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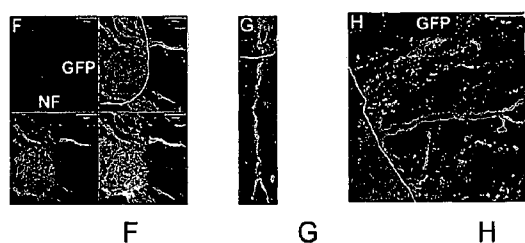
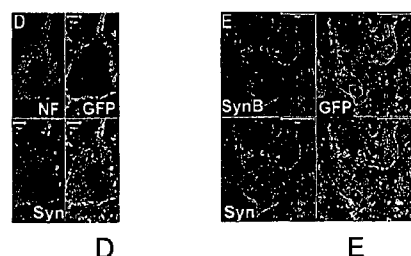
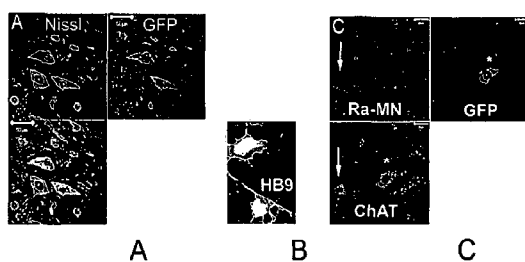
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(54) Title: METHODS FOR RESTORING NEURAL FUNCTION



(57) Abstract: The present invention features therapeutic strategies featuring cellular and pharmacological compositions and related methods to enhance functional recovery in spinal cord injury and motor neuron disease.

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METHODS FOR RESTORING NEURAL FUNCTION

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of the following U.S. Provisional Application No.:
5 60/815,896, which was filed on June 22, 2006, the entire disclosure of which is hereby
incorporated in its entirety.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

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BACKGROUND OF THE INVENTION

Spinal cord injuries and motor neuron disease frequently result in lifelong disabilities
15 from loss of motor and sensory functions. Recovery from such injuries is typically poor
because the injured CNS is an inhibitory environment for axon regeneration that severely
limits functional recovery. Neural stem cells have the potential to halt spinal motor neuron
degeneration and restore function to animals with spinal cord injury or motor neuron disease.
However, previous studies using transplanted neural stem cells have failed to show the
20 formation of functional neuronal circuits in the adult mammalian nervous system.

SUMMARY OF THE INVENTION

As described below, the present invention features therapeutic strategies featuring
cellular and pharmacological compositions and related methods to enhance functional
25 recovery in spinal cord injury and motor neuron disease.

In one aspect, the invention features a method of increasing motor function in a
subject (e.g., a human) in need thereof. The method involves administering to the subject a
cell capable of adopting a motor neuron cell fate; administering to the subject at least one
agent that stimulates axonal outgrowth in the central nervous system (CNS); and
30 administering to the subject a tropic factor, thereby increasing motor function in a subject.

In another aspect, the invention features a method of treating or preventing a
condition characterized by a reduction in motor neuron function in a subject (e.g., a human)
in need thereof. The method involves administering to the subject a cell that expresses a
motor neuron specific marker; administering to the subject at least one agent that stimulates

axonal outgrowth in the central nervous system (CNS); and administering to the subject a tropic factor, thereby increasing motor function in a subject.

In yet another aspect, the invention features a method of treating spinal muscular atrophy in a subject (e.g., a human) in need thereof. The method involves identifying the
5 subject as having spinal muscular atrophy; and administering to the subject a cell that expresses a motor neuron specific marker, thereby increasing motor function in a subject. In one embodiment, the cell is an embryonic stem cell contacted with dibutyl cyclic adenosine monophosphate prior to administration. In another embodiment, the method further involves the step of administering rolipram to the subject. In yet another embodiment, the cell is
10 contacted with retinoic acid and a chemical agonist of Shh (e.g., HhAg1.3) prior to administration to the subject. In yet another embodiment, the cell is contacted with one or more of Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, or Ciliary Neurotrophic Factor prior to administration to the subject. In yet another embodiment, the method further involves the step of administering cyclosporine, tacrolimus, or rapamycin to the subject. In
15 yet another embodiment, the method increases the number of functioning motor units (e.g., increases motor unit number estimation (MUNE) by 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100), increases the amplitude of individual motor unit, or increases the function of a limb or other organ relative to an untreated control animal.

In another aspect, the invention provides a pharmaceutical pack containing an isolated
20 cell (e.g., embryonic stem cell, neural stem cell, or a motor neuron precursor) capable of adopting a motor neuron cell fate and an agent that stimulates axonal outgrowth and/or a tropic factor. In one embodiment, the pack includes directions for using the pack to treat a condition characterized by a reduction in motor function.

In yet another aspect, the invention features a pharmaceutical pack containing an
25 agent that stimulates axonal outgrowth (e.g., a phosphodiesterase inhibitor, a functional analog of adenosine-3', 5'-cyclic monophosphate; a metabolic precursor of adenosine-3', 5'-cyclic monophosphate) and a tropic factor (e.g., glial cell derived neurotrophic factor (GDNF)). In one embodiment, the package contains an isolated cell (e.g., embryonic stem cell, neural stem cell, or a motor neuron precursor) capable of adopting a motor neuron cell
30 fate. In one embodiment, the agent that stimulates axonal outgrowth reduces myelin inhibition of axonal outgrowth. In another embodiment, the pack further contains one or more of Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, and/or Ciliary Neurotrophic Factor. In yet another embodiment, the pack further contains cyclosporine, rapamycin, or tacrolimus. In another embodiment, the pack contains directions for

identifying a patient in need of treatment (e.g., where the patient is identified as having spinal cord injury, neural avulsion, transverse myelitis, or spinal muscular atrophy; alternatively or in addition, the package further contains directions for measuring an increase in motor function.

5 In various embodiments of any of the above aspects, the cell (e.g., human or rodent cell) is an isolated embryonic stem cell, neural stem cell, or a motor neuron precursor. In other embodiments of the above aspects, the agent that stimulates axonal outgrowth acts by reducing myelin inhibition of axonal outgrowth. In other embodiments of the above aspects, the agent is selected from the group consisting of a phosphodiesterase inhibitor, a functional
 10 analog of adenosine-3', 5'-cyclic monophosphate (e.g., adenosine-3',5'-cyclic monophosphate, N⁶-Benzoyladenosine-3',5'-cyclic monophosphate, 8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate, 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate, 8-(6-Aminohexyl)aminoadenosine-3',5'-cyclic monophosphate, 8-Bromoadenosine 3',5'-cyclic monophosphate, 8-Chloroadenosine 3',5'-cyclic-
 15 monophosphate, 8-Methylaminoadenosine-3',5'-cyclic monophosphate, 8-Piperidinoadenosine-3',5'-cyclic monophosphate, N⁶,2'-O-Dibutyryladenosine-3',5'-cyclic monophosphate, N⁶,2'-O-Disuccinyladenosine-3',5'-cyclic monophosphate, 8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer, Sp-Adenosine 3',5'-cyclic monophosphorothioate triethylammonium or a functional derivative thereof) and
 20 a metabolic precursor of adenosine-3', 5'-cyclic monophosphate (e.g., adenosine-3',5'-cyclic monophosphate acetoxymethyl ester, adenosine-3',5'-cyclic monophosphorothioate acetoxymethyl ester, or a derivative thereof). In one embodiment, the cell is contacted with dibutyryl cyclic adenosine monophosphate prior to administration. In another embodiment of the above aspects, two agents of the invention are administered to the subject. In still other
 25 embodiments, one of the agents is dibutyryl cyclic adenosine monophosphate and one of the agents is rolipram. In still other embodiments of the above aspects, the agent is administered by intraspinal infusion. In still other embodiments of the above aspects, the agent is administered by systemic administration. In still other embodiments of the above aspects, the tropic factor is administered within the peripheral nervous system. In still other embodiments
 30 of the above aspects, the tropic factor is glial cell derived neurotrophic factor (GDNF). In still other embodiments of the above aspects, the tropic factor is expressed by a transplanted cell. In still other embodiments of the above aspects, the cell is contacted with retinoic acid and a chemical agonist of Shh prior to administration to a subject. In still other embodiments of the above aspects, the chemical agonist is HhAg1.3. In still other embodiments of the

above aspects, the cell is contacted with at least one of Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, or Ciliary Neurotrophic Factor prior to administration to the subject. In still other embodiments of the above aspects, the cell is administered together with one or more of Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, and Ciliary Neurotrophic Factor. In still other embodiments of the above aspects, the method further involves administering to the subject a compound that inhibits rejection of a transplanted cell. In still other embodiments of the above aspects, the compound is cyclosporine, tacrolimus, or rapamycin. In still other embodiments of the above aspects, the cell is present in a population of injected cells. In still other embodiments of the above aspects, at least 50%, 75%, 80%, 85%, 90%, 95%, or 100% of the administered cells express a motor neuron marker. In still other embodiments of the above aspects, the cell survives within the gray matter of the spinal cord for at least 3 months after administration to the subject. In still other embodiments of the above aspects, the surviving cell expresses a presynaptic marker. In still other embodiments of the above aspects, the presynaptic marker is synaptophysin, synaptobrevin, or SV2. In still other embodiments of the above aspects, the cell forms a synapse on a sensory or interneuron. In still other embodiments of the above aspects, the cell extends an axon into the white matter of the spinal cord. In still other embodiments of the above aspects, the motor neuron specific marker is selected from the group consisting of GFR α 1. In still other embodiments of the above aspects, the method increases the number of cell derived axons that reach the peripheral nervous system relative to the number in an untreated control animal, or the number of axons that reach a skeletal muscle target relative to an untreated control animal, or the number of neuromuscular junctions between transplanted cell-derived axons and host-derived muscle relative to an untreated control animal, or the number of functioning motor units or the amplitude of individual motor unit relative to an untreated control animal, or the function of a limb or other organ relative to an untreated control animal. In still other embodiments of the previous aspects, the condition is spinal cord injury, neural avulsion, transverse myelitis, or spinal muscular atrophy. In yet other embodiments, the method further involves the step of administering cyclosporine, tacrolimus, and/or rapamycin to the subject. In still other embodiments of the above aspects, the method increases the number of functioning motor units, increases the amplitude of individual motor unit, or increases the function of a limb or other organ relative to an untreated control animal. In still other embodiments of the above aspects, the cell is contacted with one or more of Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, or Ciliary Neurotrophic Factor prior to administration to the subject. In yet another embodiment, the method further

involves the step of administering cyclosporine, tacrolimus, or rapamycin to the subject. In yet another embodiment, the method increases the number of functioning motor units (e.g., increases motor unit number estimation (MUNE) by 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100), increases the amplitude of individual motor unit, or increases the function of a limb or other organ relative to an untreated control animal. Any of the aforementioned therapeutic agents may be administered alone or in combination, (e.g., two, three, four, or more), where one therapeutics is administered prior to, concurrently with, or following administration of the other(s). The administration is, preferably, within one, two, three or four days of one another, or within one, two, three, or four weeks of one another.

The invention provides cellular and pharmacological compositions to enhance functional recovery following spinal cord injury or motor neuron disease. Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Definitions

By "agent" is meant a polypeptide, peptide, nucleic acid molecule, small molecule, or mimetic.

By "analog" is meant an agent having structural or functional homology to a reference agent.

By "myelin inhibition" is meant any activity of myelin that slows or decreases axonal outgrowth. *In vitro* and *in vivo* assays for axonal outgrowth are known in the art and are described herein.

By "cell capable of adopting a motor neuron cell fate" is meant a cell (or cell derived therefrom) that (a) when grown *in vitro* or *in vivo* expresses at least one marker characteristic of a motor neuron; (b) has at least one morphological characteristic associated with a motor neuron; or (c) has at least one biological activity characteristic of a motor neuron. Motor neuron markers include, but are not limited to, choline acetyl transferase, synaptobrevin, synaptophysin, neurofibromin (NF), SV2, or expression of a reporter under HB9 promoter regulation. Morphological characteristics associated with a motor neuron include the extension of an axon or the formation of neuromuscular junction. Motor neuron biological activity includes, but is not limited to, the formation of a neuromuscular junction, synaptic activity, the innervation of a target tissue, and the generation of motor activity.

By "central nervous system" (CNS) is meant the brain or spinal cord, and cellular or molecular components thereof, including the extracellular materials and fluids.

By "central nervous system disease or injury" is meant any disease, disorder, or trauma that disrupts the normal function or connectivity of the brain or spinal cord.

By "control" is meant a standard or reference condition.

By "functional analog of adenosine-3', 5'-cyclic monophosphate" is meant any agent
5 capable of functionally or biochemically substituting for adenosine-3', 5'-cyclic monophosphate.

By "precursor of adenosine-3', 5'-cyclic monophosphate" is meant an agent from which adenosine-3', 5'-cyclic monophosphate can be biochemically derived.

By "disease" is meant any condition or disorder that damages or interferes with the
10 normal function of a cell, tissue, or organ.

By "effective amount" is meant the amount of an agent required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of an active therapeutic agent used to practice the present invention for the treatment of a CNS disease or injury varies depending upon the manner of administration, the age, body weight, and general
15 health of the subject. Ultimately, the attending clinician will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

By "enhancing axonal outgrowth" is meant increasing the number of axons or the distance of extension of axons relative to a control condition. Preferably the increase is by at least 2-fold, 2.5-fold, 3-fold or more.

By "fragment" is meant a portion of a polypeptide that has at least 50% of the
20 biological activity of the polypeptide from which it is derived. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment of a polypeptide or nucleic acid molecule may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600,
25 700, 800, 900, or 1000 nucleotides or amino acids.

By "modifies" is meant alters. In the context of the invention, an agent that modifies a cell, substrate, or cellular environment produces a biochemical alteration in a component (e.g., polypeptide, nucleotide, or molecular component) of the cell, substrate, or cellular environment.

By "motor function" is meant a biological activity mediated by a motor neuron. In
30 one embodiment, motor function is measured electrophysiologically (e.g., MUNE), by assaying muscle function, in a behavioral or functional assay, or using any other clinical parameter known in the art.

By "neuron" is meant any nerve cell derived from the nervous system of a mammal. In one embodiment, the neuron is a motor neuron.

By "phosphodiesterase inhibitor" is meant any agent that reduces the activity of a phosphodiesterase. In one embodiment, the phosphodiesterase inhibitor inhibits
5 phosphodiesterase type 4. Rolipram is an exemplary PDE type 4 inhibitor.

By "restorative CNS surgery" is meant any procedure carried out on the central nervous system to enhance neurological function.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

10 By "therapeutic delivery device" is meant any device that provides for the release of a therapeutic agent. Exemplary therapeutic delivery devices include osmotic pumps, indwelling catheters, and sustained-release biomaterials.

By "tropic factor" is meant any attractive cue.

By "variant" is meant an agent having structural homology to a reference agent but
15 varying from the reference in its biological activity. Variants provided by the invention include optimized amino acid and nucleic acid sequences that are selected using the methods described herein as having one or more desirable characteristics.

As used herein, the terms "treat," "treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that,
20 although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

As used herein, the terms "prevent," "preventing," "prevention," "prophylactic treatment" and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or
25 condition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1H provide an immunohistochemical analysis of spinal cord sections showing the survival of embryonic stem (ES) cell-derived motor neurons in the spinal cord
30 of paralyzed adult rats. Figure 1A shows lumbar spinal cord sections probed with a green fluorescent protein (GFP) antibody to define transplant-derived motor neurons and counterstained with Nissl red to identify all motor neurons within the ventral gray matter. Three months after transplantation, rats were killed and spinal cords were isolated for immunohistochemical analysis. Figure 1B shows an immunohistochemical analysis of

transplanted animals at 3 months after transplantation was performed to identify whether
transplant-derived motor neurons continued to express HB9 and GFP under control of the
HB9 promoter. Figure 1C excludes the possibility that cellular fusion had occurred and
accounted for the immunoreactivity was examined using a rat-specific motor neuron marker
5 (MO-1). An asterisk denotes a transplant-derived motor neuron, whereas the arrow denotes a
host motor neuron. Figure 1D is a section showing that transplant-derived axonal projections
were synaptophysin-positive (Syn^+) and terminated on GFP^- neurons that were
neurofilament-positive (NF^+), but choline acetyltransferase-negative (ChAT^-). Figure 1E
shows GFP^- axonal projections that were both Syn^+ and synaptobrevin-positive (SynB^+) also
10 terminated on transplant derived GFP^+ motor neurons. Figures 1F-1H show that in the
presence of inhibitors of myelin-mediated axonal repulsion, transplant-derived axons that
were NF^+ exited the spinal gray matter into surrounding white matter

Figures 2A-2F show that glial cell-derived neurotrophic factor (GDNF)-secreting
cells survive in the sciatic nerve of transplanted hosts for 6 months after transplantation.

15 Figures 2A and 2B show an immunohistochemical analysis of transplanted cells six months
after transplantation. At the time of embryonic stem (ES) cell transplantation, C17.2-GDNF
or C17.2-Bleo cells were cotransplanted into the sciatic nerves of paralyzed rats.

Immunohistochemical analysis at 6 months after transplantation showed clusters of GDNF-
secreting cells only in animals transplanted with C17.2-GDNF cells. Figure 2C shows

20 immunohistochemical analysis of sciatic nerve segments using GDNF and mouse-specific
(M2) antibodies. These studies showed that the source of GDNF after transplantation is the
C17.2-GDNF cells. Figure 2D shows that C17.2-Bleo cells also could be identified using M2
immunoreactivity. Figure 2E is a graph that shows the distribution of C17.2-Bleo or C17.2-
GDNF cells along the length of the sciatic nerve was assessed and quantitated by unbiased
25 sampling of mouse-specific (M2) immunoreactivity. The arrow denotes the site of injection
of cells into the sciatic nerve. Figure 2F is a graph showing the number of GFP^+ axons
scored for each animal ($n = 5$). The means and standard error of the means of each group are
shown. dbcAMP = dibutyryl cyclic adenosine monophosphate. Ventral roots were harvested
from transplanted animals and were subjected to confocal microscopy using neurofilament
30 (NF) and green fluorescent protein (GFP) antibodies.

Figures 3A-3I provide an immunohistochemical analysis of embryonic stem (ES)
cell-derived motor axons that reach skeletal muscle targets at 3 months after transplantation.
Figures 3A and 3B show that in Group 3 animals neurofilament-positive (NF^+) and green
fluorescent protein-positive (GFP^+) axons were identified within skeletal muscle, shown here

in the gastrocnemius muscle. Figures 3C-3E show GFP⁺ axons reaching skeletal muscle often showed clustering of acetylcholine receptors as defined by staining of skeletal muscle with rhodamine-conjugated bungarotoxin (Bgrotox). Figure 3F show GFP⁺ axons that were examined for the presence of the vesicular acetylcholine transporter (VaCHT) as a marker of synaptic maturity. Figures 3G and 3H show an immunohistochemical analysis of animals that were injected with cholera toxin B (CTB) into the quadriceps and gastrocnemius muscles. Two days later, animals were killed and spinal cords were investigated for colocalization of GFP, choline acetyltransferase (ChAT), and the retrogradely transported CTB. Arrowheads denote transplant-derived motor neurons that were retrogradely labeled; asterisks denote a host motor neuron that was retrogradely labeled with CTB. Figure 3I shows a dashed line that denotes the gray/white matter junction.

Figure 4A-4E show the formation of neuromuscular junctions (NMJs) composed of transplant-derived axons and host skeletal muscle at 6 months after transplantation. Figure 4A shows proximal (Figure 4A; quadriceps) and distal (Figure 4B; gastrocnemius) skeletal muscle were examined for the presence of NMJs composed of transplant-derived axons and host skeletal muscle. Figure 4B (left panel) shows a collapsed Z-stack montage. Figure 4B (right panel) shows a single 1 μ M confocal images with orthogonal representations (y- and z-planes, arrows) confirming the apposition of transplant-derived motor axons and host-derived postsynaptic specializations. Figures 4C-4E show sections through skeletal muscle that was harvested from limb skeletal muscle (gastrocnemius) at 6 months after transplantation. GFP⁺ axons colocalized with synaptobrevin (SynB). Figure 4D shows GFP⁺ axons colocalized with synaptophysin (Syn). Figure 4E shows GFP⁺ axons colocalized with Syn and SV2.

Figures 5A-5E show electrophysiological and behavioral analysis of transplanted animals at 6 months after transplantation demonstrating electrophysiological and functional recovery. Figure 5A shows motor unit number estimation (MUNE) of animals in Groups 4 (left), 5 (middle), and 3 (right) at 6 months after transplantation. Five to six animals in each group were examined electrophysiologically at 12 days and again at 120 days after transplantation. Each line represents the change of a single animal over that time period and the score for each group is presented at the top right of each graph. Figure 5B shows a single motor unit action potential (SMUP) as defined for the same animals in the same groups. Figure 5C is a graph showing the weight of Animals in Groups 3 to 5 as a function of time. Animals in these groups were weighed weekly after transplantation, and the group means were plotted for each group. Figure 5D shows that nine animals each from Groups 3 to 5 were scored blindly for functional recovery, defined by improvement in hind-limb grip

strength. Figure 5E is a graph showing the percentage of motor recovery exhibited by a cohort of animals transplanted with embryonic stem (ES) cell-derived motor neurons in the spinal cord bilaterally and C17.2-GDNF cells unilaterally. Animals were followed for 24 weeks and were assessed blindly for functional recovery as defined by the ability to flex the proximal leg under the animal and to push off with the foot.

DETAILED DESCRIPTION OF THE INVENTION

The invention generally features compositions and methods that are useful for treating conditions associated with a loss in motor neuron function. The invention is based, at least in part, on the observation that motor neuron function can be restored to a subject (e.g., a paralysed subject) by administering to the subject a cell having the ability to adopt a motor neuron cell fate (e.g., an embryonic stem cell treated with retinoic acid and a sonic hedge hog (SHH) agonist) in combination with a compound that enhances axonal outgrowth in the presence of myelin (e.g., a phosphodiesterase type 4 inhibitor, such as rolipram and dibutyryl cyclic adenosine monophosphate) and a tropic factor (e.g., a glial cell-derived neurotrophic factor (GDNF)) to facilitate axonal targeting. Surprisingly, cells transplanted into the gray matter of the spinal cord extended axons into the ventral roots, into the spinal white matter, and, ultimately, transplant-derived axons reached muscle targets where they formed neuromuscular junctions that were physiologically active, and mediated partial recovery from paralysis. Thus, the present invention provides the first reported anatomical and functional replacement of a motor neuron circuit within the adult mammalian host.

Therapeutic Indications

Methods of the invention address a long felt need for therapeutics useful for restoring neural function following traumatic injury of the CNS or spinal cord, motor neuron disease or, or any condition or disorder related to a loss of motor neuron function. In this regard, the therapeutic methods described herein are particularly useful for the treatment of motor neuron diseases, such as spinal muscular atrophy (SMA), amyotrophic lateral sclerosis (ALS), and transverse myelitis. SMA and ALS are fatal diseases that progressively paralyze the body, while leaving the mind intact and aware. Both of these diseases are comparatively frequent, with an annual incidence of 3 per 100,000 reported for ALS and a frequency of one in 10,000 live births observed for SMA. No cure, and little in the way of effective treatment, currently exists for these diseases. Transverse myelitis is a neurological disorder caused by inflammation affecting a segment of the spinal cord. The spinal inflammation can damage or

destroy myelin, the fatty insulating substance that covers nerve cell fibers causing scars that interrupt communications between the nerves in the spinal cord and the rest of the body.

Symptoms of transverse myelitis include a loss of spinal cord function over several hours to several weeks. Depending on the level of the spinal cord affected, patients suffering from transverse myelitis may experience loss of neural function in the neck, arms, hands, diaphragm, bowel and bladder.

Other diseases that can be treated using methods and compositions of the invention include injury to the brain and spinal cord due to trauma, ischemia, hypoxia, cerebral palsy, neurodegenerative disease, infectious disease, cancer, autoimmune disease and metabolic disorder. With respect to CNS trauma, trauma can involve a tissue insult such as an abrasion, incision, contusion, puncture, or compression. Such injuries can arise from traumatic contact of a foreign object with the head, neck, or vertebral column. Other forms of traumatic injury can arise from constriction or compression of the CNS tissue by an inappropriate accumulation of fluid (for example, a blockade or dysfunction of normal cerebrospinal fluid or vitreous humor fluid production, turnover, or volume regulation, or a subdural or intracranial hematoma or edema). Similarly, traumatic constriction or compression can arise from the presence of a mass of abnormal tissue, such as a metastatic or primary tumor. Exemplary CNS diseases or injuries include stroke, head trauma, spinal injury, hypotension, arrested breathing, cardiac arrest, Reye's syndrome, cerebral thrombosis, embolism, cerebral hemorrhage, brain tumors, encephalomyelitis, hydroencephalitis, operative and postoperative brain injury, Alzheimer's disease, Huntington's disease, Creutzfeld-Jakob disease, Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis. Thrombus, embolus, and systemic hypotension are the most common causes of cerebral ischemic episodes. Other causes of cerebral ischemia include hypertension, hypertensive cerebral vascular disease, rupture of an aneurysm, an angioma, blood dyscrasias, cardiac failure, cardiac arrest, cardiogenic shock, septic shock, head trauma, spinal cord trauma, seizure, bleeding from a tumor, or other blood loss. In other embodiments, the invention provides methods for rescuing neural connectivity related to genetic or congenital condition associated with a loss or reduction in motor function. Exemplary genetic conditions amenable to treatment with a method or composition described herein include spinal muscular atrophy and Spinal Bulbar Muscular Atrophy (SBMA), otherwise known as Kennedy's Disease.

Methods of the invention can be used to restore lost neuron function, regardless of the level of the spine at which the loss occurs. Transplantation of a cell capable of adopting a motor neuron cell fate can be made into the thoracic region of the spinal cord of a subject in

need thereof to restore function to the torso and some parts of the arms; transplantation into the lumbar region of the spinal cord can restore neural function of the hips and legs; and transplantation into the sacral region may rescue function in the groin, toes, and some parts of the legs. Scarring at one level of the spine will affect function at that level as well as at lower levels. In patients with transverse myelitis, for example, demyelination often occurs at the thoracic level. Damage at this level results in a loss of neural function in the legs, bowel and bladder. The present invention provides methods of treating CNS and motor neuron diseases and/or disorders or symptoms thereof which comprise administering a therapeutically effective amount of a pharmaceutical composition comprising a compound of the formulae herein to a subject (e.g., a mammal such as a human). Thus, in one embodiment, the invention provides is a method of treating a subject suffering from a spinal cord injury or motor neuron disease, or susceptible to such a disease or disorder or symptom thereof. The method includes the step of administering to the mammal an effective amount of a therapeutic composition (e.g., a cellular and/or pharmaceutical composition) described herein sufficient to treat the disease or disorder or symptom thereof, under conditions such that the disease or disorder is treated.

The methods herein include administering to the subject (including a subject identified as in need of such treatment) an effective amount of a composition (e.g., a cellular and/or pharmaceutical composition) described herein, or a composition described herein to produce such effect. Identifying a subject in need of such treatment can be in the judgment of a subject or a health care professional and can be subjective (e.g. opinion) or objective (e.g. measurable by a test or diagnostic method).

The therapeutic methods of the invention (which include prophylactic treatment) in general comprise administration of a therapeutically effective amount of the compounds herein, such as a composition (e.g., a cellular and/or pharmaceutical composition) of the formulae herein to a subject (e.g., animal, human) in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for a traumatic spinal cord injury or motor neuron disease, disorder, or symptom thereof. Determination of those subjects "at risk" can be made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider (e.g., genetic test, enzyme or protein marker, Marker (as defined herein), family history, and the like). The compounds herein may be also used in the treatment of any other disorders in which spinal cord injury or motor neuron disease may be implicated.

In one embodiment, the invention provides a method of monitoring treatment progress. The method includes the step of determining a level of diagnostic marker (Marker) (e.g., any target delineated herein modulated by a compound herein, a protein or indicator thereof, etc.) or diagnostic measurement (e.g., screen, assay) in a subject suffering from or
5 susceptible to a disorder or symptoms thereof associated with a traumatic spinal cord injury or motor neuron disease, in which the subject has been administered a therapeutic amount of a compound herein sufficient to treat the disease or symptoms thereof. The level of Marker determined in the method can be compared to known levels of Marker in either healthy
10 normal controls or in other afflicted patients to establish the subject's disease status. In preferred embodiments, a second level of Marker in the subject is determined at a time point later than the determination of the first level, and the two levels are compared to monitor the course of disease or the efficacy of the therapy. In certain preferred embodiments, a pre-treatment level of Marker in the subject is determined prior to beginning treatment according to this invention; this pre-treatment level of Marker can then be compared to the level of
15 Marker in the subject after the treatment commences, to determine the efficacy of the treatment.

Cellular and Pharmaceutical Compositions

The invention provides compositions useful in restoring motor neuron function to a
20 subject in need thereof. In particular, the invention provides combinations of cellular and pharmaceutical compositions useful for restoring motor neuron function to subjects have a reduction or loss of motor neuron function due to traumatic injury or disease. In one embodiment, the invention provides a cellular composition comprising a cell capable of adopting a motor neuron cell fate, such as human embryonic stem cells, a neural stem cell, or
25 a motor neuron precursor cell. In one preferred embodiment, the cellular composition comprises a human embryonic stem cell isolated using standard methods and induced to assume a motor neuron phenotype using methods known in the art and described herein. See also, for example, by Wichterle et al., Cell 110: 385-397, 2002; Soundararajan et al., J
Neurosci. 26(12):3256-68, 2006. In one embodiment, the stem cell (e.g., human embryonic
30 stem cell) is induced to assume a motor neuron fate by treating the cell with an agonist of sonic hedge hog, such as HhAg1.3 (Curis) and retinoic acid. Embryonic stem cells treated with these agents are capable of adopting a motor neuron cell fate, as characterized by the expression of motor neuron markers (choline acetyl transferase), by the extension of axons,

by the formation of neuromuscular junctions on a target muscle, and by the generation of electrophysiological activity.

Methods of the invention are useful for enhancing axonal outgrowth from the CNS to a target tissue or organ to restore function to the tissue or organ. The therapeutic efficacy of the methods of the invention can optionally be assayed by measuring (i) an increase in the number of transplanted cells having axons that reach the target tissue or organ; (ii) measuring an increase in the number of synapses or neuromuscular junctions formed with the target tissue or organ; or (iii) measuring an increase in function in the target tissue or organ as compared to a corresponding control tissue or organ (e.g., a tissue or organ that did not receive treatment). Methods for evaluating axonal outgrowth, synapse or neuromuscular junction number, or function are standard in the art and are described herein. The function of a target tissue or organ can be measured in any standard neurological or physiological assay. Strength measurements are the most straightforward way to assess reinnervation of target muscle. Muscle bulk, defined clinically or by MRI is another. Finally, electrophysiologic testing of the nerves can detect and quantify the number of functioning motor units to a limb muscle. Preferably, the number of axons that reach the target, synapses or neuromuscular junctions, or the function of the tissue or organ is increased by at least 5%, 10%, 20%, 40%, 60%, 80%, 100%, 150%, or 200% relative to a corresponding tissue or organ.

Administration

Compositions of the invention include cellular compositions comprising cells having the ability to adopt a motor neuron fate. Such cells are suitable for transplantation into the gray matter of the spine. Preferably, at least about 50%, 60%, 70%, or 75% of the cells present in a cellular composition of the invention are capable of adopting a motor neuron cell fate. More preferably, at least about 80%, 85%, 90%, 95% or even 100% of the cells present in the composition are capable of adopting a motor neuron cell fate. Induction of a motor neuron fate can be assayed by identifying the expression of one or more cell specific markers, by assaying motor neuron biological activity (e.g., formation of synapses, presence of neuromuscular junctions, electrophysiological activity, stimulation of a target muscle).

Cellular compositions of the invention can be provided directly to the spinal cord, for example, by surgical transplantation, by infusion, or by local or systemic injection (i.e. CSF or vascular delivery). In one embodiment, cells of the invention are provided to a site within the CNS where an increase in motor neuron function is desired, for example, due to disease, damage, injury, or cell death. Alternatively, cells of the invention are provided indirectly to

the CNS (e.g., spine, brain) for example, by administration into the circulatory system. If desired, the cells are delivered to a portion of the spine that innervates the tissue or organ where a restoration of neural function is desired. Advantageously, cells of the invention innervate the tissue or organ. If desired, trophic and/or differentiation agents (e.g., Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, Ciliary Neurotrophic Factor) are provided prior to, during or after administration of the cells to increase, maintain, or enhance cell survival or differentiation *in vivo*.

To facilitate axon pathfinding, tropic factors are provided at or near the target tissue or organ. In one embodiment, the tropic factor is expressed as a recombinant protein (e.g., a secreted protein) within a transplanted cell that is administered at the target site. In other embodiments, the tropic factor is released at the target site from an implanted device capable of releasing the factor at a more or less constant rate over the course of hours, days, weeks, or even months. Preferably, the tropic factor is present in an effective amount, i.e., an amount sufficient to attract the outgrowth of a motor neuron from the spine into the target tissue.

Compositions of the invention include pharmaceutical compositions comprising embryonic stem cells, neural stem cells, a motor neuron progenitor cell, or a cell expressing a tropic factor (e.g., a cell engineered to express a recombinant protein) and a pharmaceutically acceptable carrier. Administration of the cells can be autologous or heterologous. For example, cells obtained from one subject, can be administered to the same subject or a different, compatible subject. Methods for administering cells are known in the art, and include, but are not limited to, catheter administration, systemic injection, localized injection, intravenous injection, intraspinal injection or intramuscular administration). When administering a therapeutic composition of the present invention (e.g., a pharmaceutical composition), it will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion).

Cellular Compositions

Cellular compositions of the invention can be conveniently provided as sterile liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions, dispersions, or viscous compositions, which may be buffered to a selected pH. Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to administer, especially by injection. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with specific tissues. Liquid or viscous compositions

can comprise carriers, which can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like) and suitable mixtures thereof.

Sterile injectable solutions can be prepared by incorporating the cells utilized in practicing the present invention in the required amount of the appropriate solvent with various amounts of the other ingredients, as desired. Such compositions may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, dextrose, or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting, dispersing, or emulsifying agents (e.g., methylcellulose), pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts, such as "REMINGTON'S PHARMACEUTICAL SCIENCE", 17th edition, 1985, incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation.

Various additives which enhance the stability and sterility of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the cells of the invention or their progenitors.

The compositions can be isotonic, i.e., they can have the same osmotic pressure as spinal fluid, blood or lacrimal fluid. The desired isotonicity of the compositions of this invention may be accomplished using sodium chloride, or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

Viscosity of the compositions, if desired, can be maintained at the selected level using a pharmaceutically acceptable thickening agent. Methylcellulose is preferred because it is readily and economically available and is easy to work with. Other suitable thickening agents include, for example, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, carbomer, and the like. The preferred concentration of the thickener will depend upon the agent selected. The important point is to use an amount that will achieve the selected viscosity. Obviously, the choice of suitable carriers and other additives will depend on the

exact route of administration and the nature of the particular dosage form, e.g., liquid dosage form (e.g., whether the composition is to be formulated into a solution, a suspension, gel or another liquid form, such as a time release form or liquid-filled form).

A method to potentially increase cell survival when introducing the cells into a subject is to incorporate cells or their progeny (e.g., *in vivo*, *ex vivo* or *in vitro* derived cells) of interest into a biopolymer or synthetic polymer. Depending on the subject's condition, the site of injection might prove inhospitable for cell seeding and growth because of scarring or other impediments. Examples of biopolymer include, but are not limited to, cells mixed with fibronectin, fibrin, fibrinogen, thrombin, collagen, and proteoglycans. This could be constructed with or without included trophic or differentiation factors. Additionally, these could be in suspension, but residence time at sites subjected to flow would be nominal. Another alternative is a three-dimensional gel with cells entrapped within the interstices of the cell biopolymer admixture. Again, expansion or differentiation factors could be included with the cells. These could be deployed by injection via various routes described herein.

Exemplary agents that may be delivered together with a embryonic cell of the invention include, but are not limited to, Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, Ciliary Neurotrophic Factor. Other agents that may be delivered together with a cell of the invention include one or more of a sonic hedge hog agonist, retinoic acid, a phosphodiesterase type 4 inhibitor, dibutyryl cyclic adenosine monophosphate, or a derivative thereof.

Those skilled in the art will recognize that the polymeric components of the compositions should be selected to be chemically inert and will not affect the viability or efficacy of the transplanted cells as described in the present invention. This will present no problem to those skilled in chemical and pharmaceutical principles, or problems can be readily avoided by reference to standard texts or by simple experiments (not involving undue experimentation), from this disclosure and the documents cited herein.

Dosages

One consideration concerning the therapeutic use of cells capable of adopting a motor neuron fate or their progenitors or cells capable of secreting a tropic factor is the quantity of cells necessary to achieve an optimal effect. In general, doses ranging from 1 to 5×10^5 , 10^6 , or 10^7 cells may be used. However, different scenarios may require optimization of the amount of cells injected into a tissue of interest. Thus, the quantity of cells to be administered will vary for the subject being treated. In a preferred embodiment, between 10^3

to 10^9 , more preferably 10^5 to 10^7 , and still more preferably, $1, 2, 3, 4, 5, 6, 7 \times 10^7$ stem cells of the invention can be administered to a human subject. In a preferred embodiment, the number of cells is any integer between 250,000 and 10 million cells, where the bottom of the range is between 250,000 and 9,999,999, and the top of the range is between 251,000 and 10 million.

Fewer cells can be administered directly a tissue where an increase in cell number is desirable. Preferably, between 10^2 to 10^7 , more preferably 10^3 to 10^6 , and still more preferably, $1, 2, 3, 4, 5, 6, 7 \times 10^6$ cells or their progenitors can be administered to a human subject. However, the precise determination of what would be considered an effective dose may be based on factors individual to each subject, including their size, age, sex, weight, and condition of the particular subject. As few as 100, 1000, 10,000, or 100,000 cells can be administered for certain desired applications among selected patients. Therefore, dosages can be readily ascertained by those skilled in the art from this disclosure and the knowledge in the art.

A cell capable of adopting a motor neuron fate or their progenitors can comprise a purified population of such cells. As described herein, cells of the invention are identified by the expression of markers, by cellular morphology, or by biological activity. Those skilled in the art can readily determine the percentage of cells in a population using various well-known methods, such as fluorescence activated cell sorting (FACS). Preferable ranges of purity in populations comprising cells capable of adopting a motor neuron fate are about 50 to about 55%, about 55 to about 60%, and about 65 to about 70%. More preferably the purity is about 70 to about 75%, about 75 to about 80%, about 80 to about 85%; and still more preferably the purity is about 85 to about 90%, about 90 to about 95%, and about 95 to about 100%. In one embodiment, purity of cells capable of adopting a motor neuron fate or their progenitors can be determined according to the marker profile within a population. Dosages can be readily adjusted by those skilled in the art (e.g., a decrease in purity may require an increase in dosage).

The skilled artisan can readily determine the amount of cells and optional additives, vehicles, and/or carrier in compositions and to be administered in methods of the invention. Typically, any additives (in addition to the active stem cell(s) and/or agent(s)) are present in an amount of 0.001 to 50 % (weight) solution in phosphate buffered saline, and the active ingredient is present in the order of micrograms to milligrams, such as about 0.0001 to about 5 wt %; preferably about 0.0001 to about 1 wt %, still more preferably about 0.0001 to about 0.05 wt % or about 0.001 to about 20 wt %, preferably about 0.01 to about 10 wt %, and still

more preferably about 0.05 to about 5 wt %. Of course, for any composition to be administered to an animal or human, and for any particular method of administration, it is preferred to determine therefore: toxicity, such as by determining the lethal dose (LD) and LD₅₀ in a suitable animal model e.g., rodent such as mouse; and, the dosage of the composition(s), concentration of components therein and timing of administering the composition(s), which elicit a suitable response. Such determinations do not require undue experimentation from the knowledge of the skilled artisan, this disclosure and the documents cited herein. And, the time for sequential administrations can be ascertained without undue experimentation.

If desired, cells of the invention are delivered in combination with (prior to, concurrent with, or following the delivery of) agents that increase survival, enhance differentiation, and/or promote maintenance of a differentiated cellular phenotype. If desired, cells of the invention are delivered in combination with other factors that promote cell survival, differentiation, or axonal outgrowth. Such factors, include but are not limited to trophic factors, nutrients, growth factors, agents that induce differentiation, agents that enhance axonal outgrowth or pathfinding, agents that reduce an inhibitory effect of myelin, products of secretion, immunomodulators, inhibitors of inflammation, hormones, or other biologically active compounds. Trophic agents include growth factors that are known in the art to increase the survival of a motor neuron. Such factors, include those described in U.S. Patent Nos. 5,750,376 and 5,851,832, which describe methods for the *in vitro* culture and proliferation of neural stem cells. The contents of each of these references are specifically incorporated herein by reference for their description of expansion agents known in the art.

In vitro and *ex vivo* applications of the invention involve the culture of cells of the invention or their progenitors with a selected agent to achieve a desired result. Cultures of cells (from the same individual and from different individuals) can be treated with trophic or differentiation agents prior to, during, or following administration to a subject. Similarly, differentiation agents of interest can be used to direct the differentiation of an embryonic stem cell or motor neuron precursor cell, which can then be used for a variety of therapeutic applications.

Pharmaceutical Compositions

Compositions of the invention include pharmaceutical compositions that are, if desired, administered in combination with a cell of the invention. Pharmaceutical compositions of the invention include a phosphodiesterase type 4 inhibitor, a dibutyryl cyclic

adenosine monophosphate, and/or cyclosporine, FK506 (tacrolimus), and/or rapamycin. In one embodiment, the composition comprises a functional analog of adenosine-3', 5'-cyclic monophosphate or a metabolic precursor of adenosine-3', 5'-cyclic monophosphate. In another embodiment, the adenosine-3',5'-cyclic monophosphate is adenosine-3',5'-cyclic monophosphate, N⁶-Benzoyladenosine-3',5'-cyclic monophosphate, 8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate, 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate, 8-(6-Aminohexyl)aminoadenosine-3',5'-cyclic monophosphate, 8-Bromoadenosine 3',5'-cyclic monophosphate, 8-Chloroadenosine 3',5'-cyclic-

monophosphate, 8-Methylaminoadenosine-3',5'-cyclic monophosphate, 8-Piperidinoadenosine-3',5'-cyclic monophosphate, N⁶,2'-O-Dibutyryladenosine-3',5'-cyclic monophosphate, N⁶,2'-O-Disuccinyladenosine-3',5'-cyclic monophosphate, 8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer, Sp-Adenosine 3',5'-cyclic monophosphorothioate triethylammonium, or a derivative thereof. In another embodiment, the metabolic precursor of adenosine-3', 5'-cyclic monophosphate is adenosine-3',5'-cyclic monophosphate acetoxymethyl ester, adenosine-3',5'-cyclic monophosphorothioate acetoxymethyl ester, or a derivative thereof. Such agents can be provided directly to an organ of interest, such as an organ requiring an increase in neural function. Alternatively, compositions can be provided indirectly to the organ of interest, for example, by administration into the circulatory system to achieve a therapeutic effect.

Compositions can be administered to subjects in need thereof by a variety of administration routes. Methods of administration, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include intramuscular, intra-spinal, oral, rectal, topical, intraocular, buccal, intravaginal, intracisternal, intracerebroventricular, intratracheal, nasal, transdermal, parenteral, within/on implants, e.g., fibers, such as collagen, osmotic pumps, or grafts comprising cells. The term "parenteral" includes subcutaneous, intravenous, intramuscular, intraperitoneal, intragonadal or infusion. A particular method of administration involves coating, embedding or derivatizing fibers, such as collagen fibers, protein polymers, etc. with therapeutic proteins. Other useful approaches are described in Otto, D. et al., J. Neurosci. Res. 22: 83 and in Otto, D. and Unsicker, K. J. Neurosci. 10: 1912.

Screening Assays

The invention provides methods for identifying modulators, i.e., candidate or test compounds or agents (e.g., proteins, peptides, peptidomimetics, peptoids, polynucleotides, small molecules or other agents) which enhance cell survival, differentiation, axonal extension, axonal pathfinding, or neuromuscular junction formation. Agents thus identified can be used to modulate a therapeutic protocol.

The test agents of the present invention can be obtained singly or using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, e.g., Zuckermann, R.N. (1994) et al., J. Med. Chem. 37:2678-85); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) Anticancer Drug Des. 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:6909; Erb et al. (1994) Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann et al. (1994) J. Med. Chem. 37:2678; Cho et al. (1993) Science 261:1303; Carrell et al. (1994) Angew. Chem. Int. Ed. Engl. 33:2059; Carrell et al (1994) Angew. Chem. Int. Ed. Engl. 33:2061; and Gallop et al. (1994) J. Med. Chem. 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992), Biotechniques 13:412-421), or on beads (Lam (1991), Nature 354:82-84), chips (Fodor (1993) Nature 364:555-556), bacteria (Ladner, U.S. Patent No. 5,223,409), spores (Ladner U.S. Patent No. 5,223,409), plasmids (Cull et al. (1992) Proc Natl Acad Sci USA 89:1865-1869) or on phage (Scott and Smith (1990) Science 249:386-390; Devlin (1990) Science 249:404-406; Cwirla et al. (1990) Proc. Natl. Acad. Sci. 87:6378-6382; Felici (1991) J. Mol. Biol. 222:301-310; Ladner supra.).

Chemical compounds to be used as test agents (i.e., potential inhibitor, antagonist, agonist) can be obtained from commercial sources or can be synthesized from readily available starting materials using standard synthetic techniques and methodologies known to those of ordinary skill in the art. Synthetic chemistry transformations and protecting group

methodologies (protection and deprotection) useful in synthesizing the compounds identified by the methods described herein are known in the art and include, for example, those such as described in R. Larock (1989) *Comprehensive Organic Transformations*, VCH Publishers; T. W. Greene and P. G. M. Wuts, *Protective Groups in Organic Synthesis*, 2nd ed., John Wiley and Sons (1991); L. Fieser and M. Fieser, *Fieser and Fieser's Reagents for Organic Synthesis*, John Wiley and Sons (1994); and L. Paquette, ed., *Encyclopedia of Reagents for Organic Synthesis*, John Wiley and Sons (1995), and subsequent editions thereof.

Test agents of the invention can also be peptides (e.g., growth factors, cytokines, receptor ligands). Screening methods of the invention can involve the identification of an agent that increases the survival of cells or the progenitors thereof, differentiation, axonal extension, axonal pathfinding, or neuromuscular junction formation. Such methods will typically involve contacting a population of the cells with a test agent in culture or in a test animal and quantitating the number, length, or function of axons extended as a result. Comparison to an untreated control can be concurrently assessed. Where an increase in the number or length of axons extended to the target is detected relative to the control, the test agent is determined to have the desired activity.

In practicing the methods of the invention, it may be desirable to employ a purified population of cells or the progenitors thereof. A purified population of cells have about 50%, 55%, 60%, 65% or 70% purity. More preferably the purity is about 75%, 80%, or 85%; and still more preferably the purity is about 90%, 95%, 97%, or even 100%.

Increased amounts of cells or the progenitors thereof can also be detected by an increase in gene expression of genetic markers.

Differentiation is detected by assaying increases in expression of cell specific markers that are not typically expressed in the cell from which the cell of the invention is derived. An increase in the expression of a cell specific marker may be by about 5%, 10%, 25%, 50%, 75% or 100%. For example, a neuronal cell is detected by assaying for neuronal markers, such as motor neuron markers. The level of expression can be measured in a number of ways, including, but not limited to: measuring the mRNA encoded by the markers; measuring the amount of protein encoded by the markers; or measuring the activity of the protein encoded by the markers.

The level of mRNA corresponding to a marker can be determined both by *in situ* and by *in vitro* formats. The isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, polymerase chain reaction analyses and probe arrays. One diagnostic method for the detection of mRNA levels involves

contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to the mRNA encoded by the gene being detected. The nucleic acid probe is sufficient to specifically hybridize under stringent conditions to mRNA or genomic DNA. The probe can be disposed on an address of an array, e.g., an array described below. Other suitable probes
5 for use in the diagnostic assays are described herein.

In one format, mRNA (or cDNA) is immobilized on a surface and contacted with the probes, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative format, the probes are immobilized on a surface and the mRNA (or cDNA) is contacted with the probes,
10 for example, in a two-dimensional gene chip array described below. A skilled artisan can adapt known mRNA detection methods for use in detecting the level of mRNA encoded by the genetic markers described herein.

The level of mRNA in a sample can be evaluated with nucleic acid amplification, e.g., by rtPCR (Mullis (1987) U.S. Patent No. 4,683,202), ligase chain reaction (Barany (1991) Proc. Natl. Acad. Sci. USA 88:189-193), self sustained sequence replication (Guatelli et al. (1990) Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh et al. (1989) Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi et al. (1988) Bio/Technology 6:1197), rolling circle replication (Lizardi et al., U.S. Patent No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the
20 amplified molecules using techniques known in the art. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. In general, amplification primers are from about 10 to 30 nucleotides in length and flank a region from about 50 to 200 nucleotides in length. Under appropriate
25 conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

For *in situ* methods, a cell or tissue sample can be prepared/processed and immobilized on a support, typically a glass slide, and then contacted with a probe that can hybridize to mRNA that encodes the genetic marker being analyzed. In other embodiments,
30 adoption of a motor neuron fate is detected by measuring an alteration in the morphology or biological function of the cell as described in the Examples.

Expression of Recombinant Proteins

In another approach, a cell of the invention may be engineered to express a gene of whose expression promotes axonal pathfinding (e.g., a tropic factor), cell survival, neuronal differentiation, maintenance of a motor neuron cellular phenotype, or a factor that otherwise enhances the function of a transplanted motor neuron. In one embodiment, the cell is a stem cell derived motor neuron that overexpresses the receptor for GDNF (GFR α 1) so that the emerging axons are more sensitive to GDNF gradients. In another embodiment, the cell is a stem cell derived motor neuron that expresses a recombinant membrane bound semaphorin (i.e. Sema 3A) on the stem cell derived motor neuron. In another embodiment, a plexin (i.e. Plexin 2D) is administered or expressed in a cell transplanted in the peripheral nervous system as an attractive cue. Preferably, the cell expresses a tropic factor that is secreted within a target tissue, such as a muscle tissue. The gene of interest may be constitutively expressed or its expression may be regulated by an inducible promoter or other control mechanism where conditions necessitate highly controlled regulation or timing of the expression of a protein, enzyme, or other cell product. Such cells, when transplanted into a subject produce high levels of the protein to confer a therapeutic benefit. For example, the cell of the invention, its progenitor or its *in vitro*-derived progeny, can contain heterologous DNA encoding genes to be expressed, for example, in gene therapy. Insertion of one or more pre-selected DNA sequences can be accomplished by homologous recombination or by viral integration into the host cell genome. The desired gene sequence can also be incorporated into the cell, particularly into its nucleus, using a plasmid expression vector and a nuclear localization sequence. Methods for directing polynucleotides to the nucleus have been described in the art. The genetic material can be introduced using promoters that will allow for the gene of interest to be positively or negatively induced using certain chemicals/drugs, to be eliminated following administration of a given drug/chemical, or can be tagged to allow induction by chemicals, or expression in specific cell compartments.

Calcium phosphate transfection can be used to introduce plasmid DNA containing a target gene or polynucleotide into a cells and is a standard method of DNA transfer to those of skill in the art. DEAE-dextran transfection, which is also known to those of skill in the art, may be preferred over calcium phosphate transfection where transient transfection is desired, as it is often more efficient. Since the cells of the present invention are isolated cells, microinjection can be particularly effective for transferring genetic material into the cells. This method is advantageous because it provides delivery of the desired genetic material directly to the nucleus, avoiding both cytoplasmic and lysosomal degradation of the injected

polynucleotide. Cells of the present invention can also be genetically modified using electroporation.

Liposomal delivery of DNA or RNA to genetically modify the cells can be performed using cationic liposomes, which form a stable complex with the polynucleotide. For
5 stabilization of the liposome complex, dioleoyl phosphatidylethanolamine (DOPE) or dioleoyl phosphatidylcholine (DOPQ) can be added. Commercially available reagents for liposomal transfer include Lipofectin (Life Technologies). Lipofectin, for example, is a mixture of the cationic lipid N-[1-(2, 3-dioleoyloxy)propyl]-N-N-N- trimethyl ammonia chloride and DOPE. Liposomes can carry larger pieces of DNA, can generally protect the
10 polynucleotide from degradation, and can be targeted to specific cells or tissues. Cationic lipid- mediated gene transfer efficiency can be enhanced by incorporating purified viral or cellular envelope components, such as the purified G glycoprotein of the vesicular stomatitis virus envelope (VSV-G). Gene transfer techniques which have been shown effective for delivery of DNA into primary and established mammalian cell lines using lipopolyamine-coated DNA can be used to introduce target DNA into the de-differentiated cells or
15 reprogrammed cells described herein.

Naked plasmid DNA can be injected directly into a tissue comprising cells of the invention (e.g., de-differentiated or reprogrammed cells). This technique has been shown to be effective in transferring plasmid DNA to skeletal muscle tissue, where expression in
20 mouse skeletal muscle has been observed for more than 19 months following a single intramuscular injection. More rapidly dividing cells take up naked plasmid DNA more efficiently. Therefore, it is advantageous to stimulate cell division prior to treatment with plasmid DNA. Microprojectile gene transfer can also be used to transfer genes into cells either in vitro or in vivo. The basic procedure for microprojectile gene transfer was described
25 by J. Wolff in Gene Therapeutics (1994), page 195. Similarly, microparticle injection techniques have been described previously, and methods are known to those of skill in the art. Signal peptides can be also attached to plasmid DNA to direct the DNA to the nucleus for more efficient expression.

Viral vectors are used to genetically alter cells of the present invention and their
30 progeny. Viral vectors are used, as are the physical methods previously described, to deliver one or more target genes, polynucleotides, antisense molecules, or ribozyme sequences, for example, into the cells. Viral vectors and methods for using them to deliver DNA to cells are well known to those of skill in the art. Examples of viral vectors that can be used to genetically alter the cells of the present invention include, but are not limited to, adenoviral

vectors, adeno-associated viral vectors, retroviral vectors (including lentiviral vectors), alphaviral vectors (e. g., Sindbis vectors), and herpes virus vectors.

Peptide or protein transfection is another method that can be used to genetically alter de-differentiated cells or reprogrammed cells of the invention and their progeny. Peptides
5 such as Pep-1 (commercially available as Chariot™), as well as other protein transduction domains, can quickly and efficiently transport biologically active proteins, peptides, antibodies, and nucleic acids directly into cells, with an efficiency of about 60% to about 95% (Morris, M.C. et al, (2001) Nat. Biotech. 19: 1173-1176).

10 Kits or Pharmaceutical Systems

The present compositions may be assembled into kits or pharmaceutical systems for use in ameliorating a condition related to a reduction in neural function, such as spinal cord injury, or motor neuron disease. Therapeutic compositions of the invention may include one or more of: a cell capable of adopting a motor neuron fate, a phosphodiesterase type 4
15 inhibitor, dibutyryl cyclic adenosine monophosphate, a trophic factor such as Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, and Ciliary Neurotrophic Factor, and a tropic factor, such as glial cell derived neurotrophic factor, and/or cyclosporine. Cellular or pharmaceutical compositions of the invention may be supplied as agents in a kit. Kits or pharmaceutical systems according to this aspect of the invention comprise a carrier means,
20 such as a box, carton, tube or the like, having in close confinement therein one or more container means, such as vials, tubes, ampules, bottles and the like. The kits or pharmaceutical systems of the invention may also comprise associated instructions for using the agents of the invention. If desired, the instructions further include directions for carrying out a diagnostic assay (e.g., an assay to identify spinal muscular atrophy, amyotrophic lateral sclerosis, or transverse myelitis). The instructions provided in a kit according to the
25 invention may be directed to suitable operational parameters in the form of a label or a separate insert. Optionally, the kit may further comprise a standard or control information so that the test sample can be compared with the control information standard to determine if whether a consistent result is achieved.

30 The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989);

“Oligonucleotide Synthesis” (Gait, 1984); “Animal Cell Culture” (Freshney, 1987);
“Methods in Enzymology” “Handbook of Experimental Immunology” (Weir, 1996); “Gene
Transfer Vectors for Mammalian Cells” (Miller and Calos, 1987); “Current Protocols in
Molecular Biology” (Ausubel, 1987); “PCR: The Polymerase Chain Reaction”, (Mullis,
5 1994); “Current Protocols in Immunology” (Coligan, 1991). These techniques are applicable
to the production of the polynucleotides and polypeptides of the invention, and, as such, may
be considered in making and practicing the invention. Particularly useful techniques for
particular embodiments will be discussed in the sections that follow.

The following examples are put forth so as to provide those of ordinary skill in the art
10 with a complete disclosure and description of how to make and use the assay, screening, and
therapeutic methods of the invention, and are not intended to limit the scope of what the
inventors regard as their invention.

Example 1: Formation of Neuromuscular Junctions between Transplanted Embryonic 15 Stem Cells and Host Muscle

Mouse embryonic stem (ES) cells were treated with retinoic acid and a chemical
agonist of Shh, HhAg1.3 (Curis), to induce differentiation of ES cells into motor neurons
(Wichterle et al., Cell 2002; 110:385–397; Harper Proc Natl Acad Sci U S A 2004;101:7123–
7128). These ES cells were derived from a transgenic mouse that expresses GFP specifically
20 in motor neurons, driven by the motor neuron-specific HB9 promoter. The differentiating
cells were disaggregated 3.5 days after initiating the differentiation protocol and resuspended
in medium for transplantation. A total of 60,000 cells were transplanted into the ventral gray
matter of the lumbar spinal cord of 5- to 7-week-old rats that had become paralyzed after
neuroadapted Sindbis virus infection. Approximately 12,000 transplanted cells were
25 expressing GFP at this time and, therefore, were early motor neurons. In some animals, the
ES cells were resuspended in a solution containing 1 μ M dibutyryl cyclic adenosine
monophosphate (dbcAMP) before transplantation to increase their survival and their ability to
extend axons. In other animals, the phosphodiesterase type 4 inhibitor, rolipram, was
administered subcutaneously to neutralize the inhibitory effects of myelin on axonal
30 outgrowth. In still other animals, a motor axon tropic factor, GDNF, was secreted by
transplanted cells in the sciatic nerve, to attract transplanted axons toward distal targets.
Eight groups of animals treated singly or with a combination of these therapies were defined
(Table 1).

Table 1. Protocol for Transplantation of Embryonic Stem Cell-Derived Motor Neurons

Group No.	Animals at Beginning of Study, N	Cell Treatment		Pharmacological Rx		
		1	2	1	2	3
1	15	ES	C17.2-Bleo	dbcAMP	CsA	
2	15	ES	C17.2-GDNF	dbcAMP	CsA	
3	15	ES	C17.2-GDNF	dbcAMP	CsA	Rolipram
4	15	ES	C17.2-Bleo	dbcAMP	CsA	Rolipram
5	15		C17.2-GDNF	dbcAMP	CsA	Rolipram
6	15	ES	C17.2-GDNF		CsA	Rolipram
7	15	RA/ES	C17.2-GDNF	dbcAMP	CsA	Rolipram
8	15	ES	None	dbcAMP	CsA	

ES = embryonic stem; dbcAMP = dibutyryl cyclic adenosine monophosphate (1 mM); CsA = Cyclosporine (15 mg/kg/day); SQ = subcutaneously; GDNF = glial cell-derived neurotrophic factor; RA = retinoic acid; Rolipram (0.5 mg/kg/day SQ).

The outcomes of each of these groups was followed after transplantation for immunohistochemical evidence of innervation of host skeletal muscle, electrophysiological evidence of functioning motor units, and functional recovery from hind-limb paralysis. Only Group 3 received the entire cocktail of intraspinal dbcAMP, subcutaneous rolipram, C17.2-GDNF cells into the sciatic nerves, and cyclosporine to inhibit rejection of transplanted cells. Group 4 was the same except that animals were treated with C17.2-Bleo cells into the sciatic nerves instead of C17.2-GDNF. These cells expressed lower amounts of GDNF (1 ng/10⁶ cells/day vs 100 ng/10⁶ cells/day (Akerud et al., J Neurosci 2001;21:8108–8118; Llado Mol Cell Neurosci 2004;27:322–331). This allowed the importance of high GDNF in peripheral nerves. In Group 2, rolipram was omitted, and in Group 6, dbcAMP was omitted, allowing a determination of the importance of these interventions in mediating axonal outgrowth. In Group 5, the ES cell transplantation into the spinal cord was omitted. This provided for the assessment of whether the other treatments (GDNF, dbcAMP, CsA, and rolipram) induced behavioral recovery independent of ES cell-derived motor neurons. In Group 7, ES cells were transplanted that were differentiated to neural cells using retinoic acid without an agonist of Shh so that these cells formed mature neurons but not motor neurons. Animals in this group also received the other potential modifiers of reinnervation, allowing determination of the importance of motor neurons in the transplanted cells.

Example 2: Transplanted Embryonic Stem Cell-Derived Motor Neurons Survive Transplantation

The survival and integration of transplanted motor neurons at 3 and 6 months after transplantation was determined. At both time points, surviving transplant-derived motor neurons (GFP⁺) were observed within the gray matter of the spinal cord (Figure 1A). These transplant-derived motor neurons looked morphologically similar to host motor neurons and

persistently expressed GFP, which distinguished them from remaining host motor neurons. Furthermore, transplant-derived (and not host-derived) motor neurons expressed HB9 (see Figure 1B) at 3 months after transplantation. To determine whether cellular fusion of transplant-derived cells with host motor neurons occurred, the presence of rat-specific immunoreactivity within the mouse-derived GFP⁺ cells. A monoclonal antibody specific for rat motor neurons (MO-1) (Urakami J Neurosci 1990;10:620–630; Campos J Neurosci 2004;24:2090–2101) failed to identify immunoreactivity on GFP⁺ cells (see Figure 1C), indicating that cell fusion had not occurred. To determine whether surviving transplant-derived motor neurons integrated within a neural circuit, the presence of axon–soma interactions between GFP⁺ and GFP[−] cells (i.e., nonmotor neuron transplant-derived or host cells; see Figures 1D and 1E) was assayed. The presence of GFP⁺ nerve terminals that also expressed the presynaptic marker synaptophysin (Syn⁺) (see Figure 1D) on GFP[−] neurons (NF⁺), confirmed that transplant-derived collaterals formed synapses with other neurons in the spinal cord. Invariably, these other neurons were choline acetyltransferase– negative, suggesting that the targets of these transplant-derived collaterals were not motor neurons and rather were sensory or interneurons. Similarly, synaptic input from GFP[−] cells (Syn⁺ and synaptobrevin⁺) onto transplant derived motor neurons was identified (see Figure 1E), confirmed that these cells received afferent input from other neurons. As reported previously (Harper Proc Natl Acad Sci U S A 2004;101:7123–7128), few transplant derived axons extended into the spinal white matter of animals not treated with inhibitors of myelin. By contrast, in animals transplanted with embryonic stem cell–derived motor neurons and treated with both dbcAMP and rolipram (Groups 3–5), many axons extended into surrounding white matter (see Figures 1F–1H). To quantify and define the fate and survival of transplanted ES cells within the host spinal cord, an unbiased sampling of the lumbar spinal cord in three animals in each of Groups 3, 4, 6, and 7 was conducted. Transplant-derived cells were identified using a mouse specific monoclonal antibody (M2) and using neural specific polyclonal antibodies to determine neural fates within the grafted area. There was no significant difference in the total number of surviving mouse cells at 3 months after transplantation in any of the groups: 11,742 ± 640 in Group 3; 10,230 ± 730 in Group 4; 11,975 ± 780 in Group 6; and 10,654 ± 541 in Group 7. Similarly, the percentage of M2⁺ cells that coexpressed GFAP (astrocyte), CNPase (oligodendrocyte), β3-tubulin (neuron), or no neural marker did not differ among groups (Table 2).

Table 2. Fate of Transplanted Embryonic Stem Cells

Antigen	Group No. (mean % $\pm$ SEM)			
	3	4	6	7
GFAP (astrocyte)	22 $\pm$ 2	20 $\pm$ 4	19 $\pm$ 4	25 $\pm$ 5
CNPase (oligodendrocyte)	6 $\pm$ 3	8 $\pm$ 3	4 $\pm$ 1	9 $\pm$ 2
β_3 tubulin (neuron)	65 $\pm$ 13	59 $\pm$ 12	60 $\pm$ 13	51 $\pm$ 7
ChAT (motor neuron)	35 $\pm$ 7	39 $\pm$ 6	37 $\pm$ 9	2 $\pm$ 0
GABA (GABAergic neuron)	15 $\pm$ 2	12 $\pm$ 2	12 $\pm$ 3	23 $\pm$ 2
Glutamate (glutamatergic neuron)	7 $\pm$ 1	8 $\pm$ 1	5 $\pm$ 1	19 $\pm$ 6
Glycine (glycinergic neuron)	8 $\pm$ 2	2 $\pm$ 1	5 $\pm$ 1	9 $\pm$ 1
Lim1 (V0 interneuron)	10 $\pm$ 3	12 $\pm$ 3	7 $\pm$ 1	17 $\pm$ 1
Lhx3 (V2 interneuron)	13 $\pm$ 2	9 $\pm$ 1	14 $\pm$ 2	15 $\pm$ 5
Other	7 $\pm$ 3	13 $\pm$ 1	7 $\pm$ 1	15 $\pm$ 1

SEM = standard error of the mean; GFAP = glial fibrillary acidic protein; ChAT = choline acetyltransferase.

Subsets of neurons, including motor neurons, GABA⁺ neurons, glutamate⁺ neurons, glycine⁺ neurons, Lim1⁺ V0 interneurons, and Lhx3⁺ V2 interneurons were defined. As expected, only a small number of motor neurons were present in Group 7 animals (ES cells differentiated with RA only), and the percentage of motor neurons in the other groups ranged from 35 to 39%. This corresponded to approximately 4,110 $\pm$ 450 ($\pm$ standard error of the mean) surviving motor neurons per animal in Group 3, 2,553 $\pm$ 720 in Group 4, 4,430 $\pm$ 640 in Group 6, and 213 $\pm$ 32 in Group 7. Other neuron populations could also be identified in each group and did not differ among groups with the exception that nonmotor neuron populations were more frequently identified in Group 7. The 3-month survival number was slightly higher than previously reported (Harper Proc Natl Acad Sci U S A 2004;101:7123–7128), perhaps due to more efficient differentiation of ES cells before transplantation or to enhanced survival of transplanted cells. At 6 months after transplantation, 2,621 $\pm$ 321 surviving M2⁺ motor neurons were identified in Group 3 animals (n = 3) and 2,430 $\pm$ 219 in Group 6 animals (n = 3). No M2⁺/Ki67⁺ cells were observed at either 3 or 6 months (n = 6), suggesting that these cells were not undergoing cell division at those times.

Example 3: Cotransplantation of Glial Cell–Derived Neurotrophic Factor–Secreting Cells into the Sciatic Nerve Attracted Embryonic Stem Cell–Derived Axons

To further facilitate extension of ES cell–derived axons into the peripheral nervous system (PNS), GDNF-expressing cells were cotransplanted into the PNS at the time of spinal transplantation. This strategy was warranted because ES cell–derived axons that reach the ventral roots of transplanted animals fail to innervate targets (Harper Proc Natl Acad Sci U S A 2004;101:7123–7128). Suspecting that transplant derived axons may respond to tropic cues, resulting in distal growth and innervation of skeletal muscle, GDNF was chosen as a candidate tropic molecule because it is a potent stimulator of motor axonal growth (Trupp et

al., J Cell Biol 1995; 130:137–148; Hoke et al., Exp Neurol 2002;173:77–85) and because microarray data suggested that ES cell–derived motor neurons express high levels of the GDNF receptor, GFR α 1. C17.2-Bleo were transplanted (1ng GDNF/10⁶ cells/day *in vitro*), as a control cell line, or C17.2-GDNF (100 ng GDNF/10⁶ cells/day *in vitro*) cells into one or both sciatic nerves of paralyzed animals that had been cotransplanted with ES cell–derived motor neurons in the spinal cord. At 6 months after transplantation, strong GDNF immunoreactivity was detected only in the sciatic nerves of animals transplanted with C17.2-GDNF cell (Figure 2A) and not in the sciatic nerves of animals treated with C17.2-Bleo (see Figure 2B). Dual-color confocal microscopy and antibodies to GDNF and the mouse-specific M2 antibody were used to show that GDNF within the sciatic nerve originated from transplanted cells (see Figure 2C). To define the survival and migration of C17.2 cells within the sciatic nerve, immunohistochemistry was conducted using the M2 antibody along the length of the sciatic nerve (see Fig 2D). Every 30th section of sciatic nerve was analyzed from the spinal cord to below the knee (8.5cm from the spinal cord) in a subset of animals (n = 3 for each C17.2-Bleo and C17.2-GDNF). This analysis showed C17.2-Bleo or C17.2-GDNF cells in all sections of the sciatic nerve, though the highest density of cells was around the site of transplantation into the sciatic nerve (arrow denotes site of injection; see Figure 2E). There was no difference in the distribution of C17.2 cells between groups, and the estimated total number of surviving C17.2 cells in each group was similar (40,185 $\pm$ 4,235 in the C17.2-GDNF group; 43,209 $\pm$ 2,341 in the C17.2-Bleo group). To determine whether any of the experimental modifications enhanced the outgrowth of transplant derived axons as defined by the presence of GFP⁺ axons within ventral roots (see Figure 2F), all the ventral roots within 2 levels of the injection site (10 ventral roots, generated from 5 spinal levels; n = 5) were identified. Longitudinal frozen sections were generated and the number of GFP⁺ axons colocalizing with neurofibromin (NF) by immunohistochemistry were counted. Animals in Group 3 exhibited more transplant-derived axons reaching the PNS than any other group (203 $\pm$ 15 vs 123 $\pm$ 3 for Group 4 and 76 $\pm$ 11 for Group 8; *p* < 0.02). Because few transplant-derived axons were found in the ventral roots of Group 6 animals (all but dbcAMP), this indicated that locally administered dbcAMP facilitated extension of axons into the PNS, and that high levels of GDNF expression within the sciatic nerve enhanced the ability of transplant-derived axons to reach the PNS. These and subsequent data regarding outcome measures are summarized for each group in Table 3.

Table 3. Summary of Outcome Data for Each Group

Group No.	Transplant Paradigm	Transplant-Derived Axons in PNS	Behavioral Recovery	Anatomic Reinnervations	Physiological Improvement
1	No Rolipram Low GDNF	±	N	N	N/A
2	No Rolipram	±	N	N	N/A
3	Complete paradigm	Y	Y	Y	Y
4	Low GDNF	Y	N	N	N
5	No ES Cells	N	N	N	N
6	No dbcAMP	±	N	N	N/A
7	Nonmotor neuron ES cells	N	N	N	N/A
8	No GDNF No Rolipram	±	N	N	N/A

PNS = peripheral nervous system; GDNF = glial cell-derived neurotrophic factor; ES = embryonic stem; N/A = not available.

Example 4: Formation of Neuromuscular Junctions between Transplanted Motor Axons and Host Muscle

5 To determine whether ES cell-derived axons reached skeletal muscle targets by three months after transplantation, conduction confocal microscopy was used to assay fixed-frozen muscle tissue from transplanted animals in each group. Exclusively in Group 3, GFP⁺ axons within quadriceps and gastrocnemius skeletal muscle (Figure 3A) were observed. Transplant-derived axons often exhibited a branched morphology with apparent growth cones (see Fig 3A) and were strongly NF-positive (see Figs 3A, B). Clustering of the acetylcholine receptor in apposition to advancing axons was also observed (as defined by rhodamine-conjugated α -bungarotoxin; see Figures 3C–3E), though the morphology of these NMJs was simpler than that seen at 6 months, as described below. Colocalization of GFP⁺ axons with the vesicular acetylcholine transporter was also observed, indicating that these axons were active synaptically (see Figure 3F). To obtain further support for the observation that stem cell-derived axons reached skeletal muscle targets, spinal motor neurons were retrogradely labeled by injecting cholera toxin subunit b (CTB) into the quadriceps and gastrocnemius muscles of transplanted animals, and conducting immunohistochemistry. This analysis identified transplant-derived motor neurons with GFP, all motor neurons with choline acetyltransferase, and retrogradely labeled motor neurons with CTB (see Figures 3G, H). This analysis retrogradely labelled both host (see asterisk in Figure 3G) and transplant derived motor neurons (see arrowheads in Figures 3G and 3H). To determine the number of transplant-derived motor neurons capable of being retrogradely labeled from skeletal muscle, an unbiased counting of GFP⁺/CTB⁺ neurons was carried out in the spinal cord of five animals in each of Groups 1 through 5. Every fifth section was selected from the spinal cord spanning the lumbar enlargement and conducted immunohistochemistry and blind counted. This analysis showed that in each of Groups 1, 2, 4, 5, 6, 7, and 8, there were no dual-labeled

motor neurons. In Group 3, however, there were 123 ± 47 dual-labeled motor neurons per animal. Therefore, in Group 3, we have generated approximately 4,100 new motor neurons in the spinal cord, 200 new motor axons in the ventral roots, and 120 retrogradely labeled new motor neurons from skeletal muscle. At 6 months after transplantation, these studies
 5 were repeated and extended using both proximal and distal limb muscles. Within the gastrocnemius muscle of transplanted animals (Group 3), morphologically mature NMJs were observed in close apposition to GFP⁺ axons, suggesting that these axons induced the organization of postsynaptic NMJ machinery (Figure 4A). In a collapsed Z-stack image, GFP⁺ axons can be seen in close apposition to clustered acetylcholine receptors, and single-
 10 layer, confocal, microscopic images confirm with orthogonal views that they form a single NMJ composed of transplant-derived axons and host-derived muscle (see Figure 4B). Furthermore, transplant-derived axons within skeletal muscle were found to be immunoreactive to synaptic vesicle proteins synaptobrevin (see Figure 4C), synaptophysin (see Figures 4D, E), and SV2 (see Figure 4E), suggesting that they had appropriately
 15 developed presynaptic vesicle machinery.

Example 5: Electrophysiological Analyses of Transplanted Rats Show Increased Motor Unit Nerve Numbers in Hind Limbs

To determine whether these hybrid NMJs were functionally active,
 20 electrophysiological analyses of live animals was carried out to determine whether the number of functioning motor units changed over time after transplantation. Data regarding MUNE (a measure of the number of functioning motor units) and single motor unit action potential (a measure of the amplitude of individual motor units) was gathered using a preselected cohort of five to six transplanted animals in each of Groups 3 to 5. Each animal
 25 was studied in a blinded manner at 12 days after transplantation and again at 120 days after transplantation. Mean MUNE counts at baseline were similar between the groups (not statistically significant). Animals in Groups 4 (Figure 5A, left panel; $\Delta = -5 \pm 9.9$) and 5 (see Figure 5A, middle panel; $\Delta = 8 \pm 6.9$) had no significant change in MUNE. In Group 3, a significant increase in the MUNE at 120 days after transplantation was observed as compared
 30 with 12 days (see Fig 5A, right panel; $\Delta = +54 \pm 8.5$; $p < 0.04$ for intragroup change; $p < 0.01$ for intergroup difference at 120 days). This indicated that ES cell-derived motor neurons established electrically active motor units when transplanted with dbcAMP, systemically administered rolipram, and GDNF in the PNS. Single motor unit action potential increases were observed in Groups 3 and 5 between days 12 and 120 ($p < 0.05$), suggesting that GDNF

transplantation results in enhanced axonal sprouting of motor axons (see Figure 5B), and that this increase was independent of ES cell transplantation. However, it is clear that axonal sprouting mediated by GDNF was not sufficient to mediate functional recovery in the absence of transplanted ES cell-derived motor neurons.

5 The MUNE analysis provides definitive proof of the functional reestablishment of motor units. Electrophysiological examination of nerve-muscle interaction provides a quantitative measure of reinnervation of skeletal muscle, and MUNE analysis reliably track changes in function over time (Shefner et al., Muscle Nerve 2002;25:39-42). Studies in humans, as well as in animal models of amyotrophic lateral sclerosis, illustrate the sensitivity
10 of MUNE not only to early detection of abnormalities, but also to gain of function as a result of therapeutics. MUNE studies have also suggested that the total number of functioning motor units in a human limb muscle range from 65 to 479. It has also been reported that patients with progressive motor neuron diseases are asymptomatic until 70 to 80% of motor units are lost. By extension, using the highest MUNE for the large muscles of the lower
15 extremities (i.e., 479), it can be inferred that muscles that have a MUNE of approximately 100 are likely to be near normal in strength. In the transplantation paradigm in rats, an increase of approximately 50 in the MUNE was generated in the distal lower extremity. Animals with this number of functioning motor units while not altogether normal, and indeed, the hind-limb grip strength improved to approximately 50% of the preparalysis
20 strength.

Example 6: Functional Recovery of Transplanted Animals

A cohort of animals for functional recovery was followed by blindly assessing weight (see Figure 5C) and hindlimb grip strength (see Figure 5D) for up to 6 months after
25 transplantation. The code of animal grouping was broken at 6 months after transplantation, and there was a significant improvement in animal weights only in Group 3 beginning at 20 weeks after translation ($p < 0.05$ at 20 weeks; $p < 0.001$ at 24 weeks). This indicated that these animals had become more mobile in the cage and were better able to obtain food than their littermates in other groups. Indeed, hind-limb grip strength improved only in Group 3
30 with statistical distinction from other groups achieved at 18, 22, and 24 weeks ($p < 0.001$). Two representative videos of a single Group 3 rat at the time of transplantation and 24 weeks later showed that this treatment regimen rescued hind limb motor function and mobility.

Two distinct possibilities exist to explain the effects of GDNF in this regard. Perhaps the GDNF is secreted systemically, creating a general permissive environment for axonal

growth. Alternatively, the GDNF may act as a focal attractive source attracting axons distally. To distinguish between these possibilities, C17.2-GDNF cells were transplanted unilaterally into the sciatic nerve and ES cell-derived motor neurons were transplanted bilaterally into the spinal cord and functional recovery was assessed (see Figure 5E) as described above. All animals received rolipram and CsA. If GDNF functioned within a focal region of the PNS to act as an attractive cue, then asymmetric recovery should be observed ipsilateral to the transplanted C17-GDNF cells. If, however, GDNF secretion by transplanted C17.2 cells acts diffusely, then growth both ipsilateral and contralateral to the C17.2 side would be observed. Hind-limb grip strength was initially used as a readout for motor recovery as described above. However, preliminary studies showed that several animals exhibited asymmetric (ipsilateral) recovery, suggesting that unilateral grip strength measurements were unreliable in this setting. Animals were followed for 24 weeks and were assessed blindly for functional recovery as defined by the ability to flex the proximal leg under the animal and to push off with the foot. The percentage of animals that could do both at 0, 12, and 24 weeks after transplantation both ipsilateral and contralateral to the C17.2 GDNF transplantation was scored. Raters were not aware which sciatic nerve had been transplanted with C17.2-GDNF cells. Although none of the animals regained the ability to bear weight and step contralaterally to the C17.2-GDNF cells, 25% and 75% of animals regained the ability to bear weight and step ipsilateral to the C17.2-GDNF cells at 12 and 24 weeks after transplantation ($p < 0.001$). Two representative videotapes of one rat used in this analysis show that the transplanted rat recovered the ability to ambulate with the right hind limb (ipsilateral), but did not recover any hind-limb movement in the left hind limb (contralateral). Without wishing to be bound by theory, these studies suggested that GDNF acts as a focal attractive cue for ES cell-derived motor axons, and that when coadministered with dbcAMP and rolipram, facilitated the establishment of NMJs between transplant and host, resulting in physiological and behavioral recovery.

The invention features a treatment regimen that provides for the functional restoration of motor units in paralyzed adult rats using mouse ES cells. The treatment involves a combination of steps, including the directed differentiation of pluripotent stem cells or other neural progenitor cells into committed motor neuron progenitors using retinoic acid and a chemical agonist of sonic hedge hog (Shh); the stimulation of axon regeneration, by intraspinal infusion of dbcAMP and systemic administration of a phosphodiesterase 4 inhibitor; and by administering a focal attractant, such as GDNF, within the PNS. Without wishing to be bound by theory, it is likely that treatment with dbcAMP and a

phosphodiesterase 4 inhibitor reduced the inhibitory effects of myelin or altered the response of the transplanted neurons to axon guidance cues. Accordingly, the invention provides for the use of other agents (e.g., that reduce the inhibitory effects of myelin (e.g., NOGO antagonistic drugs, such as peptides, small molecules and antibodies that interfere with the inhibitory effects of NOGO). Again, without wishing to be bound by theory, it is likely that GDNF secretion directionally attracted transplant-derived axons distally, resulting in the formation of host/transplant NMJs. Accordingly, other factors which attract axons (e.g., Insulin-like growth factor (IGF-1) and hepatocyte growth factor (HGF)) are two well known motor axon tropic factors that are also useful in the methods of the invention.

Most significantly, these strategies resulted in the formation of anatomically, physiologically, and functionally active motor units between transplanted axons and host muscle. Function of the NMJ is closely tied to the function of several proteins associated with presynaptic vesicles. This indicates that functional restoration of motor units with ES cell-derived motor neurons provides a therapeutic intervention for humans with paralysis.

The results reported herein were obtained with the following materials and methods.

Antibodies

Antibodies and dilutions used in this study include: neurofilament (NF) 200KD (1:100, AB1982; Chemicon, Temecula, CA); synaptophysin (Syn; 1:100, AB9272; Chemicon); rat motor neuron-specific antibody (1:100, MO-1; Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City, IA); synatobrevin-1 (1:1,000, 104 001; Synaptic Systems, Goettingen, Germany); anti-cholera toxin antibody (1:50–100, Sigma C3062; Sigma, St. Louis, MO); rabbit anti-GDNF (1:100, sc-9010; Santa Cruz, Santa Cruz, CA); VaChT (1:1,000, AB1578; Chemicon); mouse anti-green fluorescent protein (anti-GFP; 1:100, MAB3580; Chemicon); SV2 (1:100; DSHB); rabbit anti-GFP (1:100, AB3080; Chemicon); and mouse anti-HB9 (1:50, 81.5C10/ MNR2; DSHB). For the cell fate experiments, the following antibodies were used: M2 (1:10; DSHB), α -tubulin (1:500, MMS- 435P; Covance, Princeton, NJ); choline acetyltransferase (1: 500, Ab5851; Chemicon); GABA (1:1,000, Sigma A2052; Sigma); glutamate (1:5,000, G6642; Sigma); glycine (1:50, Chemicon Ab5020; Chemicon); Lim1 (1:500, Chemicon Ab14554; Chemicon); Lhx3 (1:4,000, Chemicon Ab14555; Chemicon); GFAP (1:50, Research Diagnostics RDIPRO10555; Research Diagnostics, Minneapolis, MN); and Ki67 (1:25, Abcam Ab833). The different primary antibodies were codetected by immunofluorescence, using goat anti-rabbit and anti-mouse IgG coupled to Alexa Fluor-488 (green) and Alexa Fluor-594 (red) (1:100, Molecular Probes).

Reagents

Nissl red stain (1:100, N-21482; Molecular Probes, Eugene, OR), Nissl blue (1:100, N-21479; Molecular Probes) and tetramethylrhodamine α -bungarotoxin (1:50, T-1175; Molecular Probes) were used following the manufacturer's instructions. HhAg1.3 (Curis, Cambridge, MA) was made up as a 10mM stock in dimethylsulfoxide and was used at 1 μ M. All-*trans* retinoic acid (Sigma R-2625; Sigma) was made up as a 1mM stock in dimethylsulfoxide and used at 1 μ M. dbcAMP was obtained from Calbiochem (catalog #28745; San Diego, CA) and was used at 1 μ M. Rolipram was purchased from A.G. Scientific (R-1012-50MG; San Diego, CA) and was dissolved in 10% dimethylsulfoxide.

Animal Care

All animals were cared for and procedures performed in accordance with the Johns Hopkins Animal Care and Use Committee guidelines. Five- to 7-week-old Lewis rats (Charles River, Wilmington, MA) were used. Paralysis was induced using Neuroadapted Sindbis virus as described previously. 2,5 Cyclosporine (CsA; Calbiochem) was given at 15mg/kg mixed in the food beginning on the day before surgery/ transplantation, then every day after surgery.

Microscopy

Immunohistochemical studies were conducted by two-color confocal imaging with a Zeiss LSM510 microscope (Zeiss, Oberkochen, Germany). Images were acquired in both red and green emission channels by using an argon-krypton laser with single-channel, line-switching mode.

Transplantation of Embryonic Stem Cells

Rolipram was administered at 0.5mg/kg/day subcutaneously beginning 2 days before ES cell transplantation and continuing for 30 days after transplantation. HB9-GFP ES cells were differentiated as described previously (Wichterle et al., Cell 2002; 110:385–397; Harper Proc Natl Acad Sci U S A 2004;101:7123–7128). For differentiation of ES cells using RA alone, the 4-/4+ strategy described previously was used (Bain et al., Dev Biol 1995;168:342–357). On the day of transplantation, 10 ng/ml BDNF (Brain-Derived Neurotrophic Factor), 10 ng/ml NT3 (Neurotrophic Factor 3), and 25 ng/ml CNTF (Ciliary Neurotrophic Factor) (R&D Systems, Minneapolis, MN) were added to the culture medium. Embryoid bodies

were then disaggregated with collagenase and dispase and resuspended in serum-free media. In all groups except Group 6, the cell suspension was supplemented with dbcAMP at 1mM. Transplantation was performed at 28 days after viral inoculation, as described previously.

5 *C17.2-Glial Cell-Derived Neurotrophic Factor Cell Cultures and Preparation of Cells for Transplantation*

Two types of C17.2 neural stem cells derived from day 8 cerebellar granule cells were used in this study: C17.2-Bleo and C17.2-GDNF (Akerud et al., J Neurosci 2001;21:8108–8118; Snyder et al., Cell 1992;68:33–51). All groups received 5 μ l injections of cells at 10⁵ cells/ μ l into the sciatic nerve 4cm away from the spinal cord. Cholera toxin B (CTB) was injected in three muscles: the gluteal muscles (2.5cm from the transplantation site), quadriceps (6.5 cm from the transplantation site), and the gastrocnemius complex (10.5 cm from the injection site).

15 *Electrophysiology Measurements In Vivo*

Motor unit number estimation (MUNE) was performed using previously described methods (Shefner et al., Muscle Nerve 2002;25:39–42; Shefner et al. Muscle Nerve 2004;30:463–469). Animals were anesthetized with sodium pentobarbital (5mg/ml) injected intraperitoneally at a dose of 50mg/kg body weight. The abdomen and distal hind limbs were shaved, and animals were taped prone to a Styrofoam board. The stimulating electrodes were 0.7mm needles insulated with Teflon (Dantec sensory needle; Dantec, Skovlunde, Denmark). The cathode was placed close to the sciatic nerve at the proximal thigh, and the anode was placed subcutaneously 1cm proximally. Motor responses were recorded from a pregelled, self-adhesive surface recording strip (Nicolet Biomedical Inc., Madison, WI) cut to a length of 1.5cm and a width of about 0.5cm. This electrode was placed circumferentially around the animal's distal hind limb, and thus recorded activity in both flexor and extensor compartments. The reference electrode was a monopolar needle placed subcutaneously in the foot, 1.5 to 2cm distal to the recording electrode. Distance between stimulating and recording electrodes was 1.2 to 1.6cm. Both the right and left hind limbs were studied. Stimuli were 0.1- millisecond monophasic pulses of constant current delivered through a constant current stimulator (Medtronic Keypoint Electromyograph; Medtronic, Minneapolis, MN). Recordings were made through the same instrument. Filter settings were 300 and 3,000Hz. A maximum motor response from distal hind-limb muscles was recorded, representing the contributions of all viable motor units. Individual motor units were

stimulated with submaximal stimuli slowly increased from subthreshold levels, to determine discrete response increments representing single motor units. The individual values were averaged to yield an estimate of average single motor unit action potential amplitude. This value was divided into the peak-to-peak amplitude of the maximum compound motor action potential to yield the MUNE.

Cell Count Estimations

To estimate surviving mouse cells in the spinal cord and sciatic nerve, 50 μ M tissue sections were generated and immunohistochemistry was conducted on every 10th (spinal cord) or 30th (sciatic nerve) section. The total number of mouse-specific or neural subtype cells were counted in which both the cytoplasm and nucleus could be identified using a Hoechst counterstain in all the sections. The counted number was multiplied by 10 (spinal cord) or 30 (sciatic nerve) and 10% was subtracted as a correction for the small number of cells that would be doubly counted to arrive at an estimate for cell survival.

Behavioral Assessment

Functional recovery was assessed by evaluators blinded to treatment groups. Hind-limb grip strength was measured as described previously (Kerr et al., J Neurosci 2003;23:5131–5140). For the cohort given unilateral C17.2- GDNF cells, an animal was defined as having recovered when it had proximal and distal movement of the leg. Two evaluators performed the analysis on live or videotaped animals in a blinded way. Scores were tabulated as the number of animals that had both proximal and distal limb movement ipsilateral and contralateral to the transplantation of C17.2- GDNF cells.

Statistical Analysis

Reported values are means $\pm$ standard error of the mean. Due to the nonparametric nature of the data, nonparametric equivalent tests of analysis of variance and repeated-measures analysis of variance were used to increase the robustness of the results. The Kruskal–Wallis test was performed to analyze differences between groups at each time point, and Friedman’s nonparametric repeated-measures comparison was used to analyze differences across time within a group. Wilcoxon signed rank test was used for the comparison of two related samples. Significance was assessed at the 0.05 level.

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

5 The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

10 All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A method of increasing motor function in a subject in need thereof, the method comprising:

5 (a) administering to the subject a cell capable of adopting a motor neuron cell fate;

(b) administering to the subject at least one agent that stimulates axonal outgrowth in the central nervous system (CNS); and

10 (c) administering to the subject a tropic factor, thereby increasing motor function in a subject.

2. The method of claim 1, wherein the cell is an embryonic stem cell, neural stem cell, or a motor neuron precursor.

15 3. The method of claim 1, wherein the agent of (b) reduces myelin inhibition of axonal outgrowth.

4. The method of claim 3, wherein the agent is selected from the group consisting of:

20 (a) a phosphodiesterase inhibitor;

(b) a functional analog of adenosine-3', 5'-cyclic monophosphate; and

(c) a metabolic precursor of adenosine-3', 5'-cyclic monophosphate.

25 5. The method of claim 4, wherein the functional analog of adenosine-3',5'-cyclic monophosphate is selected from the group consisting of:

(a) adenosine-3',5'-cyclic monophosphate;

(b) N⁶-Benzoyladenosine-3',5'-cyclic monophosphate;

(c) 8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate;

(d) 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate;

30 (e) 8-(6-Aminohexyl)aminoadenosine-3',5'-cyclic monophosphate;

(f) 8-Bromoadenosine 3',5'-cyclic monophosphate;

(g) 8-Chloroadenosine 3',5'-cyclic-monophosphate;

(h) 8-Methylaminoadenosine-3',5'-cyclic monophosphate;

- (i) 8-Piperidinoadenosine-3',5'-cyclic monophosphate;
(j) N⁶,2'-O-Dibutyryladenosine-3',5'-cyclic monophosphate;
(k) N⁶,2'-O-Disuccinyladenosine-3',5'-cyclic monophosphate;
(l) 8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate, Sp-
5 isomer;
(m) Sp-Adenosine 3',5'-cyclic monophosphorothioate triethylammonium; and
(n) a functional derivative of any one of (a) to (m).

6. The method of claim 4, wherein said metabolic precursor of adenosine-3', 5'-
10 cyclic monophosphate is selected from the group consisting of:
(a) adenosine-3',5'-cyclic monophosphate acetoxymethyl ester;
(b) adenosine-3',5'-cyclic monophosphorothioate acetoxymethyl ester; and
(c) a functional derivative of any one of (a) to (b).

- 15 7. The method of claim 1, wherein the cell is contacted with dibutyryl cyclic adenosine monophosphate prior to administration.

8. The method of claim 1, wherein two agents are administered to the subject.

- 20 9. The method of claim 8, wherein one of the agents is dibutyryl cyclic adenosine monophosphate and one of the agents is rolipram.

10. The method of claim 9, wherein the agent is administered by intraspinal
infusion.

- 25 11. The method of claim 1, wherein the agent is administered by systemic administration.

12. The method of claim 1, wherein the tropic factor is administered within the
30 peripheral nervous system.

13. The method of claim 1, wherein the tropic factor is glial cell derived neurotrophic factor (GDNF).

14. The method of claim 12, wherein the tropic factor is expressed by a
5 transplanted cell.

15. The method of claim 1, wherein the cell is contacted with retinoic acid and a chemical agonist of Shh prior to administration to a subject.

10 16. The method of claim 15, wherein the chemical agonist is HhAg1.3.

17. The method of claim 15, wherein the cell is contacted with at least one of Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, or Ciliary Neurotrophic Factor prior to administration to the subject.

15 18. The method of claim 16, wherein the cell is administered together with one or more of Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, and Ciliary Neurotrophic Factor.

20 19. The method of claim 1, wherein the method further comprises administering to the subject a compound that inhibits rejection of a transplanted cell.

20. The method of claim 1, wherein the compound is cyclosporine, tacrolimus, or rapamycin.

25 21. The method of claim 1, wherein the cell is present in a population of injected cells.

22. The method of claim 1, wherein at least 75% of the administered cells express
30 a motor neuron marker.

23. The method of claim 1, wherein the cell survives within the gray matter of the spinal cord for at least 3 months after administration to the subject.

24. The method of claim 21, wherein the surviving cell expresses a presynaptic marker.

5 25. The method of claim 22, wherein the presynaptic marker is synaptophysin, synaptobrevin, or SV2.

26. The method of claim 1, wherein the cell forms a synapse on a sensory or interneuron.

10 27. The method of claim 1, wherein the cell extends an axon into the white matter of the spinal cord.

15 28. The method of claim 1, wherein the motor neuron specific marker is selected from the group consisting of GFR α 1,

29. The method of claim 1, wherein the method increases the number of cell derived axons that reach the peripheral nervous system relative to the number in an untreated control animal.

20 30. The method of claim 1, wherein the method increases the number of axons that reach a skeletal muscle target relative to an untreated control animal.

25 31. The method of claim 1, wherein the method increases the number of neuromuscular junctions between transplanted cell-derived axons and host-derived muscle relative to an untreated control animal.

30 32. The method of claim 1, wherein the method increases the number of functioning motor units or the amplitude of individual motor unit relative to an untreated control animal.

33. The method of claim 1, wherein the method increases the function of a limb or other organ relative to an untreated control animal.

34. A method of treating or preventing a condition characterized by a reduction in motor neuron function in a subject in need thereof, the method comprising:

(a) administering to the subject a cell that expresses a motor neuron specific marker;

(b) administering to the subject at least one agent that stimulates axonal outgrowth in the central nervous system (CNS); and

(c) administering to the subject an tropic factor, thereby increasing motor function in a subject.

35. The method of claim 32, wherein the condition is spinal cord injury, neural avulsion, transverse myelitis, or spinal muscular atrophy.

36. A method of treating spinal muscular atrophy in a subject in need thereof, the method comprising:

(a) identifying the subject as having spinal muscular atrophy; and

(b) administering to the subject a cell that expresses a motor neuron specific marker, thereby increasing motor function in a subject.

37. The method of claim 34 or 36, wherein the cell is an embryonic stem cell contacted with dibutyl cyclic adenosine monophosphate prior to administration.

38. The method of claim 34 or 36, further comprising the step of administering rolipram to the subject.

39. The method of claim 34, wherein the cell is contacted with retinoic acid and a chemical agonist of Shh prior to administration to the subject.

40. The method of claim 39, wherein the chemical agonist is HhAg1.3.

41. The method of claim 34 or 36, wherein the cell is contacted with Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, and Ciliary Neurotrophic Factor prior to administration to the subject.

42. The method of claim 34 or 36, further comprising the step of administering cyclosporine to the subject.

43. The method of claim 34 or 36, wherein the method increases the number of functioning motor units, increases the amplitude of individual motor unit, or increases the function of a limb or other organ relative to an untreated control animal.

44. A pharmaceutical pack comprising an isolated cell capable of adopting a motor neuron cell fate and at least one of an agent that stimulates axonal outgrowth and a tropic factor.

45. The pharmaceutical pack of claim 44, further comprising directions for using the pack to treat a condition characterized by a reduction in motor function.

46. A pharmaceutical pack comprising an agent that stimulates axonal outgrowth and a tropic factor.

47. The pharmaceutical pack of claim 46, further comprising an isolated cell capable of adopting a motor neuron cell fate.

48. The pharmaceutical pack of claim 44 or 46, wherein the agent that stimulates axonal outgrowth is selected from the group consisting of:

- (a) a phosphodiesterase inhibitor;
- (b) a functional analog of adenosine-3', 5'-cyclic monophosphate; and
- (c) a metabolic precursor of adenosine-3', 5'-cyclic monophosphate.

49. The pharmaceutical pack of claim 44 or 46, wherein the cell is an embryonic stem cell, neural stem cell, or a motor neuron precursor.

50. The pharmaceutical pack of claim 44 or 46, wherein the agent reduces myelin inhibition of axonal outgrowth.

51. The pharmaceutical pack of claim 48, wherein the functional analog of adenosine-3',5'-cyclic monophosphate is selected from the group consisting of:

- (a) adenosine-3',5'-cyclic monophosphate;
- (b) N⁶-Benzoyladenosine-3',5'-cyclic monophosphate;
- 5 (c) 8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate;
- (d) 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate;
- (e) 8-(6-Aminohexyl)aminoadenosine-3',5'-cyclic monophosphate;
- (f) 8-Bromoadenosine 3',5'-cyclic monophosphate;
- (g) 8-Chloroadenosine 3',5'-cyclic-monophosphate;
- 10 (h) 8-Methylaminoadenosine-3',5'-cyclic monophosphate;
- (i) 8-Piperidinoadenosine-3',5'-cyclic monophosphate;
- (j) N⁶,2'-O-Dibutyryladenosine-3',5'-cyclic monophosphate;
- (k) N⁶,2'-O-Disuccinyladenosine-3',5'-cyclic monophosphate;
- 15 (l) 8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer;
- (m) Sp-Adenosine 3',5'-cyclic monophosphorothioate triethylammonium; and
- (n) a functional derivative of any one of (a) to (m).

52. The pharmaceutical pack of claim 48, wherein said metabolic precursor of adenosine-3', 5'-cyclic monophosphate is selected from the group consisting of:

- 20 (a) adenosine-3',5'-cyclic monophosphate acetoxymethyl ester;
- (b) adenosine-3',5'-cyclic monophosphorothioate acetoxymethyl ester; and
- (c) a functional derivative of any one of (a) to (b).

25 53. The pharmaceutical pack of claim 44 or 46, wherein the cell is contacted with dibutyryl cyclic adenosine monophosphate prior to administration.

54. The pharmaceutical pack of claim 44 or 46, wherein one of the agents is dibutyryl cyclic adenosine monophosphate and one of the agents is rolipram.

30 55. The pharmaceutical pack of claim 44 or 46, wherein the tropic factor is glial cell derived neurotrophic factor (GDNF).

56. The pharmaceutical pack of claim 44 or 46, wherein the pack further comprises Brain-Derived Neurotrophic Factor, Neurotrophic Factor 3, and Ciliary Neurotrophic Factor.

5 57. The pharmaceutical pack of claim 44 or 46, wherein the pack further comprises cyclosporine, rapamycin, or tacrolimus.

58. The pharmaceutical pack of claim 44 or 46, wherein the pack comprises directions for identifying a patient in need of treatment.

10

59. The pharmaceutical pack of claim 58, wherein the patient is identified as having spinal cord injury, neural avulsion, transverse myelitis, or spinal muscular atrophy.

15 60. The pharmaceutical pack of claim 44 or 46, wherein the package further comprises directions for measuring an increase in motor function.

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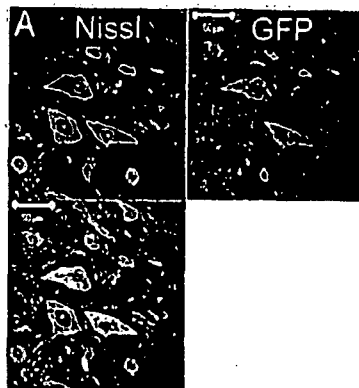


FIG. 1A



FIG. 1B

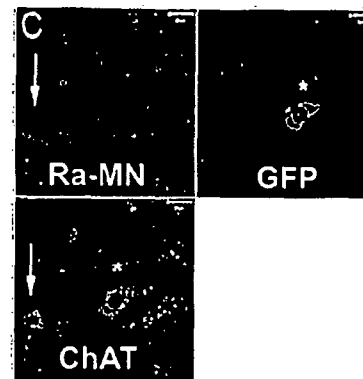


FIG. 1C

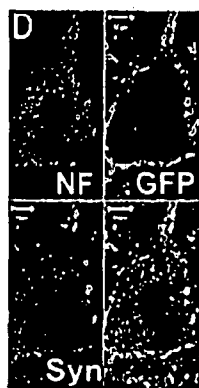


FIG. 1D

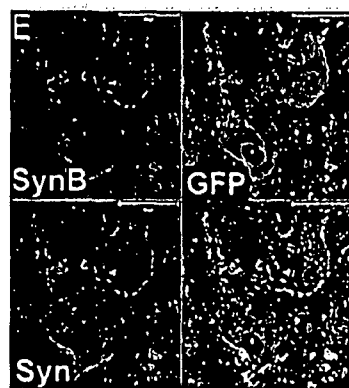


FIG. 1E

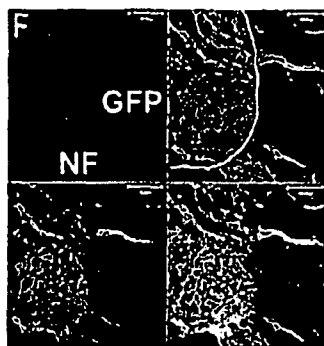


FIG. 1F



FIG. 1G

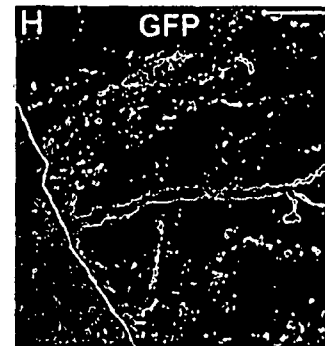


FIG. 1H

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FIG. 2B



FIG. 2D



FIG. 2A

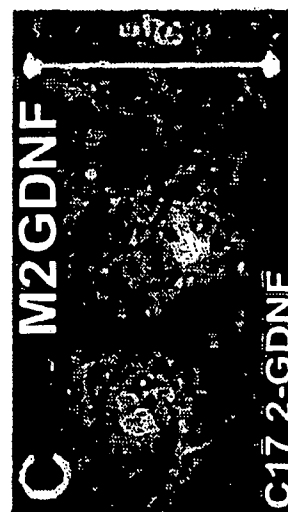


FIG. 2C

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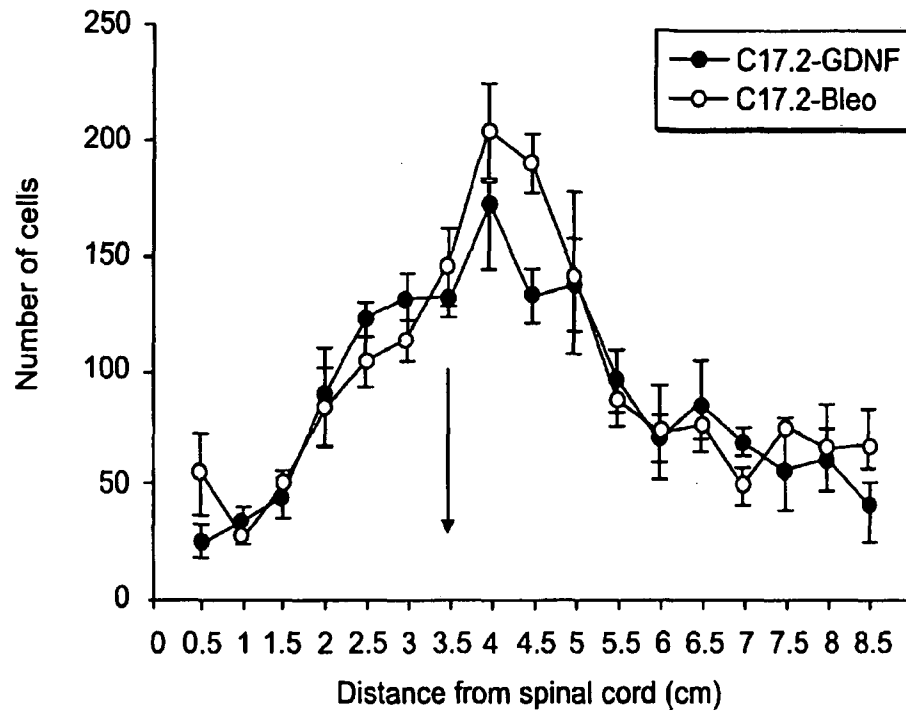


FIG. 2E

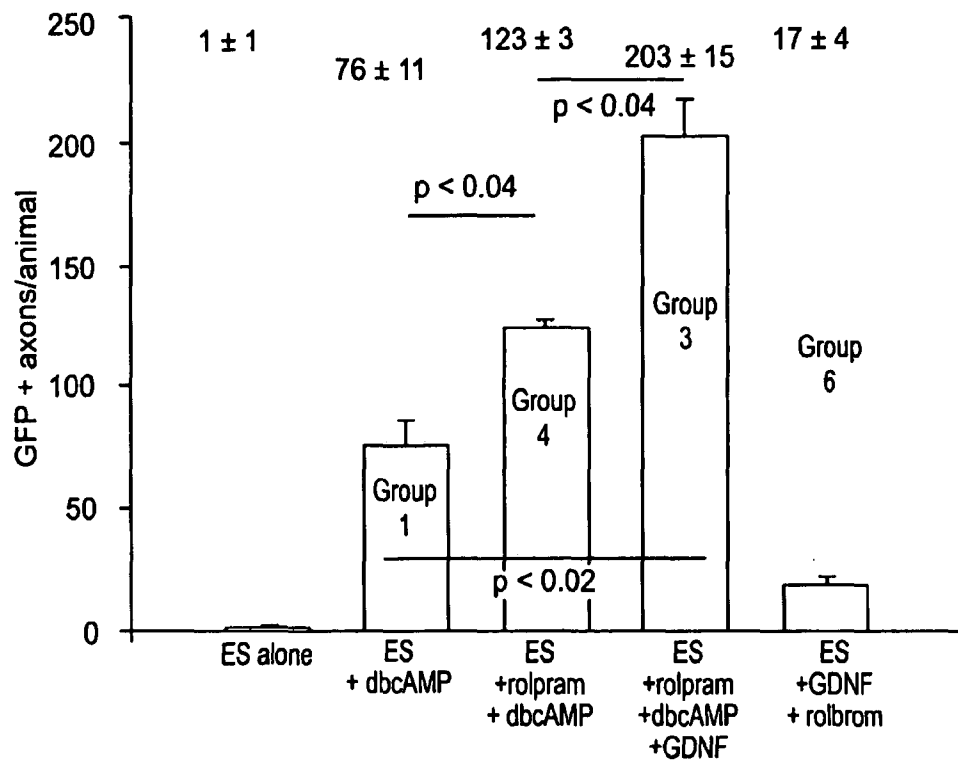


FIG. 2F

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FIG. 3A

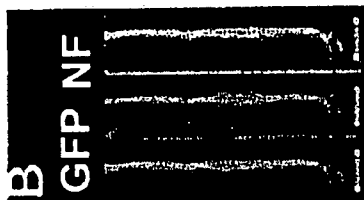


FIG. 3B

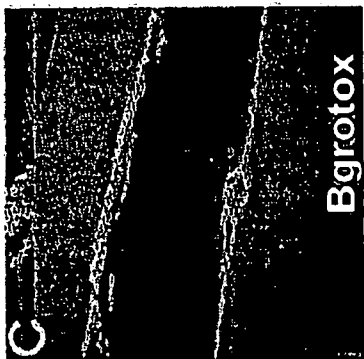


FIG. 3C

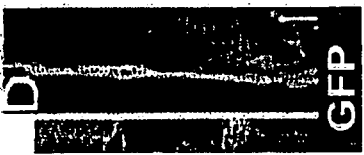


FIG. 3D

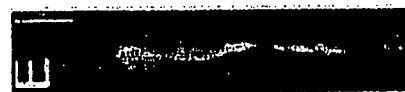


FIG. 3E



FIG. 3F

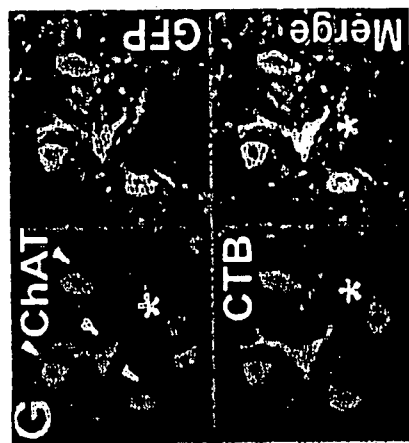


FIG. 3G

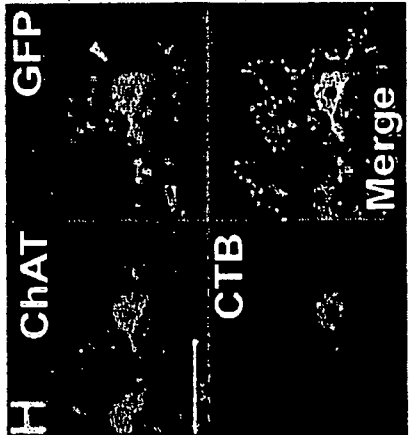


FIG. 3H

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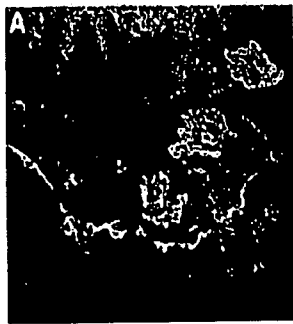


FIG. 4A

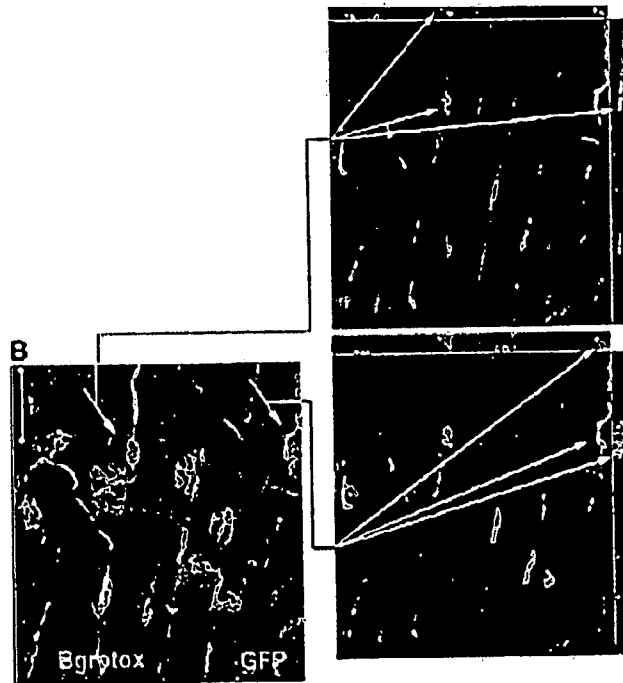


FIG. 4B

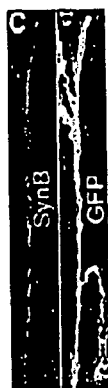


FIG. 4C

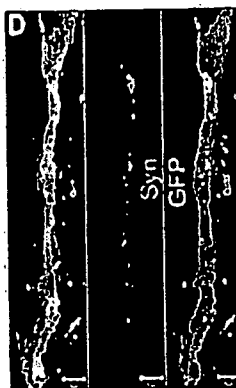


FIG. 4D

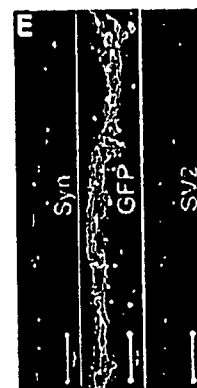


FIG. 4E

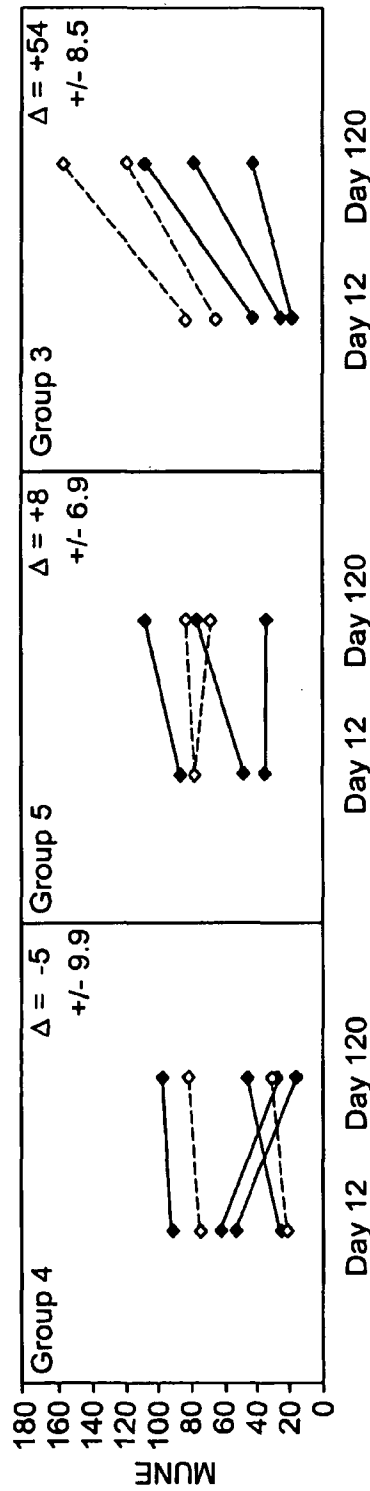


FIG. 5A

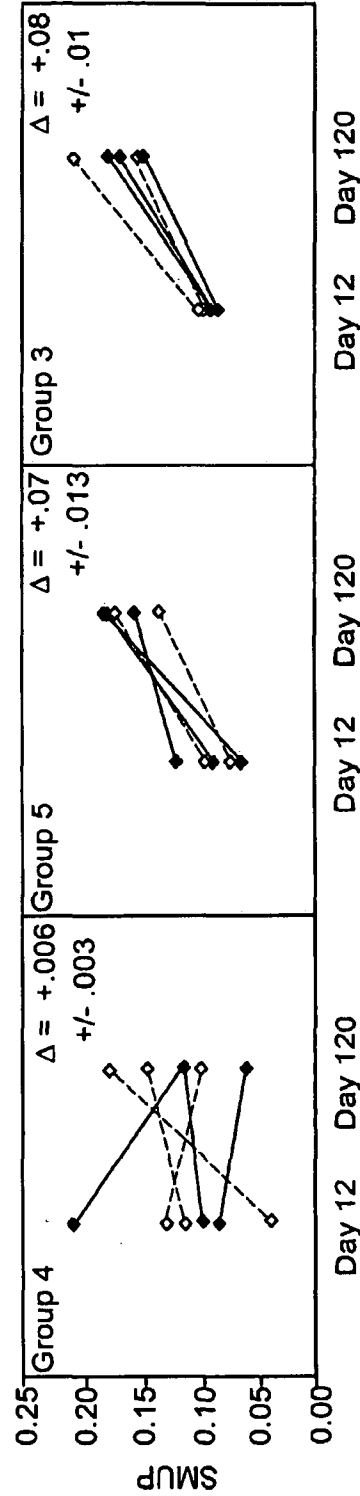


FIG. 5B

