoded by the vertebrate hedgehog (vhh) or *Shh* gene (Roelink et al 1994). Shh was shown to be capable of inducing the differentiation of floor plate and motoneurons. Interestingly, Shh could induce motoneuron differentiation in the absence of floor plate, suggesting that motoneuron differentiation does not

require floor plate-derived signals (Tanabe et al 1995). It was subsequently discovered that Shh was initially released by the notochord to induce the differentiation of floor plate. Floor plate cells subsequently released Shh which in turn induced the formation of motoneurons (Roelink et al 1995). The effect of Shh followed a classic developmental diffusion gradient where floor plate was induced by a 5-fold higher concentration than that needed for motoneuron differentiation. Exogenous application of a high dose of sonic hedgehog was capable of supressing motoneuron development and promoting further floor plate differentiation. This supports the finding of Yamada et al in 1993 that contact with notochord was necessary for floor plate induction likely due to a requirement for a high concentration of Shh. It was still unclear at this point in what part of the pathway Shh exerted an action because the progenitors were still undergoing further cell division before terminal mitosis and Islet-1 expression. It was quite obvious that Shh directly induced floor plate differentiation but it was still unclear whether it had a direct action on motoneuron differentiation or whether is caused commitment of motoneuron progenitors.

It was only in 1996 that this research was taken into a mammalian model of motoneuron development. Using knockout technology, Jessell's group demonstrated that Islet-1 expression is essential for visceral and somatic motoneurons to differentiate (Pfaff et al 1996). Motoneuron differentiation is also required for subsequent differentiation of a population of interneurons expressing the marker engrailed-1 (EN-1). Examination of the Islet-1 -/- mouse revealed that the cells that were destined to become motoneurons did not achieve any other

phenotype. Instead of resulting in an excess differentiation of another cell type, the pre-motoneurons succumbed to apoptosis.

The role of Shh has been unclear since its discovery. It was initially thought to be a differentiation factor for motoneurons, and then subsequently demonstrated to actually promote proliferation of progenitor cells (Kalyani et al 1998, Rowitch et al 1999). It was recently reported that Shh promotes the differentiation of spinal precursor cells and functions in unison with the neurotrophin NT-3 to induce motoneuron differentiation (Dutton et al 1999). This group demonstrated that Shh application alone promoted neuronal differentiation but did not increase the number of Islet-1 expressing cells. Islet-1 expression was increased after co-treatment with both Shh and NT-3, and this phenomenon was blocked using anti-bodies to NT-3. These results also supported the suggestion that Shh was simply a co-factor of motoneuron differentiation.

One of the major findings in motoneuron development arose in 1998 once again by Jessell's group (Tanabe et al 1998). Using differential screening of a cDNA library from a single Shh-induced motoneuron in the chick model, a novel homeobox gene was revealed named MNR2. MNR2 was expressed during the final division of Pax6+ motoneuron progenitors about 4-5 hours before the generation of the first post-mitotic motoneurons. At the onset of MNR2 expression, these cells are no longer dependent on Shh signaling. MNR2 represents the motoneuron-inducing transcription factor that results in the expression of later motoneuron markers Islet-1, Islet-2, Lim3 and HB9. MNR2

expression continues transiently after final mitosis of motoneurons. Presently, a mouse homologue of MNR2 remains to be discovered.

Recent work in this field has revealed further genes necessary for maintenance of a differentiated motoneuron state. The homeobox gene HB9 represses the differentiation of the V2 interneuron group to promote motoneuron differentiation (Thaler et al 1999). In HB9 knockout mice, motoneuron differentiation occurs as normal but motoneurons slowly adopt characteristics of V2 interneurons and cease to be motoneurons. In the mouse HB9 is expressed just prior to motoneuron differentiation and Islet-1 expression and persists right into adulthood. Interestingly, HB9 is expressed after terminal mitosis in the chick just prior to Islet-2 expression. This implies that despite the different time of onset, HB9 functions to maintain the motoneuron phenotype into adulthood.

The morphological and migratory characteristics of mouse motoneuron differentiation were described by Wentworth in 1984. This study traced the path and morphology of ventral root neuron differentiation from the spinal neuroepithelium by golgi impregnation techniques. At approximately E9 in the cervical spinal cord the first motoneurons differentiate from the neuroepithelium and mature through five stages of differentiation. Undifferentiated cells of the neuroepithelium (stage 1) possess no processes and constitute the most immature state. Preaxonic neuroblasts (stage 2) differentiate and migrate away from the central canal keeping a primordial process attached medially to the basal lamina during lateral migration. As the cell approaches a more lateral position, a primordial axon begins to extend laterally resulting in a bipolar

neuroblast (stage 3). As the apical (towards the neurocoel) process retracts and the axon continues to extend, the cell matures to the next developmental stage, that of a unipolar neuroblast (stage 4). As the cell begins to extend dendrites, it is termed a secondary bipolar neuron (stage 5) and subsequently a fully mature multipolar neuron. These cells assume a ventrolateral position and extend axons out the spinal ventral root.

Once motoneurons migrate to the ventral horn, a distinction is made between somatic and visceral motoneurons. This distinction was described in three developmental phases by Markham and Vaughn in the rat spinal cord (1991). It was previously suggested that somatic and visceral motoneurons are clonally related because they are both generated at the same time and form a primitive motor column. The first of these developmental phases corresponds to the entire process described by Wentworth in which all motoneurons, both autonomic and somatic, arise from the ventricular neuroepithelium and migrate to a single primordial motor column in the ventrolateral spinal cord. The second phase entails a progressive segregation of somatic and autonomic motoneurons wherein the autonomic motoneurons migrate dorsally along the lateral border of the spinal cord to the intermediate zone and achieve a more multipolar morphology. In the third phase of autonomic motoneuron development, a portion of these cells migrate from the intermediolateral region, medially towards the central canal. These processes define the genesis and migration of motoneurons during development. After this period of development, it was reported that a widespread death occurred of approximately 50% of the motoneuron population,

presumably as a result of competition for locally expressed trophic factors(Hamburger and Oppenheim 1982). This competition ultimately results in the survival of a defined population of functional motoneurons.

Neurotrophins and Motoneurons

The effects of neurotrophic factors on motoneurons have been intensely studied. It was first reported by Hamburger that motoneurons derive trophic support from their peripheral targets (i.e. muscle) (Hamburger 1958, Hollyday and Hamburger 1976). The importance of muscle has been recently underscored by a report that cre-lox mediated muscle ablation resulted in the loss of all motoneurons by embryonic day 18.5 (Grieshammer et al 1998). This indicates that muscle-derived factors and signals are essential for long-term motoneuron survival.

After motoneurons differentiate, it appears that they are independent of trophic support whether because of their small immature size or an inherent survival mechanism (Mettling et al 1995). As motoneurons mature and project to muscle, there develops a strong trophic requirement for survival. These authors suggest that this transient "trophic immunity" represents the time required for motor axons to reach peripheral targets. There are three neurotrophins (BDNF, NT-3, NT-4) that are present in the embryonic chick limb bud and exhibit survival promoting actions on motoneurons (Henderson et al 1993). NT-3 is the most highly expressed neurotrophin during embryonic and postnatal development followed by NT-4 (Griesbeck et al 1995). These authors report that BDNF is

normally expressed at low levels until peripheral nerve lesion, whereupon BDNF expression increases dramatically with concomitant down-regulation of NT-4. This would indicate that BDNF is the trk-B ligand involved in survival while NT-4 binds to trk-B in cell maintenance. The neurotrophin NT-4 was described as an activity-dependent trophic factor for motoneurons released from muscle suggesting a chronic function (Funakoshi et al 1995). The role of BDNF as a survival molecule is evident in culture where BDNF can rescue 90% of motoneurons from death after lesion (Sendtner et al 1992, Yan et al 1992, Henderson et al 1993).

The trophic factors BDNF, NT-3, NT-4 and the cytokine ciliary neurotrophic factor (CNTF) all promoted activity of ChAT in motoneurons (Wong et al 1993). This study also reported that a combination of BDNF and CNTF exhibit synergistic actions on increasing ChAT activity. The synergistic actions of BDNF and CNTF have been reported to promote motoneuron survival, neurite outgrowth and halt progression of motoneuron degeneration seen in the *wobbler* mouse model of amyotrophic lateral sclerosis (ALS) (Kato and Lindsay 1994, Mitsumoto et al 1994, Zurn et al 1996).

CNTF was first described as being expressed in very high concentrations in the peripheral nerve and localized to Schwann cells (Williams et al 1984). It was also one of the first growth factors that was demonstrated to promote survival of motoneurons in the chick (Oppenheim et al 1991, Sendtner et al 1990, Arakawa et al 1990). It was later reported that mice lacking CNTF display profound disruptions of motoneurons (Masu et al 1993). After sciatic nerve

lesion, a transient increase in CNTF concentration has been observed likely to function as a motoneuron survival factor (Sendtner 1992). This increased CNTF release is a result of Schwann cells releasing their contents, and it drops to negligible levels by one week after lesion. CNTF levels are reestablished after motor axons reconnect to peripheral targets.

Glial cell derived neurotrophic factor (GDNF) is an unrelated growth factor that displays a 75-fold more potent response than CNTF on motoneuron survival and is likely the most potent single motoneuron survival factor to date (Henderson et al 1994). Combinations of GDNF with BDNF or CNTF all promote ChAT enzyme activity (thus increasing ACh synthesis) and survival of motoneurons (Zurn et al 1996). The peripheral motoneuronopathy (*pmn*) mouse mutant is a model for early onset motoneuron disease. Facial and lumbar motoneurons are destroyed as a result of impaired axonal transport. GDNF is found in the peripheral nerve and muscle and has also been shown to attenuate motoneuron loss in the *pmn* mouse model of ALS much like BDNF and CNTF (Sagot et al 1996). Interestingly, despite such a profound effect on motoneuron survival, GDNF still cannot prevent the axon degeneration observed in the *pmn* mouse.

Motoneurons can be cultured in the absence of trophic support for only approximately 4 days on a permissive laminin substrate (Martinou et al 1989). Laminin was capable of promoting neurite outgrowth and ChAT activity but within 4 days no motoneurons survived. Recently the use of trophic "cocktails" has increased motoneuron survival dramatically. Hanson et al (1998) report that

combinations of trophic factors combined with forskolin-induced increases of cAMP increased cultured motoneuron survival for up to three weeks.

Neuromuscular Junction Development

The development of neuromuscular junctions is an elaborate process and has recently been reviewed by Sanes and Lichtman (1999). Motor axons travel directly towards distal musculature without branching and innervate a single muscle. Schwann cells migrate after motor axons in response to migratory cues expressed by the axons. In regeneration, however, Schwann cells already exist and the axon regrows through existing bands of Bungner (Riethmacher et al 1997). During primary axon extension, the motor axon paves a path to the muscle and releases trophic stimulation for Schwann cells promoting mitosis and migration. One notable motor axon-derived factor is the Schwann cell mitogen REG2 that is transported along developing axons (Livesey et al 1997). Upon reaching a muscle, the axon branches multiple times to innervate tens to hundreds of muscle fibres. Synaptic transmission occurs very shortly after a motor growth cone connects with muscle. At first, synaptic transmission is very inefficient, likely due to the immaturity of the neuromuscular junction. Within a week after initial contact, more neuromuscular junction specialization occurs resulting in a fully functional neuromuscular junction.

As a motor axon contacts the muscle, factors are released from the motor terminal, which result in clustering of ACh receptors in the postsynaptic membrane. The protein agrin has been implicated in synaptogenesis. Agrin was discovered by McMahan's group initially in the Torpedo fish and later cloned from mammals (Godfrey et al 1984, Rupp et al 1991). Agrin is expressed in motoneurons and transported down axons. It is then released from motor terminals to the synaptic cleft (McMahan 1990). In agrin knockout mice, there is a large perturbation of postsynaptic specialization. Neuromuscular junctions had few clusters or other specializations (Gautam et al 1996) and motor axons did not develop characteristic multi-fibre arborizations (Gautam et al 1996). The agrin receptor still is a subject of debate. It is thought to be the MuSK receptor because MuSK knockout mice display a similar phenotype as agrin knockouts, yet agrin apparently does not bind to MuSK. Another protein ARIA (Acetylcholine receptor inducing activity) contributes to ACh receptor synthesis in skeletal muscle (Falls et al 1993). ARIA is expressed by motoneurons and plays a role in the development and maintenance of mammalian neuromuscular junctions. Still ARIA is not specific to motoneurons and is also present at other central synapses (Sandrock et al 1995). An interesting phenomenon during ACh receptor clustering is that if ACh receptor clusters already exist on a particular myocyte, new axons will develop new sites for contact and not reconnect in apposition to previous clusters (Anderson and Cohen 1977). Immature neuromuscular junctions possess dense clusters of ACh receptors and as the neuromuscular junction matures, ACh receptor density tends to decrease from the epicentre of

the synapse (Bevan and Steinbach 1977). Initial neuromuscular synapses are very weak because of low ACh receptor density and limited neurotransmitter release (Kullberg et al 1977, Nakajima et al 1980). Postnatally, the entire motor endplate becomes raised as the postsynaptic folds form (Antony et al 1995). Quantal content of ACh is increased and the terminal does not suffer from fatigue. The myelination by Schwann cells increases the conduction velocity along axons and the efficiency of neuromuscular communication.

Multiple innervation of a single muscle fibre occurs during development and subsequently all but one input is eliminated. The selective pruning of terminal branches is competitive in that the elimination of a particular axon is dependent on the presence of another neighbour (Colman and Lichtman 1993). Several trophic factors prevent the developmental synapse elimination and may contribute to maintenance of the synapse during adulthood. These include leukemia inhibitory factor, BDNF, CNTF, bFGF, insulin-like growth factor-1 and GDNF (Kwon et al 1995, Kwon and Gurney 1996, Jordan 1996, English and Schwartz 1995, Caroni and Becker 1992, Nguyen et al 1998).

Regeneration of neuromuscular junctions is different from development in that the presence of Schwann cells is established during regeneration. Schwann cells contribute multiple protein elements to promote growth adhesion and reestablishment of synapses (Son and Thompson 1995, Son et al 1996, Sendtner et al 1992). These proteins are down-regulated after initial maturation of the neuromuscular junction and then reexpressed after lesion. (Taniuchi et al 1988, Plantinga et al 1993, You et al 1997, Matsuoka et al 1997).

What constitutes a Motoneuron?

As previously stated, there have been several preliminary reports of motoneurons differentiating from neural precursors, These studies used minimal markers to identify a motoneuron. It is difficult to identify motoneurons in this situation because there is a vague operational definition of a motoneuron. A precursor-derived motoneuron should be defined as: a neuronal cell that displays antigenic and functional characteristics of motoneurons in vivo. This would include the expression of protein markers such as ChAT, Islet-1, REG2, and the α-1c calcium channel subunit (Barber et al 1984, Ericson et al 1992, Livesey et al 1997, Jiang et al 1999). On a more functional level, precursor-derived motoneurons should also be able to extend axons towards myocytes and form cholinergic terminals on these cells. Stimulation of the presynaptic motoneuron should also be able to release ACh into the synaptic cleft and elicit contraction of the myocyte. This definition is applied to motoneuron identification in the motoneuron component of this thesis.

Project Goals

The overall goal of this thesis is to demonstrate differentiation of, and characterize two cholinergic cell types (Oligodendrocytes and Motoneurons) from multipotent neuroepithelial precursor cells. In light of the evidence of ACh responsiveness of oligodendrocyte precursors and the presence of non-neuronal ACh in other tissue types and glial cells, it is possible that oligodendrocytes also

express a cholinergic phenotype. The goal of the first project is to demonstrate that some neurosphere-derived oligodendrocytes differentiate in culture to express cholinergic markers. The hypothesis is that some oligodendrocytes are cholinergic and that ACh plays a role in oligodendrocyte differentiation. Neurospheres were cultured in various growth factors to promote optimal growth of oligodendrocytes. Immunohistochemistry demonstrated co-expression of the oligodendrocyte marker GalC with the cholinergic markers ChAT and the VAChT. Muscarinic ACh receptor blockade significantly reduced the oligodendrocyte differentiation. These findings support the hypothesis that some neurosphere-derived oligodendrocytes are cholinergic and that ACh plays a role in oligodendrocyte differentiation. This work is currently being reviewed for publication by the Journal of Comparative Neurology.

The second project described in this thesis is a characterization of motoneurons derived from neurosphere precursors. There have been some preliminary reports of motoneuron differentiation from stem cells already listed. In these studies, non-specific motoneuron markers were examined and no functional characterization was performed. The goal of the second project was to demonstrate the differentiation of functional motoneurons from neurospheres in culture and after transplantation into the sciatic nerve and provide the most comprehensive study to date characterizing these cells by various means. The hypothesis is that functional motoneurons do differentiate from neurosphere precursors in tissue culture and after transplantation into a host sciatic nerve. The functional characterization using electrophysiological and pharmacological

means were combined with immunohistochemistry for more specific motoneuronal markers. This study demonstrated that neurons differentiate from neurospheres in culture and after transplantation into the sciatic nerve, and express the motoneuronal markers ChAT, VAChT, Islet-1, REG2 and the α-1c calcium channel subunit. There was also evidence of neuromuscular junction formation using immunohistochemical and electrophysiological means. These results support the hypothesis that functional motoneurons can differentiate in culture and after transplantation. This paper will be submitted to the Journal of Neuroscience.

These two projects will be presented in this thesis as two manuscripts submitted for publication. The oligodendrocyte project will be presented first and subsequently the motoneuron work. Both projects will be summarized afterwards and general conclusions will be presented. Discussion will follow to address relevance and common themes.

Paper 1

A Population of Oligodendrocytes Derived from Multipotent Neural Precursor Cells Expresses a Cholinergic Phenotype in Culture and Responds to CNTF.

S.C. MacDonald[1], R. Simcoff[1], L.M. Jordan[1], J.G. Dodd[1], K.W. Cheng[1], and S. Hochman[1,2*]

[1]Department of Physiology, University of Manitoba, Winnipeg, MB, R3E 3J7
[2] Emory University School of Medicine, Atlanta, GA, 30322

Abstract:

Multipotent neuroepithelial precursors can proliferate in culture and differentiate into neurons, and glia. There has been little study characterizing neurosphere-derived oligodendrocytes. Previously, oligodendrocyte development has been studied using the O-2A glial progenitor cell but only oligodendrocyte markers have been examined, and other markers that might be present have not been examined including neurotransmitter synthesis enzymes. In this study, neurospheres in tissue culture differentiated with or without added trophic factors into mixed populations of neurons and glia. Regardless of trophic support, there existed a population of cells that resembled oligodendrocytes morphologically, co-expressed the oligodendrocyte protein galactocerebroside (GalC) and the acetylcholine (ACh) synthesizing enzyme choline acetyltransferase (ChAT), and did not express the neuronal marker microtubule associated protein 2 (MAP-2). Patch clamp recordings from cells with this morphology displayed only outward currents in response to depolarizing voltage steps, further supporting their oligodendroglial identity. The vesicular acetylcholine transporter, which functions to package ACh into vesicles, was also detected in $GalC^+$ oligodendrocytes. Cells cultured in the presence of the cholinergic receptor antagonist atropine showed a decrease in oligodendrocyte differentiation without apparent change in cell morphology. The effects of neurotrophins and ciliary neurotrophic factor (CNTF) on these $ChAT^+$ oligodendrocytes were examined. CNTF treatment significantly increased mean process length in oligodendrocytes as well as the

number of cholinergic oligodendrocytes differentiating from each sphere. These results suggest that ACh receptor activation plays a signaling role during the differentiation of $ChAT^+$ oligodendrocytes and that CNTF acts as a neurotrophic factor to these cells.

Text

Introduction:

Many cells of the central nervous system can release and react to growth factors in an autocrine or paracrine fashion. Oligodendrocytes are one such cell type whose primary function is the production of central nervous system myelin. Oligodendrocytes in the mouse differentiate from the ventral ventricular neuroepithelium around E14 of gestation (Warf 1991; Pringle and Richardson 1993), and produce growth factors such as neu differentiation factor (NDF) and insulin-like growth factor-1 (IGF-I). These secreted factors promote the survival, proliferation, and differentiation of oligodendrocytes by autocrine or paracrine loops (Raabe et al 1997, Shinar and McMorris 1995).

Acetylcholine (ACh) has been detected in a number of non-neuronal cell types, such as epithelial and endothelial cells, and is released in autocrine and paracrine fashion to activate ACh receptors that signal diverse cellular events including mitosis and differentiation (for review see Wessler 1999). ACh may also play a role in oligodendrocyte development (Cohen et al 1996). Cultured oligodendrocytes can express muscarinic ACh receptors. Muscarinic receptor stimulation of oligodendrocytes mobilizes intracellular calcium and has been reported to propagate Ca^{2+} waves between oligodendrocytes and to promote proliferation of oligodendrocyte progenitors. (Cohen and Almazan 1994; Simpson

and Russell 1996; Cohen et al 1996). Muscarinic receptors are down-regulated or uncoupled from their cellular effector systems as oligodendrocytes mature implying that the role of ACh is regulated developmentally (Cohen et al 1996; Kastritis and McCarthy 1993).

Existing data support the following hypothesis: In the developing spinal cord, ACh is released from spinal motoneurons, which are among the first cholinergic neurons to differentiate in the mouse spinal cord at approximately E10 (Wentworth 1984). Then, committed oligodendrocyte progenitors would respond to ACh to proliferate and subsequently differentiate from the ventricular neuroepithelium at approximately E14 (Pringle and Richardson 1993, Warf et al 1991). In light of the evidence that non-neuronal acetylcholine displays autocrine/ paracrine signaling in other systems, it is possible that oligodendrocyte precursors also synthesize their own acetylcholine to perpetuate the developmental cycle in an autocrine/paracrine fashion.

In this study we used cultures of multipotent spinal neuroepithelial precursors (neurospheres) generated in the presence of EGF and bFGF to study oligodendrocyte differentiation (Weiss et al 1996). We observed that the synthesis enzyme for acetylcholine (choline acetyltransferase, ChAT) and the vesicular acetylcholine transporter are both found in a population of differentiating oligodendrocytes, consistent with the hypothesis that oligodendrocytes express a cholinergic phenotype to sustain autocrine trophic

support with ACh. Thus, acetylcholine signaling during oligodendrocyte development may act either as a trophic factor or as a mediator of two-way cellular communication during a period prior to myelination. These results have previously been presented in abstract form (MacDonald et al 1999).

Methods:

Primary culture of precursors: Spinal cords were removed from E15- E18 CD1 albino mouse embryos sacrificed by ether anesthesia followed by cervical dislocation and dissected in a modified Hanks Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} containing KCl (4 mM), KH_2PO_4 (0.6 mM), NaCl (80 mM), $NaHCO_3$ (0.35 mM), $NaHPO_4$ (0.048 mM), D-glucose (1 mM), pH 7.3. The cords were minced and incubated in the same Ca^{2+}/Mg^{2+} -free HBSS in the presence of 100ng/ml papain (Sigma), 100ng/ml DNAse, and 1 mg/ml protease (Sigma type I) for 30 minutes. After the enzyme digestion, the tissue fragments were gently triturated and then washed in Dulbecco Modified Eagle's Medium / Ham's F12 medium (1:1) containing DNAse (1mg/ ml) for 15 minutes. The resulting cell suspension was then washed and resuspended in DMEM/F12 medium containing N2 supplement (Sigma). Dissociated cells were plated at $4x10^4$ cells per $75cm^2$ flasks in DMEM/ F12 medium containing 20ng/ml of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). After 7 days in culture, floating clusters of cells were withdrawn, centrifuged at 400 rpm and resuspended in fresh EGF/bFGF containing medium. Culture medium was half-changed every 7 days and fresh EGF/bFGF was added every 4 days.

Neural precursor cells in culture were generated using methods based on Reynolds and Weiss (Weiss et al 1996) and resulted in the formation of free floating clusters of cells that expressed the intermediate filament marker nestin

(data not shown, MacDonald et al 2000). These cells could be passaged multiple times while maintained in a proliferative state in culture with mitogens EGF and bFGF. Spheres were plated on poly-D-lysine/laminin coated culture dishes or 8 well cultures slides in DMEM/F12 or Neurobasal medium containing B27 supplement (Gibco), 2 mM glutamine and 1mM sodium pyruvate. Cultures were maintained in a 95% O_2/ 5% CO_2 filled incubator at 37°C until fixation or recording. To obtain cell differentiation for experiments, bFGF and EGF were removed from the culture medium. Trophic factors, if added, were presented in the following concentrations: BDNF (3 ng/ml), NGF (2 ng/ml), NT-3 (10 ng/ml) CNTF (20 ng/ml) and GDNF (10 ng/ml). Unless otherwise stated, culture medium was supplemented with both CNTF and BDNF to promote optimal oligodendrocyte differentiation.

Immunohistochemistry: Cultures were fixed in either 4% paraformaldehyde fix or Zamboni's fixative. Ten micron cryosections were taken from the embryos and mounted on gelatin-coated slides. All tissue was washed 5 times in PBS containing 1% FCS before primary incubation. Antibodies were diluted in PBS containing 0.8% triton-X or else PBS containing 1% FCS, 0.8% tween-20 and 0.5% horse serum: (rabbit-anti-ChAT (1:1000), goat-anti-vesicular acetylcholine transporter (1:5000), rabbit-anti-GFAP (1:1000)) (Chemicon), mouse-anti-MAP2 (1:1000)(SMI), mouse-anti-galactocerebroside (1:100; (Boeringer Mannheim),or 1:10; (a gift from Dr. B. Juurlink). Primary incubations included 1:100 horse serum (Sigma). Cultures were washed 5 times in PBS with 1% FCS between

incubations. Cultures were incubated in secondary antibodies for 90 minutes diluted as follows: donkey-anti-mouse-cy3 conjugate (1:250), donkey-anti-mouse-FITC, (1:250 Jackson); donkey-biotinylated-anti-rabbit (1:100) and streptavidin-FITC (1:100 Amersham). Cultures were washed 2 times in PBS and 3 times in 50 mM Tris-HCl before being coverslipped with a glycerin-based anti-fade medium (Vectashield; Vector Labs). For the galactocerebroside labeling, cultures were incubated in anti-GalC antibodies prior to fixation and then fixed in 4% paraformaldehyde. After fixation, cultures were treated with ice-cold methanol for 6 minutes to permeabilize the cell to cytosolic antibodies while maintaining the integrity of the GalC immunolabeling. The same procedure was then followed as described for other antibodies. Images were either photographed or digitized using a CCD digital camera or Neurolucida image analysis system.

Western Blot Analysis: Cultures were grown for 7 or 10 days and washed once in PBS before incubating in PBS without Ca^{2+}/ Mg^{2+} for 15 minutes. Cells were washed off of the dish and lysed with radioimmunoprecipitation (RIPA) buffer (containing: 0.9% NaCl, 10mM PO_4^{3-} buffer, 0.01% NP-40, 0.1% SDS, 3000 K.I.U.Aprotonin, 1 mM Sodium Orthovanadate, 0.001 PMSF) to extract total protein from the sample. Protein was loaded on a 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrophoresed for 40 minutes. The gel was then transferred to polyvinylidene difluoride (PVDF) membrane and stained with ponceau red. Non-specific sites on the membrane were blocked for 60 minutes in TBST-milk before primary incubation overnight.

Secondary incubation in goat-anti-rabbit-horseradish peroxidase was performed for 60 minutes before chemiluminescence reaction and exposure to x-ray film.

Electrophysiology: Culture dishes were perfused with HEPES-buffered recording solution consisting of (in mM): NaCl, 150; KCl, 5; HEPES, 10; $MgCl_2$, 1; $CaCl_2$,2; glucose 10; pH 7.4, 320 mOsm. Whole-cell voltage clamp recordings were performed with the Axopatch 1D amplifier (Axon Instruments). Microelectrodes were filled with solution containing (in mM): K-gluconate, 140; ethylene glycol-bis(β-amino ethyl ether) N,N,N',N',-tetraacetic acid (EGTA), 11; KOH, 35; HEPES, 10; $CaCl_2$, 1. pH was adjusted to 7.3. Data was acquired using pCLAMP 6.0 software (Axon Instruments), stored to computer, and analyzed with pCLAMP software.

Results:

Neurospheres are multipotent cell clusters that express the stem cell marker nestin during proliferation, and can be induced to differentiate into neuronal and glial phenotypes that express mature cell markers (Reynolds and Weiss 1996). We observed that neurospheres grown in culture for 7 days in the absence of mitogens resulted in cultures of neurons, astrocytes and oligodendrocytes as demonstrated by immunohistochemical detection of the specific cellular markers MAP-2, GFAP, and galactocerebroside respectively (data not shown).

Choline acetyltransferase is expressed in oligodendrocytes

When cultures were processed for the neuronal marker MAP-2 and for choline acetyltransferase (ChAT) it was revealed that a population of cells was positively immunostained for ChAT and negative for MAP-2 (Fig. 1A). Further, the $ChAT^+$ cells displayed morphology with extensive process arborization and round cell bodies that resembled cultured oligodendrocytes (Szuchet 1995). Almost all cells with oligodendrocyte morphology were $ChAT^+$ (e.g. Figs. 1,5 & 6). ChAT has traditionally been used as a marker for cholinergic neurons and has not previously been reported in oligodendrocytes. We then performed double label immunohistochemistry for ChAT and GalC and found that almost all $ChAT^+$ cells also expressed GalC (Fig. 1B,C), a myelin constituent found on the surface of oligodendrocytes. There was also a population of $GalC^+$ oligodendrocytes that did not express ChAT and a population of $ChAT^+$ cells not expressing GalC at the limits of detection.

Western blot analysis confirms specificity of the ChAT antibody

To determine whether the ChAT antibody was recognizing enzymatic ChAT, total protein was harvested from differentiated cultures grown in BDNF and CNTF, separated by SDS-PAGE for western blot. The blot was probed with the ChAT antibody (RbαChAT; Ab 143, Chemicon) and visualized on photographic film. The antibody recognized two fairly equal intensity bands at 28 and 70 KDa (Fig. 2). The presence of the 70KDa gene product confirmed that our antibody was specific and recognized ChAT in our differentiated precursor cultures (Grosman et al 1995). The protein at the 28K band is a splice variant of the ChAT gene and possesses no ChAT synthesizing activity.

Cholinergic Vesicular Transport Proteins are present in ChAT$^+$ oligodendrocytes

It is possible that oligodendrocytes synthesize ACh to package into vesicles for intercellular signaling via exoctyosis. The vesicular acetylcholine transporter (VAChT) packages acetylcholine into vesicles for neurotransmission. The VAChT is always localized in neurons expressing ChAT (Gilmor et al 1996). Dual label immunohistochemistry for the VAChT and either GalC or MAP-2 revealed that VAChT can be found in precursor-derived oligodendrocytes in culture (Fig. 3). VAChT positive cells displayed an oligodendrocyte morphology and many were positively immunostained for GalC (Fig 3A and 3B). VAChT positive cells also did not co-localize the neuronal marker MAP-2 (Data not shown). Thus, some oligodendrocytes may be capable of packaging ACh into vesicles.

Muscarinic receptor blockade affects ChAT$^+$ oligodendrocyte development

The role of ACh in oligodendrocyte development has been attributed to the intracellular Ca^{2+} mobilization and gene expression seen in response to cholinergic stimulation (Cohen and Almazan, 1994; Cohen et al, 1996). To see whether ACh has a role in oligodendrocyte development from precursor cells, neurospheres were cultured for 7 days in medium containing BDNF and CNTF to promote oligodendrocyte differentiation (see below), in the absence or presence of the broad spectrum muscarinic receptor antagonist, atropine sulfate (at 2 or 20 μM). There appeared to be no obvious effect of atropine on oligodendrocyte morphology as some oligodendrocytes in 2 and 20 μM treated cultures still differentiated and displayed long processes and diffusely spreading GalC$^+$ myelin sheets. Total number of spheres was counted in all cultures (n=32 cultures of each condition), as were the number of spheres containing GalC$^+$ oligodendrocytes. In atropine treated cultures, there was a significant decrease in the number of spheres containing oligodendrocytes as compared to untreated controls ($p < 0.001$; Fig 4). In control cultures, approximately 40% of spheres contained oligodendrocytes. This number was reduced to approximately 20% in cultures treated with atropine.

Trophic influences on neurosphere-derived oligodendrocytes

During development, trophic factors can influence the survival and differentiation of various cell types (Johe et al 1996; Barres et al 1993). Experiments at various

time intervals were conducted on precursor cells to examine whether trophic factors influence the genesis and differentiation of $ChAT^+$ oligodendrocytes. Neurospheres in culture were allowed to differentiate for 4, 7, and 14 days in the presence of a single trophic factor (Fig. 5). $ChAT^+$ cells were visible after 4 days in cultures treated with CNTF, BDNF, NGF, NT-3, or GDNF. Cultures treated with CNTF consistently yielded greater numbers of $ChAT^+$ oligodendrocytes as compared to untreated cultures or those treated with NT-3, BDNF, NGF or GDNF (Fig. 5). There were very few $ChAT^+$ cells in NT-3 treated cultures at 4 and 7 days; even untreated cultures contained more $ChAT^+$ cells. It appears that CNTF has the most significant action on increasing oligodendrocyte number.

Despite the presence of trophic factors in the culture environment, there always existed some differentiated cells that were positively immunostained for ChAT. In all trophic environments, double-labeling experiments revealed that $ChAT^+$ cells were rarely $MAP\text{-}2^+$ (Fig. 6). While there always existed $MAP\text{-}2^+$ neurons in all differentiated neurospheres, very few were $ChAT^+$.

At all time intervals there were a number of striking observations in cultures treated with CNTF. Another significant action of CNTF was on promoting process extension (Fig. 7). CNTF treated oligodendrocytes had significantly longer processes (125 μm/ process) than untreated cells or those treated with BDNF, or GDNF (all approximately 50 μm/ process ($p<0.001$); Fig. 8B). Soma diameter displayed a slight significant increase in cultures co-treated with a combination of

CNTF and BDNF ($p<0.01$)(Fig. 8C). Co-treatment with CNTF and BDNF resulted in an increased number of larger $ChAT^+$ cells (> 15 μm soma diameter) than in untreated cultures or those treated with BDNF or CNTF alone ($p<0.001$) (Fig. 8D). Thus, CNTF and BDNF together appear to promote an additive increase of differentiation of $ChAT^+$ oligodendrocytes. This is consistent with previous reports of synergistic actions of BDNF and CNTF on oligodendrocytes (Barres et al 1993).

Electrophysiological properties of oligodendrocytes

Whole cell voltage clamp recording was performed on cells with oligodendrocyte morphology in neurosphere cultures grown for 7-10 days (n= 8). Oligodendrocytes were easily identified in the culture dish for recording by their large soma diameter and long thick processes (Fig. 9). Cells possessing this characteristic morphology resembled those labeled with antibodies to both ChAT and GalC. A series of hyperpolarizing and depolarizing voltage steps from a holding potential of –80 mV demonstrated that there were no voltage-dependent inward currents in the recording population. Some 'oligodendrocytes' displayed small outward currents in response to depolarization (Fig. 9B). These electrophysiological results are consistent with those of Sontheimer et al (1988), who demonstrated voltage- dependent potassium currents in cultured oligodendrocytes isolated from the mouse spinal cord. Other cells with a neuronal morphology displayed voltage sensitive outward as well as fast inward currents in response to voltage steps. Thus, neurons are also differentiating, confirming a heterogeneity in the culture population.

Discussion:

This study reports that cells derived from multipotent neuroepithelial precursors with characteristic oligodendrocyte morphology express ChAT, a synthesis enzyme that synthesizes the neurotransmitter, ACh. These cells are MAP-2 negative, and many express the myelin marker galactocerebroside, confirming an oligodendrocyte identity.

The presence of ChAT suggests that ACh is synthesized in these cells. Non-neuronal ACh is not novel and has been reported in many different tissue types including epithelial cells of the airways, skin and muscle as well as astrocytes cultured from rodent brain (Wessler et al 1995, Klapproth et al 1997, Fu et al 1998, Wessler et al 1997). Non-neuronal ACh is implicated in the regulation of cellular processes such as mitosis, differentiation, absorption and secretion (Wessler et al 1999). Non-neuronal ACh may also govern regeneration and functional recovery after brain lesions (Björklund and Dunnett 1995), perhaps recapitulating cellular processes seen during development. We hypothesize that ACh is important to oligodendrocyte development.

We show by immunohistochemistry that a subpopulation of oligodendrocytes expresses the enzyme ChAT. The specificity of the anti-ChAT antibody is demonstrated by western blot analysis. This particular antibody (AB143) has previously been demonstrated to bind two fairly equal intensity bands at 28 and

70 KDa (Grosman et al 1995), and both bands were seen in protein samples isolated from neurosphere cultures. The smaller molecular weight protein is a splice variant of the ChAT gene and possesses no ChAT synthesizing activity. This smaller variant is also seen to co-localize with full-length 70 KDa enzymatic ChAT in cholinergic spinal neurons. The presence of the 70KDa gene product confirmed that our antibody was specific and recognized ChAT in our differentiated precursor cultures.

The electrophysiology data presented here demonstrate that the oligodendrocytes differentiating in our neurosphere cultures are similar to those obtained from primary culture of mouse spinal cord (Sontheimer et al 1988). Given our knowledge of $ChAT^+$ oligodendrocyte morphology from the immunohistochemical studies, it was relatively easy to target oligodendrocytes for recording. A limitation of the electrophysiology was our inability to identify a particular oligodendrocyte as $ChAT^+$ before recording. However, it is very likely that the oligodendrocyte recordings are from $ChAT^+$ oligodendrocytes in light of the fact that the vast majority of oligodendrocytes were $ChAT^+$.

It is possible that 'cholinergic' oligodendrocytes selectively myelinate cholinergic axons. While oligodendrocytes myelinate appropriate axons in the CNS, the mechanisms by which this occurs are not well understood (Collinson et al 1998, Duncan et al 1996). Oligodendrocytes appear not to be restricted to the size of axon they myelinate, which argues against the specificity of oligodendrocyte-

axon interaction; yet it was also shown that when oligodendrocytes myelinate several axons, they are all the same diameter (Fanarraga et al 1998). Perhaps a cholinergic oligodendrocyte may selectively myelinate multiple cholinergic axons. This may be one explanation for the cholinergic phenotype of these developing oligodendrocytes, and it may prove worthwhile to examine the expression of other neurotransmitter synthesis enzymes as well. We also demonstrated a population of oligodendrocytes that expressed galactocerebroside but not ChAT. This non-cholinergic population may represent cells at a different developmental stage or of functionally different oligodendrocyte populations.

Cholinergic oligodendrocytes differentiated regardless of the trophic support in the medium. In contrast, very few cholinergic neurons differentiated in our neurosphere cultures. In most cultures, $ChAT^+$ cells never co-localized with MAP-2 and most exhibited oligodendrocyte morphology. Clearly, our culture conditions do not support the differentiation of cholinergic neurons even though five types of cholinergic neurons differentiate in the spinal cord during development (Barber et al 1984).

Trophic factors such as CNTF have been reported to promote growth and survival of oligodendrocytes in culture (Barres et al 1993, Mayer et al 1994). In our study, the most significant action of CNTF was on promoting process extension. This is interesting, because CNTF is not expressed until approximately postnatal day 4 when active myelination is occurring (Stockli et al

1991). Perhaps CNTF expression postnatally promotes extension of oligodendrocyte processes in finding their axon targets during myelination. One observation we report was no oligodendrocytes differentiating in cultures treated with NT-3 until 14 days in culture (DIC). After 14 DIC, the number of $ChAT^+$ oligodendrocytes appears to be still relatively low while the few oligodendrocytes that have differentiated are large and well differentiated. The neurotrophin NT-3 has been described as one of the most potent mitogens for oligodendrocyte precursors and the most potent survival factor after differentiation (Kumar et al 1998; Barres et al 1993; Barres et al 1994). Thus, NT-3 may play a role in maintaining cells in an undifferentiated state and account for the extremely low yield of oligodendrocytes arising from these cultures.

As already stated, the cytokine CNTF has been demonstrated to have profound effects on differentiation, survival and growth of developing oligodendrocytes and has also been shown to arrest programmed cell death in newly differentiated oligodendrocytes (Mayer et al 1994; Louis et al 1993). While the effects of the neurotrophin BDNF are largely unknown on oligodendrocytes, BDNF has previously been shown to potentiate CNTF-mediated oligodendrocyte survival in culture (Barres et al 1993). The synergistic action of BDNF and CNTF manifested itself in this study as a dramatic increase in the number of larger $ChAT^+$ cells. These reports demonstrate a profound trophic responsiveness of oligodendrocytes and encourage further exploration of trophic factors on other oligodendrocyte characteristics such as the ability to myelinate axons.

Is ACh playing a role during oligodendrocyte differentiation?

These results describe a traditional neurotransmitter synthesis enzyme being localized to the oligodendrocyte, a non-neuronal cell that has traditionally played a more supportive role in the central nervous system. This raised the question of whether oligodendrocytes are capable of communication using acetylcholine. Our results demonstrated VAChT in differentiated oligodendrocytes. This is consistent with reports of VAChT being found always in cholinergic neurons (Gilmor et al 1996). Now it appears that VAChT is also co-localized with ChAT in precursor-derived oligodendrocytes and suggests a vesicular release of ACh from these cells. Others have shown that glia can take up choline from the extracellular space (Vernadakis 1988) to support ACh synthesis, and that the PNS equivalent to oligodendrocytes, the Schwann cells, can release ACh (Dennis and Miledi 1974). It is likely that the role of ChAT and VAChT in oligodendrocytes is to support the exocytotic release of ACh for signaling purposes. This may be involved in myelin biosynthesis as certain molecular chaperones and vesicular transport proteins are expressed during oligodendrocyte development and then cease. During differentiation, transport proteins function to traffic proteins to specific destinations on the plasma membrane as well as resorbing misfolded proteins via vesicles (Neri et al 1997). An interesting observation of their developmental study was that a specific complement of vesicular transport proteins exists in $A2B5^+$ oligodendrocyte progenitor cells (O-2A) (Neri et al 1997). Interestingly, the complement of

molecular chaperones and vesicular transport proteins changes to a different set further on in development. Thus, acetylcholine may play a role in some form of developmental feedback loop during myelin biosynthesis.

Another observation made in this study was the effect of muscarinic receptor blockade on oligodendrocyte differentiation. There was a significant reduction by 50% of oligodendrocyte differentiation in the presence of atropine. It has been previously suggested that stimulation of muscarinic ACh receptors may cause changes in oligodendrocyte progenitor development (Cohen and Almazan 1994). Muscarinic (M1 and M3) receptor stimulation results in intracellular Ca^{2+} mobilization while M2 stimulation results in decreases of cAMP formation. There are two actions to which ACh-receptor stimulation in oligodendrocyte progenitors has been attributed. Firstly, treatment of oligodendrocyte progenitors with carbachol results in activation of the immediate early gene c-fos (Cohen et al 1996). These authors suggested that the ultimate result of this would be maintained proliferation. The second action described for ACh-receptor stimulation is phosphorylation of the transcription factor, cAMP response element binding protein (CREB) which promotes differentiation of oligodendrocytes (Sato-Bigbee et al 1999). CREB phosphyorylation in immature oligodendrocytes occurs via increases in intracellular Ca^{2+} while in mature oligodendrocytes phosphorylation is attributed to β-adrenergic stimulation. High levels of CREB expression have been shown in developing oligodendrocytes. Our results demonstrating a decrease in differentiation after atropine treatment are

consistent with this second possibility. The likely mechanism for our observation is that atropine prevents CREB phosphorylation in response to M1,M3-mediated cytosolic calcium increases and thus prevents differentiation. As already mentioned, responsiveness to ACh and receptor complement on a given oligodendrocyte progenitor are developmentally regulated (Kastritsis and McCarthy 1993). Thus the action on a given cell would depend on where in the developmental pathway the cell was at the time of treatment. As neurospheres are a heterogeneous population of cells, it is not unreasonable to assume that many cells are in a proliferative stage and would be responsive to atropine. Therefore, it is possible that the oligodendrocytes that did differentiate in our atropine-treated cultures likely were already committed progenitors at the time of atropine treatment. Alternatively, perhaps they represent a population of oligodendrocytes unresponsive to ACh.

Are oligodendrocytes communicating with each other via ACh?

The notion that glial cells can communicate to other cells is not novel. It was initially shown that astrocytes propagate intracellular calcium waves to each other via gap junctions (Nedergaard 1994). These waves are capable of exciting neurons in contact with them. It was also shown that astrocytes can release glutamate upon stimulation with bradykinin or prostaglandins (Parpura et al 1994), (Bezzi et al 1998). This signaling mechanism was recently shown to be calcium dependent and has greatly supported a signaling role for glial cells.

Mature myelin contains a number of ligand-gated receptors, including muscarinic acetylcholine receptors (He et al 1996). M1, M2 and M3 muscarinic receptors are found in mature myelin with M3 receptors being the most abundant (Larocca and Almazan 1997). Stimulation of muscarinic acetylcholine receptors in oligodendrocytes causes IP3 mediated calcium waves that propagate through the cell. It was subsequently demonstrated that these waves are dependent on mitochondrial location and function (Simpson and Russell 1996). It may be that acetylcholine released from neighboring $ChAT^+$ oligodendrocytes acts via muscarinic receptors to elicit a response as one means of communication.

Taken together, these results offer a very intriguing possibility of oligodendrocyte signaling using ACh. The wide range of literature linking ACh and oligodendrocytes results in some very diverse possibilities as to the functional significance of these findings. The study of oligodendrocyte development, especially from precursor cells has implications to the fields of myelination, developmental biology and clinically to demyelinating diseases such as multiple sclerosis. Perhaps, these findings may open up deeper exploration into the study of glial to glial signaling.

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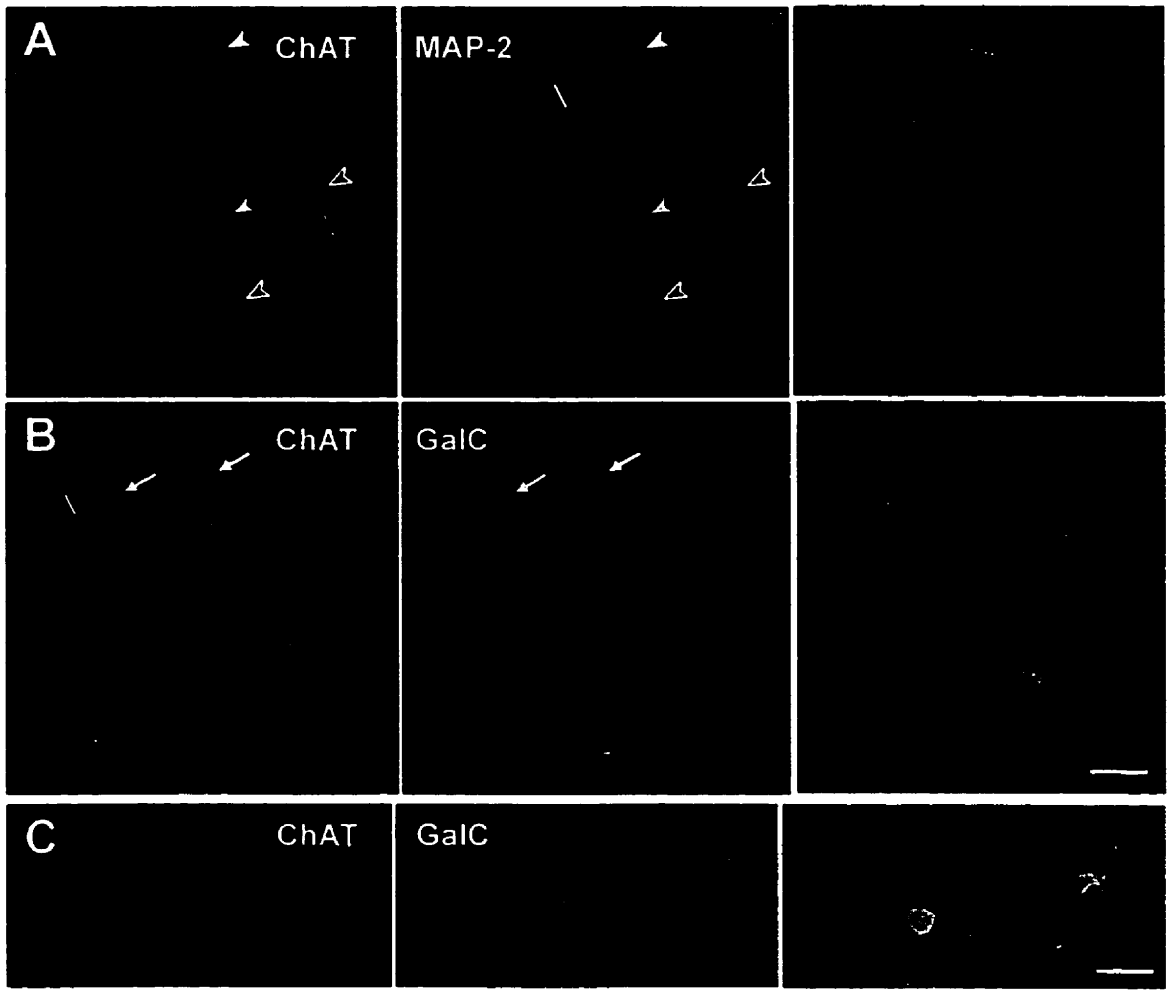
A
ChAT
MAP-2
B
ChAT
GalC
C
ChAT
GalC

Figure 1. ChAT is localized to GalC labeled oligodendrocytes or MAP-2 labeled neurons. A. While some MAP-2$^+$ cells are also ChAT$^+$ (white arrowhead), many ChAT$^+$ cells are not MAP-2 immunolabeled (black arrowheads) and the majority of MAP-2$^+$ cells are ChAT$^-$ (yellow arrowhead). B and C. A subset of oligodendrocytes expressing GalC is also labeled with antibodies to ChAT (black arrows). There are also a number of GalC$^+$ cells that do not label with ChAT antibodies (white arrows) and ChAT$^+$ cells that do not label with GalC (yellow arrows). GalC immunostaining after Triton-X permeablization results in punctate GalC labeling in B. Methanol permeablization maintains integrity of surface GalC staining in C resulting in uniform GalC labeling. The right panels on A, B, C show the immunostaining overlapped using the transparency tool in CorelDRAW 9. Neurospheres were cultured for 10 DIC in medium containing BDNF and CNTF. Scale bar is 50 μm.

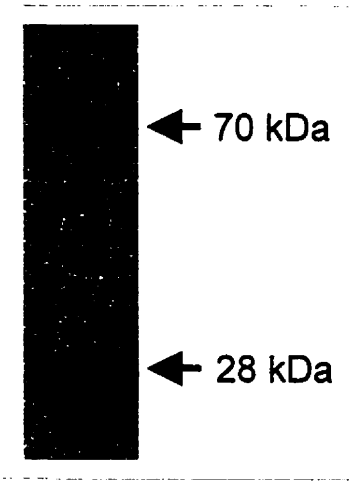
70 kDa
28 kDa

Figure 2. Western Blot analysis demonstrates that ChAT antibody 143 (Chemicon) is specific for two ChAT gene products. Ab 143 recognizes two products of ChAT gene transcription (approx 28 and 70 kDa) in differentiated neurospheres. The 70 kDa ChAT product corresponds to enzymatic ChAT.

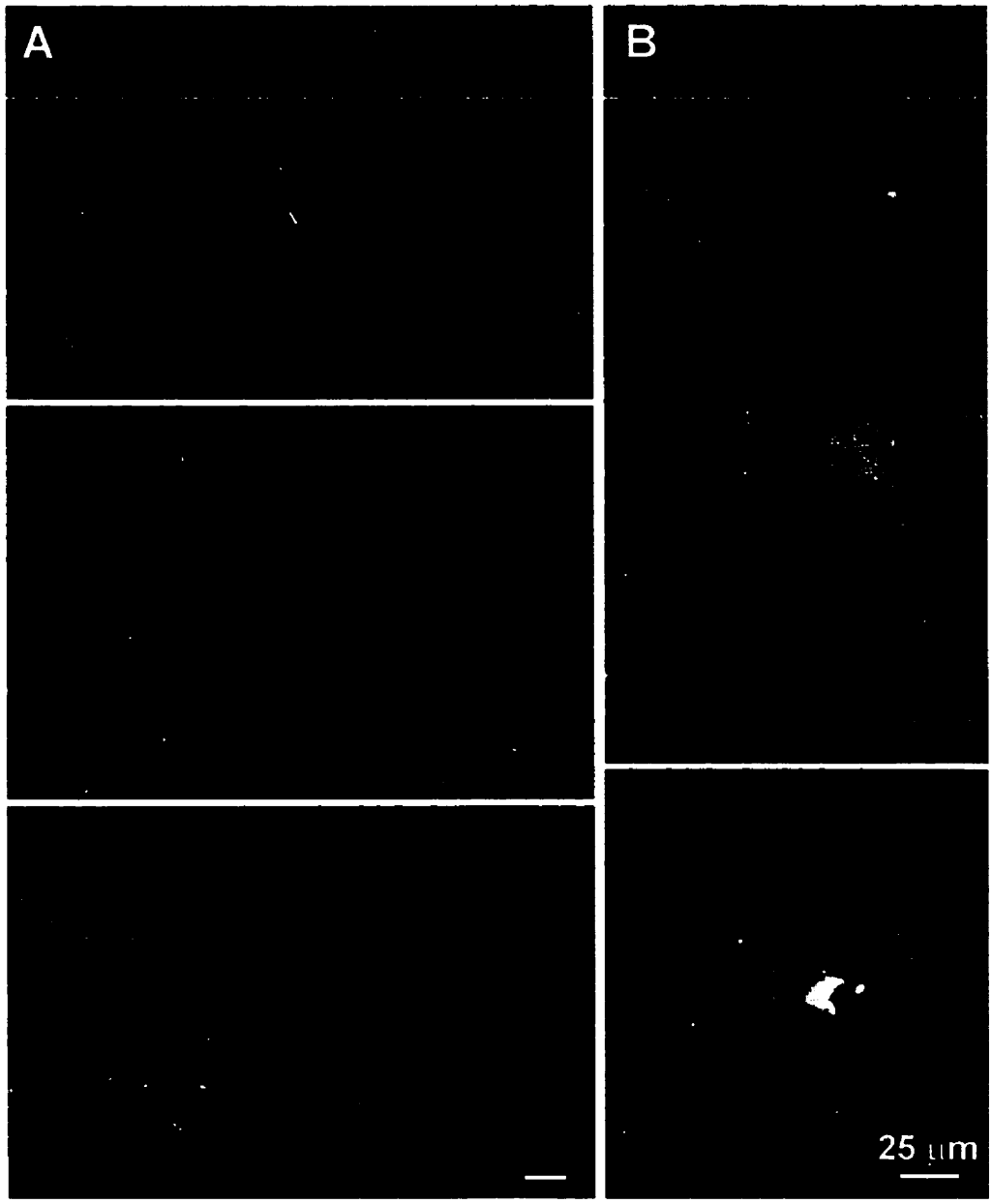
A
B
25 µm

Figure 3. The vesicular acetylcholine transporter is localized to GalC expressing oligodendrocytes. VAChT (green) and GalC (red) co-localize in several cells (A and B). Double labeling images were constructed by overlapping individual images using the transparency tool in CorelDRAW (v8). Scale bar is 25 μm in all.

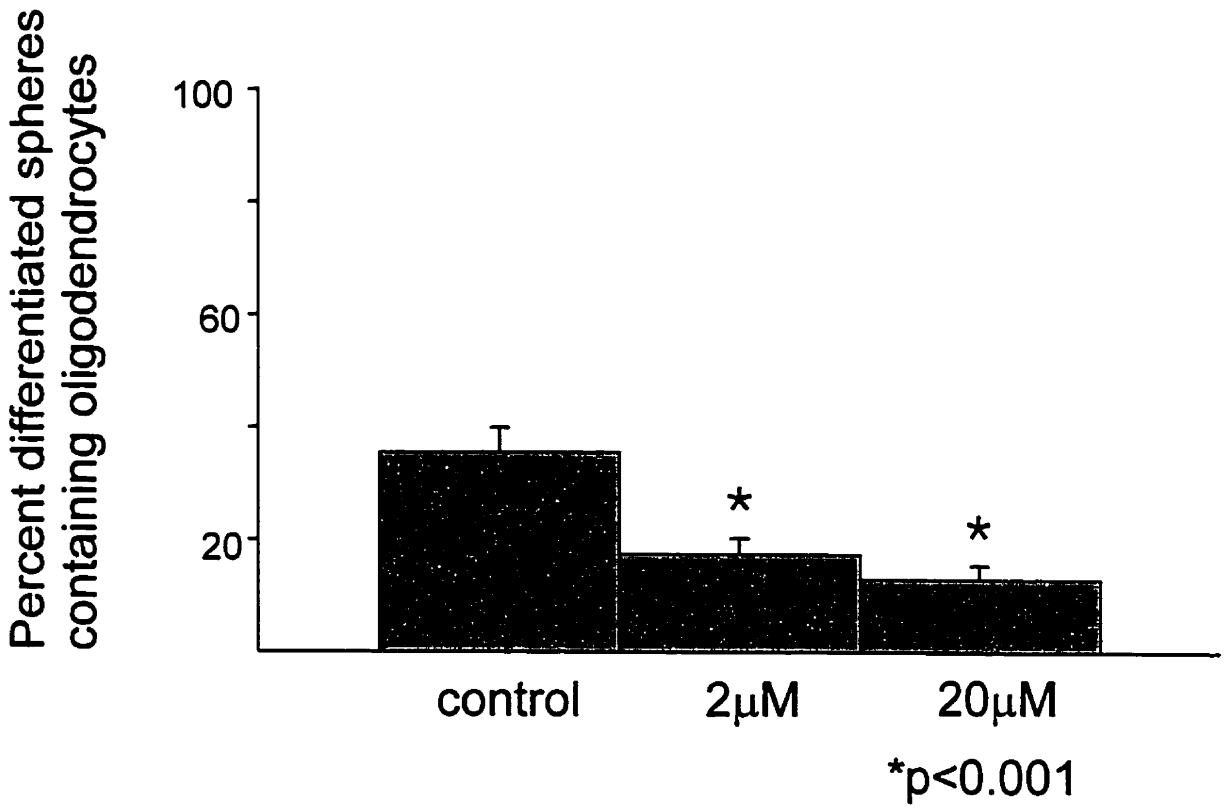
Percent differentiated spheres containing oligodendrocytes
100
60
20
*
*
control
2μM
20μM
*p<0.001

Figure 4. Histogram demonstrating the effect of muscarinic acetylcholine receptor antagonist atropine on oligodendrocyte differentiation. The number of oligodendrocyte-containing neurospheres were counted over the total number of neurospheres and expressed as a percent (n=32). 2 and 20 μM atropine was tested in comparison to control cultures. * = $p<0.001$

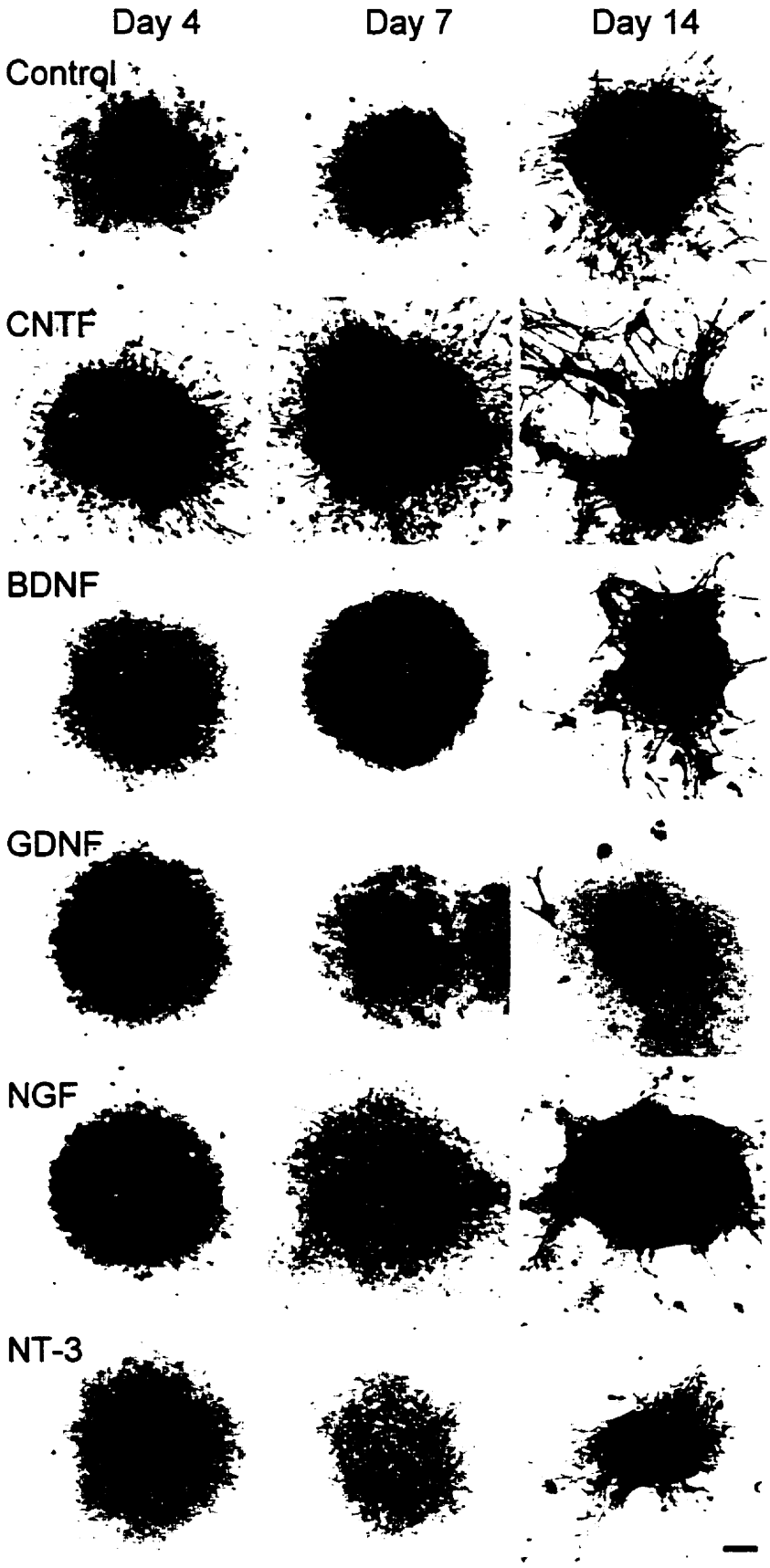
Day 4
Day 7
Day 14
Control
CNTF
BDNF
GDNF
NGF
NT-3

Figure 5. Occurrence of $ChAT^+$ oligodendrocytes in different trophic factors at 4, 7, & 14 days in culture. Images were converted to grayscale and presented as negatives using CorelDRAW (v8). Positively stained cells appear as black dots. Note that stem cell neurospheres incubated in CNTF expresses the greatest number of $ChAT^+$ oligodendrocytes, whereas there is a relative absence of $ChAT^+$ oligodendrocytes in cultures treated with NT-3. Scale bar is 100 μm.

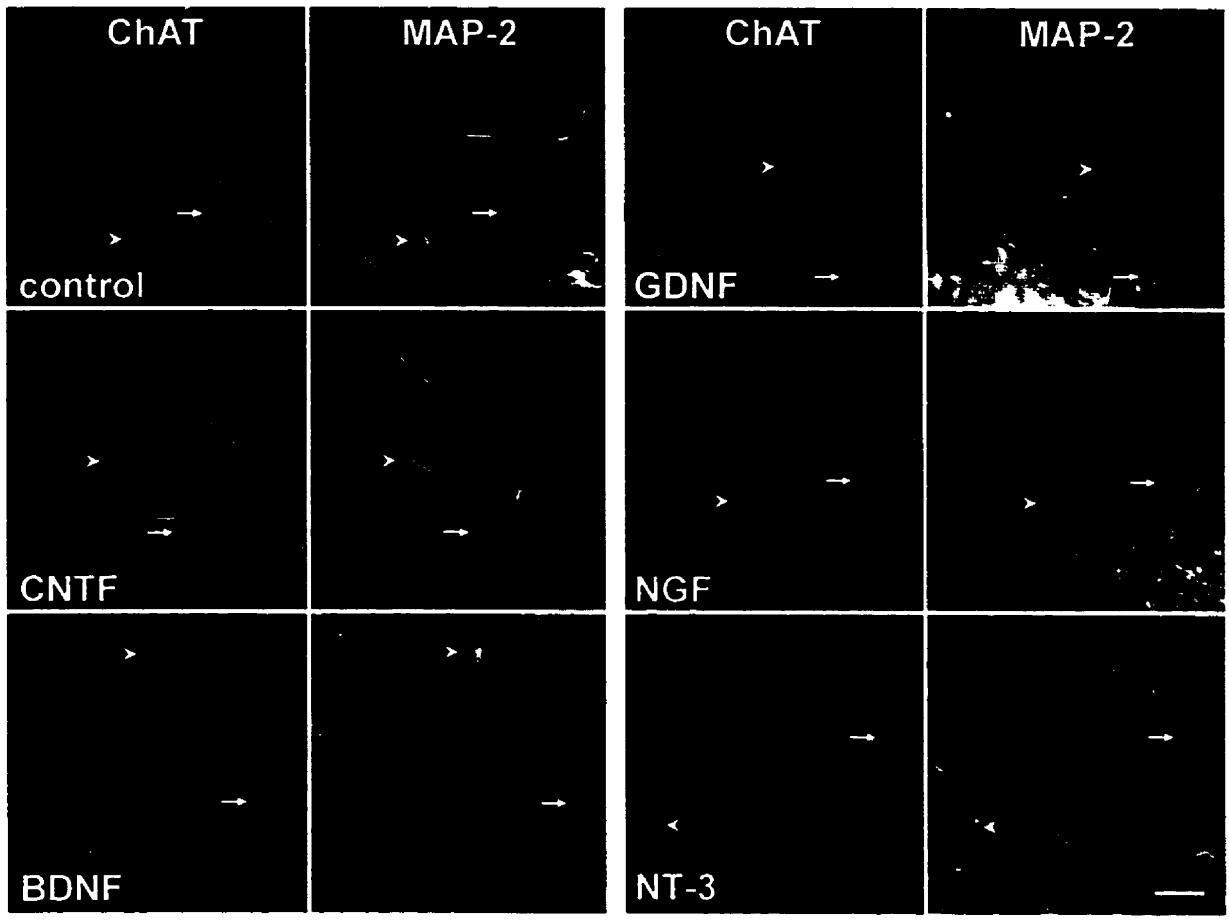
ChAT
MAP-2
ChAT
MAP-2
control
GDNF
CNTF
NGF
BDNF
NT-3

Figure 6. ChAT and MAP-2 were rarely co-localized despite trophic influence. The expression of $ChAT^+$ cells are compared to MAP-2 stained putative neurons following a 7-day incubation in the presence of various trophic factors. For all media, $ChAT^+$ cells are presented in the left panel and compared to the identical region photographed for MAP-2 staining (right panel). For a given factor, arrows identify $ChAT^+$ cells and arrowheads identify $MAP\text{-}2^+$ neurons to facilitate comparison between panels. Note that while cell morphology for $ChAT^+$ cells and $MAP\text{-}2^+$ neurons differ under the influence of the tested trophic factors, rarely were $ChAT^+$ cells double-stained with MAP-2. An example of a small double labeled cell is seen in the CNTF treated culture. Scale bar is 50 μm.

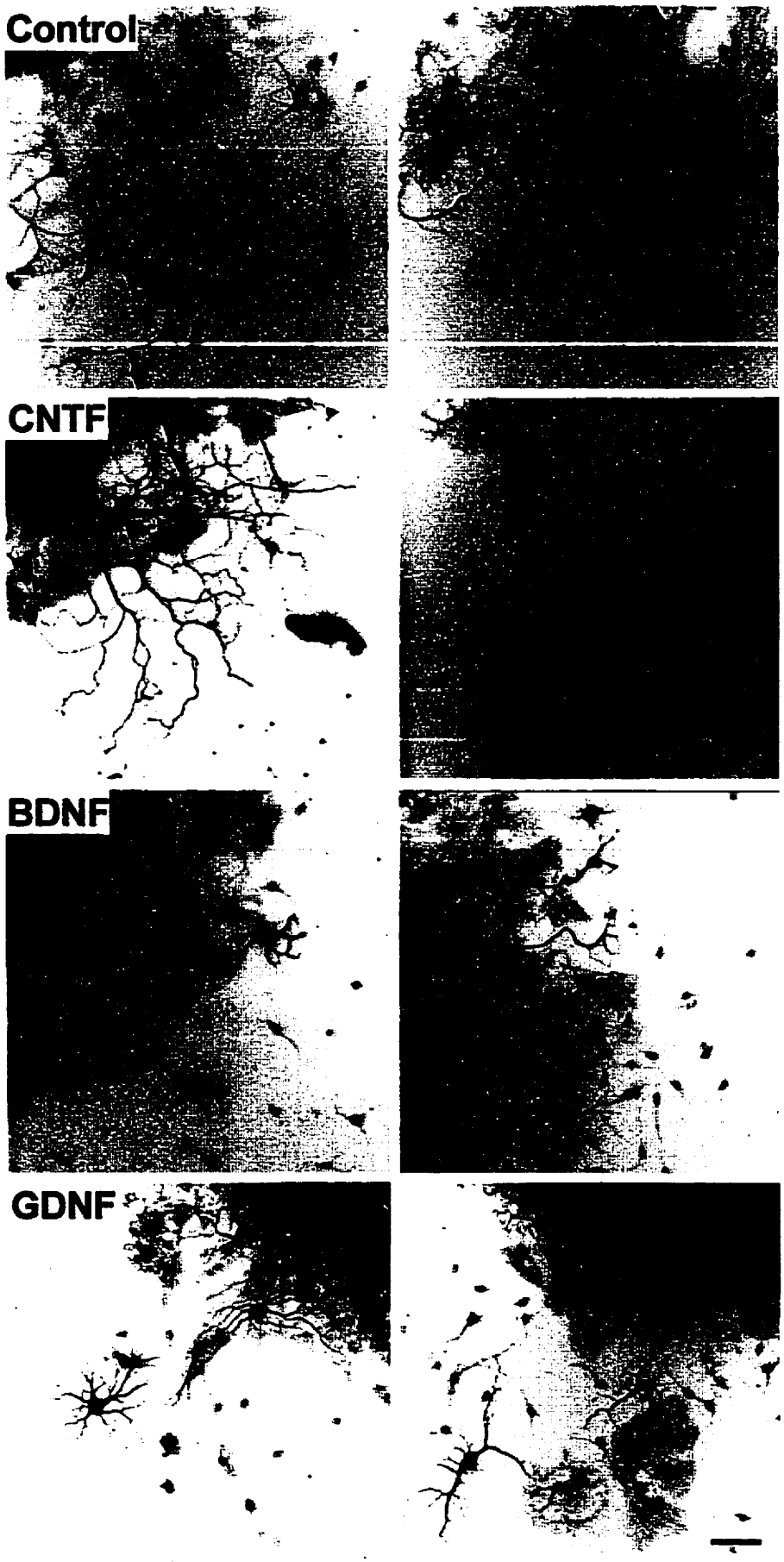
Control
CNTF
BDNF
GDNF

Figure 7. Comparison of the effects of various trophic factors on the elaboration and maturation of $ChAT^+$ oligodendrocytes. Note that CNTF supports extensive growth of $ChAT^+$ oligodendrocytes. Images were converted to grayscale and presented as negatives. Scale bar applies to all panels and is 25 μm.

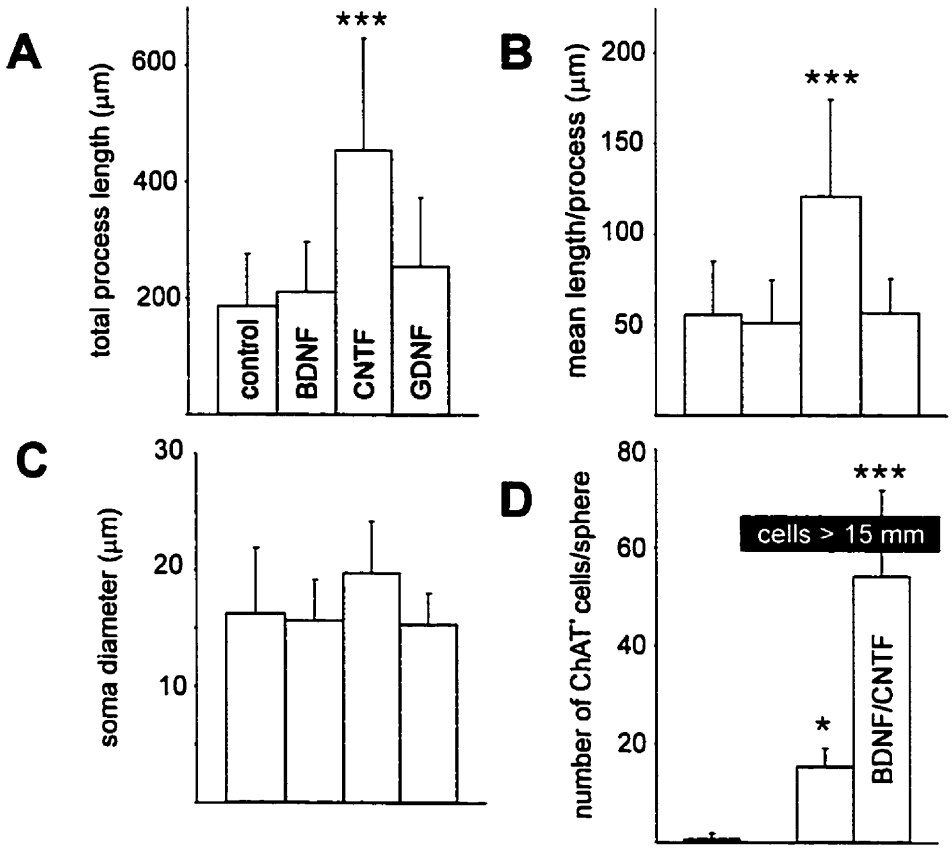
A
total process length (µm)
600
400
200
***
control
BDNF
CNTF
GDNF
B
mean length/process (µm)
200
150
100
50
***
C
soma diameter (µm)
30
20
10
D
number of ChAT cells/sphere
80
60
40
20
***
cells > 15 mm
*
BDNF/CNTF

Figure 8. A-C. Histograms quantifying the ability of various trophic factors on process length (A&B) and cell soma size (C) of $ChAT^+$ oligodendrocytes. D. When $ChAT^+$ cells having soma diameters >15 μm are compared, CNTF and BDNF appear to act synergistically to support differentiation and/or survival of these oligodendrocytes. (*$p<0.05$; *** $p<0.001$)

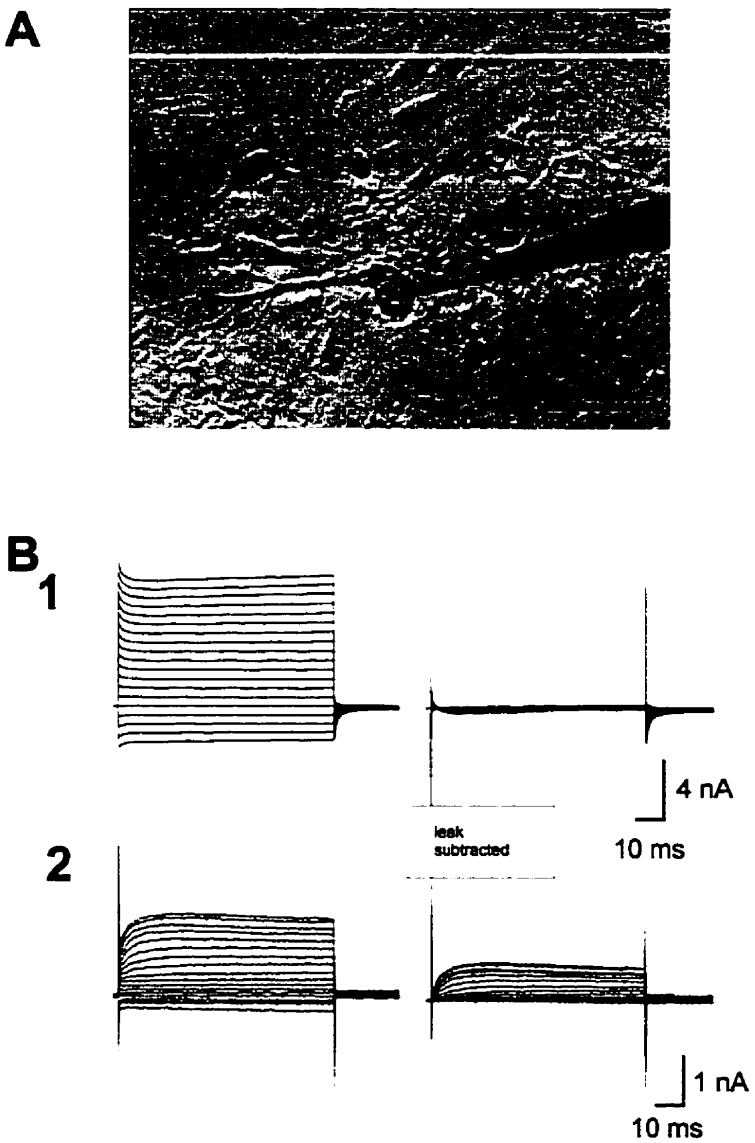
A
B
1
4 nA
leak
subtracted
10 ms
2
1 nA
10 ms

Figure 9. Electrophysiological properties of neurosphere-derived oligodendrocytes. **A.** Representative example of an oligodendrocyte in culture recorded intracellularly with a patch electrode (seen at right). Scale bar is 50 μm. **B.** The corresponding current responses to voltage steps are shown in B1 before and after leak subtraction. Note the current response is linear (ohmic). In another cell (B2) leak subtraction demonstrates the present of an outward current during depolarizing current steps. In both cells membrane voltage was stepped in 10 mV increments from –120 to +80 mV from a holding potential of –80 mV.

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Paper 2

Functional motoneurons differentiate from mouse multipotent spinal cord precursors in culture and after transplantation into lesioned sciatic nerve.

S.C. MacDonald[1], I.G. Fleetwood[2], S. Hochman[3], R. Brownstone[1*], J.G. Dodd, K.W. Cheng[1], and L.M. Jordan[1]

[1]Department of Physiology, University of Manitoba, Winnipeg, MB R3E 3J7
[2] Department of Clinical Neuroscience, University of Calgary, Calgary, AB
[3] Department of Physiology. Emory University, Atlanta, GA, 30322

Abstract:

Proliferative neural precursor cells are multipotent and can differentiate into neurons and glial cells. It was our goal to determine whether functional spinal motoneurons could differentiate from precursors in culture and after transplantation into the lesioned sciatic nerve. Cells were cultured with trophic factors or transplanted into the transected sciatic nerve. Tissue was processed immunohistochemically, and expression of the motoneuron markers ChAT, Islet-1, REG2 and the α1c calcium channel subunit was demonstrated. RT-PCR analysis also demonstrated the expression of Islet-1 in differentiated cultures. A co-culture preparation of neurospheres and skeletal myocytes was employed to show by immunohistochemistry, the formation of neuromuscular connections between precursor-derived motoneurons and myocytes. Further study of the co-culture preparation by drug perfusion revealed muscle contraction in response to the AMPA/ kainate receptor agonist kainate. Electrophysiology demonstrated end plate potentials and contraction in an isolated myocyte. As myocytes do not possess receptors for kainate, contraction must have been due to the actions of kainate on innervating motoneurons thus demonstrating the presence of neuromuscular junctions. Precursors transplanted into the sciatic nerve extended processes distally and formed cholinergic terminals in distal musculature.

These results support the hypothesis that spinal motoneurons can be generated from proliferative neural precursors grown in culture and demonstrate that they form functional neuromuscular junctions in culture as well as in vivo. These findings have relevance to cell replacement strategies currently being explored in clinical disorders such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy where selective motoneuron loss occurs.

Key Words: Spinal Cord, Precursor, Stem Cell, Co-culture, Neurosphere,

Progenitors, Development

INTRODUCTION

We have previously reported that neurosphere precursor cells can differentiate in culture into Choline acetyltransferase $(ChAT)^+$ oligodendrocytes after treatment with brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) (MacDonald et al 2000). However, it was observed in this study that a small fraction of $ChAT^+$ cells are not oligodendrocytes as they expressed the neuronal marker MAP-2. These neurons displayed a large cell diameter and multipolar morphology, and it is likely that these cells are motoneurons.

There have been previous reports attempting to derive motoneurons from neural precursor cells (Ray and Gage 1994, Kalyani et al 1997, Kalyani et al 1998, Park et al 1999). These reports examine the expression of non-specific motoneuron markers and do not demonstrate functional properties of the precursor-derived motoneurons. It has been difficult to demonstrate motoneuron differentiation from neural precursors because there is a lack of specific motoneuron markers and because motoneurons share common features with other neurons.

A motoneuron can be defined by the expression of protein markers which can be detected molecularly or histochemically. General neuronal markers such as MAP-2 should be expressed as well as the enzyme choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAChT). There are a number of LIM homeobox transcription factors also found in motoneurons. The first to be expressed during motoneuron differentiation is Islet-1, which is the first molecular

marker of terminal motoneuron differentiation in the spinal cord (Ericson et al 1992). REG2 is another protein localized to the motoneuron membrane and is transported along motor axons (Livesey et al 1997). The α1c subunit of the dihydropyridine-sensitive calcium channel has also been localized to spinal motoneurons (Jiang et al 1998). These proteins are only some of the potential markers that can be used to detect motoneurons and offer a good basis to detect motoneurons using immunohistochemistry or reverse transcriptase- polymerase chain reaction (RT-PCR).

The *sine qua non* of a motoneuron is to project axons to muscle, form neuromuscular junctions, and produce muscle contraction, thus a solely histological approach to localizing precursor-derived motoneurons may be open to criticism despite the use of multiple markers. Therefore an unequivocal identification of precursor-derived motoneurons would be their ability to form functional neuromuscular junctions. A second part of this study involved transplanting undifferentiated precursors into transected adult mouse sciatic nerve to demonstrate the ability of neural precursors to differentiate into motoneurons and form functional neuromuscular junctions in this in vivo environment.

Thus, the purpose of this study is two-fold. First, to use more specific motoneuronal markers to more convincingly identify precursor-derived motoneurons in cultures. Second, to demonstrate that similarly derived cells can

form functional neuromuscular junctions both in vitro and following transplantation in vivo.

We hypothesize that functional motoneurons can differentiate from neural precursors in culture and after transplantation into mouse sciatic nerve. By the previous definition, these cells should be able to make functional connections to skeletal muscle.

To identify and explore the functional nature of precursor-derived motoneurons, two approaches were undertaken. First, an in vitro co-culture model of precursors and skeletal myocytes has been developed to explore neuromuscular junction formation by drug responsiveness and electrophysiology. The goal of this study is to demonstrate that precursor-derived motoneurons can differentiate in culture and innervate cultured skeletal myocytes.

Neurospheres were also transplanted into the lesioned sciatic nerve to determine if differentiation into a motoneuron phenotype is possible in this environment. The peripheral nerve environment is growth permissive to motor axons and releases growth factors after lesions. Previous studies have reported successful transplantation of fetal motoneurons into the sciatic nerve (Erb et al 1993, Katsuki et al 1997). This environment may also promote the differentiation of motoneurons from neurospheres.

These results offer significant evidence that functional motoneurons differentiate from multipotent precursor cells. The findings further the field of motoneuron development and survival in vitro as well as further demonstrate the potential clinical utility of neural precursors in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS).

METHODS

Primary culture of precursor neurospheres: All animals were anaesthetized, and the experimental procedures were approved by the University of Manitoba Animal Care Committee and conformed to the standards of the Canadian Council of Animal Care. Spinal cords were removed from E16- E18 CD1 albino mouse embryos in a modified Hanks Balanced Salt Solution (HBSS) without Ca^{2+} or Mg^{2+} containing (in mM) KCl (4 mM), KH_2PO_4 (0.6 mM), NaCl (80 mM), $NaHCO_3$ (0.35 mM), $NaHPO_4$ (0.048 mM), D-glucose (1 mM), pH 7.3. The cords were minced and incubated in Ca^{2+}/Mg^{2+} free, HBSS with 100ng/ ml papain (Sigma), 100ng/ml DNAse, and 1mg/ ml protease (Sigma type I) for 30 minutes. After the enzyme incubation, the tissue was gently triturated and then washed in DMEM/F12 medium (1:1) containing DNAse (1mg/ ml) for 15 minutes. The cell suspension was then washed and resuspended in DMEM/F12 medium containing N2 supplement (Sigma). Cells were plated $4x10^4$ in uncoated $75cm^2$ flasks, and 20ng/ml of epidermal growth factor (EGF) and basic fibroblast growth

factor (bFGF) were added. After 7 days in culture, floating clusters of cells were withdrawn, centrifuged at 400 rpm, and resuspended in fresh EGF/bFGF containing medium. Half of the medium was changed every 7 days, and fresh EGF/bFGF was added every 4 days. Neurospheres were plated in Neurobasal medium containing B27 supplement (Gibco) in the absence of bFGF and EGF for 7-10 days before fixation for immunohistochemistry.

Myocyte/ Stem Cell co-culture for electrophysiology: Hindlimb muscle from CD1 mouse pups (postnatal day 1) was harvested as described above. The tissue was dissected free of remaining blood vessels, minced, and incubated in DMEM/F12 medium (1:1) containing DNAse (1mg/ml) and trypsin (1mg/ml) for 30 minutes in a 37°C incubator. After the enzyme incubation, trypsin was inactivated by DMEM/F12 containing 10% fetal calf serum (FCS). The tissue was then triturated using a series of fire polished pasteur pipettes and washed twice in growth medium consisting of DMEM/ F12 with added glutamine (2 mM), Na^+ pyruvate (1 mM), B27 supplement (1:50) (Gibco), and 5% FCS. Approximately 150μl of cell suspension was plated onto the centre of a $35mm^2$ culture dish coated with Poly-D-lysine and laminin. After 24 hours, the dish was flooded with an additional 600μl of growth medium.

After 3 days in culture, myocyte cultures were washed twice in Neurobasal or Hams F-14 medium containing glutamate and forskolin, and 8-15 multipotent stem cell neurospheres were added to the centre of the cultures. Brain-derived neurotrophic factor (BDNF) (3ng/ ml) and ciliary neurotrophic factor (CNTF)

(20ng/ ml) (Regeneron) were added to the cultures for the first 24 hours after which medium containing 2% horse serum, BDNF, CNTF and glial cell line-derived neurotrophic factor (GDNF) (10 ng/ ml)(Amgen) was used to feed cultures. Initial cultures using DMEM/F12 medium resulted in less than optimal cell health and overgrowth of proliferative fibroblasts and astrocytes. This may have been due to high levels of glutamate in the medium. To prevent possible glutamate-induced neurotoxicity we added 1 mM kynurenate to cultures upon addition of neurospheres. Neurobasal medium was used to decrease proliferation of non-neuronal cells, yet Hams F-14 medium was found to be optimal for both myocyte and neuronal growth while not resulting in culture overgrowth as quickly as Neurobasal or DMEM/ F12.

Immunohistochemistry: Cultures were fixed in 4% paraformaldehyde fix and washed 5 times in phosphate buffered saline-triton-X (0.1%) (PBST) PBST or PBS-tween-20 (0.8%) before primary incubation. The following concentrations of primary antibodies used were: Rabbit-anti-ChAT(1:1000), goat-anti-vesicular acetylcholine transporter (1:10000), (both from Chemicon); mouse-anti-microtubule associated protein 2 (MAP2) (1:1000), mouse-anti-pan-axonal (1:1000); rabbit-anti-glial fibrillary acidic protein (GFAP)(1:1000) (all from Sternberger Monoclonals Inc.); mouse-anti-Islet-1 (1:500), rat-anti-nicotinic receptor (1:500) (both from Developmental Studies Hybridoma Bank), rabbit-anti-REG2 (1:1000) (a gift from Dr. Liam Murphy); mouse-anti-α1c subunit (1:1000) (Alomone); mouse-anti-myosin (1:100) (Sigma); rabbit-anti-Glutamate receptor

(GluR) 5,6,7 (1:1000) (Pharmingen); mouse-anti-galactocerbroside (1:100) (Boehringer Mannheim). Primary incubations included 1:100 donkey serum (Sigma). Secondary antibodies were Donkey-anti-mouse-cy3 conjugate (1:250), Donkey-anti-mouse-FITC (1:250) (both from Jackson); donkey-biotinylated-anti-rabbit (1:100) and streptavidin-FITC (1:100)(both from Amersham). Betweer all antibody incubations, all cultures were washed 5 times in PBS or PBS-bovine serum albumin (1%). Final washes were in 50 mM Tris-HCl before coverslipping with a glycerine-based anti-fade medium (Vectashield, Vector Labs). Images were either photographed or digitized using a Neurolucida image analysis system.

Detection of Islet-1 expression by RT-PCR: Total RNA was extracted from differentiated neurosphere/ myocyte co-cultures at one, two and three week growth intervals using Trizol reagent (Life Technologies). Whole neonatal mouse spinal cord and adult mouse ovary RNA was also extracted to serve as positive and negative controls respectively. cDNA was synthesized using 1 μg of total RNA in a 20 μl reaction volume consisting of: First strand buffer (Gibco), 0.1M DTT (Gibco), 10 mM dNTPs, 500 ng/μl pd (N6) random hexamer primers, 20 units RNA guard, bovine serum albumin 2 μg, 1 μg total RNA and 200 units of Maloney murine leukemia virus reverse transcriptase. Reaction volume was incubated for 2 hours at 37°C and subsequently terminated by a 5 minute incubation at 65°C. The primer pair used for Islet-1 PCR was based on Kalyani et al (1998), and similar sequences were modified for the mouse genome. Sense:

5'-GCA GCA TAG GCT TCA GCA AG-3' Antisense: 5'-ATA GCA GGT CCG CAA GGT G-3'. PCR reaction consisted of PCR buffer 10 pmol sense and antisense primers 10 μl of RT reaction product, 50 μM dNTPs, 1.0 unit Taq DNA polymerase in a 100 μl volume. The reaction was run for 35 cycles and a final 7 minute incubation at 72°C at the end ensured complete extension. PCR product was electrophoresed on a 2% agarose, ethidium bromide containing gel, and bands were visualized and photographed under exposure to ultraviolet light.

Functional analysis of Co-cultures: Methods are based on Ternaux and Portalier (1993). Briefly, cultures were locally perfused with a DAD-12 fast perfusion system (Adams and List, Great Neck, NY) consisting of 12 barrels feeding into a common output manifold. Agonists kainate and acetylcholine were applied to a non-spontaneously contracting myocyte to elicit contraction. Images were then captured at a rate of 3.3 Hz on a digital camera over a 30-60 sec interval. Control solution was perfused between agonist application and also as a negative control. Contractions were then expressed graphically as a function of time. After identifying an innervated myocyte by perfusion of kainate, the myocyte was recorded from using whole cell voltage clamp techniques (Hamill et al 1981). Briefly, cultures were visualized on a Nikon inverted microscope and the culture medium was replaced with a HEPES buffered recording solution containing (in mM): HEPES(10), NaCl(150), KCl(5), MgCl2(1), CaCl2(2), Glucose(10), pH 7.4. Electrodes were pulled from borosilicate glass on a Narishige upright electrode puller with a final tip resistance of 4-6 MΩ. Intracellular recording solution

consisted of (in mM): K-gluconate(140), EGTA(11), HEPES(10), $CaCl_2$ (1), KOH (35) MgATP (4), GTP (2), pH 7.4. Recordings were aquired using pCLAMP 6.0 acquisition software and amplified through an Axopatch 1-D amplifier to computer disk. Traces were analyzed using CLAMPFIT 6.0 software.

Sciatic nerve lesion and transplantation: Methods are based on Erb et al (1993). Sixty-one adult male BalbC mice were anaesthetized with ketamine/ xylazine, and the sciatic nerve was exposed and transected. Neurospheres were dissociated and labeled 1-2 days prior to transplantation with the lipophillic fluorescent marker DiO dissolved in DMSO for 1 hour. This method was efficient in labeling precursor cells but was not complete, and there remained some transplanted cells which were unlabeled. After transection of the sciatic nerve, the proximal stump was deflected into the surrounding musculature and sutured in place. The distal nerve stump was injected with approximately 27 μL of a neurosphere suspension previously labeled with DiO and ligated to prevent leakage of the suspension. At time periods between 0 days and 7 months post-transplantation, the mice were anaesthetized and intracardially perfused with cold (4°C) pre-fix solution consisting of 0.9% NaCl, 0.1% sodium nitrite and 1000 units of heparin followed by 4% paraformaldehyde, 0.1% sodium phosphate, and 0.9% saline. The sciatic nerve was removed and post-fixed for 24 hours in fixative solution and then in 10% sucrose. Tissue was cryosectioned at 5 μm thick sections and mounted on gelatin-coated slides for immunohistochemistry as described.

RESULTS

Culture experiments:

Stem cell cultures were generated using methods based on Weiss et al. (Weiss et al 1996) and resulted in the formation of free floating clusters of cells that expressed the intermediate filament marker nestin and could be passaged multiple times while maintained in a proliferative state in culture (Fig 1A).

Precursor clusters were allowed to differentiate in the presence of BDNF (10ng/mL) and CNTF (20 ng/mL) for 7 to 10 days. In later experiments, GDNF(10 ng/ml) was also included in the culture medium to promote neuron survival and growth. Resulting cultures were fixed and initially processed for neuronal and glial markers MAP-2, GFAP and galactocerebroside which showed the existence of neurons, astrocytes and oligodendrocytes respectively with morphologies characteristic of these cell types (data not shown). All differentiated neurospheres displayed MAP-2 labeled neurons at varying frequencies.

To determine if cholinergic neurons differentiate from precursors, differentiated cell cultures were fixed and initially processed immunohistochemically for motoneuronal markers. The first marker examined was the enzyme ChAT which has been a characteristic marker of cholinergic neurons. Immunostaining for ChAT revealed the presence of large multipolar cells (Fig 1B). Most of the remaining neuronal cells were well developed while others retained a more

bipolar morphology characteristic of immature motoneurons (Wentworth 1984). All cholinergic cells were characterized by a large soma diameter (mean 21.6 ± 4.3). Treated cultures were also processed for ChAT and either MAP-2, pan-neuronal or GFAP to label neurons or astrocytes. ChAT and the MAP-2/ pan-neuronal antibodies were co-localized as well as ChAT and galactocerebroside (MacDonald et al 2000). In no instance were GFAP and ChAT antibodies colocalized. $GFAP^+$ cells were flat and displayed a diffusely projecting morphology characteristic of astrocytes (not shown). These results demonstrate cholinergic neuron differentiation from proliferative spinal cord precursor cells. All figures are representative examples of the experiments performed.

We next determined whether more specific motoneuron markers were expressed in differentiated cells. Islet-1 is part of the LIM homeobox family of transcription factors and is the first molecular indicator of motoneuron differentiation (Ericson 1992). Cultures were co-processed for ChAT and Islet-1 and revealed large cholinergic neurons displaying Islet-1 labeled nuclei (Fig 1C1, C2). There was also a population of $Chat^+$/$Islet\text{-}1^-$ cells which possessed smaller soma diameters than the large $ChAT^+$/ $Islet\text{-}1^+$ cells. This $Islet\text{-}1^-$ population may also represent another cholinergic spinal cell group. Another marker of motoneurons is the Schwann cell mitogen REG2 (Livesey et al 1997). REG2 is expressed by motoneurons and retrogradely transported along developing or regenerating motor axons. Cultures were co-processed for Islet-1 and REG2 and revealed large diameter $REG2^+$ cells which co-expressed Islet-1 in the nucleus (Fig 1D1,

D2) respectively. There were fewer REG2$^+$ neurons observed in differentiated cultures than Islet-1$^+$ cells. It is now known that REG2 only labels a subpopulation of motoneurons and thus would account for the lower number of labeled cells (Davis et al 1999). These results provide evidence for the expression of specific motoneuronal markers in cholinergic cells suggesting that some of these cholinergic neurons express the antigenic characteristics of motoneurons

To examine the time course of putative motoneuron differentiation, Islet-1 expression was also examined in neurosphere cultures after one, two and three weeks using RT-PCR technology in neurosphere/ myocyte co-cultures grown in BDNF/CNTF/GDNF and forskolin. Spinal cord and ovary RNA was harvested to serve as positive and negative controls respectively. A single strong 350bp band was present for the spinal cord and there was no band present from the ovary RNA (Figure 2). The 1 week neurosphere culture yielded a strong Islet-1 band which was weak or absent at 2 and 3 weeks. The transient expression of Islet-1 suggests that differentiating motoneurons do not survive long in culture.

Muscle-Neural Precursor Co-culture:

To determine whether functional connections could be made to muscle in support of the histochemical and molecular data, a muscle co-culture preparation was developed. Functional studies are necessary as cultured neurons occasionally express neuronal antigens but not mature electrical properties of motoneurons

that develop after making connections to muscle (Kalb and Hockfield 1994). Multipotent progenitor clusters were co-cultured with dissociated neonatal myocytes. Due to the minimal serum incorporated in the culture medium, initial cultures of myocytes required the addition of forskolin to the culture medium. Myocytes grew robustly and were phase bright with clear surface membranes and showed no striation typical of non-innervated muscle in culture. Forskolin also appeared to improve the morphology of MAP-2/ pan-axonal labeled neurons (Data not shown). Pan-axonal immunostaining revealed that the presence of nerve terminals was also increased in culture with the trophic cocktail of CNTF, BDNF and GDNF over cultures treated only with BDNF and CNTF (Data not shown). Neurons grown in the presence of forskolin possessed longer neurites and more axon terminals than their counterparts in untreated cultures. The addition of GDNF and forskolin to neurosphere cultures treated initially with BDNF and CNTF improved the growth of neurosphere-derived motoneurons.

To determine whether neuromuscular junctions formed in culture, we first looked for immunohistochemical markers of neuromuscular junctions. Immunostaining was performed using antibodies to the nicotinic receptor (NICR), as well as ChAT and the vesicular acetylcholine transporter (VAChT) to label cholinergic neurons. Nicotinic acetylcholine receptors are localized to postsynaptic sites on neuromuscular junctions and are closely apposed to cholinergic terminals from motor axons. This immunostaining resulted in the labeling of cholinergic terminals in close apposition to clusters of nicotinic receptors showing the

existence of neuromuscular junctions (Fig 3A). VAChT positive boutons were also seen in apposition to muscle fibres close to differentiated neurospheres (not shown). This was an infrequent phenomenon occurring usually 0 to 4 times per culture. There appeared to be frequent NICR clustering on myocytes but not always in apposition to a cholinergic terminal. These results support the expression of histochemical markers of neuromuscular junction formation.

To determine if the neuromuscular junctions were capable of causing muscle contraction, a pharmacological approach was employed similar to that used by Ternaux and Portalier (1993). Local perfusion of acetylcholine was used to evoke muscle contraction directly while kainate was applied to evoke contraction through excitation of a presynaptic motoneuron. There has been no report of myocytes expressing glutamate receptors, thus any kainate-evoked contraction would likely be a result of motoneuron innervation. Furthermore, myosin/ glutamate receptor immunohistochemistry was performed to demonstrate the absence of myosin and glutamate receptor co-localization in mature myotubes (data not shown). This shows that ectopic glutamate receptor expression did not occur on myocytes as an artifact of the culture preparation.

For these experiments non-spontaneously contracting myocytes were targeted and perfused with kainate in 1 week old cultures (Fig 3B1). Kainate perfusion was shown to elicit contraction in skeletal myotubes (Fig 3B2) (n=5). Kainate was washed out and contraction ceased. Acetylcholine was subsequently applied and

contraction of the original myocyte as well as neighboring myocytes was observed (Fig 3B2). After identifying an innervated myocyte by kainate perfusion, whole cell recording revealed the presence of end plate potentials capable of eliciting an action potential and contraction in the targeted myocyte (Fig 4). End plate potentials were 1-3 mV in amplitude (Fig 4B) which occasionally recruited an action potential with a long after hyperpolarization (AHP) duration (100 msec) (Figure 4C). The end plate potentials were absent during the AHP and only reappeared after the cell returned to resting membrane potential (arrows in Fig 4C). These results provide histochemical evidence of neuromuscular junctions and physiological evidence of the formation of functional contacts suggesting that precursor-derived motoneurons produce functional neuromuscular junctions in culture.

In vivo transplantation experiments:

To determine if undifferentiated neurospheres could differentiate into motoneurons after transplantation into the sciatic nerve, undifferentiated precursors were transplanted into the distal stump of a transected sciatic nerve at the time of transection. We hypothesized that trophic support in the local environment would increase after lesion and promote differentiation and/or survival of neurosphere-derived motoneurons. There is considerable fluctuation in trophic factor levels in peripheral nerve after transection, with the most relevant example being the release of CNTF into the extracellular space (Sendtner et al

1992). After different survival times, the distal sciatic nerve was harvested and processed immunohistochemically for nestin, ChAT, and the neuronal markers MAP-2/ pan-axonal. DiO labeled cells could be seen at all time points examined.

To demonstrate that the transplant remained in an undifferentiated state up to injection, sciatic nerve sections were stained for the intermediate filament protein nestin found in precursor cells. Immediately after injection (day 0), precursor grafts displayed intense nestin staining in DiO labeled cells (Fig 5A). Nestin expression of DiO labeled cells declined over time and was not detectable by 1 week post-injection. There was high background labeling of nestin in peripheral nerve sections which is consistent with the expression of nestin by Schwann cells (Hockfield and McKay 1985) At 3 days and 12 weeks post transplantation, differentiated cells displayed labeling for MAP-2 and pan-axonal neuronal markers (Fig 5B). These results indicate that nestin is expressed in the transplanted cells at the time of transplantation and then is subsequently downregulated as the cell differentiates and acquires more mature cell characteristics.

To determine whether more specific motoneuronal markers were expressed, immunohistochemistry was used to detect the motoneuronal antigens ChAT and the dihydropyridine receptor (DHP) $\alpha 1_C$ subunit. ChAT protein was found in several DiO labeled cells in the neurosphere graft at three and 12 weeks post transplantation demonstrating the existence of cholinergic neurons (Fig 6A). The

DHP $\alpha 1_C$ subunit makes up part of the L-type calcium channel and is found on motoneurons in the spinal cord (Jiang et al 1999). The α1c subunit was used as a specific motoneuron marker in place of Islet-1 because the Islet-1 antibody yielded tremendous background in sciatic nerve sections making viewing impossible. Antibodies to the α1-c calcium channel subunit were used and positively stained DiO labeled cells in the graft (Figure 6B). The pattern of labeling was very similar to that shown by Jiang et al (1999). These results demonstrate the expression of two markers found in motoneurons suggesting that motoneurons can differentiate in the sciatic nerve.

To see whether axons develop in precursor-derived neurons after transplantation, a pan-axonal antibody cocktail was used to explore the development of axons and terminals. Initially, DiO labeled processes were seen running along muscle sections (Figure 7A). Neurosphere derived-neurons were found to extend axons toward hindlimb musculature at 12 weeks, and cholinergic terminals were seen at 16 weeks post-injection (Figure 7B). An antibody to the vesicular acetylcholine transporter was also used to determine whether any of these axons and terminals were cholinergic. Cholinergic nerve terminals could be seen in apposition to hindlimb musculature in transected nerves injected with precursor cells with none observed in transected uninjected controls. An interesting histological observation was that the uninjected, transected sciatic nerves used as experimental controls were translucent and greatly deteriorated 6 months after transection much like we would expect due to Wallerian

degeneration after transection. Conversely, sciatic nerves injected with precursors had obvious substance and appeared much less deteriorated at the same time points. This is attributed to the axons developing from differentiated neurons. These results demonstrate that transplanted cells differentiate into neurons that send out axons which follow local trophic signals and project towards muscle.

DISCUSSION

The findings of this study demonstrate that motoneurons can differentiate from neural precursors both in culture and after transplantation into a host organism. These findings are significant because they include functional criteria for evaluating differentiated cells as motoneurons, which has not been previously demonstrated in the stem cell literature. We demonstrate that motoneurons can differentiate from precursors in tissue culture as well as after transplantation into the permissive environment of the sciatic nerve, in both cases making functional connections to muscle cells. The difficulty in finding markers specific only to motoneurons prompted us to use antibodies to multiple motoneuronal antigens combined with a functional analysis of neuromuscular connections to demonstrate the existence of motoneurons derived from isolated cultured neural precursors.

Immunohistochemistry of cultured neurospheres

The immunohistochemical studies reported here demonstrated that precursors differentiate into large diameter neurons which express the proteins ChAT, Islet-1 and REG2 in cultures treated with BDNF, CNTF and GDNF. Currently the yield of cells expressing these markers is very low which is consistent with previous work which has reported the existence of large diameter $ChAT^+$ or p75 positive cells from cultured neuroepithelial precursors also at low frequency (Ray and Gage 1994), (Kalyani et al 1997). In light of the report that a population of partition cells are cholinergic and also possess p75 immunoreactivity (Michael et al 1987), it becomes necessary to use additional markers to identify motoneurons. Surprisingly, we have shown that oligodendrocytes derived from neurospheres can also express ChAT (MacDonald et al 2000, and oligodendrocytes have also been shown to express the p75 receptor as well (Cassacia-Bonnefil et al 1996) demonstrating that neither of these markers can be viewed as exclusive to motoneurons. This study thus identified differentiating motoneurons using the specific motoneuronal markers, REG2 and Islet-1. REG2 is expressed in developing motor neurons and is responsive to members of the leukemia inhibitory factor family of cytokines in which CNTF belongs, and therefore we would expect it to be expressed in cultures supported with CNTF (Livesey et al 1997). It has been reported that Islet-1 is the first molecular marker of motoneuron differentiation and is expressed by all motoneurons before segregation into motoneuron subgroups (Ericson et al 1992). We believe that the

co-localization of Islet-1 with both REG2 and ChAT strongly supports the existence of motoneurons in these cultures.

Both Islet-1 and REG2 are also expressed in the dorsal root ganglia (DRG) which may complicate ascribing a motoneuron identity. However, DRG neurons possess a remarkably different morphology and are much more spherical and unipolar or bipolar in shape. ChAT has not traditionally been reported in the DRG (Barber et al 1984; Schoenen et al 1989) but there is a recent report demonstrating ChAT-like immunoreactivity in the DRG suggesting that ChAT may exist where it was previously absent (Sann et al 1995). The neurons in this study still do not demonstrate a morphology consistent with DRG neurons suggesting that they are unlikely cholinergic DRG neurons.

Functional study and efficiency of the co-culture preparation

The co-culture preparation used in this study was based on previously published techniques on muscle cell culture (Ternaux and Portalier 1993). The addition of forskolin to the co-cultures was observed to have a great effect on promoting myocyte health and morphology. This is consistent with the known actions of forskolin increasing the intracellular concentration of cAMP and promoting cell survival. Forskolin has also been previously reported to promote survival of cultured motoneurons and in combination with other trophic factors can promote motoneuron survival for up to three weeks in culture (Hanson et al 1998). We have previously shown that the combination of BDNF and CNTF promotes the

growth of cholinergic oligodendrocytes (MacDonald et al 2000). The addition of both forskolin and GDNF in co-culture with skeletal myocytes appears to promote the growth and survival of motoneurons.

We were unable to record from the innervating motoneuron with intracellular recording methods due to the complex nature of the co-culture preparation. It was not possible to identify a motoneuron and innervated myocyte because of the cell density and debris of the culture. Neuronal processes could be followed for short distances from the soma but the connections to muscle could not be visualized prior to fixation and immunostaining. The drug perfusion technique is an efficient way of observing myocyte contraction visually, and to find cells that are innervated. It was difficult to locate kainate-responsive myocytes in this study because of inherent spontaneously contracting myocytes.

When combined with immunohistochemistry for VAChT and the nicotinic acetylcholine receptor, the drug perfusion data supported the existence of functional neuromuscular junctions in culture. The myocyte whole cell recording data demonstrated convincingly that neuromuscular junctions are able to form and give end plate potentials to cultured myocytes. Action potentials and contraction in cultured myocytes were recorded in response to kainate supporting muscle activation indirectly by activation of motoneurons. This finding is supported by previous work demonstrating the ability of motoneurons to form neuromuscular junctions in culture (Michikawa et al 1991). These results were

likely the most significant findings of this study as they demonstrated that myocytes could be functionally innervated by precursor-derived neurons after differentiation.

Immunohistochemistry in transplanted sciatic nerve

Motoneuron differentiation in the adult sciatic nerve was a striking outcome of these experiments. This result suggests that the sciatic nerve environment contains factors which can direct motoneuron development. It is curious that the precursors could differentiate into motoneurons in a region of the host organism which normally does not express differentiation cues. There appeared to be motoneurons differentiating despite the absence of any classical motoneuron differentiation factor such as sonic hedgehog or bone morphogenic protein-2. This would suggest that a default program exists allowing some motoneuron differentiation which subsequently relies on local trophic support or that they derive the appropriate support from the neurospheres themselves. Thus it was suggested that the differentiating motoneurons would have a high trophic requirement in order to survive after differentiation. On the assumption that motoneuron progenitors existed in the transplant and would differentiate regardless of local differentiation cues, we decided to transplant the cells into an environment rich in support factors for motoneurons after they differentiated. The peripheral nerve environment seemed to be a logical location since the trophic support of the Schwann cells and terminal myocytes functions to maintain motoneuron axon health and motoneuron survival in the normal adult (Sendtner

et al 1992). The peripheral nerve milieu is also much more permissive to axon growth in comparison to the hostile growth environment of the central nervous system where most of the transplantation work to date has occurred.

Motoneuron survival has been previously reported after transplantation into the transected sciatic nerve and it has been accompanied by successful reinnervation of the hindlimb musculature (Erb et al 1993, Katsuki et al 1997). These groups used immunohistochemical, dye tracing and electrophysiological methods to demonstrate restoration of neuromuscular junctions. These studies encouraged us to use this approach in our own model. Immunohistochemistry of the sciatic nerve sections in our sciatic nerves injected with neural precursors demonstrated the presence of neurons, cholinergic cells, and more specifically motoneurons using antibodies to MAP-2, ChAT, VAChT and the dihydropyridine receptor subunit α1c respectively. This was coincident with the decrease in expression of the precursor cell marker nestin in DiO labeled cells, indicating that differentiation of precursors into more mature neuronal phenotypes had occurred. This is supported by the report that nestin is rapidly downregulated in CNS progenitors as they differentiate (Dahlstrand et al 1995).

In order to determine whether motoneurons differentiated from neural precursors can functionally innervate muscle in vivo, it is necessary to examine the transplanted sciatic nerve using electrophysiology. Preliminary electrophysiological analysis performed on transplanted sciatic nerves 31 weeks

post transplantation indicated positive EMG responses with nerve stimulation. Stimulus-contraction coupling of ankle muscles was also visually observed in response to the stimuli. Subsequent stimulation of lesioned uninjected sciatic nerves resulted in no coordinated ankle movement (personal observations). This preliminary observation supports the existence of functional neuromuscular junction formation in transplanted sciatic nerves and encourages further study. Although an important starting point, the immunohistochemical identification of motoneuronal antigens is not sufficient to identify cells as motoneurons. As we report here, the cells must also grow axons towards muscle, form cholinergic terminals and functional connections with muscle in order to be classified as motoneurons.

These results convincingly demonstrate the differentiation of functional spinal motoneurons from neuroepithelial precursors in culture and also upon transplantation into the adult sciatic nerve which normally supports survival and maintenance of motor axons. We conclude that functional motoneurons are able to differentiate from proliferative neuroepithelial precursors and innervate muscle. While these results demonstrate that it is possible to obtain functional motoneurons from neurospheres, the growth conditions need to be optimized before any further characterization. Approaches are currently being explored to increase the yield of motoneurons differentiating in culture, and after transplantation for further characterization. This work is relevant to conditions such as amyotrophic lateral sclerosis and spinal muscular atrophy where selective motoneuron loss occurs.

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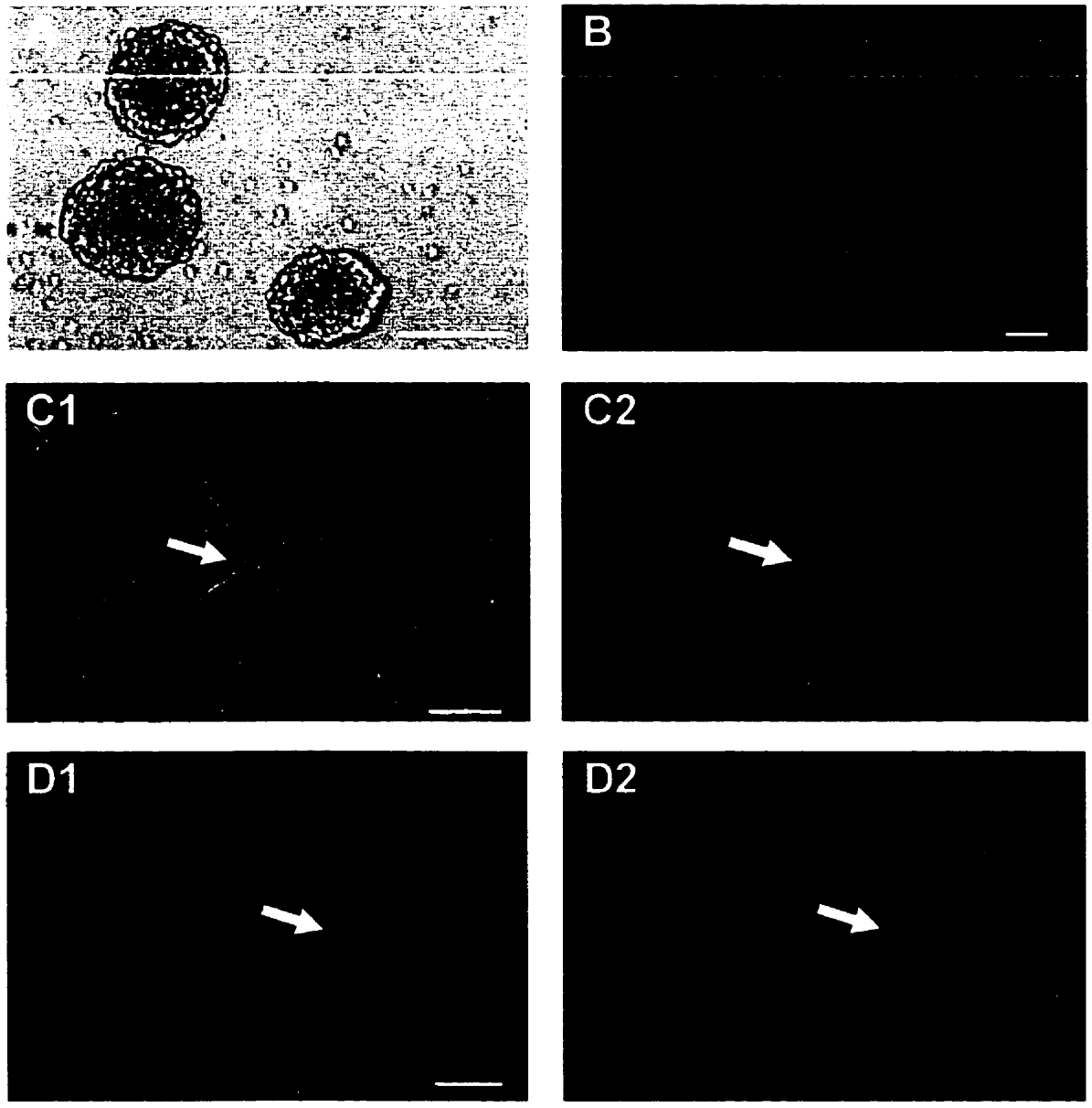

B
C1
C2
D1
D2

Figure 1: Immunohistochemical detection of motoneuron antigens in culture. Phase contrast micrograph of proliferative neural precursor clusters grown in tissue culture (A). After plating on a permissive substrate in the absence of mitogens, some precursors differentiate into large diameter ChAT+ neurons (B, C1). Immunohistochemistry was used to identify more specific markers of motoneuron differentiation and growth. A subpopulation of ChAT+ neurons expresses the LIM homeobox transcription factor, Islet-1 (C2). An example of a Reg-2 immunoreactive neuron (D1) which also expresses Islet-1 (D2) is also shown. Scale bars: (A) 100 μm ;(B) 15 μm; (C) and (D) 50 μm.

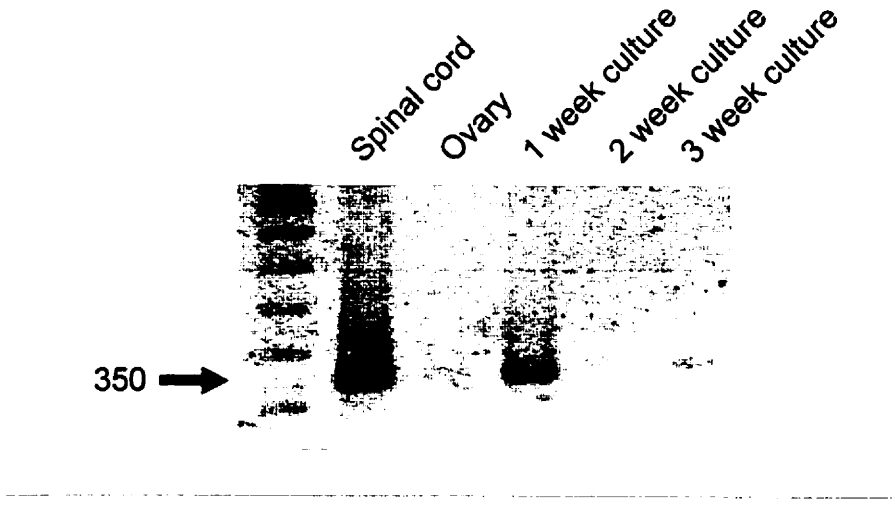

Spinal cord
Ovary
1 week culture
2 week culture
3 week culture
350

Figure 2: Islet-1 is expressed in differentiated neuroepithelial cultures. Islet-1 RT-PCR product is electrophoresed on an agarose gel and visualized under ultraviolet illumination. First lane corresponds to 100bp marker. Second lane displays a strong 350bp band derived from spinal cord RNA. RNA harvested from a 1 week old culture displays a similar band. A faint band is visible from a 3 week old culture. No band was visible in Ovary RNA or 2 week old culture RNA.

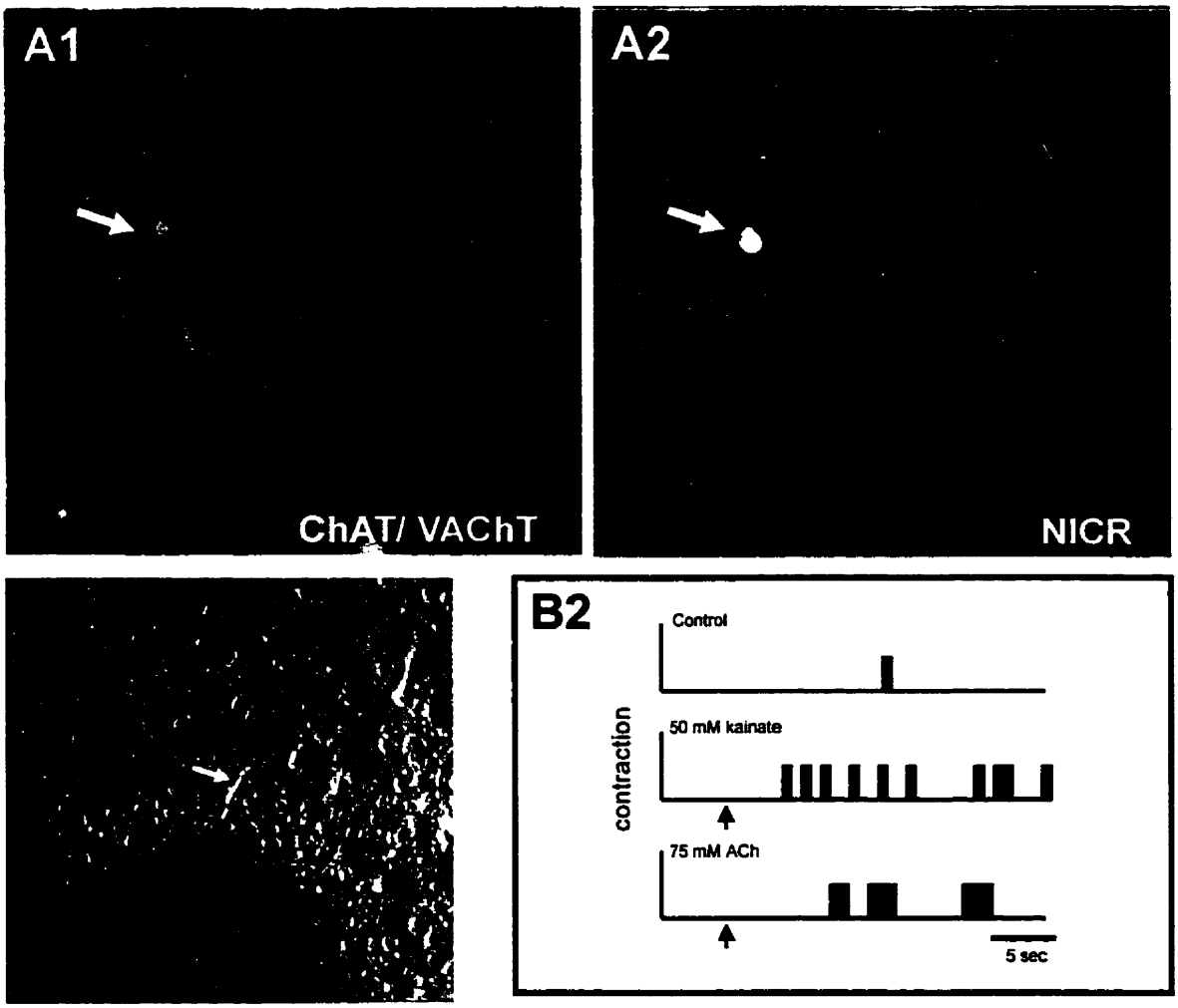
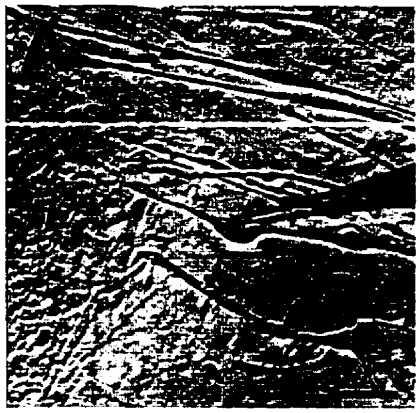
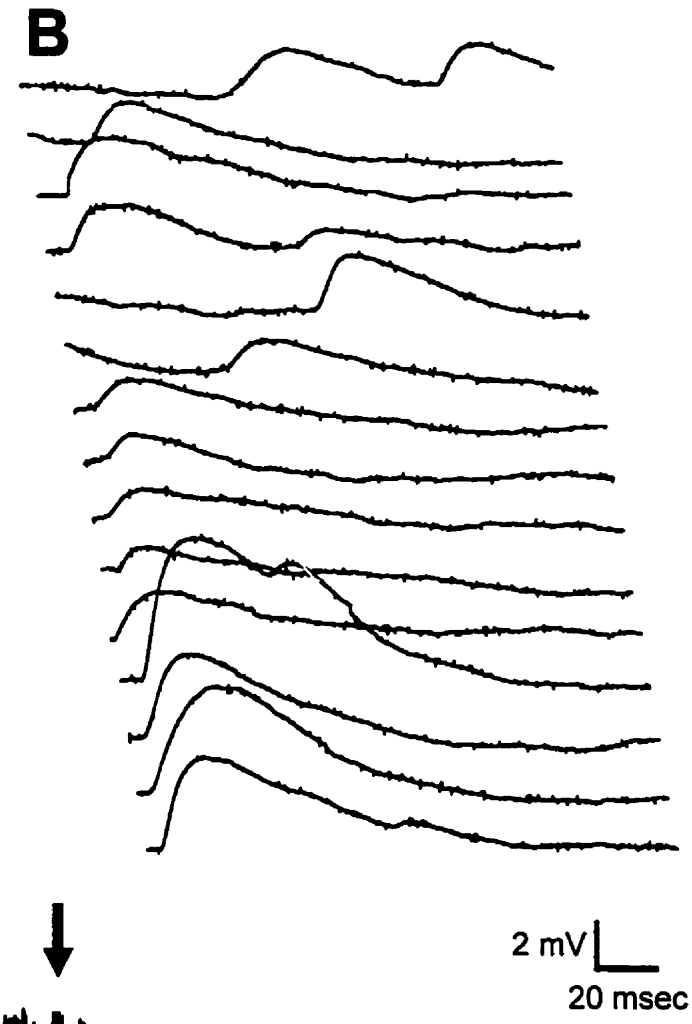

A1
ChAT/ VAChT
A2
NICR
B2
Control
50 mM kainate
contraction
75 mM ACh
5 sec

Figure 3: Neuromuscular junctions develop in a precursor/ myocyte co-culture. An example of a cholinergic axon and terminal (A1) in apposition with a cluster of nicotinic acetylcholine receptors (A2) as demonstrated using ChAT/ VAChT and Nicotonic receptor immunohistochemistry. Phase contrast micrograph of a precursor cluster/ myocyte co-culture depicts a myocyte in close apposition to a differentiating cluster (white arrow, B1). A contraction profile (B2) shows that with perfusion of kainate or ACh (black arrows), the myocyte contracted. Kainate application caused contraction only in the innervated myocyte while ACh perfusion caused contraction in all neighbouring myocytes as well.




B
2 mV
20 msec

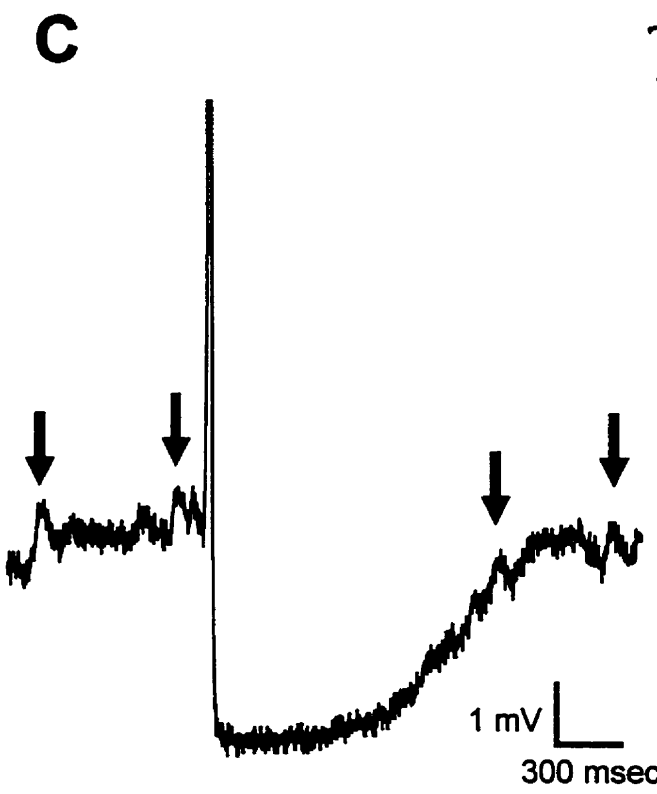

C
1 mV
300 msec

Figure 4: Electrophysiological responsiveness of a skeletal myocyte in co-culture. Phase contrast micrograph of a precursor/ myocyte co-culture (A). The differentiating neurosphere is seen at the left and marked by the letter n. Long healthy myocytes are seen running along the substrate and the recording electrode is seen on the targeted myocyte. This particular cell was seen to contract in response to 50 μM kainate and ceased to contract upon washout of the agonist. End plate potentials are seen in B in response to low concentration of kainate in the recording medium. There were no spontaneous end plate potentials seen in the absence of kainate. End plate potentials are also demonstrated before and after an action potential in the myocyte (black arrows, C).

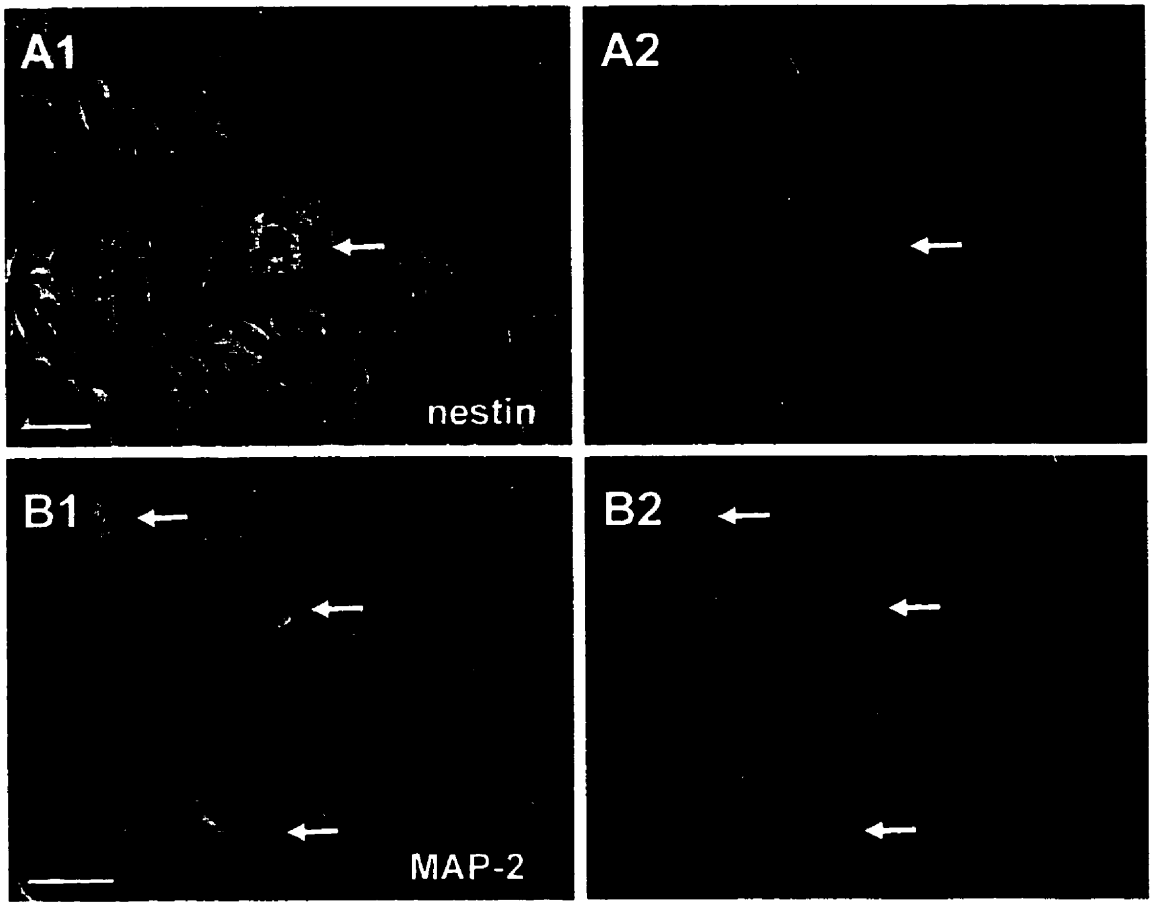

A1
nestin
A2
B1
MAP-2
B2

Figure 5: Expression of Nestin and MAP-2 in transplanted sciatic nerves. Sections of adult mouse sciatic nerve after transplantation of neural precursors. Neural precursors were traced using DiO during the transplantation procedure (A2, B2). One day after transplantation of precursors, intense nestin immunoreactivity is seen in cells at the graft site (white arrows, A1). As early as 3 days post transplantation, MAP-2^{+} neurons are seen at the graft site (white arrows, B1). Scale bars (A) 15 μm; (B) 25 μm

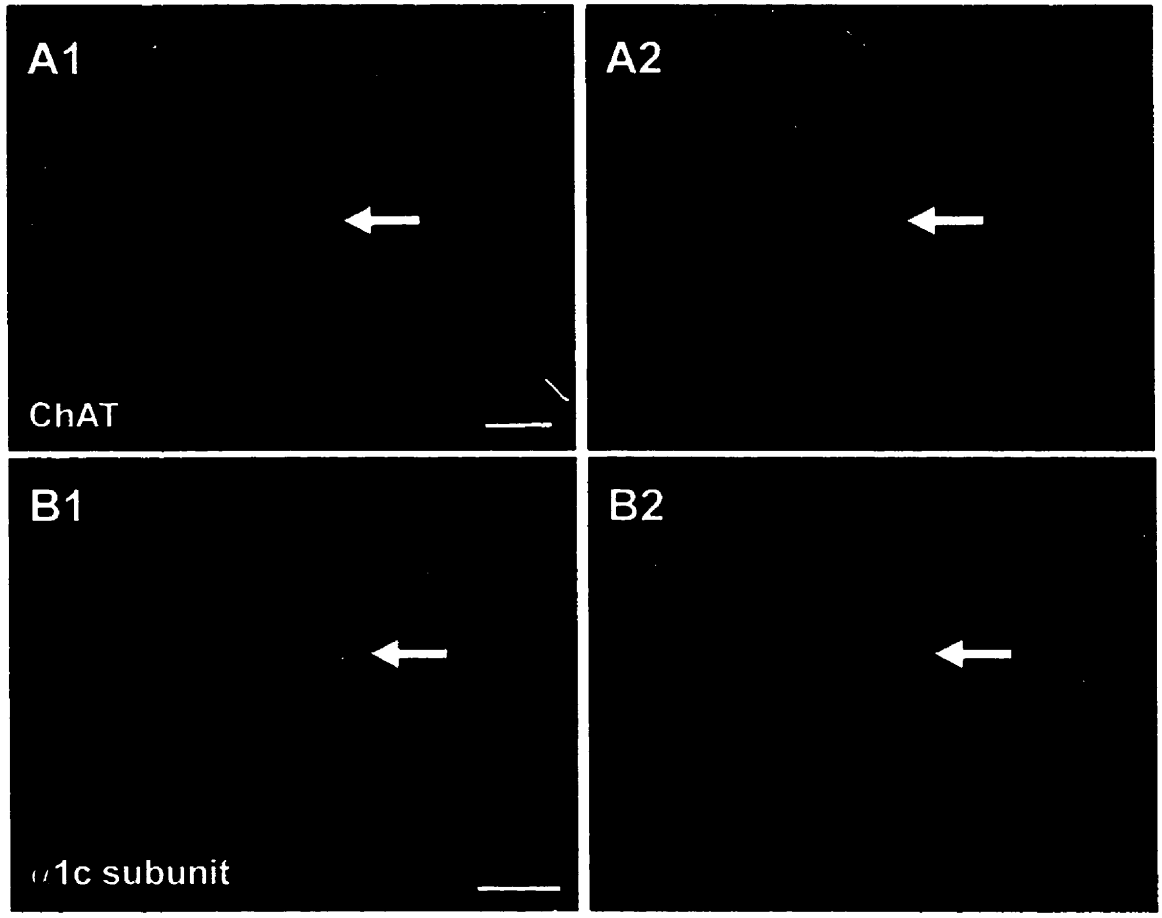

A1
A2
ChAT
B1
B2
α1c subunit

Figure 6: Motoneuron markers are expressed in transplanted sciatic nerve. Sections of adult mouse sciatic nerve after cell transplantation. DiO labelling is shown in (A2, B2). Expression of ChAT (A1) at 12 weeks and the $\alpha 1_C$ subunit of the dihydropyridine receptor (B1) at 4 weeks give evidence that motoneurons are differentiating in the cell-transplanted sciatic nerve. Scale bars (A) 20 μm (B) 10 μm

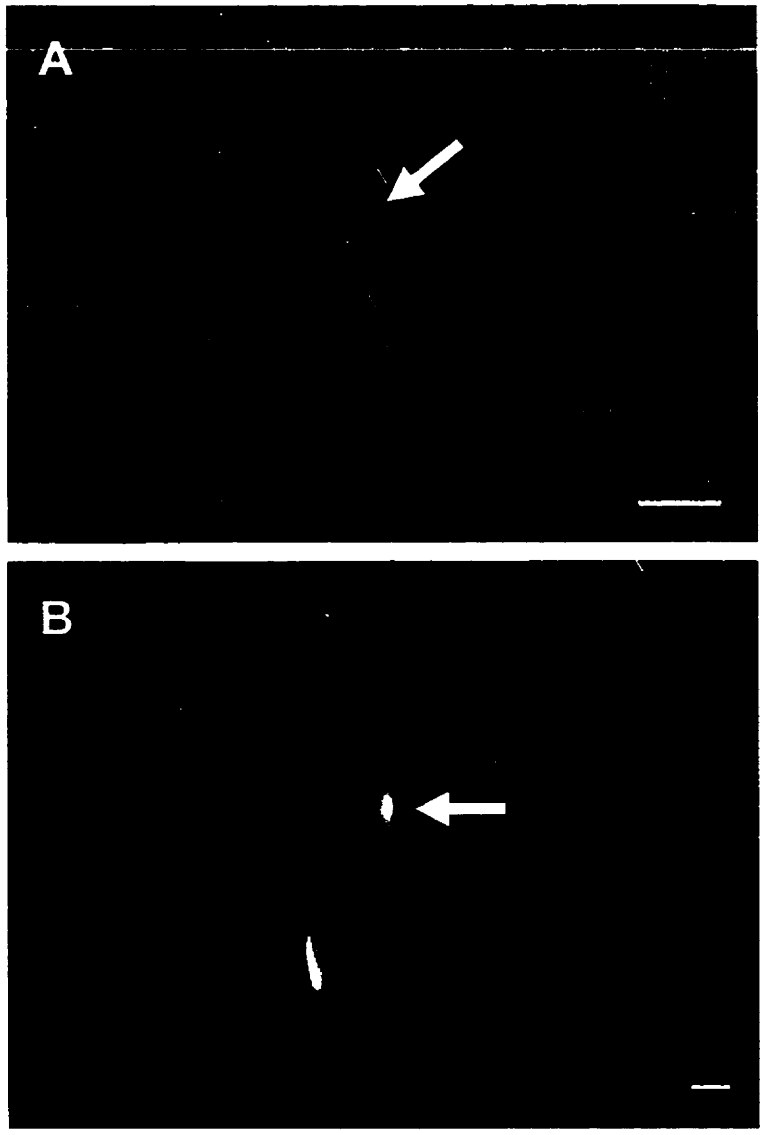

A
B

Figure 7. Precursor cells send axons to distal muscles. At 12 weeks after transplantation, DiO labeled axon in A (arrow) runs along musculature attached to distal end of sciatic nerve section. Cholinergic terminals labeled with VAChT antibodies are seen on muscle tissue at distal end of sciatic nerve section at 16 weeks post-transplantation. Scale bars (A) 25 μm (B) 5 μm

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Summary of Results

Paper 1

- Neurospheres treated with CNTF and BDNF differentiate into oligodendrocytes expressing a cholinergic phenotype.
- Treatment of neurospheres with the muscarinic ACh receptor antagonist atropine yields reduced oligodendrocyte differentiation.
- Treatment of neurospheres with CNTF yields the most oligodendrocyte differentiation and promotes the most extensive process outgrowth.

Paper 2

Tissue Culture:

- Neurospheres treated with CNTF, BDNF, GDNF and forskolin differentiate in culture into neurons that express ChAT, Islet-1, and REG2.
- Differentiated neurons extend cholinergic processes to co-culture myocytes and possess cholinergic terminals in apposition to nicotinic acetylcholine receptor clusters.
- Kainate application to neurosphere-muscle co-cultures elicits end plate potentials, action potentials and muscle contraction.

Transplantation:

- Neurospheres transplanted into the transected mouse sciatic nerve differentiate into neurons expressing ChAT and the α1c calcium channel subunit.
- Neurosphere-derived neurons extend processes towards distal musculature and form cholinergic terminals.

Conclusions

Paper 1

The results suggest that oligodendrocytes differentiate from neurosphere precursors and display a cholinergic phenotype. Optimal oligodendrocyte differentiation and growth occurred with CNTF treatment. Treatment with the muscarinic receptor antagonist atropine, decreased oligodendrocyte differentiation. This evidence supports the hypothesis that some oligodendrocytes are cholinergic and that ACh plays a role in oligodendrocyte differentiation likely through an autocrine/ paracrine mechanism.

Paper 2

The results suggest that neurons differentiate from proliferative neurosphere precursors that display the antigenic properties of motoneurons. Co-culture with skeletal myocytes resulted in the antigenic and functional development of neuromuscular junctions. Transplantation of neurospheres into the lesioned sciatic nerve resulted in the differentiation of neurons expressing motoneuron markers that projected to hindlimb muscles and formed cholinergic terminals. This evidence supports the hypothesis that functional motoneurons can differentiate from neurosphere precursors in tissue culture and after transplantation into the host sciatic nerve.

Discussion

These studies demonstrate the differentiation of two cholinergic cell types from proliferative neuroepithelial precursors isolated from the mouse spinal cord. The first paper furthers the field of oligodendrocyte research by demonstrating the expression of a traditional neurotransmitter synthesis enzyme (ChAT) in oligodendrocytes. This study also demonstrates the trophic response of this population, as well as a mechanism of action during oligodendrocyte differentiation. Cholinergic oligodendrocytes flourish in the culture conditions used in this study yet a small number of cholinergic neurons are still observed. The second study examines the possibility that this small fraction of cholinergic neurons represents a population of motoneurons. This study furthers the field of precursor-derived motoneurons by demonstrating that motoneurons can differentiate from neurospheres and attain functional characteristics such as forming connections to muscle. The culture conditions of the second study differ from the first in that they include exogenous trophic factor support and co-culture with muscle for tissue-derived trophic support to support the growth of motoneurons.

Paper 1

One of the main findings of the oligodendrocyte manuscript was the demonstration of a classical neurotransmitter synthesis enzyme expressed in oligodendrocytes. It does not appear that ChAT is the only synthesis enzyme

expressed in oligodendrocytes. As this study was being developed it became evident that other enzymes such as dopamine-β-hydroxylase and tyrosine hydroxylase were also localized in neurosphere-derived oligodendrocytes (C. Svendsen; A. Gloster personal communications) The neurosphere preparation is an ideal preparation for studying neural cell development in a very easily manipulated environment. In light of the fact the these enzymes are not expressed in mature oligodendrocytes in vivo, their existence during differentiation of the neurospheres suggests that the expression is developmentally regulated. To give insight into the developmental nature of this phenomenon, it would be prudent to explore the expression of these enzymes in oligodendrocyte precursors from E14 and postnally through the myelination process into the adult. In conjunction with specific markers for oligodendrocyte precursors at different stages it would also be possible to see if ChAT is expressed in the O-2A progenitor, for example, co-localized with A2B5 or PDGFRα, or else O4 expressing cells. We know from this work that O1(GalC)$^+$ cells express ChAT and we assume that O10$^+$ cells do not, as they are fully mature oligodendrocytes. Other histochemical techniques such as in situ hybridization, or in situ RT-PCR may again be useful in localizing the mRNAs encoding different neurotransmitter synthesis enzymes for increased specificity.

In view of the preponderance of evidence in the literature regarding the responsiveness of oligodendrocytes to ACh as well as the existence of non-neuronal ACh, it is almost predictable that oligodendrocytes would in fact synthesize ACh. There have even been reports in the squid giant axon

preparation of ACh release from Schwann cells acting in an autocrine manner (Lieberman and Sanzenbacher 1992). Schwann cells from the squid giant axon preparation are thought to receive glutamate excitation and release ACh for an autocrine response (Lieberman and Sanzenbacher 1992). Recently oligodendrocyte progenitors were reported to receive glutamatergic stimulation in the rat hippocampus (Bergles et al 2000). This finding may indicate that a similar mechanism exists in the mammal. It may be that the autocrine response would result in activity-dependent development or possibly the release of some growth factor.

It has been reported that oligodendrocytes myelinate specific sizes of axons (Fanarraga 1998). It is possible that a transmitter phenotype specifically exists or develops during, or just prior to myelination. For example, an oligodendrocyte contacts a small sized cholinergic axon and besides myelinating other small sized axons, also seeks out cholinergic small sized axons. It has been suggested that neurospheres in culture differentiate down the lineage pathway of the CNS region they are isolated from. This would suggest that spinal cord neurospheres differentiate into spinal cord oligodendrocytes that would express ChAT to myelinate the wide range of cholinergic spinal axons. Further study of different mechanisms may shed light on the physiological action of ACh on oligodendrocyte development.

Another plausible extension of this work would be an exploration of the mechanism of ACh release from oligodendrocytes. Other groups have demonstrated that non-quantal release of ACh may be one mechanism (Dennis

and Miledi 1974). There also appears to be conflicting evidence regarding the effects of ACh on oligodendrocyte membrane potential. One group has reported that cholinergic receptor stimulation has no effect on membrane potential (Gilbert et al 1984), yet it has also been reported that ACh receptor stimulation inhibits an inwardly rectifying potassium conductance (Karschin et al 1994). It may be useful to study cholinergic oligodendrocytes and see if they respond to ACh stimulation while non-cholinergic oligodendrocytes do not respond.

To confirm that the oligodendrocyte ChAT is functional, it may also be useful to perform a ChAT assay and measure the synthesis of ChAT in a purified oligodendrocyte culture. While not really necessary, this would confirm that the ChAT seen with immunohistochemistry is indeed synthesizing active ACh. To see if oligodendrocytes are in fact releasing ACh it might also be useful to employ the patch clamp recording technique called "sniffing", which entails a membrane patch on an electrode tip that is brought close to a given cell. The cell is stimulated and neurotransmitter release from the cell is measured via the membrane "microphone" at the tip of the electrode. The sniffing technique would also circumvent the need for a ChAT assay.

Paper 2

This study represents the most comprehensive study to date characterizing spinal motoneurons derived from multipotent spinal cord precursor cells. It also includes functional characterization of neurosphere-derived motoneurons. While the evidence presented here makes a strong case for motoneuron differentiation from neural precursors, there are other approaches

that also may be pursued to further support these findings. Further functional characterization will give even more evidence of motoneuron differentiation and also of functional application. It has been reported that neuroepithelial precursors differentiate into neurons with immature electrical properties (Liu et al 1999). This is not suprising in a relatively hostile culture environment but presents an even stronger impetus to show that mature functional innervation is possible in order to illustrate the clinical utility of stem cells. The perfusion analysis of the co-culture preparation is effective for eliciting the muscle contraction with kainate but recording the response by video capture did not permit for an ideal representation of the response.

One method to perhaps give a more descriptive illustration would be to perform calcium imaging during perfusion of kainate and acetylcholine. Preliminary experiments studying Fluo-3 activation in the co-culture preparation have revealed much the same results as the perfusion experiments with video capture. Kainate application resulted in a profound increase in intracellular calcium in a targeted myocyte as well as activation of the neighbouring neurosphere (personal observation). This result also demonstrates that other laboratory approaches can be applied to further demonstrate neuromuscular junctions. It would be useful to use a curarizing agent to block the kainate-evoked Ca^{2+} increase as an experimental control and thus give definitive experimental proof of neuromuscular differentiation. It would also be prudent to further examine functional recovery of the transected mouse sciatic nerve.

The histological evidence supports the reinnervation of host muscle by neurosphere-derived motoneurons, and preliminary experiments of nerve stimulation evokes ankle movement, further supporting this hypothesis. A definitive experiment to demonstrate functional reinnervation would be to stimulate the transplanted nerve, record from the distal muscle and then apply a curarizing agent to block the response.

We call neurosphere stem cells "multipotent", but to date no comprehensive catalogue of neuronal subtypes has been created. By definition, a multipotent CNS stem cell should be able to generate ALL neuronal subtypes. Presently, there has been some reports of co-localization of neuronal markers such as MAP-2, Tujl, or NSE with neurotransmitter synthesis enzymes. Different neuronal phenotypes have been demonstrated, and now this study offers another phenotype to be incorporated into that catalogue. These results therefore support the multipotent nature of the neurospheres and demonstrate that another defined cell type can differentiate.

The Islet-1 antibody used in this study proved difficult to work with. The labeling with Islet-1 was highly dependent on fixation procedure and extent of permeablization. Even then, Islet-1 labeling resulted in labeled nuclei with varying degrees of intensity. This finding was consistent with the results of Vult von Steyern (1999) who also report different degrees of Islet-1 labeling. The Islet-1 antibody also tended to yield very high background labeling of whole, undifferentiated precursor cells, rendering any attempt at Islet-1 quantification impossible. The undifferentiated cells were about the size of an Islet-1^{+} nucleus

or smaller. Thus using conservative parameters, any Islet-1 labeling had to be nuclear and culture background had to be quite low before the labeling could be considered authentic. For this reason RT-PCR was used to definitively demonstrate Islet-1 expression in differentiated cultures. The development of novel molecular approaches such as in situ RT-PCR are now becoming more advanced and yield reliable results (Gey et al 1999). Islet-1 primers have proven to be specific (Kalyani et al 1998; MacDonald et al 2000), and the in situ RT-PCR approach may yield more uniformly visible Islet-1 labeling.

It is known that islet-1 is also expressed in dorsal root ganglion (DRG) neurons (Thor et al 1991). It could therefore be suggested that the islet-1 expressing cells are dorsal root ganglion neurons and not motoneurons. DRG neurons are mostly unipolar or bipolar in shape and display a morphology very different than those described in this study. Even neurons derived from DRG precursors appear rounder and possess one or two very fine processes (Namaka et al 2000). Cholinergic expression in the DRG has not been reported consistent with the notion that our islet-1/ChAT$^+$ cells are not DRG neurons (Barber et al 1984). In order to demonstrate that these cells are not DRG neurons, it may be worthwhile to perform double label immunohistochemistry for Islet-1 and a protein exclusive to the DRG such as PKCε.

In the field of stem cell biology, a common concern that arises is the possibility that primary neurons and not neurons derived from the precursor cells are being cultured. It is highly unlikely that the motoneurons seen in this study are primary motoneurons for a number of reasons. Firstly, the neurospheres are

passaged several times and each time dissociated and replated. Any differentiated motoneurons require substrate adherence for survival and would display a propensity to attach to the floor of the culture flask. Secondly, after dissociation, the cell suspension results in cells 2-5 μm in diameter. This is a very large difference from the large soma size seen in the motoneurons in differentiated cell culture. Thirdly, it has been demonstrated that the trophic requirements of differentiated motoneurons are extremely demanding (Hanson et al 1998). For the period of months that the neurospheres are kept in a proliferative phase in bFGF and EGF, the lack of any neurotrophins, CNTF or GDNF would surely result in the death of primary motoneurons. The very fragile nature of primary motoneurons in culture also would not permit the cryopreservation procedure of our neurospheres which exposes the precursors to a relatively harsh freeze/thaw cycle. For these reasons, it is unlikely that the motoneurons in this study are residual motoneurons from primary culture.

With the increasing evidence that motoneurons can be derived from neurospheres, it would be beneficial to direct efforts into increasing the motoneuron yield, or characterizing mechanisms of differentiation in culture and in the sciatic nerve. Current work being done is taking advantage of the transient extracellular CNTF increase after sciatic transection. This work aims to determine whether CNTF is the primary differentiation factor in the sciatic nerve or whether it simply promotes survival of motoneurons on a default differentiation pathway.

Attempts to increase yield can involve using novel growth factors during either the proliferation or the differentiation phase. Adding factors such as CNTF,

thyroid hormone or retinoic acid during the proliferation phase was reported to increase the commitment of a progenitor cell to a specific lineage (Johe et al. 1996). This culture preparation offers a simple model to study new motoneuron differentiation factors as they are discovered. For example, the discovery of the bone morphogenetic proteins may offer new insight into the differentiation of neuroepithelial precursors. The recent finding of the differentiating effects of NT-3 coapplied with Shh suggests that co-factors may be required to increase motoneuron differentiation (Dutton et al 1999). As further aspects of motoneuron differentiation are revealed, this preparation will increase in usefulness for the study of motoneuron development and therapeutic use in cell replacement strategies.

Limitations of the co-culture preparation

The co-culture preparation used in the motoneuron study was effective in demonstrating functional neuromuscular junctions but was not ideal for further characterization. There were few functional neuromuscular junction development that resulted in a narrow window for characterization. As demonstrated by the Islet-1 RT-PCR, there is substantial loss of Islet-1 expression and presumably motoneurons after 1 week in culture. The preparation also had to be optimized such that the myocytes were in culture for enough time to mature and display contractile properties while the neurospheres could not be maintained in co-culture for times over one week because of degeneration of the motoneurons. It was not possible to use established co-culture techniques of spinal cord tissue

and myocytes because the spinal cord tissue used in the literature had already differentiated. The preparation had to be hardy enough to support the survival and growth requirements of the myocytes while at the same time promoting the differentiation of neurospheres. Initial use of Neurobasal and DMEM/ F12 culture media were conducive to neurosphere-differentiation or myocyte growth respectively but not both together. The use of Ham's F-14 media appeared to circumvent this problem and promote the health of myocytes and differentiation of neurospheres. The addition of forskolin also dramatically increased the health and substance of myocytes in co-culture.

The spontaneous contraction of isolated myocytes also presented problems and confusion during the functional analysis of the cells. Contractions of myocytes in response to kainate had to be reproduced to verify the responsiveness. In cases where a positive result was not reproducible in a subsequent attempt, the cell was excluded. The nature of the drug perfusion experiments was also such that only one myocyte could be targeted for each culture dish. After capturing the computer video file, other cells of the culture dish were assumed to be already over excited by kainate application to the dish.

Also due to the wide array of cells in the co-culture and overwhelming numbers of proliferative astrocytes and fibroblasts, it was difficult to visualize individual cells and follow axons to neighbouring myocytes during live viewing. In every instance tissue fixation and processing was required before cells could be visualized. The extent of cell projections and interactions with muscle was always determined retrospectively. Another point is that neuronal axons could also easily

extend for several fields of view in length making it difficult to stimulate a motoneuron soma and record visually or electrophysiologically from a nearby myocyte. Under high power objective it appeared that boutons could be seen on some myocytes in live culture, but these units could also have been simply cellular debris adhering to the cell membrane. The immunohistochemical processing later revealed cholinergic terminals on myocytes.

Common Themes of Neurosphere Precursors

Neurosphere precursors offer an easily isolated cell preparation for the study of CNS cell differentiation and limitless cell supply for transplantation studies, but this preparation is not without limitations. The primary limitation of neurospheres is the heterogeneity of the cell population. The asymmetric division of stem cells and progenitors produces clones which are each unique and yield highly variable results. This preparation is suited more towards questions possessing a yes/no answer rather than questions with highly quantitative results. This will be discussed in more detail later. Another confounding factor is that neurospheres rarely undergo complete differentiation. After neurospheres are cultured for a period of days to weeks, there always exist masses of small cells without processes at the site of sphere attachment. There is always spreading of the sphere but also a large number of undifferentiated cells. This is

consistent with Vult von Steyern et al (1999) and Johe et al (1997), who also report incomplete differentiation.

The process of passaging neurospheres is another method used frequently in stem cell biology which displays limitations to its usefulness. Cell passaging is one method that is used to perform clonal analysis and demonstrate stem cell characteristics. In this approach a single clone is dissociated and plated into 96-well plates with a density of one cell per well. Neurosphere precursor cells grow optimally in contact with other cells as demonstrated by their nature to form "spheres". In isolating cells from the group, local and contact-derived trophic signals are absent and frequently the cells die. Dissociating and replating cells in a group cell suspension results in some reclumping of the precursors (also seen by C. Svendsen, personal communication). This is problematic when attempting to identify a single neurosphere as being derived from a single initial stem cell. These limitations are important to the study of stem cell biology and clonal analysis of neurosphere precursors. Taken into the appropriate context, these limitations can be addressed while undertaking differentiation studies.

The Problem of Quantification

The question of quantifying yield of motoneurons presents a precarious situation. It was not reasonable to calculate percent yields of Islet-1 or even ChAT labeled cells in differentiated cultures. For a number of reasons, many precursors remained undifferentiated, neurons and glial cells yield finite numbers of non-mitotic cells, while differentiated astrocytes continued to proliferate the

longer the neurospheres were left in culture. This observation makes quantification (already a very time consuming task) also a very unreliable means of expressing cell counts. Quantification studies have been performed in the past where total cell number has been counted as well as specific cell types (Johe et al 1996). These authors reported the effects of specific growth factors on driving differentiation of an entire cell clone into neurons or glial cells. These studies can be useful in demonstrating a general trend in a situation where most if not all of a clone generates a particular cell type. However, in situations where only a fraction of the total number of cells yield a particular cell type (e.g. motoneurons), this method is hardly effective and would be misleading as the proliferative cells continued to outnumber the non-mitotic cells of interest. One means of circumventing this problem would be to compare neurospheres of approximately the same size in different culture conditions. This, however, would still not account for the intra-sphere variability of an extremely heterogeneous group of cells.

A Common Motoneuron/Oligodendrocyte Progenitor?

These data describe a developmental phenomenon of ChAT expression in oligodendrocytes. ChAT is classically found only in cholinergic neurons such as motoneurons. The presence of ChAT in these two cells demonstrates a common feature between oligodendrocytes and motoneurons, and, as previously stated, is not the only similarity between these two cell types (Shh induction, location of origin, etc). ChAT appears to be differentially expressed in oligodendrocytes and

motoneurons as well as other cholinergic neurons: motoneurons increasingly express ChAT as they mature, while oligodendrocytes express ChAT only during development. There has been discussion in the field of oligodendrocyte development about a common progenitor cell that yields both motoneurons and oligodendrocytes (Richardson et al 1997). It has been suggested that a common progenitor cell exists that yields motoneuron progeny between E10 and E13 and then subsequently oligodendrocyte progeny. There are several lines of evidence to support this theory (recently reviewed in Richardson et al 2000). Both cell types differentiate from the ventral spinal neuroepithelium in response to the same concentration of Shh. After both groups differentiate, a widespread cell death occurs as a result of competition for local trophic factors. The location of both progenitor cells is also influenced by the transcription factor pax6 (Ericson et al 1997). It has been proposed that a population of neuroepithelial precursors generates motoneurons and the oligodendrocyte precursors at a later time point (Sun et al 1998). These authors discussed several models of oligodendrocyte differentiation and deemed the common progenitor model the most likely model. As already mentioned, motoneurons differentiate from the neuroepithelium at approximately E10. Motoneuron progenitors express MNR2 but not the PDGFRα. Oligodendrocyte progenitors differentiate from the neuroepithelium starting at E12.5 at the end of motoneuron differentiation. Interestingly, in the Danforth's short tail mutant (which lacks a notochord and floor plate, i.e. no Shh is produced) there is a lack of oligodendrocyte progenitor cells and gross disruption in motoneuron formation. It was subsequently reported that the same

concentration of Shh can induce both motoneuronal or oligodendrocyte differentiation at different developmental time points. The PDGFRα is not expressed in motoneurons until they are mature whereupon PDGF acts to maintain cell survival (Vignais et al 1995). However, a mouse mutant called patch exists with a defective PDGFRα gene. This mutation is embryonic lethal and results in a 65% decrease in overall motoneuron numbers as well as stunted growth of the remaining motoneurons (Li et al 1996). During development, PDGFRα expression is localized solely to oligodendrocyte progenitors and then expression is terminated upon oligodendrocyte maturation and myelin synthesis. This differential expression of PDGFRα (like ChAT in this thesis) may be relevant in the quest to identify a common motoneuron/oligodendrocyte progenitor.

The dm-20/plp gene yields two different products by splicing (Timsit et al 1992). These authors present a comprehensive report on plp/dm-20 in the developing mouse. Both plp and dm-20 proteins are indistinguishable by current immunohistochemical methods, but the use of in situ hybridization and polymerase chain reaction has allowed further analysis of their expression. Plp has long been recognized as one of the major proteins in myelin and is expressed at postnatal day 2 in the mouse when the period of active myelination is occurring. The *jimpy* mouse mutant (which lacks the *dm-20/plp* gene) demonstrates gross deficits in oligodendrocyte development and myelination. Interestingly, the alternate splice variant dm-20, is first expressed by neuroepithelial cells at E9.5 in the mouse. It has been suggested that dm-20 functions to guide oligodendrocyte progenitor cell commitment, but this

suggestion was later refuted because oligodendrocyte progenitors do not differentiate until approximately E14, after motoneuron differentiation is complete.

There exist two other members of this gene family, M6a and M6b, which display more sequence homology to dm-20 than plp, and also have similar distribution patterns (Yan et al 1996). The M6a protein is also localized to neurons at E10 in the brain and spinal cord and is believed to be involved in neurite extension. M6a is expressed in only differentiated post-migratory cells and thus is among the earliest markers of neuronal differentiation but not a marker of a progenitor cell. The M6b product is localized to cells of the ependymal zone of the spinal cord and later in neurons and glial cells. This raises the notion that dm-20 or perhaps M6b may be markers of the common motoneuron/ oligodendrocyte progenitor cell. It is important to note that motoneuron development appears to proceed normally in the *jimpy* mouse. This suggests that while dm-20 expression may be a marker for a motoneuron/ oligodendrocyte precursor, it is not essential for motoneuron differentiation. In contrast, the other gene product, plp, is a marker for oligodendrocytes and also essential for oligodendrocyte differentiation.

It has also been reported that dm-20 was highly expressed in sciatic nerve extracts(Timsit et al1992). The authors attributed its expression to Schwann cells, but it may be that the dm-20 was originally contained in motor axons. It is thought that dm-20 is not restricted to an oligodendrocyte lineage, and it has been shown that dm-20 mRNA is downregulated in PC12 cells as they

differentiate into neurons (Ikenaka 1992). dm-20 also appears to be localized to neuronal precursor cells, again supporting the notion that it may indicate a neuronal progenitor in vivo as well.

The differential expression the PDGFRα may be one factor that determines the fate of the common motoneuron/ oligodendrocyte progenitor. Assuming that all motoneuron/ oligodendrocyte progenitors express DM-20, while only those ultimately becoming oligodendrocytes expressing the PDGFRα. Ubiquitous PDGFRα stimulation would induce oligodendrocyte differentiation. The PDGFA-/- mouse demonstrates gross deficits in myelination and oligodendrocyte differentiation supporting this hypothesis. If one could generate a dm-20 or M6b green fluorescent protein expression vector for transgenic application with the *patch* PDGFRα -/- mouse would possibly solve this riddle. The subsequent harvest of fluorescent PDGFRα-/- cells could proliferate in culture under mitogenic immortalization like neurospheres for differentiation. Under these conditions, could a motoneuron progenitor cell then be derived that would yield only motoneurons?

Conclusions

We conclude that neurosphere precursor cells can differentiate into oligodendrocytes expressing cholinergic cell markers and respond to CNTF treatment. Cholinergic stimulation of oligodendrocyte precursors contributes to oligodendrocyte differentiation as evidenced by muscarinic blockade. These

results support the hypothesis of the existence of cholinergic oligodendrocytes releasing acetylcholine to promote oligodendrocyte differentiation.

The second study concludes that functional motoneurons can differentiate from neurosphere precursors in culture and after transplantation into the transected sciatic nerve. Neurosphere precursors can differentiate into neurons exhibiting antigenic properties of motoneurons, and make functional connections to myocytes.

These studies offer significant insight into the fields of stem cell biology, as well as oligodendrocyte and motoneuron differentiation. There is also relevance to the study of clinical disorders such as multiple sclerosis, ALS and spinal muscular atrophy in which a loss of oligodendrocytes or motoneurons occurs.

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