

Functional motor neurons differentiating from mouse multipotent spinal cord precursor cells in culture and after transplantation into transected sciatic nerve

STEPHEN C. MACDONALD, PH.D., IAN G. FLEETWOOD, M.D., SHAWN HOCHMAN, PH.D.,
JANICE G. DODD, PH.D., GAVIN K. W. CHENG, PH.D., LARRY M. JORDAN, PH.D.,
AND ROBERT M. BROWNSTONE, M.D., PH.D.

Department of Physiology, University of Manitoba, Winnipeg, Manitoba; and the Departments of Surgery (Neurosurgery), and Anatomy and Neurobiology, Dalhousie University, Halifax, Nova Scotia, Canada

Object. One of the current challenges in neurobiology is to ensure that neural precursor cells differentiate into specific neuron types, so that they can be used for transplantation purposes in patients with neuron loss. The goal of this study was to determine if spinal cord precursor cells could differentiate into motor neurons both in culture and following transplantation into a transected sciatic nerve.

Methods. In cultures with trophic factors, neurons differentiate from embryonic precursor cells and express motor neuronal markers such as choline acetyltransferase (ChAT), Islet-1, and REG2. Reverse transcription–polymerase chain reaction analysis has also demonstrated the expression of Islet-1 in differentiated cultures. A coculture preparation of neurospheres and skeletal myocytes was used to show the formation of neuromuscular connections between precursor cell–derived neurons and myocytes both immunohistochemically and electrophysiologically. Following various survival intervals, precursor cells transplanted distal to a transection of the sciatic nerve differentiated into neurons expressing the motor neuron markers ChAT and the $\alpha_1.1.2$ (class C, L-type) voltage-sensitive Ca^{++} channel subunit. These cells extended axons into the muscle, where they formed cholinergic terminals.

Conclusions. These results demonstrate that motor neurons can differentiate from spinal cord neural precursor cells grown in culture as well as following transplantation into a transected peripheral nerve.

KEY WORDS • spinal cord • stem cell • neural progenitor cell • developmental study • myocyte

ONE of the current challenges in neurobiology is to ensure that neural precursor cells differentiate into specific neuron types, so that they can be used for transplantation purposes in patients with neuronal loss. For example, the capacity to replace nigral dopaminergic neurons in patients with Parkinson disease or somatic motor neurons in patients with motor neuron diseases with stem cells could lead to viable treatments for patients with these diseases. The goal of this study was to determine if spinal cord precursor cells could differentiate into motor neurons both in culture and following transplantation into a transected sciatic nerve.

In an earlier study, it was reported that neurosphere precursor cells can differentiate in culture into ChAT-positive oligodendrocytes after treatment with BDNF and CNTF.¹⁸ In that study, however, it was observed that a small fraction of ChAT-positive cells were neurons because they expressed the neuron marker MAP-2. These neurons displayed a large cell diameter with a multipolar structure, and it was hypothesized that these cells were motor neurons.

The pursuit of stem cell–derived motor neurons is not novel. There have been previous studies in which attempts have been made to derive motor neurons from neural precursor cells;^{13,14,21,22} however, in those studies researchers examined the expression of nonspecific motor neuron markers and did not demonstrate the functional properties of precursor cell–derived motor neurons. It has been difficult to demonstrate motor neuron differentiation from neural precursor cells because of the paucity of specific motor neuron markers. Most recently, however, progress has been made in the *in vitro* differentiation of motor neurons from mouse embryonic stem cells.³¹ The physiological characteristics of these cells have not been studied.

A motor neuron can be defined, in part, by the expression of protein markers detectable by molecular or immunohistochemical methods. General neuron markers such as

Abbreviations used in this paper: ACh = acetylcholine; BDNF = brain-derived neurotrophic factor; bFGF = basic fibroblast growth factor; ChAT = choline acetyltransferase; CNTF = ciliary neurotrophic factor; DiO = 3,3'-dioctadecylcarbocyanine perchlorate; DMEM = Dulbecco modified Eagle medium; DRG = dorsal root ganglion; EGF = epidermal growth factor; GDNF = glial cell line–derived neurotrophic factor; GFAP = glial fibrillary acidic protein; HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MAP-2 = microtubule-associated protein-2; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RT = reverse transcription; VAChT = vesicular ACh transporter.

Motor neuron differentiation from precursor cells

MAP-2 should be expressed, as well as cholinergic cell markers such as the enzyme ChAT and VACHT. There are a number of LIM homeobox transcription factors also found in motor neurons. The first molecular marker of terminal motor neuron differentiation in the spinal cord is Islet-1.⁶ Another protein localized to the motor neuron membrane is REG2, which is transported along the axons of motor neurons.¹⁶ The $\alpha_1.2$ (class C, L-type) subunit of the voltage-sensitive Ca^{++} channel has also been localized to spinal motor neurons.¹² These are some of the markers that can be used to detect motor neurons by using immunohistochemical analysis or RT-PCR.

The sine qua non of a motor neuron is the projection of axons to muscle, forming neuromuscular junctions able to effect muscle contraction. Thus, a solely histological approach to localizing precursor-derived motor neurons may be open to criticism, despite the use of multiple markers, and unequivocal identification of precursor cell-derived motor neurons would rely on their ability to form functional neuromuscular junctions. In looking for motor neuron differentiation, it is therefore necessary to demonstrate the formation of neuromuscular junctions.

The hypothesis examined in this study is that spinal cord-derived progenitor cells will differentiate into motor neurons both in vitro and following transplantation into a divided sciatic nerve. To demonstrate appropriate differentiation, it is not only necessary to show that the cells express appropriate markers, but also to demonstrate that neuromuscular junctions are formed.

To identify and explore the functional nature of precursor cell-derived motor neurons, two approaches were undertaken. First, an in vitro coculture model of precursor cells and skeletal myocytes was developed to explore neuromuscular junction formation by testing drug responsiveness and electrophysiological responses. The goal of these experiments was to demonstrate that precursor cell-derived motor neurons can differentiate in culture and innervate cultured skeletal myocytes.

Neurospheres were also transplanted into the sectioned sciatic nerve to determine if differentiation into a motor neuron phenotype is possible in this environment. This was thought to be plausible because the peripheral nerve environment is growth permissive to motor axons and releases growth factors after lesions.²⁷ Authors of previous studies have reported successful transplantation of embryonic motor neurons and fetal spinal cord preparations into the sciatic nerve, including the development of neuromuscular junctions.^{5,15} In addition to a supportive role for differentiated motor neurons, it was hypothesized that this environment would also promote the differentiation of motor neurons from neurospheres.

The results demonstrate differentiation of precursor cells into motor neurons both in vitro and following transplantation into a divided sciatic nerve, thus providing evidence that functional motor neurons can differentiate from multipotent precursor cells. This work has previously been presented in abstract^{7,18} and thesis¹⁷ form.

Materials and Methods

All experiments were performed after anesthesia had been induced in the animals. 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.

Primary Culture of Precursor Neurospheres

The culture methods were based on those described by Weiss, et al.;²⁹ they were slightly modified as described later in this paper. The spinal cords were removed from E16-E18 CD1 albino mouse embryos in a modified Hanks balanced salt solution without Ca^{++} or Mg^{++} , which contained the following substances (in mM): KCl (4), KH_2PO_4 (0.6), NaCl (80), $NaHCO_3$ (0.35), $NaHPO_4$ (0.048), and D-glucose (1) at a pH of 7.3. The spinal cords were minced and incubated in Ca^{++} and Mg^{++} -free Hanks balanced salt solution with 100 ng/ml papain (Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada), 100 ng/ml DNase, and 1 mg/ml protease (Type I, Sigma-Aldrich Canada, Ltd.) for 30 minutes. After the enzyme incubation, the tissue was gently triturated and washed in DMEM-F12 medium (1:1) containing DNase (1 mg/ml) for 15 minutes. The cell suspension was then washed and resuspended in DMEM-F12 medium containing N2 supplement (Sigma-Aldrich Canada, Ltd.). Afterward, the cells (4×10^4) were plated in uncoated 75-cm² flasks, and 20 ng/ml of EGF and bFGF were added. After 7 days in culture, floating clusters of cells were withdrawn, centrifuged at 400 rpm, and resuspended in fresh medium containing EGF and bFGF. Half the medium was changed every 7 days, and fresh EGF and bFGF were added every 4 days. The neurospheres were plated in Neurobasal medium containing B27 supplement (Invitrogen Life Technologies, Burlington, ON, Canada) in the absence of bFGF and EGF for 7 to 10 days before fixation for immunohistochemical analysis.

Myocyte-Precursor Cell Coculture for Electrophysiological Investigation

Hindlimb muscles from CD1 mouse pups were harvested on postnatal Day 1. Each tissue sample was dissected free of remaining blood vessels, minced, and incubated in DMEM-F12 medium (1:1) containing DNase (1 mg/ml) and trypsin (1 mg/ml) for 30 minutes in a 37°C incubator. After the enzyme incubation, the trypsin was inactivated by addition of DMEM-F12 medium containing 10% fetal calf serum. The tissue was then triturated using a series of fire polished Pasteur pipettes and washed twice in growth medium consisting of DMEM-F12 medium with added glutamine (2 mM), Na^+ pyruvate (1 mM), B27 supplement (1:50) (Invitrogen Life Technologies), and 5% fetal calf serum. Approximately 150 μ l of cell suspension was plated onto the center of a 35-mm² culture dish that had been coated with poly-D-lysine and laminin. After 24 hours, the dish was flooded with an additional 600 μ l of growth medium.

After 3 days in culture, the myocyte cultures were washed twice in Neurobasal or Hams F-14 medium containing glutamate and forskolin, and 8 to 15 multipotent precursor cell neurospheres were added to the center of the cultures. Brain-derived neurotrophic factor (3 ng/ml) and CNTF (20 ng/ml; Regeneron Pharmaceuticals, Inc., Tarrytown, NY) were added to the cultures for the first 24 hours, after which medium containing 2% horse serum, BDNF, CNTF, and GDNF (10 ng/ml; Amgen Canada, Inc., Mississauga, ON, Canada) was used to feed the cultures. Initial cultures in which DMEM-F12 medium was used resulted in less than optimal cell health and overgrowth of proliferative fibroblasts and astrocytes. This may have been due to the high levels of glutamate in the medium. To prevent possible glutamate-induced neurotoxicity, 1 mM kynurenate was added to the cultures on addition of the neurospheres. This seemed to improve cell health, but did not prevent the rapid overgrowth of glia in the cultures. Neurobasal medium was used to decrease the proliferation of nonneuronal cells; nevertheless, Hams F-14 medium was found to be optimal for both myocyte and neuron growth while not resulting in culture overgrowth as quickly as Neurobasal or DMEM-F12 medium.

Immunohistochemical Analysis of Cultured Cells

Cultures were fixed in a solution of 4% paraformaldehyde and washed five times in PBS-Triton-X (0.1%) or PBS-Tween-20 (0.8%) before primary incubation. The primary antibodies used (and their concentrations) were as follows: Rabbit anti-ChAT (1:1000)

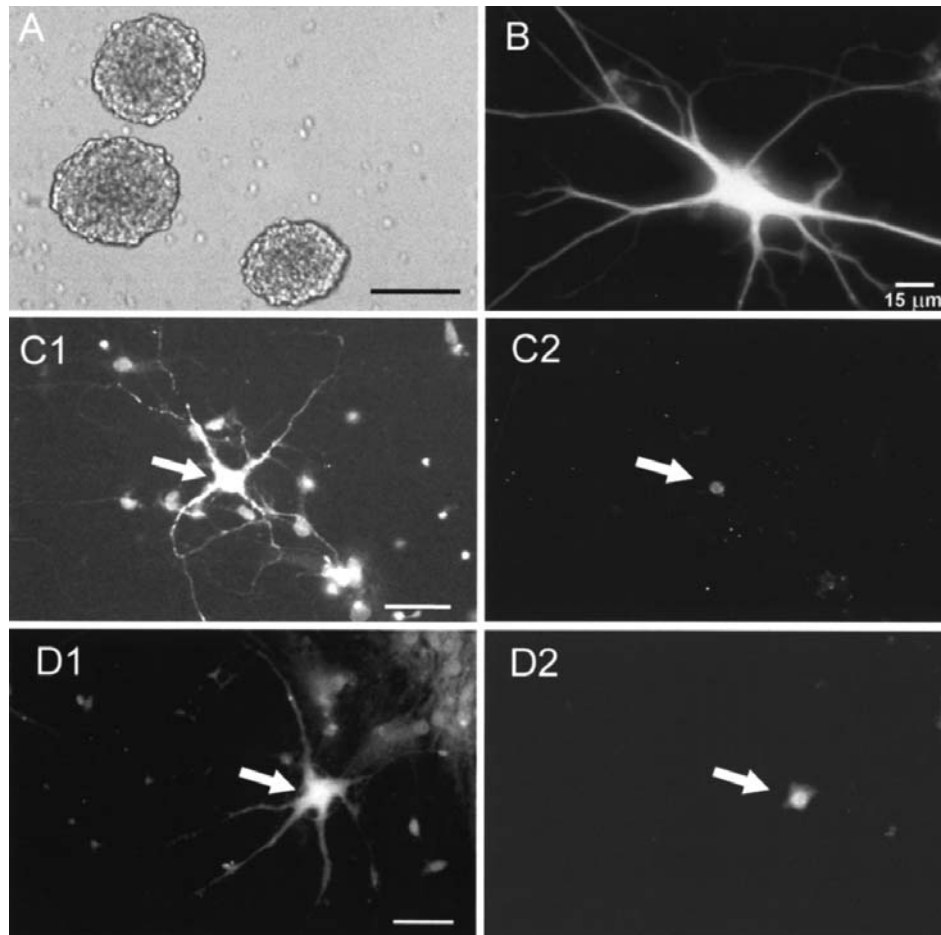


FIG. 1. Immunohistochemical detection of motor neuron antigens in tissue culture. Phase-contrast micrograph demonstrating clusters of proliferative neural precursor cells grown in tissue culture (A). After they were plated on a permissive substrate in the absence of mitogens, some precursor cells differentiate into large-diameter ChAT-positive neurons (B and C1 [arrow]). Immunohistochemical analysis was used to identify more specific markers of motor neuron differentiation and growth. A subpopulation of ChAT-positive neurons expresses the LIM homeobox transcription factor, Islet-1 (arrow in C2). An example of an REG2-immunoreactive neuron (arrow in D1), which also expresses Islet-1 (arrow in D2), is also shown. Bars = 100 μ m (A); 15 μ m (B); and 50 μ m (C1–D2).

and goat anti-VACHT (1:10000) (both from Chemicon International, Mississauga, ON, Canada); mouse anti-MAP-2 (MAP2A,B; 1:1000), mouse anti-panaxonal antibodies (1:1000), and rabbit anti-GFAP (1:1000) (all from Sternberger Monoclonals, Inc., Lutherville, MD); mouse anti-Islet-1 (1:500) and rat anti-nicotinic receptor (1:500) (both from Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); rabbit anti-REG2 (1:1000; a gift from Dr. Liam Murphy, University of Manitoba, Winnipeg, MB, Canada); mouse anti- α 1c (α 1.2) subunit (1:1000; Alomone Labs, Jerusalem, Israel); mouse anti-myosin (1:100; Sigma-Aldrich Canada, Ltd.); rabbit anti-glutamate receptors 5, 6, and 7 (1:1000; BD Biosciences Pharmingen, San Diego, CA); and mouse anti-galactocerebroside (1:100; Boehringer Mannheim, Laval, QC, Canada). Primary incubations included donkey serum (dilution 1:100; Sigma-Aldrich Canada, Ltd.). Secondary antibodies included the following (and their concentrations): donkey anti-mouse/cy3 conjugate (1:250) and donkey anti-mouse/fluorescein isothiocyanate (1:250) (both from Jackson Laboratories, Bar Harbor, ME); and donkey biotinylated anti-rabbit (1:100) and streptavidin-fluorescein isothiocyanate (1:100) (both from Amersham Biosciences, Inc., Baie d'Urfé, QC, Canada). Between all antibody incubations, all cultures were washed five times in PBS or in PBS-bovine serum albumin (1%). Final washes were conducted in 50 mM Tris-HCl before the specimens together with a glycerine-based antifade medium were

protected with a coverslip (Vectashield; Vector Laboratories Canada, Inc., Burlington, ON, Canada). Images were either photographed or digitized using a Neurolucida image analysis system (MicroBrightField, Inc., Williston, VT).

Detection of Islet-1 Expression by RT-PCR Analysis

Total RNA was extracted from differentiated neurosphere-myocyte cocultures at 1-, 2-, and 3-week growth intervals by using Trizol reagent (Invitrogen Life Technologies). Whole neonatal mouse spinal cord and adult mouse ovary RNAs were also extracted to serve as positive and negative controls, respectively. Complementary DNA was synthesized using 1 μ g of total RNA in a 20- μ l reaction volume consisting of the following: First-strand buffer and 0.1 M 1,4-dithiothreitol (both from Invitrogen Life Technologies), 10 mM deoxynucleoside triphosphates, 500 ng/ μ l of random hexamer (N6) primers, 20 U of RNA guard, 2 μ g bovine serum albumin, 1 μ g total RNA, and 200 U of Moloney murine leukemia virus reverse transcriptase. The reaction volume was incubated for 2 hours at 37°C and, subsequently, the reaction was terminated by a 5-minute incubation at 65°C. The primer pair used for the Islet-1 PCR was based on that proposed by Kalyani, et al.,¹⁴ and similar sequences were modified for the mouse genome: sense, 5'-GCAGCATAGGCTTCAGCAAG-3'; antisense, 5'-ATAGCAGGTCCGCAAGGTG-3'.

Motor neuron differentiation from precursor cells

The PCR included PCR buffer (Pharmacia Canada, Mississauga, ON, Canada), 1 pmol sense and antisense primers, 10 μ l of RT reaction product, 50 μ M deoxynucleoside triphosphates, and 1 U of Taq DNA polymerase (Pharmacia Canada) in a 100 μ l volume. The reaction was run for 35 cycles (60 seconds at 95°C, 45 seconds at 58°C, and 45 seconds at 72°C) followed by a final 7-minute incubation at 72°C to ensure complete extension. The PCR product was electrophoresed on a 2% agarose ethidium bromide-containing gel, and the bands were visualized and photographed under exposure to ultraviolet light.

Functional Analysis of Cocultures

The methods we used are based on those outlined by Ternaux and Portalier.²⁸ Briefly, cultures were locally perfused (DAD-12 fast perfusion system; Adams and List, Great Neck, NY) consisting of 12 barrels feeding into a common output manifold. Agonists kainate and ACh were applied to a nonspontaneously contracting myocyte to elicit contraction. Images were then captured at a rate of 3.3 Hz on a digital camera over a 30 to 60-second interval. Control solution was perfused between agonist applications and was used 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 using whole-cell voltage-clamp techniques.⁸ Briefly, the cultures were visualized with the aid of an inverted microscope (Nikon Canada, Inc., Mississauga, ON, Canada) and the culture medium was replaced with a HEPES-buffered recording solution containing the following components (in mM): HEPES (10), NaCl (150), KCl (5), MgCl₂ (1), CaCl₂ (2), and glucose (10) at a pH of 7.4. Electrodes were pulled from borosilicate glass on an upright electrode puller (Narishige Scientific Instrument Lab, Tokyo, Japan) with a final tip resistance of 4 to 6 M Ω . The intracellular recording solution consisted of the following components (in mM): K-gluconate (140), ethyleneglycol-bis(β -aminoethylether)-N,N',N',-tetraacetic acid (11), HEPES (10), CaCl₂ (1), KOH (35) Mg adenosine triphosphate (4), and guanosine triphosphate (2) at a pH of 7.4. Recordings were acquired using pCLAMP (version 6.0) acquisition software (Axon Instruments, Inc., Union City, CA) and amplified through an Axopatch 1-D amplifier (Axon Instruments, Inc.) to a computer disk. Traces were analyzed using CLAMPFIT software (version 6.0; Axon Instruments, Inc.).

Sciatic Nerve Transection and Transplantation

The methods we used for this study were based on those proposed by Erb, et al.⁵ Sixty-one adult male BalbC mice were anesthetized with ketamine and xylazine. The sciatic nerve was exposed and transected. Neurospheres were dissociated and labeled 1 to 2 days before transplantation with the lipophilic fluorescent marker DiO, which had been dissolved in dimethyl sulfoxide for 1 hour. This method was efficient for labeling precursor cells, but was not complete, and there remained some transplanted cells that were not labeled. After transection of the sciatic nerve, the proximal stump was deflected into the surrounding musculature and sutured into a muscle bed away from the distal nerve stump. The distal nerve stump was injected with approximately 27 μ l of the neurosphere suspension previously labeled with DiO and was ligated to prevent leakage of the suspension. At time periods between 0 days and 7 months posttransplantation, the mice were again anesthetized and were perfused by intracardiac administration of cold (4°C) prefix solution, consisting of 0.9% NaCl, 0.1% sodium nitrite, and 1000 U of heparin, followed by 4% paraformaldehyde, 0.1% sodium phosphate, and 0.9% saline. The sciatic nerve and the distal muscle were removed and postfixed for 24 hours in this latter fixative solution, after which they were placed in a 10% sucrose solution. The tissue was cryosectioned into 5- μ m-thick slices and mounted on gelatin-coated slides for immunohistochemical analysis in the manner described for the *in vitro* experiments. At the time of the animal's death, both sciatic nerves were harvested and the nonexperimental nerve served as a control. Various controls were used including nonsectioned, nontransplanted nerves, transected nontransplanted nerves, and transected nerves injected with neurosphere-free BDNF and CNTF-impregnated media.

Results

Culture Experiments

Precursor cell cultures were generated using methods based on those described by Weiss, et al.,²⁹ resulting 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).

There have been previous reports of growth and survival-promoting actions of BDNF, CNTF, and GDNF on motor neurons.^{10,25} Clusters of precursor cells were allowed to differentiate in the presence of BDNF (10 ng/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. The resulting cultures were fixed and initially processed for the neuron and glia markers MAP-2, GFAP, and galactocerebroside, which demonstrated the existence of neurons, astrocytes, and oligodendrocytes, respectively, with the morphological characteristics of all these cell types.¹⁸ All differentiated neurospheres displayed MAP-2-labeled neurons at varying frequencies.

One of the defining markers of cholinergic neurons is the enzyme ChAT, which synthesizes ACh. To determine if cholinergic neurons differentiate from precursor cells, cell cultures were processed immunohistochemically for ChAT (68 cultures). Immunostaining for ChAT revealed the presence of large multipolar cells (Fig. 1B). Most of the neuronal ChAT-positive cells were multipolar, whereas some retained the more bipolar morphological characteristics of immature motor neurons.³⁰ All cholinergic cells were characterized by a large soma diameter (mean 21.6 ± 4.3 μ m). Double-label immunohistochemical analysis was performed for ChAT and either MAP-2/panneuronal antibodies, galactocerebroside, or GFAP to label neurons, oligodendrocytes, or astrocytes, respectively. In addition to finding the colocalization of ChAT and galactocerebroside, which demonstrated differentiation into cholinergic oligodendrocytes,¹⁷ ChAT and the MAP-2/panneuronal antibodies were also colocalized, indicating that some neurons were cholinergic. In no instance were GFAP and ChAT antibodies colocalized. The GFAP-positive cells were flat and displayed a diffusely projecting structure characteristic of astrocytes (data not shown). These results demonstrate cholinergic neuron differentiation from proliferative spinal cord precursor cells.

Additional immunohistochemical examination of differentiated cultures was necessary by using antibodies to more specific motor neuron markers. Islet-1 is part of the LIM homeobox family of transcription factors and is the first molecular indicator of motor neuron differentiation.⁶ Cultures that were coprocessed for ChAT and Islet-1 revealed large cholinergic neurons displaying Islet-1-labeled nuclei (Fig. 1C1 and C2; 15 cultures). There was also a population of ChAT and Islet-1-positive cells that possessed smaller soma diameters than the large ChAT and Islet-1-positive cells. This Islet-1 population may represent another cholinergic spinal neuronal or oligodendroglial group. Another marker of motor neurons is the Schwann cell mitogen REG2.¹⁶ Expressed by motor neurons, REG2 is retrogradely transported along developing or regenerating motor axons. Cultures were coprocessed for Islet-1 and REG2 and revealed large-diameter REG2-positive cells that coex-

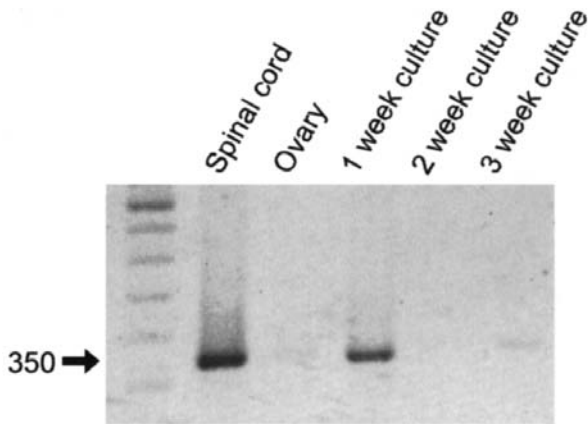


FIG. 2. Expression of Islet-1 in differentiated neuroepithelial cultures. The Islet-1 RT-PCR product was electrophoresed on an agarose gel and visualized under ultraviolet illumination. The first lane corresponds to the 100-bp marker. The second lane displays a strong 350-bp band derived from spinal cord RNA. The RNA that was harvested from a 1-week-old culture displays a similar band. A faint band is visible for the RNA obtained from a 3-week-old culture, but no band was visible for ovary RNA or for RNA obtained from a 2-week-old culture.

pressed Islet-1 in the nucleus (six cultures; Fig. 1D1 and D2). There were fewer REG2-positive neurons observed in differentiated cultures than Islet-1-positive cells. It is known that REG2 only labels a subpopulation of motor neurons and this would account for the lower number of labeled cells.⁴ These results provide evidence for the expression of specific motor neuron markers in cholinergic cells, suggesting that some of these cholinergic neurons express the antigenic characteristics of motor neurons.

To examine the time course of putative motor neuron differentiation, Islet-1 expression was also examined in neurosphere cultures after 1, 2, and 3 weeks by applying RT-PCR technology to neurosphere–myocyte cocultures grown in medium containing BDNF, CNTF, GDNF, and forskolin (four cultures). Neonatal spinal cord and adult ovary RNA were harvested to serve as positive and negative controls, respectively. A single strong 350-bp band was present for the spinal cord and there was no band present from the ovary RNA (Fig. 2). The 1-week-old 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 culture conditions are not favorable for long-term survival of motor neurons.

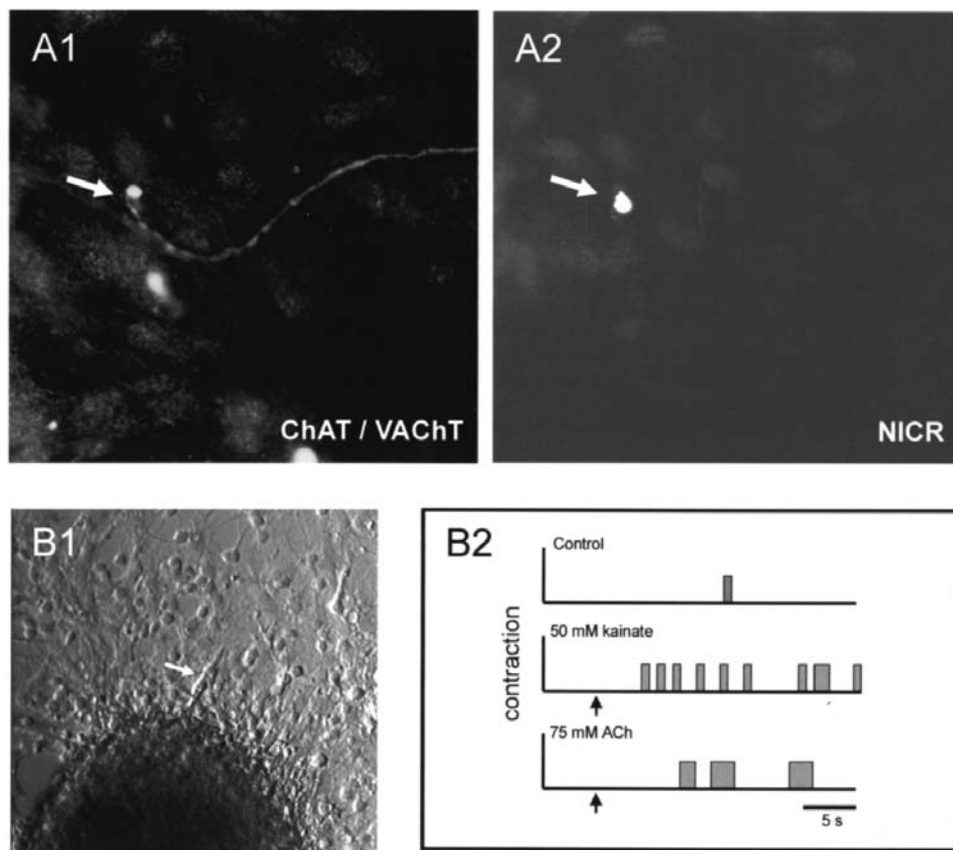


FIG. 3. Development of neuromuscular junctions in a precursor cell–myocyte coculture. An example of a cholinergic axon and terminal (arrow in A1) in apposition to a cluster of nicotinic ACh receptors (NICR; arrow in A2) is demonstrated using ChAT–VACHT and nicotinic receptor immunohistochemical analysis. Phase-contrast micrograph (B1) of a precursor cell cluster–myocyte coculture depicting a myocyte in close apposition to a differentiating cluster (white arrow). A contraction profile (B2) showing that the myocyte contracted in response to a perfusion of kainate or ACh (black arrows). Kainate application caused contraction only in the innervated myocyte, whereas an ACh perfusion caused contraction in all neighboring myocytes as well.

Motor neuron differentiation from precursor cells

Muscle–Neural Precursor Cell Coculture

As stated earlier, to be satisfied that these cells were indeed motor neurons, it is necessary to demonstrate functional connections with muscle. A muscle coculture preparation was, therefore, developed to determine whether functional connections could be made to muscle, in support of the immunohistochemical and molecular data indicating motor neuron differentiation. Clusters of multipotent progenitor cells were cocultured with dissociated myocytes from P0–P1 mouse pups, and the preparation was adjusted to optimize growth and differentiation of both myocytes and neurospheres. The addition of forskolin to the culture medium improved the differentiation and growth of myocytes (56 cultures), and also appeared to increase the number of MAP-2/panaxon-labeled neurons (data not shown). The neurons found in BDNF/CNTF-treated cultures with added forskolin had longer neurites and more axon terminals than those found in BDNF/CNTF-treated cultures without forskolin. The addition of GDNF to the culture medium containing BDNF and CNTF increased the number of nerve terminals, but neither the length of the neurites nor the number of the neurons was increased (data not shown). Thus, the addition of GDNF and/or forskolin to neurosphere cultures treated initially with BDNF and CNTF was one means by which this preparation was optimized to improve the growth of neurosphere-derived motor neurons.

To determine whether neuromuscular junctions formed in culture, the cultures were immunohistochemically labeled with markers for neuromuscular junctions. Immunostaining was performed using antibodies to the nicotinic receptor, as well as to ChAT and VAcHT to label cholinergic somata and axon terminals, respectively (16 cultures). *In vivo*, nicotinic ACh receptors are normally localized to postsynaptic sites on neuromuscular junctions and are closely apposed to cholinergic terminals from motor axons. The immunostaining of cultures revealed labeling of cholinergic terminals in close apposition to clusters of nicotinic receptors, showing the anatomical existence of neuromuscular junctions (Fig. 3A). In some cultures, VAcHT-positive boutons were also seen in apposition to muscle fibers located close to differentiated neurospheres, suggesting that cholinergic synapses were present on the myocytes. The rare (zero–four times/culture) occurrence of these boutons indicates that either the culture conditions were not optimal for neuromuscular development or that the detection methods were not ideal. These results provide anatomical evidence that neuromuscular junctions are formed *in vitro*.

To determine if the neuromuscular junctions were capable of effecting muscle contraction, we applied a pharmacological approach similar to that used by Ternaux and Portier.²⁸ Local perfusion of ACh was used to evoke muscle contraction directly, whereas kainate was applied to evoke contraction through excitation of a presynaptic motor neuron. To be satisfied that the glutamate was acting on the neurons and not on the myocytes, a double-label immunohistochemical investigation for myosin and the glutamate receptors 5, 6, and 7 was performed (four cultures). No instances of double labeling were seen; therefore, kainate-induced contraction was a sign of synaptic activation of the myocytes.

For these experiments, nonspontaneously contracting myocytes were targeted and perfused with kainate in

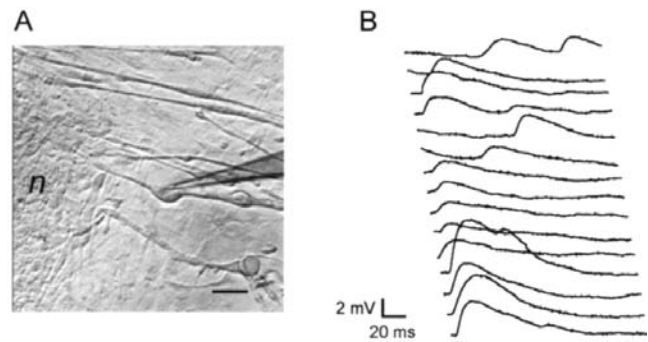


FIG. 4. Electrophysiological responsiveness of a skeletal myocyte in coculture. A: Phase-contrast micrograph showing a precursor cell–myocyte coculture. The differentiating neurosphere is seen on the left side of the panel and is marked by the letter n. Long healthy myocytes are seen running along the substrate, and the recording electrode is seen on the targeted myocyte. This particular cell was seen to contract in response to 50 μ M kainate and ceased to contract after the agonist was washed out. Bar = 50 μ m. B: End-plate potentials in response to a low concentration of kainate in the recording medium. No spontaneous end-plate potentials were observed in the absence of kainate.

1-week-old cultures (Fig. 3B1). Kainate perfusion was shown to elicit contraction in skeletal myotubes (Fig. 3B2; five). Kainate was washed out and the contraction ceased. Acetylcholine was subsequently applied and the contraction of the original myocyte as well as those of neighboring myocytes were 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). The end-plate potentials were 1 to 3 mV in amplitude (Fig. 4B), which occasionally recruited an action potential with a long posthyperpolarization duration (100 msec). The end-plate potentials were absent during the posthyperpolarization period and only reappeared after the cell returned to a resting membrane potential (data not shown). These results provide physiological evidence of the formation of functional contacts, indicating that, *in vitro*, spinal multipotent precursor cells can differentiate into neurons that express motor neuron antigens and can develop axons that can innervate myocytes and produce functional neuromuscular junctions.

In Vivo Transplantation Experiments

The next step was to determine if undifferentiated neurospheres could differentiate into motor neurons after transplantation into the transected sciatic nerve. The hypothesis to be tested was that the local environment would be appropriate not only for the survival of transplanted precursor cells, but also for the differentiation of these cells into motor neurons. There is considerable fluctuation in trophic factor levels in peripheral nerve after transection; for example, CNTF is released into the extracellular space.²⁷ Sixty-one BalbC mice were used in this part of the study. Neurospheres were transplanted into transected sciatic nerves, which were then harvested at the following intervals: Day 0 (four nerves); Days 1 through 5 (six nerves); 1 week (six nerves); 2 weeks (three nerves); 3 weeks (six nerves); 4

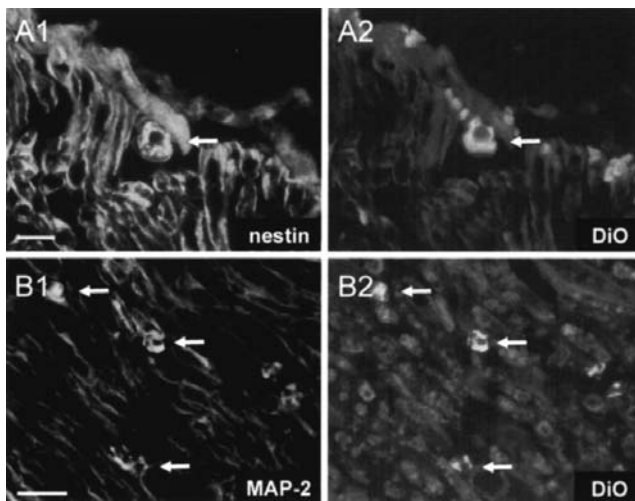


FIG. 5. Expression of nestin and MAP-2 in transplanted sciatic nerves shown in sections of adult mouse sciatic nerve after transplantation of neural precursor cells. Neural precursor cells were traced using DiO (arrows) during the transplantation procedure (A2 and B2). One day after transplantation of precursor cells, intense nestin immunoreactivity is seen in cells at the graft site (arrows in A1). As early as 3 days posttransplantation, MAP-2-positive neurons are seen at the graft site (arrows in B1). Bars = 15 μm (A1 and A2) and 25 μm (B1 and B2).

weeks (seven nerves); 5 weeks (one nerve); 6 weeks (two nerves); 8 weeks (five nerves); 10 weeks (five nerves); 12 weeks (three nerves); 15 weeks (two nerves); 24 weeks (two nerves); and 31 weeks (one nerve). The remaining eight mice either served as controls or did not survive the experimental procedure.

It was first demonstrated that DiO-labeled cells could be seen at all survival times; these cells survived at least 7 months following transplantation. To demonstrate that the transplant remained in an undifferentiated state at the time of injection, sciatic nerve sections were stained for the intermediate filament protein nestin, which is found in precursor cells. Immediately after injection (Day 0), precursor cell grafts displayed intense nestin staining in DiO-labeled cells (Fig. 5A). The nestin expression of DiO-labeled cells declined over time and was not detectable by 1 week postinjection. There was high background labeling of nestin in peripheral nerve sections, which is consistent with the expression of nestin by Schwann cells.¹¹

At 3 days and 12 weeks posttransplantation, differentiated cells displayed labeling for MAP-2 and panaxonal neuron markers (Fig. 5B). These results indicate that at least some precursor cells differentiate along neuronal pathways following transplantation.

To determine whether more specific motor neuron markers were expressed, immunohistochemical analysis was used to detect the motor neuron antigens ChAT and the voltage-sensitive Ca^{++} channel $\alpha_1.2$ subunit. The ChAT labeling was revealed in several DiO-labeled cells in the neurosphere graft at 2, 3, 4, 8, 10, and 12 weeks posttransplantation, demonstrating the existence of cholinergic neurons at several time points (Fig. 6A). The $\alpha_1.2$ subunit constitutes a portion of an L-type Ca^{++} channel and is found on motor neurons in the spinal cord.¹² This subunit was used as a specific motor neuron marker in place of Islet-1 because

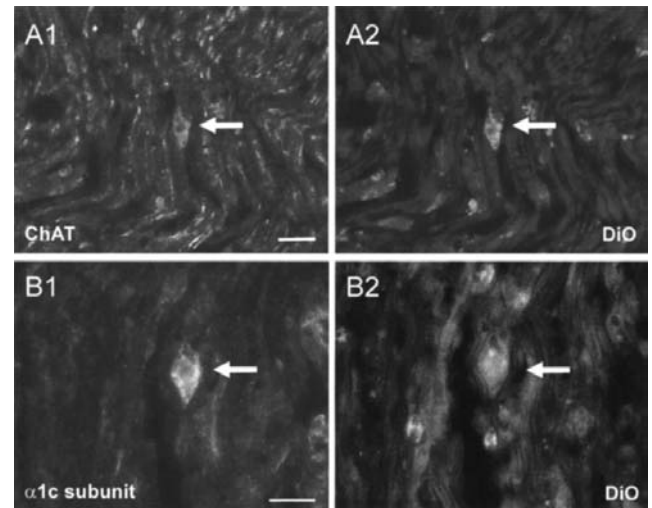


FIG. 6. Expression of motor neuron markers in transplanted sciatic nerve shown in sections of adult mouse sciatic nerve after cell transplantation. The DiO labeling is marked by arrows (A2 and B2). Expression of ChAT (arrow in A1) at 12 weeks and the $\alpha_1.2$ (class C) subunit of the L-type Ca^{++} channel (arrow in B1) at 4 weeks give evidence that motor neurons are differentiating in the cell-transplanted sciatic nerve. Bars = 20 μm (A1 and A2) and 10 μm (B1 and B2).

the Islet-1 antibody produced a tremendous background of excess labeling in sciatic nerve sections, making interpretation impossible. Antibodies to the $\alpha_1.2$ Ca^{++} channel subunit were used and positively stained DiO-labeled cells in the graft (Fig. 6B). The punctate cytosolic pattern of labeling was very similar to that shown by Jiang, et al.¹² These results demonstrate the expression of two markers found in motor neurons, suggesting that precursor cells can differentiate along the path of a motor neuron following transplantation into the transected sciatic nerve.

To determine if these differentiated cells had axons, a panaxonal antibody cocktail was used. This demonstrated that the implanted cells developed axonal processes (Fig. 7A). On examination of the triceps surae muscle, it was clear that DiO-labeled processes extended down the nerve to appose muscle fibers by 12 weeks following implantation (Fig. 7A). In addition, these fibers developed cholinergic terminals, as demonstrated by labeling with anti-VACHT by 16 weeks posttransplantation (Fig. 7B). As expected, no native cholinergic terminals could be identified at this interval in control animals with a transected, nontransplanted sciatic nerve (data not shown). Furthermore, there were macroscopic structural differences between the transplanted nerve and the control sectioned nerve. The transected nontransplanted nerves were translucent and difficult to identify after several months. Nevertheless, the nerves with the precursor cell implants were substantial, readily identifiable, easily dissectible, and amenable to manipulation. These results demonstrate that precursor cells transplanted into the distal portion of a divided sciatic nerve can differentiate into neurons that do the following: 1) express a motor neuron phenotype; 2) develop axons extending toward muscle; and 3) develop cholinergic terminals. These transplanted neurons also appear to maintain the viability of the distal nerve.

Discussion

The findings of this study demonstrate that motor neurons can differentiate from neural precursor cells both in culture and after transplantation into a transected sciatic nerve. The cells' identity as motor neurons has been confirmed by demonstrating that they are cholinergic neurons that express markers specific for motor neurons. Additionally, they develop axonal processes and synapse with muscle. Synaptic excitation of myocytes has been demonstrated in coculture conditions. The difficulty in finding markers specific to motor neurons prompted the use of antibodies to multiple motor neuron antigens combined with a functional analysis of neuromuscular connections to demonstrate the existence of motor neurons derived from isolated cultured neural precursor cells.

Differentiation of Cultured Neurospheres

The immunohistochemical studies reported here demonstrate that precursor cells differentiate into large-diameter neurons that 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 (< 1%). This is consistent with findings of previous studies in which the authors also reported a low frequency of large-diameter ChAT-positive or p75-positive cells derived from cultured neuroepithelial precursor cells.^{13,22} In light of the report that a population of partition cells is cholinergic and also possesses p75 immunoreactivity,¹⁹ it becomes necessary to use additional markers to identify these cells as motor neurons. Surprisingly, in a previous report it was shown that oligodendrocytes derived from neurospheres can also express ChAT.¹⁸ Oligodendrocytes have been shown to express the p75 receptor as well,² demonstrating that neither of these markers can be viewed as specific for motor neurons. In this study we therefore identified differentiating motor neurons in culture by using the specific motor neuron markers REG2 and Islet-1. The marker REG2 is expressed in developing motor neurons and is responsive to members of the leukemia-inhibitory factor family of cytokines to which CNTF belongs. It is therefore not surprising to see REG2 expressed in cultures supported with CNTF.¹⁶ It has been reported that Islet-1 is the first molecular marker of motor neuron differentiation and is expressed by all motor neurons before segregation into motor neuron subgroups.⁶ The colocalization of Islet-1 with both REG2 and ChAT demonstrates the development of motor neuron phenotypes in these cultures.

A possibility for consideration, however, is that these cells could be DRG neurons. Both Islet-1 and REG2 are also expressed in the DRG. The marker ChAT has not traditionally been reported in the DRG,^{1,24} but there is a recent report demonstrating ChAT-like immunoreactivity in the DRG.²³ Nonetheless, DRG neurons possess a remarkably different structure than motor neurons, being much more spherical and either unipolar or bipolar in shape. The neurons in this study do not demonstrate such morphological characteristics. Furthermore, the relationship of these neurons to myocytes would preclude them from being primary afferents (see the following section).

A recent study has convincingly demonstrated motor neuron differentiation from embryonic stem cells in vitro.³¹

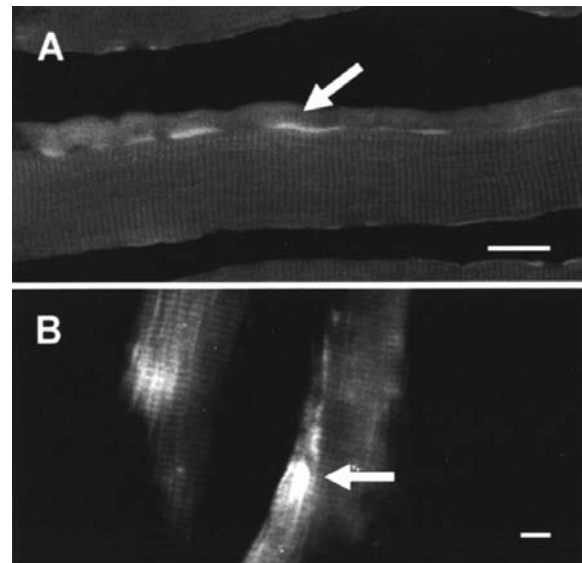


FIG. 7. Precursor cells sending axons to distal muscles. A: At 12 weeks after transplantation, a DiO-labeled axon (arrow) runs along the musculature attached to the distal end of a sciatic nerve section. B: Cholinergic terminals labeled with VAcHT antibodies (arrow) are seen on muscle tissue at the distal end of the sciatic nerve section at 16 weeks posttransplantation. Bars = 25 μ m (A) and 5 μ m (B).

In that study the investigators used a combination of retinoic acid and a sonic hedgehog agonist to recapitulate normal motor neuron development. The neurons generated in this fashion expressed molecular markers of motor neuron identity and, following implantation into an embryonic chick spinal cord at the time of normal motor neuron development, developed axons that made synaptic contacts with intercostal muscles. The physiological characteristics of these neurons have not yet been studied.

Synaptic Connections in Myocyte–Precursor Cell Cocultures

Although the cells appear to be motor neurons anatomically, to be identified definitively as motor neurons they must have the capacity to innervate muscle and effect muscle contraction. To examine this in vitro, a coculture preparation based on that described by Ternaux and Portalier²⁸ was used. Michikawa, et al.,²⁰ also have demonstrated that motor neurons can form neuromuscular junctions in culture. The addition of forskolin to the cocultures was observed to have a great effect on promoting myocyte health and structure. Myocyte length increased up to 700% and displayed more robust contractile responses, as observed with the aid of a light microscope. This is consistent with the known actions of forskolin such as increasing the intracellular concentration of cyclic adenosine monophosphate and promoting cell survival. Forskolin also has been previously reported to promote survival of cultured motor neurons and, in combination with other trophic factors, can promote motor neuron survival for up to 3 weeks in culture.⁹ The addition of both forskolin and GDNF to a coculture with skeletal myocytes appears to promote the growth and survival of motor neurons.⁹

The anatomical evidence demonstrates the formation of

cholinergic (VAcHT-positive) terminals in close apposition to nicotinic receptors on the myocytes. The cell density and debris of the culture made it impossible to identify individual motor neurons innervating myocytes in the living culture and to target these neurons for intracellular recording. Nevertheless, the myocyte whole-cell recording experiments revealed end-plate potentials in response to kainate, demonstrating that neuromuscular junctions are able to form in these cocultures. We therefore used a drug-perfusion technique modeled after that reported by Ternaux and Portalier²⁸ to demonstrate functional connections between the neurons and the myocytes. Myocyte contraction can be seen visually. Kainate application, which had no demonstrable direct effect on the myocytes, led to synaptically evoked myocyte contraction, demonstrating the formation of functional synapses in coculture. In summary, these results demonstrate that precursor cells can differentiate into neurons with motor neuron phenotypes that can functionally innervate myocytes.

Differentiation in Transplanted Sciatic Nerve

The fact that the precursor cells could differentiate into motor neurons following transplantation into the transected sciatic nerve indicates that the sciatic nerve environment contains factors that can direct motor neuron development. It is curious that the precursor cells could differentiate into motor neurons in a region of the host organism that normally does not contain those cells. The absence of any classic motor neuron differentiation factor such as sonic hedgehog would suggest that either a default program exists, allowing some motor neuron differentiation, or that the transplanted cells derive appropriate support from the neurospheres themselves. It was hypothesized that, because the trophic support of Schwann cells and terminal myocytes functions to maintain the health of motor neuron axons and the survival of motor neurons in the healthy adult,²⁶ this environment may hold sufficient cues to promote survival and effect differentiation. The peripheral nerve milieu is also much more permissive to axon growth than the hostile growth environment of the central nervous system.

Motor neuron survival has been reported after transplantation of embryonic motor neurons or fetal spinal cord preparations into the transected sciatic nerve, and it has been accompanied by successful reinnervation of the hindlimb musculature.^{5,15} In these studies the researchers used immunohistochemical, dye tracing, and electrophysiological methods to demonstrate restoration of neuromuscular junctions. The results of these studies led us to use this approach in the current study. Immunohistochemical analysis of the sections from the sciatic nerves injected with neural precursor cells demonstrated the presence of neurons, cholinergic cells, and, more specifically, motor neurons by using antibodies to MAP-2, ChAT, and VAcHT, and the $\alpha_1.2$ voltage-sensitive Ca^{++} channel subunit. This was coincident with a decrease in the expression of the precursor cell marker nestin in DiO-labeled cells. Nestin is rapidly downregulated in progenitor cells of the central nervous system as they differentiate,³ indicating that differentiation of precursor cells into more mature neuron phenotypes had occurred.

Although an important starting point, the immunohistochemical identification of motor neuron antigens is not sufficient to identify cells as motor neurons. The cells must also grow axons toward muscle and form cholinergic ter-

minals and functional connections with muscle to be classified as motor neurons. To determine whether motor neurons differentiated from neural precursor cells can functionally innervate muscle *in vivo*, it would be necessary to study the transplanted sciatic nerve electrophysiologically. Preliminary studies performed 31 weeks posttransplantation indicated positive electromyographic responses in both the triceps surae and tibialis anterior muscles in response to nerve stimulation. Stimulus-contraction coupling of ankle muscles was also observed visually. Subsequent stimulation of transected, nontransplanted sciatic nerves resulted in no ankle movement (personal observations). This preliminary observation supports the existence of functional neuromuscular junction formation in transplanted sciatic nerves and encourages the pursuit of further study.

These results demonstrate the differentiation of functional spinal motor neurons from neuroepithelial precursor cells in culture and also on transplantation into the adult sciatic nerve. Although these results demonstrate that it is possible to obtain functional motor neurons from neurospheres, the growth conditions *in vitro* need to be optimized to increase the yield of motor neurons.³¹ Furthermore, the factors that promote survival and differentiation following transplantation must be identified.

Acknowledgments

The authors acknowledge the generous donation of BDNF and CNTF (Regeneron Pharmaceuticals) and GDNF (Amgen) used in this study. The REG2 antibody was kindly donated by Dr. Liam Murphy. We gratefully acknowledge Mike Sawchuk for his expert assistance, and Eleanor Ling, Carolyn Gibbs, Jacquie Schwartz, Shirley Frederickson, and Maria Setterbom for their excellent technical support.

References

1. Barber RP, Phelps PE, Houser CR, et al: The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: an immunocytochemical study. **J Comp Neurol** **229**:329-346, 1984
2. Casaccia-Bonnel P, Carter BD, Dobrowsky RT, et al: Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. **Nature** **383**:716-719, 1996
3. Dahlstrand J, Lardelli M, Lendahl U: Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. **Brain Res Dev Brain Res** **84**:109-129, 1995
4. Davis DR, Livesey FJ, De Felipe C, et al: Changing patterns in Reg-2 immunoreactivity in sensory neurones following peripheral nerve axotomy. **Soc Neurosci Abstr** **25**:1265, 1999 (Abstract)
5. Erb DE, Mora RJ, Bunge RP: Reinnervation of adult rat gastrocnemius muscle by embryonic motoneurons transplanted into the axotomized tibial nerve. **Exp Neurol** **124**:372-376, 1993
6. Ericson J, Thor S, Edlund T, et al: Early stages of motor neuron differentiation revealed by expression of homeobox gene *Islet-1*. **Science** **256**:1555-1560, 1992
7. Fleetwood IG, MacDonald SC, Sawchuk MA, et al: Survival and differentiation of spinal cord stem cells transplanted into transected sciatic nerves of adult mice. **Soc Neurosci Abstr** **24**:68, 1998 (Abstract)
8. Hamill OP, Marty A, Neher E, et al: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. **Pflügers Arch** **391**:85-100, 1981

Motor neuron differentiation from precursor cells

9. Hanson MG Jr, Shen S, Wiemelt AP, et al: Cyclic AMP elevation is sufficient to promote the survival of spinal motor neurons in vitro. **J Neurosci** **18**:7361–7371, 1998
10. Henderson CE, Phillips HS, Pollock RA, et al: GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. **Science** **266**:1062–1064, 1994
11. Hockfield S, McKay RD: Identification of major cell classes in the developing mammalian nervous system. **J Neurosci** **5**:3310–3328, 1985
12. Jiang Z, Rempel J, Li J, et al: Development of L-type calcium channels and a nifedipine-sensitive motor activity in the postnatal mouse spinal cord. **Eur J Neurosci** **11**:3481–3487, 1999
13. Kalyani A, Hobson K, Rao MS: Neuroepithelial stem cells from the embryonic spinal cord: isolation, characterization, and clonal analysis. **Dev Biol** **186**:202–223, 1997
14. Kalyani AJ, Piper D, Mujtaba T, et al: Spinal cord neuronal precursors generate multiple neuronal phenotypes in culture. **J Neurosci** **18**:7856–7868, 1998
15. Katsuki M, Atsuta Y, Hirayama T: Reinnervation of denervated muscle by transplantation of fetal spinal cord to transected sciatic nerve in the rat. **Brain Res** **771**:31–36, 1997
16. Livesey FJ, O'Brien JA, Li M, et al: A Schwann cell mitogen accompanying regeneration of motor neurons. **Nature** **390**:614–618, 1997
17. MacDonald SC: **Oligodendrocytes and Motoneurons: Two Cholinergic Cell Types Derived From Multipotent Spinal Neuroepithelial Precursor Cells**. Thesis. Winnipeg, Canada: University of Manitoba, 2000
18. MacDonald SC, Simcoff R, Jordan LM, et al: A population of oligodendrocytes derived from multipotent neural precursor cells expresses a cholinergic phenotype in culture and responds to ciliary neurotrophic factor. **J Neurosci Res** **68**:255–264, 2002
19. Michael GJ, Kaya E, Averill S, et al: TrkA immunoreactive neurons in the rat spinal cord. **J Comp Neurol** **385**:441–455, 1997
20. Michikawa M, Kobayashi T, Tsukagoshi H: Early events of chemical transmission of newly formed neuromuscular junctions in monolayers of human muscle cells co-cultured with fetal rat spinal cord explants. **Brain Res** **538**:79–85, 1991
21. Park KI, Liu S, Flax JD, et al: Transplantation of neural progenitor and stem cells: developmental insights may suggest new therapies for spinal cord and other CNS dysfunction. **J Neurotrauma** **16**:675–687, 1999
22. Ray J, Gage FH: Spinal cord neuroblasts proliferate in response to basic fibroblast growth factor. **J Neurosci** **14**:3548–3564, 1994
23. Sann H, McCarthy PW, Mader M, et al: Choline acetyltransferase-like immunoreactivity in small diameter neurones of the rat dorsal root ganglion. **Neurosci Lett** **198**:17–20, 1995
24. Schoenen J, Delree P, Leprince P, et al: Neurotransmitter phenotype plasticity in cultured dissociated adult rat dorsal root ganglia: an immunocytochemical study. **J Neurosci Res** **22**:473–487, 1989
25. Sendtner M, Holtmann B, Kolbeck R, et al: Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. **Nature** **360**:757–759, 1992
26. Sendtner M, Kreutzberg GW, Thoenen H: Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. **Nature** **345**:440–441, 1990
27. Sendtner M, Stockli KA, Thoenen H: Synthesis and localization of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration. **J Cell Biol** **118**:139–148, 1992
28. Ternaux JP, Portalier P: Influence of tongue myoblasts on rat dissociated hypoglossal motoneurons in culture. **Int J Dev Neurosci** **11**:33–48, 1993
29. Weiss S, Dunne C, Hewson J, et al: Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. **J Neurosci** **16**:7599–7609, 1996
30. Wentworth LE: The development of the cervical spinal cord of the mouse embryo. I. A Golgi analysis of ventral root neuron differentiation. **J Comp Neurol** **222**:81–95, 1984
31. Wichterle H, Lieberam I, Porter JA, et al: Directed differentiation of embryonic stem cells into motor neurons. **Cell** **110**:385–397, 2002

Manuscript received August 15, 2002.

This work was supported by a University of Manitoba Research Development Fund grant to Dr. Brownstone, and by Canadian Institutes of Health Research operating Grant No. MGP-37755 to Dr. Jordan.

Address reprint requests to: Robert M. Brownstone, M.D., Ph.D., Division of Neurosurgery, 1796 Summer Street, Room 3809, Halifax, Nova Scotia B3H 3A7, Canada. email: Rob.Brownstone@dal.ca.