The present disclosure relates generally to compositions and methods for treating a subject with a neurodegenerative disease and/or disorder such as an injured spinal cord, stroke, or ALS. Such methods include administering to a subject in need thereof, a therapeutically effective amount of one or more immunosuppressive drugs and/or a therapeutically effective amount of neural stem cells. Treatment with the one or more immunosuppressive drugs and neural stem cells may be effective to ameliorate one or more signs or symptoms of a neurodegenerative disease and/or disorder such as an injured spinal cord, stroke, or ALS.
COMPOSITIONS COMPRISING AN IMMUNOSUPPRESSIVE DRUG AND/OR NEURAL STEM CELLS AND METHODS OF USING SAME FOR THE TREATMENT OF NEURODEGENERATIVE DISEASES AND/OR DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of priority to U.S. Provisional Application No. 61/772,336 filed March 4, 2013 and to U.S. Provisional Application No. 61/887,584 filed October 7, 2013, both of which are incorporated herein by reference in their entirety.

FIELD

[0002] The present invention relates generally to compositions and methods for treating neurodegenerative diseases and/or disorders such as spinal cord injuries, stroke, or amyotrophic lateral sclerosis.

BACKGROUND

[0003] Cell replacement therapies (CRT) are rapidly gaining traction as viable treatments for neurodegenerative diseases and/or disorders including, for example, acute and chronic spinal cord injury (SCI), stroke, and amyotrophic lateral sclerosis (ALS). However, sites of neurodegeneration are a highly inflammatory, inhospitable environment for cell growth leading to low survival of the grafted cells (e.g., less than 5%). Thus, in order to deliver an effective dose of cells, the final dose must be injected at least 20 times. This, in turn, requires a much larger scale of cell manufacturing which poses further regulatory and economic obstacles. Failure to demonstrate reproducible administration of effective doses of cell therapy prevents approval for use by government and other regulatory agencies such as the Food and Drug Administration.

[0004] There is, therefore, a need for improved methods of treating neurodegenerative diseases and/or disorders with a regenerative therapy.

SUMMARY

[0005] The present disclosure provides compositions and methods for treating (e.g., a subject with a neurodegenerative disease and/or disorder) a neurodegenerative disease and/or disorder such as an injured spinal cord including, for example, an acute spinal cord injury, stroke, or ALS. Such methods include administering to a subject, in need thereof, a therapeutically effective amount of one or more immunosuppressive drugs and/or a therapeutically effective amount of spinal cord-derived neural stem cells. Treatment with
the one or more immunosuppressive drugs and spinal cord-derived neural stem cells may be effective to ameliorate one or more signs or symptoms of a neurodegenerative disease and/or disorder such as an injured spinal cord including, for example, an acute spinal cord injury, stroke, or ALS.

[0006] The present disclosure provides methods of treating a subject with a neurodegenerative disease or disorder, the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject; and administering a therapeutically effective amount of neural stem cells to one or more sites of the neurodegenerative disease or disorder.

[0007] In some embodiments of each or any of the above or below mentioned embodiments, the neural stem cells are spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are embryonic spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are fetal spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the fetal spinal cord-derived neural stem cells are obtained from a fetus having a gestational age of about 5 to about 20 weeks. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are human spinal cord-derived neural stem cells.

[0008] In some embodiments of each or any of the above or below mentioned embodiments, the one or more immunosuppressive drugs include one or more of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and/or sirolimus. In some embodiments of each or any of the above or below mentioned embodiments, the one or more immunosuppressive drugs are tacrolimus and mycophenolate mofetil. In some embodiments of each or any of the above or below mentioned embodiments, the one or more immunosuppressive drugs are tacrolimus, mycophenolate mofetil, and methylprednisolone.

[0009] In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of the one or more immunosuppressive drugs are administered intravenously, orally, or as a bolus injection. In some embodiments of each or any of the above or below mentioned embodiments, tacrolimus is administered both as a bolus injection and intravenously, wherein mycophenolate mofetil is administered orally, and wherein methylprednisolone is administered as a bolus injection.

[0010] In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of tacrolimus is a toxic amount.
In some embodiments of each or any of the above or below mentioned embodiments, the neurodegenerative disease or disorder is a spinal cord injury. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord injury is a traumatic spinal cord injury or an ischemic spinal cord injury.

In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are injected into one or more areas of the spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are administered to about 5 to about 50 sites in the injured spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the one or more sites are separated by a distance of approximately 100 microns to about 5000 microns.

In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells differentiate into neurons that engraft in vivo into the injured spinal cord of the subject. In some embodiments of each or any of the above or below mentioned embodiments, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the injured spinal cord of the subject.

In some embodiments of each or any of the above or below mentioned embodiments, the neurodegenerative disease or disorder is ALS. In some embodiments of each or any of the above or below mentioned embodiments, the ALS is associated with neurodegeneration in the brain and/or spinal cord.

In some embodiments of each or any of the above or below mentioned embodiments, the methods further comprise administering to the subject a therapeutically effective amount of neural stem cells to one or more sites of neurodegeneration of the brain and/or spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are administered to about 5 to about 50 sites in the brain and/or spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the one or more sites are separated by a distance of approximately 100 microns to about 5000 microns.

In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells differentiate into neurons that engraft in vivo into the brain and/or spinal cord of the subject. In some embodiments of each or any of the above or below mentioned embodiments, at least 20%, 30%, 40%, 50%,
60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the spinal cord and/or brain of the subject.

[0017] In some embodiments of each or any of the above or below mentioned embodiments, the neurodegenerative disease or disorder is stroke.

[0018] In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are injected into one or more areas of neurodegeneration in the brain. In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are administered to about 5 to about 50 sites in the brain. In some embodiments of each or any of the above or below mentioned embodiments, the one or more sites are separated by a distance of approximately 100 microns to about 5000 microns.

[0019] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells differentiate into neurons that engraft in vivo into the brain of the subject. In some embodiments of each or any of the above or below mentioned embodiments, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the brain of the subject.

[0020] In some embodiments of each or any of the above or below mentioned embodiments, the subject is human.

[0021] In some embodiments of each or any of the above or below mentioned embodiments, the methods further comprise expanding the fetal spinal cord-derived neural stem cells to form an expanded spinal cord-derived neural stem cell population. In some embodiments of each or any of the above or below mentioned embodiments, expanding the fetal spinal cord-derived neural stem cells includes culturing the fetal spinal cord-derived neural stem cells in the absence of serum. In some embodiments of each or any of the above or below mentioned embodiments, expanding the fetal spinal cord-derived neural stem cells includes exposing the fetal spinal cord-derived neural stem cells to at least one growth factor. In some embodiments of each or any of the above or below mentioned embodiments, the growth factor is selected from the group consisting of bFGF, EGF, TGF-alpha, aFGF and combinations thereof.

[0022] The present disclosure also provides methods for increasing engraftment of one or more neural stem cells administered to a subject with a neurodegenerative disease or disorder, the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject.
In some embodiments of each or any of the above or below mentioned embodiments, the neurodegenerative disease or disorder is a spinal cord injury, stroke, or ALS.

In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are embryonic spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are fetal spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the fetal spinal cord-derived neural stem cells are obtained from a fetus being gestational age of about 5 to about 20 weeks. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are human spinal cord-derived neural stem cells.

In some embodiments of each or any of the above or below mentioned embodiments, the one or more immunosuppressive drugs include one or more of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and/or sirolimus. In some embodiments of each or any of the above or below mentioned embodiments, the one or more immunosuppressive drugs are tacrolimus and mycophenolate mofetil. In some embodiments of each or any of the above or below mentioned embodiments, the one or more immunosuppressive drugs are tacrolimus, mycophenolate mofetil, and methylprednisolone.

In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of the one or more immunosuppressive drugs are administered intravenously, orally, or as a bolus injection. In some embodiments of each or any of the above or below mentioned embodiments, tacrolimus is administered both as a bolus injection and intravenously, wherein mycophenolate mofetil is administered orally, and wherein methylprednisolone is administered as a bolus injection.

In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of tacrolimus is a toxic amount.

In some embodiments of each or any of the above or below mentioned embodiments, the subject is human.

The present disclosure also provides methods of treating a subject with an injured spinal cord, the method comprising: administering a therapeutically effective amount of spinal cord-derived neural stem cells to one or more areas of the injured spinal cord; and administering a therapeutically effective amount of at least one immunosuppressive drug to the subject.
[0030] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are embryonic spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are fetal spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the fetal spinal cord-derived neural stem cells are obtained from a fetus being a gestational age of about 5 to about 20 weeks. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are human spinal cord-derived neural stem cells.

[0031] In some embodiments of each or any of the above or below mentioned embodiments, the immunosuppressive drug is at least one of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and/or sirolimus. In some embodiments of each or any of the above or below mentioned embodiments, the immunosuppressive drug is tacrolimus and mycophenolate mofetil.

[0032] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord injury is traumatic spinal cord injury or ischemic spinal cord injury.

[0033] In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are injected into one or more areas of the injured spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are administered to about 5 to about 50 sites in the injured spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the one or more sites are separated by a distance of approximately 100 microns to about 5000 microns.

[0034] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells differentiate into neurons that engraft in vivo into the injured spinal cord of the subject. In some embodiments of each or any of the above or below mentioned embodiments, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the injured spinal cord of the subject.

[0035] In some embodiments of each or any of the above or below mentioned embodiments, the subject is human.

[0036] In some embodiments of each or any of the above or below mentioned embodiments, the method further comprise expanding the fetal spinal cord-derived neural stem cells to form an expanded spinal cord-derived neural stem cell population. In some
embodiments of each or any of the above or below mentioned embodiments, expanding the fetal spinal cord-derived neural stem cells includes culturing the fetal spinal cord-derived neural stem cells in the absence of serum. In some embodiments of each or any of the above or below mentioned embodiments, expanding the fetal spinal cord-derived neural stem cells includes exposing the fetal spinal cord-derived neural stem cells to at least one growth factor. In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of the expanded neural stem cell population to one or more areas of the injured spinal cord of the subject; and administering at least one immunosuppressive drug to the subject.

[0037] The present disclosure also provides methods of treating an injured spinal cord in a subject, the method comprising: obtaining at least one neural stem cell from spinal cord tissue of a human; expanding the at least one neural stem cell to form an expanded neural stem cell population; concentrating the expanded neural stem cell population; administering a therapeutically effective amount of the expanded neural stem cell population to one or more areas of the injured spinal cord of the subject; and administering at least one immunosuppressive drug to the subject.

[0038] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are embryonic spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are fetal spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the fetal spinal cord-derived neural stem cells are obtained from a fetus being a gestational age of about 5 to about 20 weeks. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are human spinal cord-derived neural stem cells.

[0039] In some embodiments of each or any of the above or below mentioned embodiments, the immunosuppressive drug is at least one of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and/or sirolimus. In some embodiments of each or any of the above or below mentioned embodiments, the immunosuppressive drug is tacrolimus and mycophenolate mofetil.

[0040] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord injury is traumatic spinal cord injury or ischemic spinal cord injury.

[0041] In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are injected into one or more areas of the injured spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective
amount of spinal cord-derived neural stem cells are administered to about 5 to about 50 sites in the injured spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the one or more sites are separated by a distance of approximately 100 microns to about 5000 microns.

[0042] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells differentiate into neurons that engraft in vivo into the injured spinal cord of the subject. In some embodiments of each or any of the above or below mentioned embodiments, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the injured spinal cord of the subject.

[0043] In some embodiments of each or any of the above or below mentioned embodiments, the subject is human.

[0044] In some embodiments of each or any of the above or below mentioned embodiments, the methods further comprise expanding the fetal spinal cord-derived neural stem cells to form an expanded spinal cord-derived neural stem cell population. In some embodiments of each or any of the above or below mentioned embodiments, expanding the fetal spinal cord-derived neural stem cells includes culturing the fetal spinal cord-derived neural stem cells in the absence of serum. In some embodiments of each or any of the above or below mentioned embodiments, expanding the fetal spinal cord-derived neural stem cells includes exposing the fetal spinal cord-derived neural stem cells to at least one growth factor. In some embodiments of each or any of the above or below mentioned embodiments, the growth factor is selected from the group consisting of bFGF, EGF, TGF-alpha, aFGF and combinations thereof.

[0045] The present disclosure also provides methods for increasing the efficacy of a stem cell therapy in a subject with an acute spinal cord injury, the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject.

[0046] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are embryonic spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are fetal spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the fetal spinal cord-derived neural stem cells are obtained from a fetus being a gestational age of about 5 to about 20 weeks. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are human spinal cord-derived neural stem cells.
[0047] In some embodiments of each or any of the above or below mentioned embodiments, the immunosuppressive drug is at least one of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and/or sirolimus. In some embodiments of each or any of the above or below mentioned embodiments, the immunosuppressive drug is tacrolimus and mycophenolate mofetil.

[0048] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord injury is traumatic spinal cord injury or ischemic spinal cord injury.

[0049] In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are injected into one or more areas of the injured spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are administered to about 5 to about 50 sites in the injured spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the one or more sites are separated by a distance of approximately 100 microns to about 5000 microns.

[0050] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells differentiate into neurons that engraft in vivo into the injured spinal cord of the subject. In some embodiments of each or any of the above or below mentioned embodiments, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the injured spinal cord of the subject.

[0051] In some embodiments of each or any of the above or below mentioned embodiments, the subject is human.

[0052] The present disclosure also provides methods for increasing engraftment of one or more stem cells in a subject with an acute spinal cord injury, the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject.

[0053] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are embryonic spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are fetal spinal cord-derived neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the fetal spinal cord-derived neural stem cells are obtained from a fetus being a gestational age of about 5 to about 20 weeks. In some embodiments of each or
any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells are human spinal cord-derived neural stem cells.

[0054] In some embodiments of each or any of the above or below mentioned embodiments, the immunosuppressive drug is at least one of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and/or sirolimus. In some embodiments of each or any of the above or below mentioned embodiments, the immunosuppressive drug is tacrolimus and mycophenolate mofetil.

[0055] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord injury is traumatic spinal cord injury or ischemic spinal cord injury.

[0056] In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are injected into one or more areas of the injured spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of spinal cord-derived neural stem cells are administered to about 5 to about 50 sites in the injured spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the one or more sites are separated by a distance of approximately 100 microns to about 5000 microns.

[0057] In some embodiments of each or any of the above or below mentioned embodiments, the spinal cord-derived neural stem cells differentiate into neurons that engraft in vivo into the injured spinal cord of the subject. In some embodiments of each or any of the above or below mentioned embodiments, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the injured spinal cord of the subject.

[0058] In some embodiments of each or any of the above or below mentioned embodiments, the subject is human.

[0059] The present disclosure also provides method of treating a subject with amyotrophic lateral sclerosis (ALS) comprising: administering to the subject a composition comprising at least one immunosuppressive drug in an amount effective to alleviate one or more symptoms of ALS in the subject.

[0060] In some embodiments of each or any of the above or below mentioned embodiments, the one or more immunosuppressive drugs are one or more of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and/or sirolimus. In some embodiments of each or any of the above or below mentioned embodiments, the one or more immunosuppressive drugs are tacrolimus and mycophenolate mofetil. In some embodiments of each or any of the above or below
mentioned embodiments, the one or more immunosuppressive drugs are tacrolimus, mycophenolate mofetil, and methylprednisolone.

[0061] In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of the one or more immunosuppressive drugs are administered intravenously, orally, or as a bolus injection. In some embodiments of each or any of the above or below mentioned embodiments, mycophenolate mofetil is administered orally, and wherein methylprednisolone is administered as a bolus injection.

[0062] In some embodiments of each or any of the above or below mentioned embodiments, the therapeutically effective amount of tacrolimus is a toxic amount.

[0063] In some embodiments of each or any of the above or below mentioned embodiments, the ALS is associated with neurodegeneration in the brain and/or spinal cord.

[0064] In some embodiments of each or any of the above or below mentioned embodiments, the methods further comprise administering to the subject a therapeutically effective amount of neural stem cells to one or more sites of neurodegeneration of the brain and/or spinal cord.

[0065] In some embodiments of each or any of the above or below mentioned embodiments, the subject is a human.

[0066] The present disclosure provides methods and compositions for treating a subject with amyotrophic lateral sclerosis (ALS). Such methods include administering to a subject, in need thereof, a composition comprising at least one immunosuppressive drug in an amount therapeutically effective to alleviate one or more symptoms of ALS in the subject.

[0067] The present disclosure also provides methods for reducing rate of functional decline in a subject with ALS by administering to the subject a composition comprising at least one immunosuppressive drug in an amount therapeutically effective to reduce the rate of functional decline in the subject.

[0068] The present disclosure provides methods for alleviating one or more symptoms of ALS in a subject in need thereof comprising administering to the subject a composition comprising at least one immunosuppressive drug in an amount therapeutically effective to alleviate one or more of the symptoms of ALS in the subject.

[0069] The present disclosure also provides methods for treating a subject with ALS comprising of injecting at least one neural stem cell into one or more areas of spinal cord of the subject and administrating to the subject a composition comprising at least one immunosuppressive drug thereby to alleviate one or more symptoms of ALS in the subject.
[0070] In some embodiments of each or any of the above or below mentioned embodiments, the subject is treated with at least one immunosuppressive drug in an amount sufficient to reduce rejection of the neural stem cells prior to the injection of the neural stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the subject is treated with at least one immunosuppressive drug in an amount therapeutically effective to reduce rejection of the neural stem cells after the injection of the neural stem cells.

[0071] In some embodiments of each or any of the above or below mentioned embodiments, the neural stem cells are isolated from mammalian fetal spinal cord. In some embodiments of each or any of the above or below mentioned embodiments, the neural stem cells are derived from mammalian embryonic stem cells. In some embodiments of each or any of the above or below mentioned embodiments, the neural stem cells are derived from mammalian induced pluripotent stem cells.

[0072] In some embodiments of each or any of the above or below mentioned embodiments, a therapeutically effective amount of an immunosuppressive drug or a combination of immunosuppressive drugs is administered to treat ALS. In some embodiments of each or any of the above or below mentioned embodiments, the immunosuppressive drug may include, for example, methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, sirolimus, or any combinations thereof.

[0073] In some embodiments of each or any of the above or below mentioned embodiments, the subject is a mammal, for example, a human.

[0074] These and other embodiments of the invention are described in further detail herein below.

**BRIEF DESCRIPTION OF THE FIGURES**

[0075] The foregoing summary, as well as the following detailed description of the disclosure, will be better understood when read in conjunction with the appended figures. For the purpose of illustrating the disclosure, shown in the figures are embodiments which are presently preferred. It should be understood, however, that the disclosure is not limited to the precise arrangements, examples and instrumentalities shown.

[0076] Figure 1 shows extensive human cell survival at 4 weeks after grafting at the T12 contusion site in an immunosuppressed minipig. hNUMA- human specific nuclear antigen; HO14-human specific axonal neurofilament; NeuN-neron specific marker.

[0077] Figure 2 shows disease progression as measured using ALS functional rating scale-revised (ALSFRS-R) (top panels), disease progression as measured by force vital
capacity (FVC) (middle panels), and disease progression as measured by hand-held dynamometry (HHD) (bottom panels).

[0078] Figure 3 shows disease progression as measured using electrical impedance myography (EIM).

DETAILED DESCRIPTION

[0079] Sites of neurodegeneration are often a highly inflammatory, inhospitable environment for cell growth. As such, it has been recommended that a cell replacement therapy (CRT) should not begin until approximately 7-10 days after an injury (e.g., an injury that led to neurodegeneration) in order to maximize cell survival. However, longer wait times often lead to greater glial scar formation and less therapeutic benefit to CRT. In an effort to overcome the drawbacks of existing CRT, the inventors have surprisingly shown that a CRT (e.g., a therapeutically effective amount of neural stem cells) has increased survival and increased engraftment in an acutely injured spinal cord when such CRT is accompanied by treatment of the subject with one or more immunosuppressive drugs (e.g., an immunosuppressive drug with anti-inflammatory effects) including, for example, a combination of one or more of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and sirolimus. Preferred combinations of immunosuppressive agents include tacrolimus and mycophenolate mofetil, and optionally methylprednisolone. However, any immunosuppressive drug is contemplated for in the present disclosure and includes any drug aimed at decreasing or preventing activity of the subject's immune response. The methods of the present disclosure may thus be used to treat a neurodegenerative disease or disorder such as an acute traumatic spinal cord injury, an acute ischemic spinal cord injury, stroke, or amyotrophic lateral sclerosis (ALS). The disclosed methods may be used to replace or complement other pharmaceutical approaches used in the treatment of spinal cord injury.

[0080] Notably, prior tests with immunosuppressive drugs (e.g., an anti-inflammatory drug) such as minocycline demonstrated that such drugs were unable to slow a human neurodegenerative disease or disorder particularly, amyotrophic lateral sclerosis (ALS). In fact, not only were such drugs unable to slow neurodegeneration they instead led to a faster rate of neurodegeneration. See, e.g., Gordon et al. (2007) Lancet Neurology 6:1045-53. Thus, the finding disclosed herein that certain immunosuppressive drugs (e.g., certain combination of immunosuppressive drugs) used by themselves or in combination with the administration of neural stem cells can not only slow neurodegeneration but also reverse neurodegeneration was entirely unexpected as such drugs have been considered ineffective for the treatment of neurodegenerative diseases or disorders.
A neurodegenerative disease or disorder is a disease or medical condition associated with neuron loss or dysfunction. Examples of neurodegenerative diseases or disorders include neurodegenerative diseases, central nervous system injuries or dysfunctions. Neurodegenerative diseases include, for example, Alzheimer's disease or other dementia, aging, multiple sclerosis (MS), schizophrenia, macular degeneration, glaucoma, diabetic retinopathy, peripheral neuropathy, Huntington's disease, amyotrophic lateral sclerosis, and Parkinson's disease. CNS injuries include, for example, cerebrovascular events like strokes (e.g., hemorrhagic strokes, focal ischemic strokes or global ischemic strokes), ocular ischemia, and dural sinus thrombosis; traumatic brain or spinal cord injuries (e.g., injuries caused by a brain or spinal cord surgery or physical accidents); concussion; injury caused by drugs, (e.g., chemotherapeutics, recreational drugs, and neuroleptics); coronary artery bypass graft (CABG) surgery; and ischemia at child birth. CNS dysfunctions include, for example, depression, epilepsy, neurosis and psychosis.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the progressive deterioration of motor neurons which often results in fatal paralysis. ALS has an incidence rate of 2 per 100,000 people. Roughly 5-10% of cases of ALS are inherited while the majority of cases, known as sporadic ALS, do not display any conventional hereditary pattern and have no recognized cause. Disease progression is typically rapid with patients often dying within a few years of onset. Common symptoms associated with the deterioration and eventual loss of motor neurons include loss of control over voluntary movements, weakening and shrinking of muscles, fatigue, clumsiness, spasticity, and convulsions. As the disease progresses, patients begin to lose use of their limbs and ultimately become paralyzed. At late stages of the disease, patients have difficulty swallowing and chewing. Patients often die as a result of respiratory failure attributable to a weakening of the diaphragm muscles.

It is known that the outcome of immunosuppressive therapies is strongly affected by numerous factors including the combination of drugs used, side effects, the general condition of the patient, and the disease progression. Use of a combination of immunosuppressive drugs allows for the exploitation of the specific differences in the mechanism of action of each drug without requiring extremely high doses of any single immunosuppressive drug that can result in unwanted side effects.

Combinations of immunosuppressive drugs, administered according to a specified dosing regimen, have been shown by the inventors to effectively increase engraftment of transplanted cells (e.g., a CRT) in an acutely injured spinal cord. Specifically, such combinations may include tacrolimus, mycophenolate mofetil, and...
methylprednisolone. Tacrolimus is preferably administered as a bolus injection (e.g., about 0.1 mg/kg) and then at a first continuous dose (e.g., steady state plasma levels of 50-60 ng/ml) for the first fourteen days after the CRT followed by a second continuous dose that is half the dose of the first dose (e.g., steady state plasma levels of 25-35 ng/ml) until termination of administration of the drug. In some embodiments, the first continuous dose of tacrolimus is a toxic dose. Additionally, mycophenolate mofetil is preferably administered at a dose for the first fourteen days after the CRT after which its administration is terminated. Further, methylprednisolone is preferably administered as a single dosage after the CRT and Depo Medrol may optionally be administered for a period of seven days.

[0085] The present disclosure provides methods of treating a subject (e.g., a human) with a neurodegenerative disease or disorder, the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject; and administering a therapeutically effective amount of neural stem cells (e.g., spinal cord-derived neural stem cells) to one or more sites of the neurodegenerative disease or disorder. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone. It will be understood that the one or more immunosuppressive drugs may be administered prior to, at the same time, and/or after the administration of neural stem cells.

[0086] The present disclosure also provides methods of treating a subject (e.g., a human) with an injured spinal cord (e.g., a traumatic spinal cord injury or an ischemic spinal cord injury), the method comprising: administering a therapeutically effective amount of neural stem cells (e.g., spinal cord-derived neural stem cells) to one or more areas of the injured spinal cord; and administering a therapeutically effective amount of at least one immunosuppressive drug to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone. It will be understood that the one or more immunosuppressive drugs may be administered prior to, at the same time, and/or after the administration of neural stem cells.

[0087] The present disclosure also provides methods of treating a subject (e.g., a human) with a stroke (e.g., a subject that suffered from/ experienced a stroke), the method comprising: administering a therapeutically effective amount of neural stem cells (e.g., spinal cord-derived neural stem cells) to one or more areas of neurodegeneration in the brain; and administering a therapeutically effective amount of at least one immunosuppressive drug to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally
methylprednisolone. It will be understood that the one or more immunosuppressive drugs may be administered prior to, at the same time, and/or after the administration of neural stem cells.

[0088] The present disclosure also provides methods of treating a subject (e.g., a human) with ALS, the method comprising: administering a therapeutically effective amount of neural stem cells (e.g., spinal cord-derived neural stem cells) to one or more areas of neurodegeneration in the brain and/or spinal cord; and administering a therapeutically effective amount of at least one immunosuppressive drug to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone. It will be understood that the one or more immunosuppressive drugs may be administered prior to, at the same time, and/or after the administration of neural stem cells.

[0089] The present disclosure also provides methods of treating a neurodegenerative disease or disorder in a subject (e.g., a human), the method comprising: obtaining at least one neural stem cell (e.g., a spinal cord-derived neural stem cell) of a human; expanding the at least one neural stem cell to form an expanded neural stem cell population; concentrating the expanded neural stem cell population; administering a therapeutically effective amount of the expanded neural stem cell population to one or more sites of neurodegeneration in the subject; and administering one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone. It will be understood that the one or more immunosuppressive drugs may be administered prior to, at the same time, and/or after the administration of neural stem cells.

[0090] The present disclosure also provides methods of treating an injured spinal cord (e.g., a traumatic spinal cord injury or an ischemic spinal cord injury) in a subject (e.g., a human), the method comprising: obtaining at least one neural stem cell (e.g., a spinal cord-derived neural stem cell) of a human; expanding the at least one neural stem cell to form an expanded neural stem cell population; concentrating the expanded neural stem cell population; administering a therapeutically effective amount of the expanded neural stem cell population to one or more areas of the injured spinal cord of the subject; and administering one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone. It will be understood that the one or more immunosuppressive drugs may be administered prior to, at the same time, and/or after the administration of neural stem cells.
The present disclosure also provides methods of treating a stroke (e.g., neurodegeneration associated with a stroke) in a subject (e.g., a human), the method comprising: obtaining at least one neural stem cell (e.g., a spinal cord-derived neural stem cell) of a human; expanding the at least one neural stem cell to form an expanded neural stem cell population; concentrating the expanded neural stem cell population; administering a therapeutically effective amount of the expanded neural stem cell population to one or more areas of neurodegeneration in the brain of the subject; and administering one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone. It will be understood that the one or more immunosuppressive drugs may be administered prior to, at the same time, and/or after the administration of neural stem cells.

The present disclosure also provides methods of treating ALS in a subject (e.g., a human), the method comprising: obtaining at least one neural stem cell (e.g., a spinal cord-derived neural stem cell) of a human; expanding the at least one neural stem cell to form an expanded neural stem cell population; concentrating the expanded neural stem cell population; administering a therapeutically effective amount of the expanded neural stem cell population to one or more areas of neurodegeneration in the brain and/or spinal cord of the subject; and administering one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone. It will be understood that the one or more immunosuppressive drugs may be administered prior to, at the same time, and/or after the administration of neural stem cells.

The present disclosure also provides methods for increasing the efficacy of a stem cell therapy in a subject (e.g., a human) with a neurodegenerative disease or disorder, the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone.

The present disclosure also provides methods for increasing the efficacy of a stem cell therapy in a subject (e.g., a human) with a spinal cord injury (e.g., a traumatic spinal cord injury or an ischemic spinal cord injury), the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone.
[0095] The present disclosure also provides methods for increasing the efficacy of a stem cell therapy in a subject (e.g., a human) with a stroke (e.g., a subject that suffered from/ experienced a stroke), the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone.

[0096] The present disclosure also provides methods for increasing the efficacy of a stem cell therapy in a subject (e.g., a human) with ALS, the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone.

[0097] The present disclosure also provides methods for increasing engraftment of one or more neural stem cells (e.g., spinal cord-derived neural stem cells) in a subject (e.g., a human) with a neurodegenerative disease or disorder, the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone.

[0098] The present disclosure also provides methods for increasing engraftment of one or more neural stem cells (e.g., spinal cord-derived neural stem cells) in a subject (e.g., a human) with a spinal cord injury (e.g., a traumatic spinal cord injury or an ischemic spinal cord injury), the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone.

[0099] The present disclosure also provides methods for increasing engraftment of one or more neural stem cells (e.g., spinal cord-derived neural stem cells) in a subject (e.g., a human) with a stroke (e.g., a subject that suffered from/ experienced a stroke), the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject. In an embodiment, the one or more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone.

[0010] The present disclosure also provides methods for increasing engraftment of one or more neural stem cells (e.g., spinal cord-derived neural stem cells) in a subject (e.g., a human) with ALS, the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject. In an embodiment, the one or
more immunosuppressive agents may include tacrolimus, mycophenolate mofetil, and optionally methylprednisolone.

[00101] Neural stem cells contemplated for use in the present disclosure include spinal cord-derived neural stem cells such as embryonic spinal cord-derived neural stem cells or fetal spinal cord-derived neural stem cells such as from a fetus being a gestational age of about 5 to about 20 weeks. In an embodiment, the neural stem cells are human neural stem cells. The neural stem cells are able to differentiate into neurons that engraft in vivo into one or more sites of neurodegeneration in the subject. Preferably, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the injured spinal cord of the subject.

[00102] The methods of the present disclosure may comprise expanding the neural stem cells to form an expanded neural stem cell population. In an embodiment, expanding the neural stem cells includes culturing the neural stem cells in the absence of serum. In a further embodiment, expanding the neural stem cells includes exposing the neural stem cells to at least one growth factor including, for example, a growth factor selected from the group consisting of bFGF, EGF, TGF-alpha, aFGF and combinations thereof.

[00103] Immunosuppressive drugs contemplated for use in the present disclosure include methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, sirolimus, or any combination thereof. In a preferred embodiment, the immunosuppressive drugs include tacrolimus and mycophenolate mofetil.

[00104] The neural stem cells (e.g., a therapeutically effective amount of neural stem cells) may be injected into one or more sites (i.e., areas) of neurodegeneration and may be administered to about 5 to about 50 sites of neurodegeneration. In an embodiment, the one or more sites may be separated by a distance of approximately 100 microns to about 5000 microns.

[00105] The present disclosure also provides methods for reducing a rate of functional decline in a subject (e.g., a human) with ALS comprising: administering to the subject a composition comprising at least one immunosuppressive drug in an amount effective to reduce the rate of functional decline in the subject. In an embodiment, the effective amount of the one or more immunosuppressive drugs are administered intravenously, orally, or as a bolus injection. In a further embodiment, tacrolimus including, for example, a toxic dose of tacrolimus, is administered both as a bolus injection and intravenously, wherein mycophenolate mofetil is administered orally, and wherein methylprednisolone is administered as a bolus injection.

[00106] The present disclosure also provides methods for alleviating one or more symptoms of ALS in a subject (e.g., a human) with ALS comprising: administering to the
subject a composition comprising at least one immunosuppressive drug in an amount effective to alleviate one or more of the symptoms of ALS in the subject. In an embodiment, the effective amount of the one or more immunosuppressive drugs are administered intravenously, orally, or as a bolus injection. In a further embodiment, tacrolimus including, for example, a toxic dose of tacrolimus, is administered both as a bolus injection and intravenously, wherein mycophenolate mofetil is administered orally, and wherein methylprednisolone is administered as a bolus injection.

[00107] The present disclosure also provides methods of treating a subject (e.g., a human) with ALS comprising: injecting at least one neural stem cell into one or more areas of the spinal cord and/or brain of the subject and administrating to the subject a composition comprising at least one immunosuppressive drug thereby to alleviate one or more symptoms of ALS in the subject. In an embodiment, the effective amount of the one or more immunosuppressive drugs are administered intravenously, orally, or as a bolus injection. In a further embodiment, tacrolimus including, for example, a toxic dose of tacrolimus, is administered both as a bolus injection and intravenously, wherein mycophenolate mofetil is administered orally, and wherein methylprednisolone is administered as a bolus injection.

[00108] A combination of therapies may be required to effectively slow or treat the pathogenic processes of ALS. Thus, the disclosure provides methods and compositions for treating ALS aimed at stopping or at least reducing the disease progression caused by an autoimmune process through the treatment with an immunosuppressive therapy alone or in combination with a cell-based therapy including, for example, neural stem cells.

[00109] The present disclosure also provides one or more immunosuppressive agents including, for example, tacrolimus, mycophenolate mofetil, and optionally methylprednisolone useful for treating any known disease or disorder such as an inflammatory disease or disorder.

[00110] In some embodiments, "treating" or "treatment" of a disease, disorder, or condition such as a spinal cord injury includes at least partially: (1) preventing the disease, disorder, or condition, i.e., causing the clinical symptoms of the disease, disorder, or condition not to develop in a mammal that is exposed to or predisposed to the disease, disorder, or condition but does not yet experience or display symptoms of the disease, disorder, or condition; (2) inhibiting the disease, disorder, or condition, i.e., arresting or reducing the development of the disease, disorder, or condition or its clinical symptoms; or (3) relieving the disease, disorder, or condition, i.e., causing regression of the disease, disorder, or condition or its clinical symptoms.
In some embodiments, "effective amount," as used herein, refers to the amount of spinal cord-derived neural stem cells that is required to confer a therapeutic effect on the subject. A "therapeutically effective amount," as used herein, refers to a sufficient amount of spinal cord-derived neural stem cells being administered which will relieve to some extent one or more of the symptoms of the disease, disorder, or condition being treated. In some embodiments, the result is a reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. For example, in some embodiments, an "effective amount" for therapeutic uses is the amount of the spinal cord-derived neural stem cells required to provide a clinically significant decrease in disease symptoms without undue adverse side effects. In some embodiments, an appropriate "effective amount" in any individual case is determined using techniques, such as a dose escalation study. The term "therapeutically effective amount" includes, for example, a prophylactically effective amount. In other embodiments, an "effective amount" of spinal cord-derived neural stem cells is an amount effective to achieve a desired pharmacologic effect or therapeutic improvement without undue adverse side effects. In other embodiments, it is understood that "an effect amount" or "a therapeutically effective amount" varies from subject to subject, due to variation in metabolism, age, weight, general condition of the subject, the condition being treated, the severity of the condition being treated, and the judgment of the prescribing physician.

The term "in need of treatment" as used herein refers to a judgment made by a caregiver that a patient requires or will benefit from treatment. This judgment is made based on a variety of factors that are in the realm of a caregiver's expertise, but that includes the knowledge that the patient exhibits a clinical symptom or manifestation of ALS.

As used herein, the term "alleviate" or "alleviating" or "ameliorating" refers to lightening or lessening the severity of a symptom, condition, or disorder. It is understood that, in certain circumstances, a treatment can alleviate a symptom or condition without treating the underlying disorder.

As used herein, the term, "neural stem cell" or "NSC" refers to a multipotential stem cell that can be functionally defined according to their capacity to differentiate into each of the three major cell types of the central nervous system (CNS): neurons, astrocytes, and oligodendrocytes. As used herein, the term "stem cell" refers to an undifferentiated cell that is capable of self-renewal, meaning that with each cell division at least one daughter cell will also be a stem cell. NSCs can also refer to neural or neuronal progenitors, or neuroepithelial precursors.

In one embodiment, the NSCs are multipotent such that each cell has the capacity to differentiate into a neuron, astrocyte or oligodendrocyte. In another
embodiment, the NSCs are bipotent such that each cell has the capacity to differentiate into two of the three cell types of the CNS. In another embodiment, the NSCs include at least bipotent cells generating both neurons and astrocytes in vitro and include at least unipotent cells generating neurons in vivo.

[0016] Growth conditions can influence the differentiation direction of the cells toward one cell type or another, indicating that the cells are not committed toward a single lineage. In culture conditions that favor neuronal differentiation, cells, particularly from human CNS, are largely bipotent for neurons and astrocytes and differentiation into oligodendrocytes is minimal. Thus, the differentiated cell cultures of the disclosed methods may give rise to neurons and astrocytes.

[0017] In an embodiment, the NSCs are isolated from the CNS. As used herein, the term "isolated" with reference to a cell, refers to a cell that is in an environment different from that which the cell naturally occurs (e.g. where the cell naturally occurs in an organism) and the cell is removed from its natural environment.

[0018] NSCs may be isolated from an area which is naturally neurogenic for a desired population of neurons and from embryonic, fetal, post-natal, juvenile or adult tissue. The desired population of cells may include the cells of a specific neuronal phenotype which can replace or supplement such phenotype lost or inactive in the course of disease progression. In an embodiment, the NSCs are isolated from the subventricular zone (SVZ) or from the subgranular zone of the dentate gyrus (DG). In preferred embodiments, the NSCs are isolated from the spinal cord in which neurogenesis of ventral motor-neurons is substantial and obtained at a gestational age of human fetal development during which neurogenesis of ventral motor-neurons is substantial.

[0019] Accordingly, in an embodiment, NSCs are isolated from the spinal cord at a gestational age of about 6.5 to about 20 weeks. Preferably, NSCs are isolated from the spinal cord at a gestational age of about 7 to about 9 weeks. In another embodiment the NSCs are isolated from embryonic spinal cord tissue. In yet another embodiment, neural stem cells are isolated from a human. It should be appreciated that the proportion of the isolatable NSC population can vary with the age of the donor. Expansion capacity of the cell populations can also vary with the age of the donor.

[00120] The NSCs of the ventral midbrain, for example, are distinct from the NSCs obtained from the spinal cord at the same gestational stage. In particular, the NSCs from the ventral midbrain exclusively give rise to tyrosine-hydroxylase-expressing dopaminergic neurons, whereas NSCs from the spinal cord exclusively generate acetylcholine-producing cholinergic neurons. Both cell types, however, simultaneously generate the more ubiquitous glutamate- and GABA-producing neurons. Therefore, in an embodiment, the
disclosed methods include obtaining NSCs from the spinal cord to treat conditions ameliorated or attenuated, at least in part, by the implantation of acetylcholine-producing cholinergic neurons.

[00121] NSCs can also be isolated from post-natal and adult tissues. NSCs derived from post-natal and adult tissues are quantitatively equivalent with respect to their capacity to differentiate into neurons and glia, as well as in their growth and differentiation characteristics. However, the efficiency of in vitro isolation of NSCs from various post-natal and adult CNS can be much lower than isolation of NSCs from fetal tissues which harbor a more abundant population of NSCs. Nevertheless, as with fetal-derived NSCs, the disclosed methods enable at least about 30% of NSCs derived from neonatal and adult sources to differentiate into neurons in vitro. Thus, post-natal and adult tissues can be used as described above in the case of fetal-derived NSCs.

[00122] In an embodiment, human fetal spinal tissue is dissected under a microscope. A region of tissue corresponding to the lower cervical/upper thoracic segments is isolated. The NSCs are isolated, pooled, and expanded on poly-D-lysine coated culture vessels in a media containing fibronectin and basic fibroblast growth factor (bFGF; FGF-2). Cells are expanded and then concentrated to the desired target cell density of about 10,000 cells per microliter in a medium free of preservative and antibiotics. Concentrated cells may be used fresh for implantation or frozen for later use.

[00123] In an embodiment, the NSCs are derived from embryonic stem cells or induced pluripotent stem cells. As used herein, the term "embryonic stem cell," refers to a stem cell isolated from the developing embryo which can give rise to all of the cells of the body (e.g., cells of the ecto-, meso-, and/or endo-dermal cell lineages). The term "induced pluripotent stem cell," as used herein, refers to a stem cell derived from a somatic cell (e.g., a differentiated somatic cell) that has a higher potency than the somatic cell. Embryonic stem cells and induced pluripotent stem cells are capable of differentiation into more mature cells (e.g., neural stem cells or neural progenitor cells). Methods employed for growing and differentiating embryonic or induced pluripotent stem cells into NSCs in vitro can, for example, be such as those described in Daadi et al., PLoS One. 3(2):e1644 (2008).

[00124] In an embodiment, the NSCs can be diluted with an acceptable pharmaceutical carrier. The term "pharmaceutically acceptable carrier" as used herein refers to a diluent, adjuvant, excipient, or vehicle with which the cells of the disclosure are administered and which is approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. Such pharmaceutical carriers can be liquids, such as
water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical carriers can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea, and the like. When administered to a patient, the neural stem cells and pharmaceutically acceptable carriers can be sterile. Water is a useful carrier when the cells are administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers also include excipients such as glucose, lactose, sucrose, glycerol monostearate, sodium chloride, glycerol, propylene, glycol, water, ethanol and the like. The present compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The present compositions advantageously may take the form of solutions, emulsion, sustained-release formulations, or any other form suitable for use. The selection of a suitable carrier is within the skill of the ordinary artisan.

[00125] Various neuronal subtypes can be obtained from manipulation of embryonic stem cells expanded in culture. Thus, specific neuronal subtypes, based on the disclosed methods, can be isolated and purified from other irrelevant or unwanted cells to improve the result, as needed, and can be used for treatment of cognitive dysfunction.

[00126] The NSCs in the disclosed methods can be derived from one site and transplanted to another site within the same subject as an autograft. Furthermore, the NSCs in the disclosed methods can be derived from a genetically identical donor and transplanted as an isograft. Still further, the NSCs in the disclosed methods can be derived from a genetically non-identical member of the same species and transplanted as an allograft. Alternatively, NSCs can be derived from non-human origin and transplanted as a xenograft. With the development of powerful immunosuppressants, allograft and xenograft of non-human neural precursors, such as neural precursors of porcine origin, can be grafted into human subjects.

[00127] A sample tissue can be dissociated by any standard method. In one embodiment, tissue is dissociated by gentle mechanical trituration using a pipette and a divalent cation-free buffer (e.g. saline) to form a suspension of dissociated cells. Sufficient dissociation to obtain largely single cells is desired to avoid excessive local cell density.

[00128] For successful commercial application of NSCs, maintaining robust and consistent cultures that have stable expansion and differentiation capacities through many successive passages is desirable. As described above, the culture methods can be optimized to achieve long-term, stable expansion of an individual cell line of NSCs from different areas and ages of CNS development while maintaining their distinct progenitor properties. In one embodiment, stem cells can be cultured according to the methods set
U.S. 7,544,511, the entireties of which are incorporated by reference herein.

[00129] In an embodiment, the NSCs of the disclosed methods can include pre-
differentiated cells for transplantation. For maximum yield of the cells and for simplicity of
the procedure, a confluent culture is harvested for transplantation which comprises
primarily a population of undifferentiated cells. It should be appreciated, however, that a
minor population of cells just starting to differentiate spontaneously can also exist due to
the increased cell density.

[00130] In an embodiment, the NSCs are concentrated in a solution such as the
clinically usable, hibernation or freezing solutions described above. In an embodiment, the
NSCs are concentrated to an appropriate cell density which can be the same or different
from the cell density for administration of the cells. In an embodiment, the cell density for
administration can vary from about 1,000 cells per microliter to about 1,000,000 cells per
microliter depending upon factors such as the site of the injection, the minimum dose
necessary for a beneficial effect, and toxicity side-effect considerations.

[00131] Low cell survival of donor cells using known methods has necessitated the
delivery of a large quantity of cells to a relatively small area in order to attempt effective
treatment. Injection volume, however, is hydrostatic pressure exerted on the host tissue
and the prolonged injection time associated with high injection volumes exacerbates
surgical risk. Additionally, over-injection of donor cells leads to compression and
subsequent injury of the host parenchymal tissue. In attempting to compensate for volume
constraints, known methods have required preparation of high cell density suspensions for
the injections. However, a high cell density promotes tight clustering of the transplanted
cells and inhibits cell migration or spreading preventing effective treatment beyond a limited
area and compromising seamless integration into the host tissue.

[00132] In contrast, as a result of improved survival in vivo of the cells prepared by the
disclosed methods, fewer number of cells are needed per injection. In fact, up to three to
four times the number of injected cells have been shown to exist after six months from the
time of injection demonstrating significant quantitative survival using the disclosed
methods. Also, because of the quantitative survival, reproducible administration of desired
cell doses can be achieved. Accordingly, in one embodiment, the NSCs are concentrated
to a density of about 1,000 to about 1,000,000 cells per microliter. In one embodiment, the
NSCs are concentrated to a density of about 2,000 to about 80,000 NSCs per microliter. In
another embodiment, about 5,000 to about 50,000 NSCs per microliter have been used for
effective engraftment. In another embodiment, about 10,000 to 30,000 NSCs per microliter
are used. In a preferred embodiment, the NSCs are concentrated to a density of about 70,000 NSCs per microliter.

[00133] In another embodiment, the NSCs are concentrated to a density of about 1,000 to about 10,000 cells per microliter, about 10,000 to about 20,000 cells per microliter, about 20,000 to about 30,000 cells per microliter, about 30,000 to about 40,000 cells per microliter, about 40,000 to about 50,000 cells per microliter, about 50,000 to about 60,000 cells per microliter, about 60,000 to about 70,000 cells per microliter, about 70,000 to about 80,000 cells per microliter, about 80,000 to about 90,000 cells per microliter, or about 90,000 to about 100,000 cells per microliter.

[00134] In another embodiment, the NSCs are concentrated to a density of about 100,000 to about 200,000 cells per microliter, about 200,000 to about 300,000 cells per microliter, about 300,000 to about 400,000 cells per microliter, about 400,000 to about 500,000 cells per microliter, about 500,000 to about 600,000 cells per microliter, about 600,000 to about 700,000 cells per microliter, about 700,000 to about 800,000 cells per microliter, about 800,000 to about 900,000 cells per microliter, about 900,000 to about 1,000,000 cells per microliter.

[00135] In another embodiment, the NSCs can be delivered to a treatment area suspended in an injection volume of less than about 100 microliters per injection site. For example, in the treatment of cognitive dysfunction of a human subject where multiple injections may be made, an injection volume of 0.1 and about 100 microliters per injection site can be used. In preferred embodiments, the NSCs can be delivered to a treatment area suspended in an injection volume of about 1 microliter per injection site.

[00136] In an embodiment, the disclosed methods include injecting NSCs at a cell density of about 1,000 to about 10,000 cells per microliter, about 10,000 to about 20,000 cells per microliter, about 20,000 to about 30,000 cells per microliter, about 30,000 to about 40,000 cells per microliter, about 40,000 to about 50,000 cells per microliter, about 50,000 to about 60,000 cells per microliter, about 60,000 to about 70,000 cells per microliter, about 70,000 to about 80,000 cells per microliter, about 80,000 to about 90,000 cells per microliter, or about 90,000 to about 100,000 cells per microliter into to one or more areas of the spinal cord of the subject.

[00137] In some embodiments, the disclosed methods include injecting NSCs at a cell density of about 100,000 to about 200,000 cells per microliter, about 200,000 to about 300,000 cells per microliter, about 300,000 to about 400,000 cells per microliter, about 400,000 to about 500,000 cells per microliter, about 500,000 to about 600,000 cells per microliter, about 600,000 to about 700,000 cells per microliter, about 700,000 to about 800,000 cells per microliter, about 800,000 to about 900,000 cells per microliter, or about
900,000 to about 1,000,000 cells per microliter into to one or more areas of the spinal cord of the subject.

[00138] In an embodiment, the disclosed methods include injecting NSCs at a cell density of about 5,000 to about 50,000 cells per microliter. In preferred embodiments, the disclosed methods include injecting NSCs at a cell density of about 70,000 cells per microliter.

[00139] In an embodiment, the disclosed methods include multiple injections of NSCs at a total cell number of about 4,000 to about 40,000 cells, about 40,000 to about 80,000 cells, about 80,000 to about 120,000 cells, about 120,000 to about 160,000 cells, about 160,000 to about 200,000 cells, about 200,000 to about 240,000 cells, about 240,000 to about 280,000 cells, about 280,000 to about 320,000 cells, about 320,000 to about 360,000 cells, or about 360,000 to about 400,000 cells introduced into one or more areas of the spinal cord of the subject.

[00140] In some embodiments, the disclosed methods include multiple injections of NSCs with a total cell number of about 400,000 to about 800,000 cells, about 800,000 to about 1,200,000 cells, about 1,200,000 to about 1,600,000 cells, about 1,600,000 to about 2,000,000 cells, about 2,000,000 to about 2,400,000 cells, about 2,400,000 to about 2,800,000 cells, about 2,800,000 to about 3,200,000 cells, about 3,200,000 to about 3,600,000 cells, or about 3,600,000 to about 4,000,000 cells introduced into one or more areas of the spinal cord of the subject.

[00141] The volume of media in which the expanded NSCs are suspended for delivery to a treatment area can be referred to herein as the injection volume. The injection volume depends upon the injection site and the degenerative state of the tissue. More specifically, the lower limit of the injection volume can be determined by practical liquid handling of viscous suspensions of high cell density as well as the tendency of the cells to cluster. The upper limit of the injection volume can be determined by limits of compression force exerted by the injection volume that are necessary to avoid injuring the host tissue, as well as the practical surgery time.

[00142] Any suitable device for injecting the cells into a desired area can be employed in the disclosed methods. In an embodiment, a syringe capable of delivering sub-microliter volumes over a time period at a substantially constant flow rate is used. The cells can be loaded into the device through a needle or flexible tubing or any other suitable transfer device.

[00143] In another embodiment, the cells are injected at between about 2 and about 5 sites in the brain. In an embodiment, the cells are injected at between about 5 and about 10 sites in the brain. In an embodiment, the cells are injected at between about 10 to
about 30 sites in the brain. In an embodiment, the cells are injected at between about 10 to about 50 sites in the brain. At least two of the sites can be separated by a distance of approximately 100 microns to about 5,000 microns. In an embodiment, the distance between injection sites is about 400 to about 600 microns. In an embodiment, the distance between injections sites is about 100 to about 200 microns, about 200 to about 300 microns, about 300 to about 400 microns, about 400 to about 500 microns, about 500 to about 600 microns, about 600 to about 700 microns, about 700 to about 800 microns, about 800 to about 900 microns, or about 900 to about 1,000 microns. In an embodiment, the distance between injection sites is about 1,000 to about 2,000 microns, about 2,000 to about 3,000 microns, about 3,000 to about 4,000 microns, or about 4,000 to about 5,000 microns. The distance between injections sites can be determined based on generating substantially uninterrupted and contiguous donor cell presence throughout the spinal cord tissue and based on the average volume of injections demonstrated to achieve about 2-3 month survival in animal models such as rats or pigs. The actual number of injections and distance between injections in humans can be extrapolated from results in animal models.

[00144] The NSCs of the disclosed methods can generate large numbers of neurons in vivo. When the NSCs are not overtly pre-differentiated prior to transplant, the NSCs can proliferate up to two to four cell divisions in vivo before differentiating, thereby further increasing the number of effective donor cells. Upon differentiation, the neurons secrete specific neurotransmitters. In addition, the neurons secrete into the milieu surrounding the transplant in vivo growth factors, enzymes and other proteins or substances which are beneficial for different conditions. Accordingly, a variety of conditions can be treated by the disclosed methods because of the ability of the implanted cells to generate large numbers of neurons in vivo and because the cognitive dysfunction may be caused by or result in missing elements including neuron-derived elements. Therefore, subjects suffering from cognitive dysfunctions due to lack of such neuron-derived elements, such as growth factors, enzymes and other proteins, can be treated effectively by the disclosed methods.

[00145] In an embodiment, the composition comprising an amount of NSCs may be administered to a subject in accordance with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, intravenous, subcutaneous, intra-articular, intrasynovial, or intrathecal routes. Intracerebrospinal, intrathecal, intravenous, intraperitoneal, or subcutaneous administration of the cells is preferred, with intracerebrospinal, intrathecal, or intravenous routes being particularly preferred; however, other cell administration paradigms well known in the art can be used.
In an embodiment, introducing the therapeutically effective amount of the NSCs includes injecting at least a portion of the therapeutically effective amount into a plurality of areas of the recipient spinal cord. In an embodiment, the desired injection site includes at least one area of the spinal cord. In an embodiment, the cells are implanted into at least one specific segment or region of the spinal cord such as the cervical, thoracic or lumbar region of the spinal cord. In the lumbar region, for example, only five pairs of nerve roots traverse the bony canal of vertebrae with each pair of nerve roots exiting the spine at each lumbar level distributed over a wide area. Due to a lower density of nerve roots in the lumbar region of the spinal cord, the lumbar region is particularly well-suited for providing a safe site for injection of cells. In an embodiment, the cells are implanted in the intermediate zone of the spinal cord parenchyma.

In an embodiment, the NSCs can be delivered to or near the ventral horn of the gray matter of various spinal segments from cervical to lumbar.

In an embodiment, the areas include doral horn.

In an embodiment, the areas include intrathecal space.

In one embodiment, compositions of the NSCs of the invention are formulated as an injectable formulation and comprise, for example, an aqueous solution or suspension of the active ingredient suitable for intracerebrospinal delivery. When preparing the composition for injection, particularly for intracerebrospinal delivery, a continuous phase can be present that comprises an aqueous solution of tonicity modifiers, buffered to a pH below about 7, or below about 6, for example about 2 to about 7, about 3 to about 6 or about 3 to about 5. The tonicity modifiers can comprise, for example, sodium chloride, glucose, mannitol, trehalose, glycerol, or other pharmaceutical agents that render osmotic pressure of the formulation isotonic with blood. Alternatively, when a larger quantity of the tonicity modifier is used in the formulation, it can be diluted prior to injection with a pharmaceutically acceptable diluent to render the mixture isotonic with blood.

In some embodiments of any of the aforementioned methods, the composition comprising NSCs is administered once. In some embodiments of any of the aforementioned methods, administration of an initial dose the composition comprising NSCs is followed by the administration of one or more subsequent doses. Examples of dosing regimens (e.g., an interval between the first dose and one or more subsequent doses) that can be used in the methods of the disclosure include an interval of about once every week to about once every 12 months, an interval of about once every two weeks to about once every 6 months, an interval of about once every month to about once every 6 months, an interval of about once every 3 months to about once every 6 months. In some embodiments,
administration is monthly, every two months, every three months, every four months, every five months, every six months, or upon disease recurrence.  

[00152] In an embodiment, the NSCs are injected at between about 5 and about 50 sites. In an embodiment, the NSCs are injected at between about 10 to about 30 sites on each side of the cord. At least two of the sites can be separated by a distance of approximately 100 microns to about 5000 microns. In an embodiment, the distance between injection sites is about 400 to about 600 microns. The distance between injections sites can be determined based on generating substantially uninterrupted and contiguous donor cell presence throughout the spinal segments and based on the average volume of injections demonstrated to achieve about 2 to 3 month survival in animal models such as rats or pigs. In an embodiment, the NSCs are injected along both sides of the midline of the spinal cord to span the length of at least several lumbar segments useful for treating a symptom such as spasticity/rigidity or motor neuron survival. The actual number of injections in humans can be extrapolated from results in animal models. 

[00153] The methods of the present disclosure include administration of one or more immunosuppressive drugs prior to, concurrent with, or after the injection of the NSCs. 

[00154] In some embodiments, the NSCs and immunosuppressive drug may be co-administered. The NSCs and immunosuppressive drug which make up the therapy may be a combined dosage form or in separate dosage forms intended for substantially simultaneous administration. The NSCs and immunosuppressive drug may also be administered sequentially, with either the NSCs or immunosuppressive drug being administered by a regimen calling for multiple step administration. Thus, a regimen may call for sequential administration of the NSCs and immunosuppressive drug with spaced-apart administration of the separate, active agents. The time period between the multiple administration steps may range from, for example, a few minutes to several hours to days, depending upon the properties of the NSCs and immunosuppressive drug such as potency, solubility, bioavailability, plasma half-life and kinetic profile of the therapeutic compound, as well as depending upon the effect of food ingestion and the age and condition of the subject. Circadian variation of the target molecule concentration may also determine the optimal dose interval. The NSCs and immunosuppressive drug whether administered simultaneously, substantially simultaneously, or sequentially, may involve a regimen calling for administration of the NSCs by intravenous route and the immunosuppressive drug by an oral route, a percutaneous route, an intravenous route, an intramuscular route, or by direct absorption through mucous membrane tissues, for example. Whether the neural stem cells and immunosuppressive drug are administered orally, by inhalation spray, rectally, topically, buccally (for example, sublingual), or
parenterally (for example, subcutaneous, intramuscular, intravenous and intradermal injections, or infusion techniques), separately or together, each such therapeutic compound will be contained in a suitable pharmaceutical formulation of pharmaceutically-acceptable excipients, diluents or other formulations components.

[00155] Any immunosuppressive drug is contemplated for in the present disclosure. As used herein, the term "immunosuppressive drug" denotes any drug aimed at decreasing or preventing activity of the subject's immune response.

[00156] Immunosuppressive drugs act to inhibit the proliferation and activity of all or a substantial portion of the immune cells within the body. Many immunosuppressive drugs function by inhibiting a step in the interleukin 2 (IL-2) signaling pathway. IL-2 is a cytokine that regulates the growth, proliferation, and activation of lymphocytes. For example, the immunosuppressive drug tacrolimus, considered one of the most potent immune system suppressors, is a calcineurin-dependent inhibitor that blocks IL-2 production and reduces proliferation of T-cells. Another immunosuppressive drug sirolimus acts in a calcineurin-independent fashion to inhibit the response of T- and B-cells to IL-2. Still other immunosuppressive drugs, such as mycophenolate mofetil and prednisolone, function by inhibiting key enzymes required for T- and B-cell growth or by binding to glucocorticoid receptors, respectively. It is well-recognized in the field that many immunosuppressive drugs have high inter- and intra-patient variability and require routine dosage adjustments to maintain appropriate trough levels for therapeutic concentrations.

[00157] In an embodiment of any of the above-described methods, the immunosuppressive drug comprises methylprednisolone. For methylprednisolone, an effective amount can range from about 4 to 1,000 mg per dose. A preferred dosage of methylprednisolone may be about 125 mg administered intravenously immediately prior to surgery. Methylprednisolone also goes by the trade names Medrol® and Solu-Medrol®. Effective dosages will also vary, as recognized by those skilled in the art, dependent on route of administration, excipient usage, whether methylprednisolone is given as a combination therapy with another immunosuppressive drug, and also depends heavily on the individual patient. Determining the appropriate dosage of an immunosuppressive drug are customary methods to physicians skilled in the art.

[00158] In an embodiment of any of the above-described methods, the immunosuppressive drug comprises prednisone. For prednisone, an effective amount can range from about 5 to 70 mg per dose. A preferred dosage of prednisone may be 60 mg delivered orally and tapered to 0 mg over 1 month. Effective doses will also vary, as recognized by those skilled in the art, dependent on route of administration, excipient
usage, whether the prednisone is given as a combination therapy with another immunosuppressive drug, and also depends heavily on the individual patient.

[00159] In an embodiment of any of the above-described methods, the immunosuppressive drug comprises basiliximab. For basiliximab, an effective amount can range from about 10 to 20 mg per dose. A preferred dosage of basiliximab may be 20 mg delivered intravenously, one dose given during transplantation and one given on postoperative day 4. Basiliximab also goes by the trade name Simulect®. Effective doses will also vary, as recognized by those skilled in the art, dependent on route of administration, excipient usage, whether the basiliximab is given as a combination therapy with another immunosuppressive drug, and also depends heavily on the individual patient.

[00160] In an embodiment of any of the above-described methods, the immunosuppressive drug comprises tacrolimus. For tacrolimus, an effective amount can range from about 0.03 to 0.3 milligrams per kilogram per dose. Tacrolimus also goes by FK-506, fujimycin or trade names Prograf®, LCP-Tacro™, Advagraf®, and Protopic®. Effective doses will also vary, as recognized by those skilled in the art, dependent on route of administration, excipient usage, whether the tacrolimus is given as a combination therapy with another immunosuppressive drug, and also depends heavily on the individual patient. In an embodiment, a toxic dose of tacrolimus is administered to a subject.

[00161] Trough concentrations of tacrolimus are assessed to establish the appropriate dosing regimen. Therapeutic doses of tacrolimus have been reported to be 10-20 ng/mL while doses greater than 20 ng/mL are associated with neurotoxicity. A preferred dosage of tacrolimus may maintain trough concentrations of about 4 to 8 ng/mL delivered orally twice a day.

[00162] In an embodiment of any of the above-described methods, the immunosuppressive drug comprises mycophenolate mofetil. For mycophenolate mofetil, an effective amount can range from about 1000 to 2000 milligrams per dose. A preferred dosage of mycophenolate mofetil may be 1,000 mg given orally twice a day. Mycophenolate mofetil also goes by mycophenolic acid and the trade name CellCept®. The salt mycophenolate sodium may also be used. Mycophenolate sodium goes by the trade name Myfortic®. Effective doses will also vary, as recognized by those skilled in the art, dependent on route of administration, excipient usage, whether the mycophenolate mofetil is given as a combination therapy with another immunosuppressive drug, and also depends heavily on the individual patient.

[00163] In an embodiment of any of the above-described methods, the immunosuppressive drug comprises sirolimus. For sirolimus, an effective amount can range from about 1 to 20 milligrams per dose. Sirolimus also goes by the name rapamycin
and the trade name Rapamune®. Effective doses will also vary, as recognized by those skilled in the art, dependent on route of administration, excipient usage, whether the sirolimus is given as a combination therapy with another immunosuppressive drug, and also depends heavily on the individual patient.

The dosage may be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment. The compositions may be given as a bolus dose, to maximize the circulating levels for the greatest length of time after the dose. Continuous infusion may also be used after the bolus dose.

In some embodiments, compositions used in the methods described herein further comprise a pharmaceutically acceptable excipient. As used herein, the term "excipient" refers to any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, that are compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. The compositions may also contain other active compounds providing supplemental, additional, or enhanced therapeutic functions. The pharmaceutical compositions may also be included in a container, pack, or dispenser together with instructions for administration.

A pharmaceutical composition is formulated to be compatible with its intended route of administration. Methods to accomplish the administration are known in the art. "Administration" is not limited to any particular delivery system and may include, without limitation, parenteral (including subcutaneous, intravenous, intramedullary, intraarticular, intramuscular, or intraperitoneal injection), rectal, topical, transdermal, or oral (for example, in capsules (e.g., as, powder, granules, microtabiet, micropellets, etc.), suspensions, or tablets).

Administration to an individual may occur in a single dose or in repeat administrations, and in any of a variety of physiologically acceptable salt forms, and/or with an acceptable pharmaceutical carrier and/or additive as part of a pharmaceutical composition. Physiologically acceptable salt forms and standard pharmaceutical formulation techniques and excipients are well known to persons skilled in the art.

In one embodiment of the present disclosure, an immunosuppressive drug is administered daily (or 1 to 5 times daily), weekly, or monthly. Illustratively, the composition is administered three times a week for five weeks and then weekly for an additional five weeks, and such administration achieves, for example, suppression of ALS.

A dosage and dosage regimen may be administered to provide the optimal desired response (e.g., therapeutic response). The dose of an immunosuppressive drug may be measured in units of mg/kg of patient body weight. Alternatively, the dose of an
immunosuppressive drug is measured in units of mg/kg of patient lean body weight (e.g., body weight minus body fat content), in units of mg/m² of patient body surface area, or in units of mg per dose (e.g., a fixed dose) administered to a patient. Any measurement of dose can be used in conjunction with the compositions and methods of the invention and dosage units can be converted by means standard in the art.

[00170] The method comprises the administration of an immunosuppressive drug of the present invention to a subject in need thereof. In one embodiment, the dosage regimen of immunosuppressive drug corresponds to once-a-day or twice-a-day dosages, and can include, for example, about 0.0001 mg/kg, about 0.0005 mg/kg, about 0.001 mg/kg, about 0.01 mg/kg, about 0.05 mg/kg, about 0.1 mg/kg, about 0.5 mg/kg, about 1 mg/kg, about 2 mg/kg, about 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 30 mg/kg, about 40 mg/kg, about 50 mg/kg, about 60 mg/kg, about 70 mg/kg, about 80 mg/kg, about 90 mg/kg, about 100 mg/kg, about 110 mg/kg, about 120 mg/kg, about 130 mg/kg, about 140 mg/kg, about 150 mg/kg, about 160 mg/kg, about 170 mg/kg, about 180 mg/kg, about 190 mg/kg, about 200 mg/kg, about 220 mg/kg, about 240 mg/kg, about 250 mg/kg, about 500 mg/kg, about 750 mg/kg, or about 1,000 mg/kg (by body weight of the subject) dose of an immunosuppressive drug of the present invention, and can be modified in accordance with a variety of factors. These specific mg/kg amounts can vary, for example, from about 0.01% to about 20% or more, depending on the application and desired therapeutic result.

Other factors include the type of subject, the age, weight, sex, diet, and medical condition of the subject and the severity of the disease. Thus, the dosage regimen actually employed can vary widely and therefore deviate from the dosage regimen set forth above.

[00171] An immunosuppressive drug for use in any of the aforementioned methods may be administered in one or more doses (e.g., an initial dose optionally followed by one or more subsequent doses). Those skilled in the art will appreciate that dosages are generally higher and/or frequency of administration greater for initial treatment as compared with maintenance regimens. In certain embodiments, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more or eleven or more subsequent doses of the antibody are administered. The aforementioned dosage amounts refer to mg (immunosuppressive drug)/kg (weight of the individual to be treated).

[00172] An immunosuppressive drug thereof for use in any of the aforementioned methods may also be administered as a fixed dose, independent of a dose per subject weight ratio.

[00173] In some embodiments, the immunosuppressive drug is administered in one or more fixed doses of about 1000 mg or less, 500 mg or less, or 250 mg or less, 100 mg or
less, 90 mg or less, 80 mg or less, 70 mg or less, 60 mg or less, 50 mg or less, 40 mg or less, 30 mg or less, 20 mg or less, or 10 mg or less of immunosuppressive drug. In some embodiments, the immunosuppressive drug is administered in one or more doses of at least 0.01 mg, at least 0.5 mg of immunosuppressive drug, at least 1 mg of immunosuppressive drug, or at least 10 mg of immunosuppressive drug. In some embodiments, the immunosuppressive drug thereof is administered in one or more doses of 1 mg to 100 mg of immunosuppressive drug.

[00174] In certain embodiments, the fixed dose immunosuppressive drug is from about 1 mg to about 10 mg, about 1 mg to about 25 mg, about 10 mg to about 50 mg, about 10 mg to about 100 mg, about 25 mg to about 100 mg, about 50 mg to about 100 mg, about 100 mg to about 150 mg, about 150 mg to about 200 mg, about 200 mg to about 250 mg, about 250 mg to about 500 mg, about 500 mg to about 1000 mg. In some embodiments, the fixed dose of immunosuppressive drug thereof is less than 100 mg.

[00175] In various embodiments, dosage units of the present invention contain, for example, about 1 mg to about 2000 mg, about 0.001 mg to about 750 mg, about 0.01 mg to about 500 mg, about 0.1 mg to about 300 mg or about 1 mg to about 100 mg of an immunosuppressive drug of the present invention. Illustratively, such unit dosage forms can contain about 0.001 mg, or about 0.01 mg, or about 0.1 mg, or about 1 mg, or about 2 mg, or about 5 mg, or about 10 mg, or about 15 mg, or about 20 mg, or about 30 mg, or about 40 mg, or about 50 mg, or about 60 mg, or about 70 mg, or about 80 mg, or about 90 mg, or about 100 mg, or about 110 mg, or about 120 mg, or about 130 mg, or about 140 mg, or about 150 mg, or about 160 mg, or about 170 mg, or about 180 mg, or about 190 mg, or about 200 mg, or about 300 mg, or about 400 mg, or about 500 mg, or about 750 mg, or about 1000 mg of an immunosuppressive drug of the present invention.

[00176] Illustratively, dosage units each contain about 0.01 mg, about 0.1 mg, about 1 mg, about 5 mg, about 10 mg, about 15 mg, about 20 mg, about 40 mg, about 80 mg, about 100 mg, about 250 mg, about 500 mg, or about 1000 mg of an immunosuppressive drug of the present invention. The dosage unit form can be selected to accommodate the desired frequency of administration used to achieve the specified daily dosage. In one embodiment, a composition of the invention will be administered to a subject in an amount
sufficient to about 0.1 to about 15 mg, about 0.5 to about 10 mg, and or about 1 to about 5 mg of the active agent, for example methylprednisolone, prednisone, sirolimus, etc.

[00177] In some embodiments of any of the aforementioned methods, the immunosuppressive drug is administered once to treat or prevent ALS. In some embodiments of any of the aforementioned methods, administration of an initial dose of immunosuppressive drug is followed by the administration of one or more subsequent doses. Examples of dosing regimens (e.g., an interval between the first dose and one or more subsequent doses) that can be used in the methods of the disclosure include an interval of about once every week to about once every 12 months, an interval of about once every two weeks to about once every 6 months, an interval of about once every month to about once every 6 months, an interval of about once every month to about once every 3 months, or an interval of about once every 3 months to about once every 6 months. In some embodiments, administration is monthly, every two months, every three months, every four months, every five months, every six months, or on recurrence of ALS.

[00178] The disclosure also provides dosing regimens for use in any of the aforementioned methods, wherein the dosing regimens comprise more than one dosing interval for administration of the immunosuppressive drug. In some embodiments, the dosage regimen comprises at least two (e.g., two, three, four, five, six) different dosing intervals for administration of the immunosuppressive drug. In some embodiments, the dosage regimen comprises two different dosing intervals for administration of the immunosuppressive drug, wherein a first dosing interval comprises administration of one or more doses of immunosuppressive drug thereof and a second dosing interval comprises administration of one or more doses of the immunosuppressive drug thereof, and wherein the first dosing interval is shorter in time than the second dosing interval. For example, the first dosing interval may be days or weeks, and the second dosing interval may be months. In some embodiments, the first dosing interval is about 5 days to about 28 days, about 7 days to about 21 days, about 12 days to about 16 days, or about 14 days. In some embodiments, the second dosing interval is about 1 month to about 3 months, about 1 month to about 2 months, or about 1 month.

[00179] In some embodiments of any of the aforementioned methods, the dose can be escalated or reduced to maintain a constant dose in the blood or in a tissue. In related embodiments, the dose is escalated or reduced by about 2%, 5%, 8%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 95% in order to maintain a desired level of the immunosuppressive drug.
[00180] In some embodiments of any of the aforementioned methods, the immunosuppressive drug is administered to a subject such that the interval between doses is a time sufficient to maintain a plasma concentration of said immunosuppressive drug in the subject at a level of at least about 0.1 µg/mL, at least about 0.3 µg/mL, at least about 1 µg/mL or at least about 2 µg/mL. In some embodiments, these plasma concentration values refer to values obtained for an individual that is treated with the immunosuppressive drug in accordance with the disclosure herein.

[00181] In some embodiments of any of the aforementioned methods, administration of an initial dose of the immunosuppressive drug is followed by the administration of one or more subsequent doses, and wherein said one or more subsequent doses are in an amount that is approximately the same or less than the initial dose.

[00182] In some embodiments of any of the aforementioned methods, administration of an initial dose of the immunosuppressive drug is followed by the administration of one or more subsequent doses, and wherein at least one of the subsequent doses is in an amount that is more than the initial dose.

[00183] In some embodiments of any of the aforementioned methods, an immunosuppressive drug is administered, wherein administration of an initial dose of the immunosuppressive drug is followed by the administration of one or more subsequent doses, and wherein the plasma concentration of said immunosuppressive drug in the human is permitted to decrease below a level of about 0.1 µg/mL, about 0.07 µg/mL, about 0.05 µg/mL, about 0.03 µg/mL or about 0.01 µg/mL for a period of time greater than about 1 week and less than about 6 months between administrations during a course of treatment with said initial dose and one or more subsequent doses. In some embodiments, the plasma concentration values refer to values obtained for an individual that is treated with immunosuppressive drug in accordance with the disclosure herein.

[00184] The amount of immunosuppressive drug necessary to elicit a therapeutic effect can be experimentally determined based on, for example, the absorption rate of the immunosuppressive drug into the blood serum or the bioavailability of the immunosuppressive drug. It is understood, however, that specific dose levels of the immunosuppressive drug of the present invention for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, and diet of the subject (including, for example, whether the subject is in a fasting or fed state), the time of administration, the rate of excretion, the drug combination, the severity of the diabetes mellitus and form of administration. Treatment dosages generally may be titrated to optimize safety and efficacy. Typically, dosage-effect relationships from in vitro and/or in vivo tests initially can provide useful guidance on the
proper doses for subject administration. Studies in animal models generally may be used for guidance regarding effective dosages for treatment of diabetic disorders or diseases in accordance with the present invention. In terms of treatment protocols, it should be appreciated that the dosage to be administered will depend on several factors, including the particular immunosuppressive drug that is administered, the route administered, the condition of the particular subject, etc. Generally speaking, one will desire to administer an amount of the immunosuppressive drug for a period of time that elicits a desired therapeutic effect, for example, lowering blood glucose level to acceptable levels, or improvement or elimination of symptoms, and other indicators as are selected as appropriate measures by those skilled in the art. Determination of these parameters is well within the skill of the art.

[00185] In some embodiments, the composition comprising the immunosuppressive drug may be administered to a subject in accordance with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous, intraperitoneal, or oral administration of the immunosuppressive drug is preferred, with intravenous or oral routes being particularly preferred.

[00186] In one embodiment, an immunosuppressive drug of the invention is formulated as an injectable formulation and comprises, for example, an aqueous solution or suspension of the active ingredient suitable for intravenous delivery. When preparing the immunosuppressive drug for injection, particularly for intravenous delivery, a continuous phase can be present that comprises an aqueous solution of tonicity modifiers, buffered to a pH below about 7, or below about 6, for example about 2 to about 7, about 3 to about 6 or about 3 to about 5. The tonicity modifiers can comprise, for example, sodium chloride, glucose, mannitol, trehalose, glycerol, or other pharmaceutical agents that render osmotic pressure of the formulation isotonic with blood. Alternatively, when a larger quantity of the tonicity modifier is used in the formulation, it can be diluted prior to injection with a pharmaceutically acceptable diluent to render the mixture isotonic with blood.

[00187] In another embodiment, the immunosuppressive drug of the present invention is administered by intravenous (IV) infusion or intra-arterial administration over a desired period (for example, bolus injection, 5 min, 15 min, 30 min, 1 hr, 2 hr, 3 hr, 6 hr, 24 hr, 48 hr, 72 hr or 96 hour infusions). In one embodiment of the present invention the period of administration is no greater than about 3 hours.

[00188] In another embodiment of the present invention, the immunosuppressive drug of the invention is in the form of solid dosage forms, for example tablets (including but not
limited to swallowable tablets, chewable tablets, suspension tablets, etc.), capsules, caplets, troches, losenges, powders, granules, etc. Solid compositions are illustratively prepared by mixing the therapeutic agent with a pharmaceutical excipient to form a solid preformulation composition containing a homogeneous mixture of the therapeutic agent and excipient. When referring to these preformulation compounds as homogeneous, it is meant that the agents are substantially evenly distributed throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms, such as tablets, pills and capsules. This solid preformulation is then subdivided into unit dosage forms of the type described herein.

[00189] Compressed tablets are solid dosage forms prepared by compacting a formulation containing the immunosuppressive drug and excipient selected to aid the processing and improve the properties of the product. The term "compressed tablet" generally refers to a plain, uncoated tablet for oral ingestion, prepared by a single compression or by pre-compaction tapping followed by a final compression.

[00190] The solid dosage forms of the present invention may be coated or otherwise compounded to provide a dosage form affording the advantage of improved handling or storage characteristics. For example, a tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former.

[00191] An immunosuppressive drug of the present invention can further comprise one or more pharmaceutically acceptable excipients. Suitable excipients are any of those commonly used excipients in pharmaceutics and should be selected on the basis of compatibility with the pharmaceutical agent and the release profile properties of the desired dosage form. Any suitable excipient can be present in a composition of the invention in an amount of about 1% to about 80%, about 2% to about 70%, about 3% to about 60%, about 4% to about 50%, or about 5% to about 40%, by weight.

[00192] Illustrative classes of pharmaceutical excipients include binders, disintegrants, filling agents, surfactants, solubilizers, stabilizers, preservatives, lubricants, wetting agents, diluents, tableting agents, glidants, etc.

[00193] In one embodiment, a composition of the invention comprises a preservative. Illustrative preservatives include benzalkonium chloride, propylparaben, butylparaben, chlorobutanol, benzyl alcohol, phenol, sodium benzoate, or EDTA.

[00194] Illustrative binders include acacia, alginic acid and salts thereof, cellulose derivatives, methylcellulose, hydroxyethyl cellulose, hydroxypropyl cellulose, magnesium aluminum silicate, polyethylene glycol, gums, polysaccharide acids, bentonites, hydroxypropyl methylcellulose, gelatin, polyvinylpyrrolidone, polyvinylpyrrolidone/vinyl acetate copolymer, crospovidone, povidone, polymethacrylates,
hydroxypropylmethylcellulose, hydroxypropylcellulose, starch, pregelatinized starch, ethylcellulose, tragacanth, dextrin, microcrystalline cellulose, sucrose, or glucose, and the like.

[00195] Illustrative disintegrants (also referred to as disintegration agents) include starches, pregelatinized corn starch, pregelatinized starch, cellulosics, cross-linked carboxymethylcellulose, sodium starch glycolate, crospovidone, cross-linked polyvinylpyrrolidone, croscarmellose sodium, a calcium, a sodium alginate complex, clays, alginates, gums, or sodium starch glycolate, and any disintegration agents used in solid preparations.

[00196] Illustrative filling agents include lactose, calcium carbonate, calcium phosphate, dibasic calcium phosphate, calcium sulfate, microcrystalline cellulose, cellulose powder, dextrose, dextrates, dextran, starches, pregelatinized starch, sucrose, xylitol, lactitol, mannitol, sorbitol, sodium chloride, polyethylene glycol, and the like.

[00197] Illustrative surfactants include sodium lauryl sulfate, sorbitan monooleate, polyoxyethylene sorbitan monooleate, poloxamers, poloxamers, bile salts, glyceryl monostearate, Pluronic™ line (BASF), and the like.

[00198] Illustrative solubilizers include citric acid, succinic acid, fumaric acid, malic acid, tartaric acid, maleic acid, glutaric acid sodium bicarbonate and sodium carbonate and the like.

[00199] Illustrative stabilizers such as antioxidation agents, buffers, or acids, and the like, can also be utilized.

[00200] Illustrative lubricants include magnesium stearate, calcium hydroxide, talc, sodium stearyl fumarate, hydrogenated vegetable oil, stearic acid, glyceryl behapate, magnesium, calcium and sodium stearates, stearic acid, talc, waxes, Stearowet, boric acid, sodium benzoate, sodium acetate, sodium chloride, DL-leucine, polyethylene glycols, sodium oleate, or sodium lauryl sulfate, and the like.

[00201] Illustrative wetting agents include oleic acid, glyceryl monostearate, sorbitan monooleate, sorbitan monolaurate, triethanolamine oleate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monolaurate, sodium oleate, or sodium lauryl sulfate, and the like.

[00202] Illustrative diluents include lactose, starch, mannitol, sorbitol, dextrose, microcrystalline cellulose, dibasic calcium phosphate, sucrose-based diluents, confectioner's sugar, monobasic calcium sulfate monohydrate, calcium sulfate dihydrate, calcium lactate trihydrate, dextrates, inositol, hydrolyzed cereal solids, amylose, powdered cellulose, calcium carbonate, glycine, or bentonite, and the like.
Illustrative anti-adherents or glidants include talc, corn starch, DL-leucine, sodium lauryl sulfate, and magnesium, calcium, or sodium stearates, and the like.

Illustrative pharmaceutically compatible carriers include acacia, gelatin, colloidal silicon dioxide, calcium glycerophosphate, calcium lactate, maltodextrin, glycerine, magnesium silicate, sodium caseinate, soy lecithin, sodium chloride, tricalcium phosphate, dipotassium phosphate, sodium stearoyl lactylate, carrageenan, monoglyceride, diglyceride, or pregelatinized starch, and the like.


In making compositions of the present invention, the individual components can be mixed with a pharmaceutically acceptable excipient, diluted by the excipient or enclosed within a capsule, sachet, paper or other container.

When an excipient serves as a diluent, it can be a solid, semi-solid or liquid material, which acts as a vehicle, carrier or medium for the active ingredient. Thus, the compositions can be in the form of a tablet, pill, powder, lozenge, sachet, cachet, elixir, troche, suspension, emulsion, solution, syrup, aerosol (as a solid or in a liquid medium), soft and hard gelatin capsule, sterile packaged powder, dispensable powder, granule, or liquid.

In one embodiment of the present invention, the manufacturing processes may employ one or a combination of methods: (1) dry mixing, (2) direct compression, (3) milling, (4) dry or non-aqueous granulation, (5) wet granulation, or (6) fusion. Lachman *et al.*, *The Theory and Practice of Industrial Pharmacy* (1986). Such tablets may also comprise film coatings, which disintegrate upon oral ingestion or upon contact with diluent.

Subjects undergoing treatment with an immunosuppressive drug disclosed herein can be routinely monitored by any of the methods well known in the art to determine the effectiveness of therapy. Continuous analysis of such data permits modification of the treatment regimen during therapy so that optimal effective amounts of immunosuppressive drug of the present invention are administered at any point in time, and so that the duration of treatment can be determined as well. In this way, the treatment regimen/dosing schedule can be rationally modified over the course of therapy so that the lowest amount of an immunosuppressive drug exhibiting satisfactory effectiveness is administered, and so that administration is continued only so long as is necessary to successfully treat the condition or disorder.
The present methods can also be used in combination ("combination therapy") with another pharmaceutical agent(s) that is indicated for treating spinal cord injury or for helping to manage the symptoms associated with spinal cord injury including, for example, pain.

As used herein, the phrase "combination therapy" refers to the administration of at least one NSC and/or at least one immunosuppressive drug in conjunction with another pharmaceutical agent(s).

The phrase "combination therapy" embraces the administration of a composition of the present invention in conjunction with another pharmaceutical agent that is indicated for treating or preventing spinal cord injury in a subject, as part of a specific treatment regimen intended to provide a beneficial effect from the co-action of these therapeutic agents for the treatment of ALS. The beneficial effect of the combination includes, but is not limited to, pharmacokinetic or pharmacodynamic co-action resulting from the combination of therapeutic agents. Administration of these therapeutic agents in combination typically is carried out over a defined time period (usually substantially simultaneously, minutes, hours, days, weeks, months or years depending upon the combination selected). "Combination therapy" generally is not intended to encompass the administration of two or more of these therapeutic agents as part of separate monotherapy regimens that incidentally and arbitrarily result in the combinations of the present invention. "Combination therapy" is intended to embrace administration of these therapeutic agents in a sequential manner, that is, where each therapeutic agent is administered at a different time, as well as administration of these therapeutic agents, or at least two of the therapeutic agents, in a substantially simultaneous manner. Substantially simultaneous administration can be accomplished, for example, by administering to the subject a single injection, tablet or capsule having a fixed ratio of each therapeutic agent or in multiple, single injections, capsules, or tablets for each of the therapeutic agents. Sequential or substantially simultaneous administration of each therapeutic agent can be implemented by any appropriate route. For example, the composition of the present invention can be administered orally, percutaneously, intravenously, intramuscularly, and/or directly absorbed through mucosal membranes while the other therapeutic agent or agents of the combination can be administered by any appropriate route for that particular agent or agents, including, but not limited to, an oral route, a percutaneous route, an intravenous route, an intramuscular route, or by direct absorption through mucous membrane tissues. The sequence in which the therapeutic agents are administered is not narrowly critical. "Combination therapy" also can embrace the administration of the therapeutic agents as described above in further combination with other biologically active ingredients, such as,
but not limited to: (1) antiinflammatory agents, such as a steroidal or nonsteroidal antiinflammatory drug, and/or a 5-lipoxygenase inhibitor; (2) agents for reducing muscle pain, such as, for example, baclofen, dantrolene, tizanidine, ibuprofen, or acetaminophen; (3) agents to manage cognition, such as, for example, cholinesterase inhibitors (such as donepezil, galantamine, rivastigmine) or memantine; (4) mood regulating agents, such as for depression or anxiety (such as detromethorphan and quinidine); (5) agents for regulating sleep disorders; and with non-drug therapies, such as, but not limited to, surgery.

[00213] The therapeutic compounds which make up the combination therapy may be a combined dosage form or in separate dosage forms intended for substantially simultaneous administration. The therapeutic compounds that make up the combination therapy may also be administered sequentially, with either therapeutic compound being administered by a regimen calling for two step administration. Thus, a regimen may call for sequential administration of the therapeutic compounds with spaced-apart administration of the separate, active agents. The time period between the multiple administration steps may range from, for example, a few minutes to several hours to days, depending upon the properties of each therapeutic compound such as potency, solubility, bioavailability, plasma half-life and kinetic profile of the therapeutic compound, as well as depending upon the effect of food ingestion and the age and condition of the subject. Circadian variation of the target molecule concentration may also determine the optimal dose interval. The therapeutic compounds of the combined therapy whether administered simultaneously, substantially simultaneously, or sequentially, may involve a regimen calling for administration of one therapeutic compound by oral route and another therapeutic compound by an oral route, a percutaneous route, an intravenous route, an intramuscular route, or by direct absorption through mucous membrane tissues, for example. Whether the therapeutic compounds of the combined therapy are administered orally, by inhalation spray, rectally, topically, buccally (for example, sublingual), or parenterally (for example, subcutaneous, intramuscular, intravenous and intradermal injections, or infusion techniques), separately or together, each such therapeutic compound will be contained in a suitable pharmaceutical formulation of pharmaceutically-acceptable excipients, diluents or other formulations components.

[00214] Without further description, it is believed that one of ordinary skill in the art may, using the preceding description and the following illustrative examples, make and utilize the agents of the present disclosure and practice the claimed methods. The following working examples are provided to facilitate the practice of the present disclosure, and are not to be construed as limiting in any way the remainder of the disclosure.
EXAMPLES

Example 1: Expansion of Human Neural Stem Cells

[00215] Spinal cord from at least one donor of gestational age of approximately 7-8.5 weeks was obtained. A single contiguous tissue of the spinal cord was dissociated in Ca\(^{++}\) and Mg\(^{++}\)-free phosphate buffered saline using mechanical trituration. The resulting cell suspension was then seeded into tissue culture plates pre-coated with both poly-L-ornithine or poly-D-lysine and human fibronectin or other extracellular matrix proteins. Tissue culture-treated plates or flasks were then incubated with 100 μg/ml poly-D-lysine for 1 hour at room temperature. They were then washed three times with water and dried. They were then incubated with 25 mg/ml for 5 minutes at room temperature. Sometimes, 10 mg/ml fibronectin for 1 hour at room temperature was used. Sometimes, 1 mg/ml fibronectin for 18 hours at 37°C was used. Culture media consisting of N2 (DMEM/F12 plus insulin, transferrin, selenium, putrescine, and progesterone) was supplemented with 1 human recombinant basic fibroblast growth factor (bFGF). In an embodiment, a range of 0.1 ng/ml-100 ng/ml can be used. In an embodiment, optimally, 10 ng/ml of bFGF was used.

[00216] The resulting initial culture consists of post-mitotic neurons and proliferative NSCs in a monolayer. Subsequently, after approximately five to about twenty days in culture, the dividing, nestin-positive, NSCs dominate the culture over the non-dividing neurons or the slowly-dividing glia. Under these culture conditions, NSCs are selectively favored for expansion. The expanding NSC population was passaged by mild enzymatic treatment, such as using trypsin. The cells were then cultured in media free of serum or substantially free of serum. Although low concentration of serum may be tolerated by the cells, it is best to avoid exposing the cells to serum since serum contains many cytokines such as LIF and CNTF which promote glial differentiation of the NSCs. Thus, during passage, the enzyme used was stopped by adding specific enzyme inhibitor, such as trypsin inhibitor, rather than serum. At each passage, the number of harvested cells were counted, and a fraction was re-seeded for further expansion. Using this method, human NSCs can be expanded beyond 10\(^{18}\) -fold increase in population while maintaining their growth and differentiation properties. During the expansion, almost all cells express nestin, the \textit{in vivo} marker of mitotic neuroepithelial cells, and are absent of antigens of differentiated neurons and glia such as type 3-beta tubulin and GFAP. The cells were also negative by immunostaining for PSA-NCAM, a possible marker of committed neuronal progenitors, 04 and GalC, markers of oligodendrocytes, and RC2, a marker of radial glia. Thus, determined by immunostaining, the NSCs stably maintain their expression of antigen profile throughout the prolonged expansion period.
Example 2: Differentiation of Human Spinal Cord Neural Stem/Progenitor Cells

At any point during expansion of the NSCs, the cultures can be differentiated by withdrawal of the mitogen in the culture such as bFGF. Differentiation of NSCs ensues within about 1-3 days after the removal of mitogen, and distinct heterogeneous cell morphologies are apparent. By approximately day 4-7 of differentiation, neuron-specific antigens, such as MAP2c, tau, and type III beta-tubulin, can be visualized by immunostaining. By approximately day 12-14, elongated, fasciulated axonal processes are evident throughout the culture along with clear polarization of subcellular protein trafficking. By approximately day 28, synaptic proteins, such as synapsin and synaptophysin, localize into axon terminals, appearing as punctate staining. Additional feeder layer of astrocytes can be provided to further promote long-term maturation of the neurons. Differentiation of human spinal NSCs generates mixed cultures of neurons and glia wherein the neurons robustly express neuron-specific antigens such as tau, MAP2ab and type3 beta tubulin and comprises approximately 50% of the culture. Additionally, the culture spontaneously generates long, bundled, axon cables that stretch for several centimeters. A significant proportion of the neurons are GABAergic with cholinergic motor neurons also being present in the culture. Presence of significant GABA neurons in culture predicts usefulness of the human spinal NSCs for treating various neurological conditions caused by decreased GABA production in certain circuitry. Likewise, presence of cholinergic neurons demonstrates that the human spinal NSCs are capable of motor neuron differentiation and predicts their usefulness for treating various motor neuron diseases caused by gradual degeneration of motor neurons. For treatment, the NSCs may be expanded with or without further phenotype-enhancing conditions, harvested, and injected into a neural area of deficiency.

Example 3: Treatment of a Subject with an Acute Spinal Cord Injury

Isoflurane-anesthetized adult Gottingen-Minnesota minipigs (n=10) underwent 2-level laminectomies (L2-L5) followed by L3 spinal contusion using a 5-mm-diameter circular bar (peak force of 2.5kg at a velocity of 3cm/sec). At 24 hours postinjury, animals received 12 bilateral injections of hNPCs targeted in and around the injury epicenter. After cell grafting, animals receive a bolus (0.1mg/kg) injection of tacrolimus (5mg/ml, i.v.). One arm of a double lumen femoral vein catheter was then connected to a 7 day chronic infusion pump and tacrolimus was continuously delivered (0.15 mg/kg/day) in saline for the first 14 days and then 0.075mg/kg/day thereafter until termination. The dose may be adjusted to achieve steady state plasma level of 50-60 ng/ml for the first 14 days
and then 25-35 ng/ml thereafter until termination. Additionally, mycophenolate mofetil (MFF; Cellcept; 500 mg tablets) was administered orally (1g/kg B.I.D.) in drinking water for first 14 days after injections of hNPCs. Furthermore, just after cell grafting animals received methylprednisolone (30mg/kg i.v.) as a single bolus and then Depo-Medrol (40mg i.m./7days).

[00220] During recovery, motor and sensory function were periodically monitored for 4 weeks. After survival, the presence of grafted cells was confirmed after staining spinal cord sections with a combination of human-specific (hNUMA, H014, hNSE, hSYN) or non-specific (DCX, MAP2, CHAT, GFAP, APC) antibodies (see, Figure 1).

[00221] In all cell-grafted animals, hNUMA positive cells were readily identified. Numerous terminally differentiated grafted neurons with extensive axodendritic sprouting were seen; these exhibited hNSE and H014 immunoreactivity. Similarly, a high density of hSYN-positive terminals derived from grafted neurons and residing in the vicinity of host neurons were also seen. A moderate degree of inflammatory change, as evidenced by the appearance of reactive astrocytes and microglia, was also identified. These data demonstrate that, using this immunosuppression protocol, xenograft cells grafted into the acutely injured spinal cord can survive despite the inflammatory, post-traumatic environment.

Example 4: Treatment of a Subject with an Acute Spinal Cord Injury

[00222] Studies are conducted to determine the effects treating acute spinal cord injury in a subject. For example, a multicenter, randomized, double-blind, placebo-controlled study is undertaken to evaluate treatment with a weight-based or fixed dose of spinal cord-derived neural stem cells (NSCs) in human subjects with a spinal cord injury. More specifically, a clinical study was performed to examine the efficacy and safety of introducing a therapeutically effective amount of spinal cord-derived NSCs to at least one area of the spinal cord of the human subject and a therapeutically effective amount of at least one immunosuppressive drug.

[00223] Surgery is performed using standard anesthetic and monitoring techniques. For each human subject 10 μL of the live NS1566 NSC cell suspension is microinjected into each site at a rate of 5 μL/min over 2 minutes. Each injection contains approximately 100,000 cells (about 10,000 cells per microliter). The needle tip is left in place for 1 minute postinfusion to reduce suspension reflex. The process is completed over 5 to 10 distinct injections using precise stereotaxic coordinates. Control patients received sterile vehicle (hibernation buffer) at the same stereotaxic coordinates. All patients are monitored and cared for following injections by standard post operative care procedures.
Once the NSCs are present in the afflicted individual, they engraft and differentiate and thereby help treat the cognitive dysfunction. One advantage of this method is that it may be repeated, as needed, and thereby alleviate some or all of the cognitive dysfunction in the human subject. Optionally, cells may be differentiated into appropriate cell types in vitro before transplantation.

Example 5: Treatment of subjects with ALS with immunosuppressive therapy and NSCs

Each subject with ALS received a two-level, bilateral laminectomy at vertebral level T11 and T12. Six subjects (#1 - #6) received five unilateral or five bilateral injections of NSCs. Each injection contained approximately 100,000 cells in an 8.5 to 10 microliter volume. NSCs were injected for more than a 2-minute period and the injection needle was kept in place for an additional minute to prevent reflux during needle exit. Total surgical time, from first incision to closing, ranged between 3 to 4.5 hours. Proper postoperative care was standard for patients undergoing laminectomy with an intradural procedure.

Subjects received an intravenous dose of methylprednisolone (125 mg) immediately prior to surgery. Subjects were treated post-operatively with an oral dose of 60 mg of prednisone, tapering to 0 over 1 month. Two doses of basiliximab (20 mg) were delivered intravenously, one on day 1 and the other on day 4. Tacrolimus was given twice a day to maintain a trough level of 4-8 ng/ml, and mycophenolate mofetil was given at 1,000 mg orally twice a day. Tacrolimus and mycophenolate were administered for the duration of the treatment so long as they were well tolerated.

Subjects were assessed prior to treatment with baseline laboratory studies that included standard clinical examinations, laboratory measures of metabolic and hematologic function, screening for the presence of CMV or of HLA antibodies, hand-held dynamometry (HHD) to assess upper and lower extremity strength, forced vital capacity (FVC), ALS Functional Rating Scale-Revised (ALSFS-R), Electrical impedance myography (EIM), bladder ultrasound, and MRI scans of the brain and entire spinal cord. Each subject was evaluated monthly for at least three months prior to surgery to generate a pre-treatment slope of disease progression and re-evaluated during the week prior to beginning treatment. The re-evaluation also included standard preoperative laboratories, electrocardiogram, and chest x-ray. Subjects were evaluated at two and four weeks after implantation and then monthly thereafter. Standard clinical examinations and questionnaires were administered at each visit, along with laboratory measures of metabolic and hematologic function, serum levels for tacrolimus, and screening for reactivation of CMV by PCR in those patients with serological evidence of pre-existing infection. Subjects were followed monthly or bimonthly with standard clinical examination,
HHD, ALSFRS-R, FVC, EIM, and bladder ultrasounds. MRI scans of the surgical region were performed at least every 6 months.

[00228] Subject treated with neural stem cells and immunotherapy showed no precipitous decline in function after surgery (see, Figures 2 and 3).

Example 6: Treatment of subjects with immunosuppressive therapy alone

[00229] Studies are conducted to determine the effects of a composition comprising at least one immunosuppressive drug in subjects with ALS. For example, a multicenter, randomized, double-blind, placebo-controlled study is undertaken to evaluate treatment with a weight-based or fixed dose of a composition comprising at least one immunosuppressive drug in human subjects diagnosed with ALS. More specifically, a clinical study was performed to examine the efficacy and safety of a composition comprising at least one immunosuppressive drug. The composition is effective to treat ALS, reduce the rate of function decline of the subject, or alleviate symptoms of ALS. Subjects are evaluated with standard clinical examinations, laboratory measures of metabolic and hematologic function, serum levels of immunosuppressive drugs, screened for reactivation of CMV or presence of HLA antibodies, ALSFRS-R, HHD, FVC, EIM, bladder ultrasound, and MRI scans of the surgical site.

[00230] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the disclosure are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[00231] The terms "a," "an," "the" and similar referents used in the context of describing the disclosure (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein is intended merely to better illuminate the disclosure and does not pose a limitation on the scope of the disclosure otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the disclosure.
Groupings of alternative elements or embodiments of the disclosure disclosed herein are not to be construed as limitations. Each group member can be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group can be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

Certain embodiments of this disclosure are described herein, including the best mode known to the inventors for carrying out the disclosure. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the disclosure to be practiced otherwise than specifically described herein. Accordingly, this disclosure includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the disclosure unless otherwise indicated herein or otherwise clearly contradicted by context.

Specific embodiments disclosed herein can be further limited in the claims using consisting of or and consisting essentially of language. When used in the claims, whether as filed or added per amendment, the transition term "consisting of" excludes any element, step, or ingredient not specified in the claims. The transition term "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s). Embodiments of the disclosure so claimed are inherently or expressly described and enabled herein.

It is to be understood that the embodiments of the disclosure disclosed herein are illustrative of the principles of the present disclosure. Other modifications that can be employed are within the scope of the disclosure. Thus, by way of example, but not of limitation, alternative configurations of the present disclosure can be utilized in accordance with the teachings herein. Accordingly, the present disclosure is not limited to that precisely as shown and described.
[00236] While the present disclosure has been described and illustrated herein by references to various specific materials, procedures and examples, it is understood that the disclosure is not restricted to the particular combinations of materials and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art. It is intended that the specification and examples be considered as exemplary, only, with the true scope and spirit of the disclosure being indicated by the following claims. All references, patents, and patent applications referred to in this application are herein incorporated by reference in their entirety.
CLAIMS

1. A method of treating a subject with a neurodegenerative disease or disorder, the method comprising:
   - administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject; and
   - administering a therapeutically effective amount of neural stem cells to one or more sites of the neurodegenerative disease or disorder.

2. The method of claim 1, wherein the neural stem cells are spinal cord-derived neural stem cells.

3. The method of claim 2, wherein the spinal cord-derived neural stem cells are embryonic spinal cord-derived neural stem cells.

4. The method of claim 2, wherein the spinal cord-derived neural stem cells are fetal spinal cord-derived neural stem cells.

5. The method of claim 4, wherein the fetal spinal cord-derived neural stem cells are obtained from a fetus having a gestational age of about 5 to about 20 weeks.

6. The method of claim 2, wherein the spinal cord-derived neural stem cells are human spinal cord-derived neural stem cells.

7. The method of claim 1, wherein the one or more immunosuppressive drugs include one or more of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and/or sirolimus.

8. The method of claim 1, wherein the one or more immunosuppressive drugs are tacrolimus and mycophenolate mofetil.

9. The method of claim 1, wherein the one or more immunosuppressive drugs are tacrolimus, mycophenolate mofetil, and methylprednisolone.

10. The method of claim 9, wherein the therapeutically effective amount of the one or more immunosuppressive drugs are administered intravenously, orally, or as a bolus injection.
11. The method of claim 10, wherein tacrolimus is administered both as a bolus injection and intravenously, wherein mycophenolate mofetil is administered orally, and wherein methylprednisolone is administered as a bolus injection.

12. The method of any one of claims 7 - 11, wherein the therapeutically effective amount of tacrolimus is a toxic amount.

13. The method of claim 1, wherein the neurodegenerative disease or disorder is a spinal cord injury.

14. The method of claim 13, wherein the spinal cord injury is a traumatic spinal cord injury or an ischemic spinal cord injury.

15. The method of claim 13, wherein the therapeutically effective amount of spinal cord-derived neural stem cells are injected into one or more areas of the spinal cord.

16. The method of claim 15, wherein the therapeutically effective amount of spinal cord-derived neural stem cells are administered to about 5 to about 50 sites in the injured spinal cord.

17. The method of claim 16, wherein the one or more sites are separated by a distance of approximately 100 microns to about 5000 microns.

18. The method of claim 15, wherein the spinal cord-derived neural stem cells differentiate into neurons that engraft in vivo into the injured spinal cord of the subject.

19. The method of claim 15, wherein at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the injured spinal cord of the subject.

20. The method of claim 1, wherein the neurodegenerative disease or disorder is ALS.

21. The method of claim 20, wherein the ALS is associated with neurodegeneration in the brain and/or spinal cord.

22. The method of claim 21 further comprising administering to the subject a therapeutically effective amount of neural stem cells to one or more sites of neurodegeneration of the brain and/or spinal cord.
23. The method of claim 22, wherein the therapeutically effective amount of spinal cord-derived neural stem cells are administered to about 5 to about 50 sites in the brain and/or spinal cord.

24. The method of claim 23, wherein the one or more sites are separated by a distance of approximately 100 microns to about 5000 microns.

25. The method of claim 22, wherein the spinal cord-derived neural stem cells differentiate into neurons that engraft in vivo into the brain and/or spinal cord of the subject.

26. The method of claim 22, wherein at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the spinal cord and/or brain of the subject.

27. The method of claim 1, wherein the neurodegenerative disease or disorder is stroke.

28. The method of claim 27, wherein the therapeutically effective amount of spinal cord-derived neural stem cells are injected into one or more areas of neurodegeneration in the brain.

29. The method of claim 28, wherein the therapeutically effective amount of spinal cord-derived neural stem cells are administered to about 5 to about 50 sites in the brain.

30. The method of claim 29, wherein the one or more sites are separated by a distance of approximately 100 microns to about 5000 microns.

31. The method of claim 28, wherein the spinal cord-derived neural stem cells differentiate into neurons that engraft in vivo into the brain of the subject.

32. The method of claim 28, wherein at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the spinal cord-derived neural stem cells are capable of generating neurons in the brain of the subject.

33. The method of claim 1, wherein the subject is human.
34. The method of claim 4 further comprising expanding the fetal spinal cord-derived neural stem cells to form an expanded spinal cord-derived neural stem cell population.

35. The method of claim 34, wherein expanding the fetal spinal cord-derived neural stem cells includes culturing the fetal spinal cord-derived neural stem cells in the absence of serum.

36. The method of claim 34, wherein expanding the fetal spinal cord-derived neural stem cells includes exposing the fetal spinal cord-derived neural stem cells to at least one growth factor.

37. The method of claim 36, wherein the growth factor is selected from the group consisting of bFGF, EGF, TGF-alpha, aFGF and combinations thereof.

38. A method for increasing engraftment of one or more neural stem cells administered to a subject with a neurodegenerative disease or disorder, the method comprising: administering a therapeutically effective amount of one or more immunosuppressive drugs to the subject.

39. The method of claim 38, wherein the neurodegenerative disease or disorder is a spinal cord injury, stroke, or ALS.

40. The method of claim 38, wherein the spinal cord-derived neural stem cells are embryonic spinal cord-derived neural stem cells.

41. The method of claim 38, wherein the spinal cord-derived neural stem cells are fetal spinal cord-derived neural stem cells.

42. The method of claim 41, wherein the fetal spinal cord-derived neural stem cells are obtained from a fetus being a gestational age of about 5 to about 20 weeks.

43. The method of claim 41, wherein the spinal cord-derived neural stem cells are human spinal cord-derived neural stem cells.
44. The method of claim 38, wherein the one or more immunosuppressive drugs include one or more of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and/or sirolimus.

45. The method of claim 38, wherein the one or more immunosuppressive drugs are tacrolimus and mycophenolate mofetil.

46. The method of claim 38, wherein the one or more immunosuppressive drugs are tacrolimus, mycophenolate mofetil, and methylprednisolone.

47. The method of claim 38, wherein the therapeutically effective amount of the one or more immunosuppressive drugs are administered intravenously, orally, or as a bolus injection.

48. The method of claim 47, wherein tacrolimus is administered both as a bolus injection and intravenously, wherein mycophenolate mofetil is administered orally, and wherein methylprednisolone is administered as a bolus injection.

49. The method of any one of claims 44 - 48, wherein the therapeutically effective amount of tacrolimus is a toxic amount.

50. The method of claim 38, wherein the subject is human.

51. A method of treating a subject with amyotrophic lateral sclerosis (ALS) comprising: administering to the subject a composition comprising at least one immunosuppressive drug in an amount effective to alleviate one or more symptoms of ALS in the subject.

52. The method of claim 51, wherein the one or more immunosuppressive drugs are one or more of methylprednisolone, prednisone, basiliximab, tacrolimus, mycophenolate mofetil, and/or sirolimus.

53. The method of claim 51, wherein the one or more immunosuppressive drugs are tacrolimus and mycophenolate mofetil.

54. The method of claim 51, wherein the one or more immunosuppressive drugs are tacrolimus, mycophenolate mofetil, and methylprednisolone.
55. The method of claim 54, wherein the therapeutically effective amount of the one or more immunosuppressive drugs are administered intravenously, orally, or as a bolus injection.

56. The method of claim 55, wherein tacrolimus is administered both as a bolus injection and intravenously, wherein mycophenolate mofetil is administered orally, and wherein methylprednisolone is administered as a bolus injection.

57. The method of any one of claims 52 - 56, wherein the therapeutically effective amount of tacrolimus is a toxic amount.

58. The method of claim 51, wherein the ALS is associated with neurodegeneration in the brain and/or spinal cord.

59. The method of claim 58 further comprising administering to the subject a therapeutically effective amount of neural stem cells to one or more sites of neurodegeneration of the brain and/or spinal cord.

60. The method of claim 51, wherein the subject is a human.
Figure 3

Months pre/post surgery

Phase (degrees)
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61P 25/00 (2014.01)
USPC - 435/368

According to International Patent Classification (IPC) or to both national classification and IPC.

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 35/30, 38/00; A61P 25/00, 25/04 (2014.01)
USPC - 424/93.6, 93.7; 435/368, 375

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 35/30; C12N 5/0623 (2014.02)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Pubmed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

Further documents are listed in the continuation of Box C.

- Special categories of cited documents:
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier application or patent but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed
  - "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  - "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  - "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Date of the actual completion of the international search: 19 May 2014

Date of mailing of the international search report: 28 MAY 2014

Authorized officer: Blaine R. Copenheaver
PCT Hapodesh: 571-272-4500
PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)